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Suppressible and Nonsuppressible Autocrine Mast Cell Tumors Are Distinguished by Insertion of an Endogenous Retroviral Element (IAP) into the Interleukin 3 Gene

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Summary

After v-H-*ras* expression, the interleukin 3 (IL-3)–dependent PB-3c mast cells progress in vivo to two different classes of IL-3 autocrine tumors. Class I tumors show a germline configuration of the IL-3 gene and represent more than 90% of tumors analyzed so far. Somatic cell fusion of class I tumor lines with the nontumorigenic parental PB-3c resulted in loss of oncogenic IL-3 expression by a posttranscriptional mechanism with concomitant tumor suppression. Class II tumors arise rarely and contain an insertion in one IL-3 allele. This alteration was linked to enhanced IL-3 gene transcription. For one tumor, the insertion was shown to be an endogenous retroviral element (intracisternal A-particle). Cell hybrids of class II tumors with PB-3c remained IL-3 independent, expressed IL-3, and formed tumors rapidly. These results suggest that the v-H-*ras* oncogene synergizes with a recessive and a dominant lesion in class I and II tumors, respectively, both of which lead to the autocrine production of IL-3.

Tumorigenesis is viewed as a multistep process with sequential acquisition of genetic lesions described as dominant gain-of-function and recessive loss-of-function (1). Constitutive activation of *ras* proto-oncogenes by point mutations represents the former type and has been detected in numerous human malignancies including leukemias (2–4). As a GTP/GDP binding protein, p21 *ras* is thought to function analogously to other G proteins in the context of signal transduction (5). Upon stimulation of hemopoietic cells with IL-2, IL-3, GM-CSF, or via the TCR, inactive GDP-p21 *ras* is converted to the activated GTP state by GDP-GTP exchange (6, 7). The GTPase-activating proteins such as GAP and NF-1 counter-regulate by increasing the low intrinsic GTPase of p21 *ras* (8). Upon GTP hydrolysis, p21 *ras* returns to the inactive GDP state thereby completing the *ras* activation cycle. Although the immediate effector molecule(s) is still elusive, recent evidence places *ras* upstream of a kinase cascade including c-*raf*, kinases of MAP kinase, and the ribosomal S6 kinase p90 to transmit signals to the nucleus (9). Since oncogenic point mutations lock p21 in the active GTP state (2), *ras* transformation is thought to result from the constitutive activation of signaling pathways. However, as shown experimentally in transfection assays, activating mutations in *ras* oncogenes alone are not sufficient to induce malignant transformation, but require further cooperating alterations (2, 10, 11). This agrees well with the detection of activating *ras* mutations in some still preneoplastic conditions, such as the myelodysplastic syndrome (12). Conversely, suppression of *ras* oncogene–transformed tumor cells by cell fusion or DNA transfection has been shown to operate without affecting the expression of the oncogene (11, 13–16). Although the biochemical aspects of p21 *ras* and its modulator molecules become more defined, the interplay of *ras* with complementing alterations in oncogenic transformation is not fully understood.

To investigate the role of *ras* in hemopoietic malignancy, we have focused on a murine tumor model, in which establishment of an autocrine IL-3 loop is the hallmark of tumors generated from the IL-3–dependent mast cell line PB-3c in response to v-H-*ras* oncogene expression (17). Work in our and other laboratories (18–20) has shown that primary mast cells can be a physiological source of IL-3 and other cytokines after immunological stimulation via IgE receptors. This response can be mimicked pharmacologically by treatment with calcium ionophores. In PB-3c mast cells, IL-3 expression by calcium ionophore appears to result from posttranscriptional stabilization of a constitutive IL-3 transcript (21). After the removal of the stimulus, negative control resumes by rapidly degrading the IL-3 mRNA. This physiological response is altered by *ras* oncogenes. In the presence of oncogenic *ras*, IL-3 hyperinduction by calcium ionophores and slowed-down decay of IL-3 mRNA after removal of the inducer, was observed (22, 23). Oncogenic IL-3 expression in autocrine tumor lines might thus result from escape from negative control either by alterations in the IL-3 gene or by loss of *trans*-acting

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suppression. Initial evidence for the latter was obtained by somatic cell fusion. When somatic cell hybrids between the tumor cells and the nontumorigenic parental PB-3c were formed, downregulation of IL-3 expression and partial tumor suppression was observed (16). In this report, we present evidence that oncogenic induction of IL-3 expression in autocrine mast cell tumors can in fact involve two different mechanisms. Whereas the more frequent alteration is recessive in somatic cell fusion, the other behaves dominantly and shows enhanced IL-3 gene transcription linked to the insertion of an endogenous retroviral element.

Materials and Methods

Cell Lines and Tissue Culture. PB-3c is a cloned, IL-3–dependent, nontumorigenic mast cell line from murine bone marrow (24). The V2 line was obtained by ZIP-*ras-neo* infection of PB-3c. The IL-3–producing, autocrine tumor lines V2D1 and V4D6 have been described (17). 8V4-T12, 15V4-5T2, 22V4-T1, and R56VT are tumor lines derived from inoculation with the ZIP-*ras-neo*–infected PB-3c subclones 8V4, 15V4, 22V4 (23), and R56V (Banholzer, R., unpublished data). PB-3c H is a hygromycin B–resistant derivative of PB-3c selected after infection with the ZH2 retrovirus (Muser, J., unpublished data). All cell lines were cultured in IMDM containing 10% (vol/vol) of FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 50 μg 2-ME. Saturating amounts of conditioned medium from the IL-3–producing X63-mIL-3 line (25) were added to IMDM for growth of the IL-3–dependent cell lines. Hybrid lines were generated by polyethylene glycol fusion and hygromycin/G418 selection in the presence of IL-3 as described (16). The DNA content of the hybrid cells was compared with the parental cells by FACS® analysis (Becton Dickinson & Co., Mountain View, CA) using ethidium bromide or propidium iodide staining and found to contain the double DNA content (results not shown).

Northern Blot Analysis. 5×10^7 cells were harvested and total RNA was extracted (26). From 400 μg of total RNA, a mRNA-enriched preparation was obtained by single passage through oligo-dT cellulose and precipitated, followed by electrophoresis in 1.1% (g/vol) agarose 0.66 M formaldehyde gel in MOPS buffer pH 5.9 (27), blotting, and hybridization as described previously (23). The IL-3 probe was generated by in vitro transcription (Boehringer Mannheim, Rotkreuz, Switzerland) from a pSP6-vector containing a 398-bp IL-3 cDNA fragment (28). IL-6 cDNA probe, generously provided by Dr. J. vanSnick (Ludwig-Institute for Cancer, Brussels, Belgium) and the chicken β-actin probe (0.57-kb PstI fragment) were labeled using a random-priming kit (Boehringer Mannheim). Quantitation of IL-3 mRNA levels, and normalization to actin levels were done using a phosphoimager (Molecular Dynamics, Sunnyvale, CA).

Southern Blot Analysis. Preparation and overnight digestion of total genomic DNA, electrophoresis separation, transfer onto nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH), hybridization to the 1.9-kb HindIII fragment of the genomic IL-3 gene subcloned from the 8.6-kb EcoRI fragment (29) and labeled by random priming, was done according to standard procedures.

Nuclear Run-on Analysis. Nuclei were isolated from 10^7 of the indicated cells and stored at −70°C, transcription reactions at 26°C, isolation of ^{32}P-labeled RNA (30), hybridization to the indicated denatured DNA probes in 50% formamide 5× SSC 0.1% SDS at 50°C, and washing were done as described in detail elsewhere (22).

Inverse PCR and DNA Sequencing. Two independent genomic DNA preparations (5 μg) of the V4D6 tumor line were digested overnight with HindIII. After phenol extraction and precipitation, a ligation reaction at a DNA concentration of 10 ng/ml was set up overnight at room temperature using T4-DNA ligase. After inactivation at 65°C for 10 min, the sample was digested with ApaI for 5 h before heat inactivation at 65°C. Using 50 ng of DNA, a PCR reaction was performed in 100 μl overlayed with paraffin, using 20 pmoles of the primers M422 5′-CCTCCATAGCAAACC-ACAAAC-3′ and M423 5′-ACCACCAGCATCCACACCATG-3′, and 2.5 U of AmpliTaq (Perkin-Elmer Cetus, Norwalk, CT) in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM dNTP, for 35 cycles at 95°C for 1 min, 60°C for 2 min, and 72°C for 3 min. After phenol extraction and precipitation, two major bands of 1.9 and 0.5 kb were separated on a 0.7% low-melting agarose electrophoresis gel. The 0.5-kb band was excised and ligated blunt-ended into the pSK+Bluescript vector cut with SmaI. Two independent inserts corresponding to each DNA preparaton were sequenced in both directions using the Sequenase® kit (United States Biochemical, Cleveland, OH).

Cell Proliferation and Viability. Proliferation and viability of cells in the presence and absence of IL-3 was monitored by the MTT method (31), by [^{3}H]thymidine incorporation, or by trypan blue color exclusion, where indicated.

Tumor Formation. To analyze tumor formation and latency, cells were washed twice in ice-cold PBS and 10^5 or 10^6 cells/animal were injected subcutaneously into 8–12 wk-old female syngeneic DBA/2 mice. Mice were examined twice per week for tumor formation up to 42 wk.

Results

IL-3 Gene Rearrangement Distinguishes Two Tumor Classes with Autocrine IL-3 Loop. Upon progression to tumor cells in syngeneic animals, v-H-*ras*–expressing PB-3c cells change from an IL-3–dependent to an IL-3–producing autocrine state. In this ongoing study in our laboratory, the IL-3 gene locus was analyzed in over 40 independently formed tumors by Southern blot to detect potential alterations. The majority, however, showed a germline configuration like the precursor cell PB-3c. So far, only four tumor lines showed a detectable alteration of one IL-3 allele. As shown here by hybridization with a probe containing the IL-3 promoter and part of the first exon, the unaltered IL-3 gene is detected in PB-3c or a ZIP-*ras-neo*–infected subclone R56V as a single band of 1.9 kb after digestion with HindIII (Fig. 1, lanes *1* and *3*) or of 6.6 kb with SphI, respectively (Fig. 1, lanes *2* and *4*). Three tumor lines without detectable alterations such as V2D1, 15V-T2, and 8V4-T12 were termed class I (Fig. 1, lanes *5–10*), whereas tumor lines with one rearranged allele, apparent as two bands in the SphI digestion, such as tumor lines V4D6, R56VT, and the 22V4-T1, were termed class II tumors (Fig. 1, lanes *12*, *14*, and *16*). The weaker intensity of the rearranged allele of 12 kb in 22V4-T1 is compatible with one altered and three nonrearranged alleles, as chromosome typing has indicated a near tetraploidy for the IL-3–dependent precursor clone 22 (data not shown). With respect to the occurrence of the rearrangement, it is interesting to note that R56VT and 22V4-T1 are the only two tumors obtained from infected PB-3c subclones R56V and 22V4 in 25 inoculation experiments with 5×10^6 or 10^7 cells injected. The injected subclones express v-H-*ras* because of infection with the ZIP-

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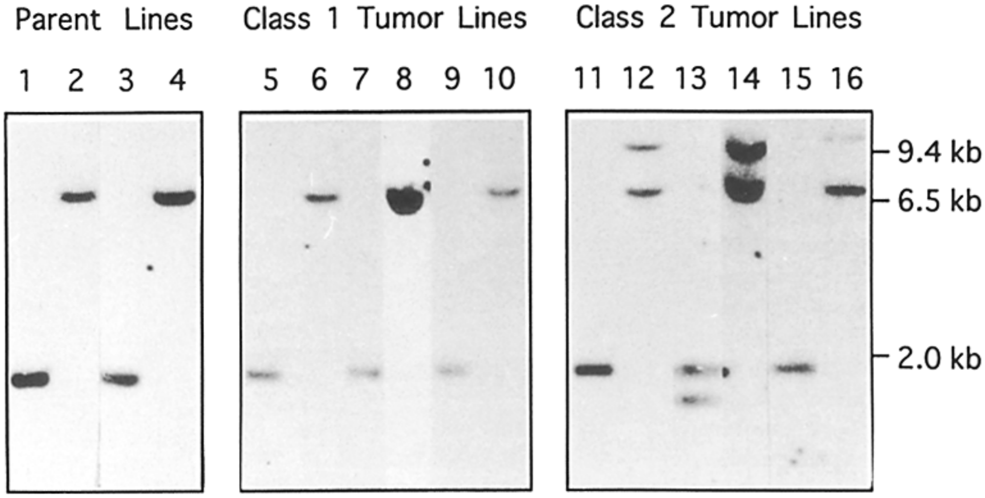

Figure 1. Southern blot analysis of the IL-3 gene. DNA was prepared from the indicated cells, digested with HindIII (odd numbers) or SphI (even numbers) and hybridized to the genomic 1.9-kb HindIII fragment of IL-3 encompassing the promoter region up to the exon 1. Lanes (*1* and *2*) PB-3c; (*3* and *4*) R56V, a v-H-*ras* expressing subclone of PB-3c; (*5* and *6*) V2D1; (*7* and *8*) 15V-T2; (*9* and *10*) 8V4-T12; (*11* and *12*) R56VT; (*13* and *14*) V4D6; (*15* and *16*) 22V4-T1.

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                   1                                                                                                            100
      <-(-252)IL-3 U3-region->                                          GRE        Enhancer1  Z-DNA                               *
V4D6     gtgggaggaagTGTTGGGAGC CGCCCCCACA TTCACCGTTA CAAGATGGCG CTGACATCCT GTGTTCTAAG TGGTAAACAA ATAATCTGCG CATGTGCCAA GGGTATCTTA
WEHI3B             ---------- ---C------ ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
ANGI               ---------- ---G------ ---------- ---------- ------.G-- ---------- ---------- ---------- ---------- ----------
MOS                ---------- ---G------ -------G-- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                   101  *                CRE                              CAAT                             TATA           200
V4D6               TGACTACTTG TGCTCTGCCT TCCCCGTGAC GTCAACTCGG CCGATGGGCT GCAGCCAATC AGGGAGTGAC ACGTCCGAGG CGAAGGAGAA TGCTCCTTAA
WEHI3B             ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
ANGI               ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
MOS                ---------- ---------- ---------- -------..- G-CGAT---- ---------- ---------- ---------- ---------- ----------

                   201                 R-region->                                                                     polyA  304
V4D6               GAGGGACGGG G.TTTCGTTT TCTCTCTCTC TTGCTTCTTG CTCTCTTGCT TCTTGCTCTC TTGCTTCCTG CACCCTGGCT CCTGAAGATG TAAGAAATAA AGCTT
WEHI3B             ---------- -T-------- ---------- ---------- -------... .......... .......... .......... .......... .......... .....
ANGI               ---------- -.-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----
MOS                ---------- -.-------- ----G----- ---------- -------... .......... ..-------C G--------- ---------- ---------- -----
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Figure 2. Nucleotide sequence of downstream insertion junction in class II tumor V4D6. The IL-3 promoter fragment up to the novel HindIII site in V4D6 was cloned by inverse PCR (see Materials and Methods). The sequence revealed the presence of a 5'LTR of an IAP inserted at position (*−252*) of the IL-3 transcription start. The U3 and R-region boundaries are indicated. Typical regulatory sequence motifs found are the glucocorticoid response element (*GRE*), cAMP response element (*CRE*), the CAAT and TATA box in the U3-region, and three repeats of the duodecamer CTCTCTTGCTTC sequence and the poly(A)+ site in the R-region as indicated. (*) Two positions in the enhancer 2 of relevance for bidirectional transcription (49). A comparison for the LTR of the IAP in the IL-3/Hox2.4 gene of WEHI-3B (41, 50), for the angiotensinogen IAP (33), and the rc-*mos* IAP (51) are given. (- - -) Identical nucleotides; (· · ·) gaps.

ras-neo vector, but do not show the IL-3 insertion before the singular tumor formation (Fig. 1, compare lanes *4* and *12*). In contrast, typical class I tumors are generated from the *ras*-expressing PB-3c subclones 8V4 and 15V4 after a long latency of 9–14 wk, but with a high tumor incidence of 80–100% under the same conditions (17, 23).

Insertion of an Intracisternal A-particle (IAP)[1] *in the IL-3 Promoter of the Class II Tumor V4D6.* A more extensive analysis of the rearrangement produced a restriction pattern compatible with an IAP (32), an endogenous retroviral element, that had inserted into the IL-3 locus (data not shown). The double band detected in the class II tumor line V4D6 after HindIII digestion indicated that this insertion was in proximity of the IL-3 promoter (Fig. 1, lane *13*). Cloning by inverse PCR after HindIII digestion and circularization (see Materials and Methods) allowed the identification of the 5'LTR sequence of an IAP that had inserted at position (−252) of the IL-3 transcription start site (Fig. 2). The orientation was head-to-head with respect to the transcriptional orientation of the IL-3 gene. Analysis of the nucleotide sequence showed the presence of the typical IAP-LTR sequence motifs in the U3-region (see legend to Fig. 2). Comparison with other IAP-LTRs revealed that the sequence of the U3- and R-region was almost identical to an IAP-LTR detected previously in the angiotensinogen locus (33). Interestingly, the 5'LTR of the V4D6-IAP was found to be inserted only 9 bp upstream of the previously described IAP–IL-3 insertion site detected in WEHI-3B (34), an IL-3–producing myelomonocytic leukemia line, but differed in stretches of the R-region (Fig. 2). From the SphI digestion (Fig. 1), and other restriction digestions (data not shown), the inserted element was estimated to be about 5 kb in size.

[1] *Abbreviation used in this paper:* IAP, intracisternal A-particle.

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Enhanced IL-3 Gene Transcription in Class II Tumors. Assuming an IAP enhancer insertion mechanism, we searched for functional alteration at the transcriptional level. The rates of IL-3 gene transcription of class II tumor lines V4D6 and R56VT, class I tumors, and the IL-3–dependent precursor lines were compared in a nuclear run-on assay. Relative to the actin signal as activity control, the signals for IL-3 transcription were indeed increased in the class II tumors V4D6 and R56VT over the levels detected in the nonrearranged precursors PB-3c and R56 (Fig. 3, *A* and *B*) or the v-H-*ras*–expressing lines V2 or R56V (Fig. 3, compare *G* and *H* with *C* and *D*). Furthermore, the rate of IL-3 gene transcription in class II tumors appeared to be higher than in the class I tumor lines V2D1 and 15V-T2 (Fig. 3, *E* and *F*; see also Fig. 6, *A* and *C*). As expected, expression of v-H-*ras* from the ZIP-*ras-neo* vector leads to a dramatic increase in transcription signal for *ras* (Fig. 3, *A* and *B* vs *C* and *D*). Potential effects of the v-H-*ras* expression on IL-3 gene transcription are currently under investigation. Transcription of IL-6 was included as a control for a cytokine of different chromosomal location which is already expressed in PB-3c cells, but is irrelevant for proliferation (our own unpublished data; see also Figs. 4 and 7). Taken together, these results indicate that the DNA rearrangement in class II tumors is associated with an enhanced transcription of the IL-3 gene.

IL-3 Expression of Class II Tumors Is Dominant in Somatic Cell Hybrids with PB-3c. We have previously shown that the autocrine IL-3 expression of the class I tumor line V2D1 was recessive as it could be downregulated by somatic cell fusion with the nontumorigenic, IL-3–dependent parental line PB-3c (16). Since the p21 *ras* expression levels were not affected, this experiment suggested the loss of a negative regulator of IL-3 expression in autocrine tumor formation which could be supplied in *trans* by fusion with the untransformed PB-3c. It was therefore of interest to compare the effect of somatic cell fusion on the IL-3 expression of class II tumors. Class I and II cell hybrids were generated between the IL-3–independent G418-resistant tumor cells of V2D1 or V4D6 and a hygromycin-resistant subline of PB-3c, respectively. After selection with G418 and hygromycin in the presence of IL-3, resistant hybrids, termed PB3c/V2D1 and PB3c/V4D6 were analyzed for IL-3 expression by Northern blotting of poly(A)$^+$ enriched RNA. The results in Fig. 4 show that IL-3 expression of the class I tumor line V2D1 is reduced to trace amounts in the hybrid PB3c/V2D1 (Fig. 4, lanes *3* and *4*), whereas IL-3 expression in the class II hybrid is only slightly reduced. Quantitation of β-actin normalized IL-3 expression by storage phosphor technique indicated an approximately fourfold reduction in class II hybrid PB3c/V4D6, whereas the signal in the class I hybrid PB3c/V2D1 was nearly 20-fold reduced. In contrast, cell fusion had no significant effect on β-actin or IL-6 expression. The nontumorigenic PB-3c do not express IL-3 mRNA, as indicated by Northern blotting (Fig. 4, lane *1*), and reverse PCR (data

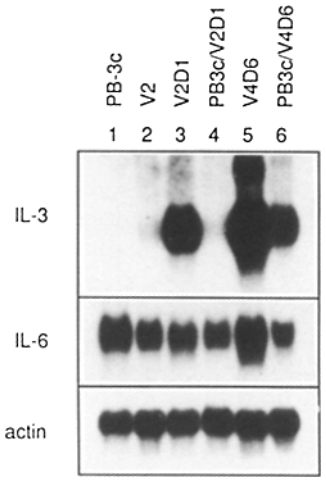

Figure 4. IL-3 mRNA expression in somatic cell hybrids. Poly(A)$^+$ enriched RNA from 400 μg of total RNA were extracted from the indicated cell lines, analyzed by Northern blotting, and hybridized to IL-3 cDNA, IL-6 cDNA, and β-actin (Materials and Methods).

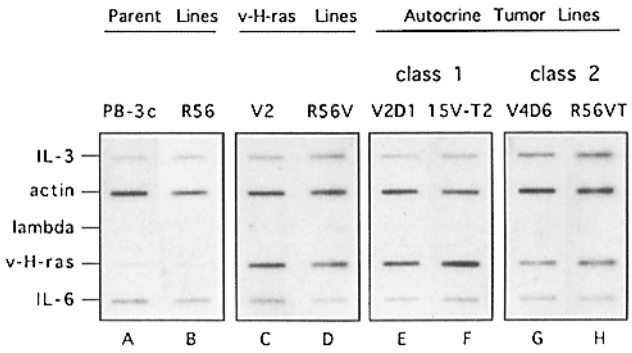

Figure 3. Nuclear run-on analysis. The indicated cDNA probes, and lambda DNA as unspecific control, were spotted on nitrocellulose filters and hybridized to labeled nuclear RNA isolated from 10^7 nuclei of the indicated cell lines, as described. PB-3c (*A*); R56, a subclone of PB-3c (*B*); V2, ZIP-*ras-neo*–infected PB-3c (*C*); R56V, ZIP-*ras-neo*–infected R56 (*D*); V2D1, class I tumor derived from V2 (*E*); 15V-T2, class I tumor derived from 15V4 (*F*); V4D6, a class II tumor derived from ZIP-*ras-neo* infected PB-3c (*G*); and R56VT, a class II tumor derived from R56V (*H*).

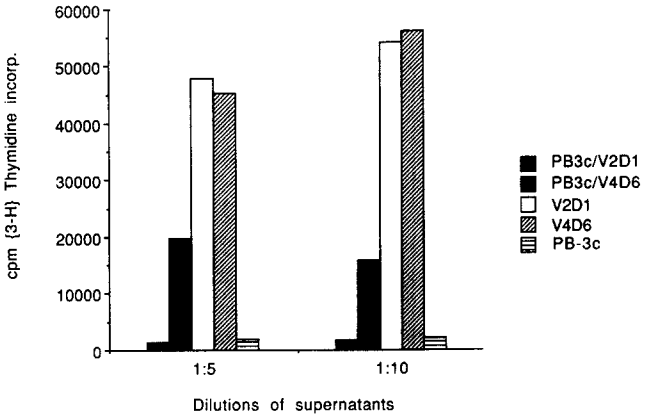

Figure 5. Mitogenic activity in supernatants of tumor and cell hybrids. Cells were washed three times and resuspended in IL-3–free IMDM/10% FCS at 10^5 cell/ml and incubated. The supernatants were taken after 3 d, sterile-filtered (0.2 μm diameter pore size), and tested in two dilutions for mitogenic activity on an IL-3–dependent subclone of PB-3c.

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not shown). A low level of IL-3 expression appears to follow the expression of v-H-*ras* in the still IL-3–dependent V2 line (Fig. 4, lane *2*), as described previously (23). Mitogenic activity in supernatants of the tumors and respective hybrids were tested in 1:5 and 1:10 dilution on an IL-3–responsive subline of PB-3c. The results agree with the pattern of IL-3 mRNA expression (Fig. 5) showing a reduction in the class II hybrid in comparison with the respective tumor, whereas the class I hybrid was at the background level of PB-3c. To examine the effect of somatic cell fusion on IL-3 gene transcription, in particular whether a transcriptional turnoff was responsible for the observed downregulation of IL-3 expression in class I hybrids, both classes of tumors and the respective hybrid lines were compared in a nuclear run-on experiment (Fig. 6). The rate of IL-3 gene transcription was examined using an IL-3 cDNA fragment and a nonoverlapping genomic fragment containing the transcription start up to the HindIII site in exon 1 of IL-3. Given the comparable levels of the β-actin signal, almost similar rates of transcription were detected in V2D1 and its respective hybrid PB3c/V2D1. The autoradiograms were analyzed by densitometric scanning and the rates normalized to actin are given for comparison (Fig. 6). Taking the transcription rate detected by the IL-3 cDNA in the class I tumor V2D1 as 1.0, the difference to the corresponding signal of 1.2 in the class I hybrid was considered to be insignificant. This suggested that the dramatic downregulation of IL-3 mRNA expression in class I hybrid PB3c/V2D1 (Fig. 4) was not due to a transcriptional turnoff (Fig. 6, *A* and *B*), but appears to involve negative posttranscriptional regulation. In the class II hybrid PB3c/V4D6, however, a 35% reduction of the transcription rate compared with the class II tumor was observed (Fig. 6, *C* and *D*). The rate of IL-3 gene transcription in the class II tumor was about fourfold higher than in the class I tumor. The 5′-IL-3 probe showed comparable results, yet at a two- to threefold higher level. The reason for this is unclear, but might involve a polarity effect of RNA-polymerase II transcription in the in vitro reaction.

Class II Hybrids Proliferate without Exogenous IL-3 and Form Tumors Rapidly. The pattern of IL-3 mRNA expression was also reflected in cell proliferation in vitro and tumorigenicity in syngeneic animals. The class I tumor V2D1 (Fig. 7, open triangles), the class II tumor V4D6 (Fig. 7, closed triangles), and the class II hybrid PB3c/V4D6 (Fig. 7, closed diamonds) grew in the absence of exogenous IL-3 in vitro and formed tumors in the animal within 4–6 wk (Fig. 7 and Table 1). In contrast, the class I hybrid PB-3c/V2D1 (Fig. 7, open diamonds) required exogenous IL-3 for growth and showed partial or total tumor suppression in the animal, depending on whether 10^5 or 10^6 cells were inoculated (Table 1). PB-3c cells infected with a retrovirus conferring hygromycin resistance required exogenous IL-3 (Fig. 7, pentangles) and were nontumorigenic (Table 1). These results suggest that, in contrast to the recessive class I tumor cells, the IL-3 rearrangement detected in class II tumor cells is associated with a dominant tumor phenotype that could not be reverted by cell fusion. In support of this hypothesis, cell fusion of other class I tumor lines 15V-T2 and 8V4-T12 to PB-3c resulted in IL-3 dependence of cell viability, whereas proliferation and viability of class II tumor lines R56VT and 22V4-T1 and their respective hybrids proved to be IL-3 independent (Table 2). Mitogenic activity could not always be detected in the supernatants of all IL-3–independent cell lines correlating with the fact that the levels of IL-3 mRNA expression were low in these tumor lines (Table 2, and data not shown).

Discussion

Somatic cell fusion as a well-established tool in cancer research has helped to classify tumorigenic alterations into dom-

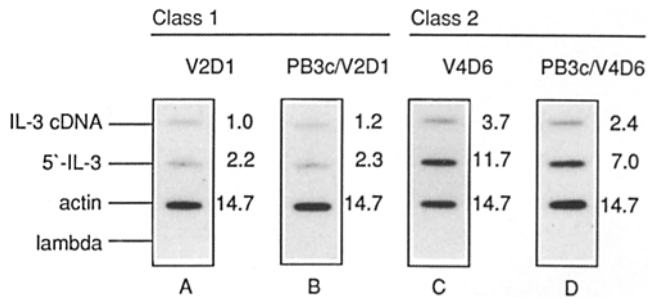

Figure 6. Comparison of IL-3 transcription in tumors and cell hybrids by nuclear run-on assay. The assay was carried out as described (in Materials and Methods). To detect the IL-3 transcription rate, an IL-3 cDNA fragment and the 5′-IL-3 probe, a nonoverlapping genomic IL-3 fragment containing the transcription start and part of exon 1, were compared relative to the β-actin cDNA signal, whereas lambda DNA served as control for unspecific DNA binding. For comparison, the relative transcriptional activity is given after the intensities were quantitated by densitometric scanning, and normalized to the areas of the corresponding actin signal. The activity of the IL-3 cDNA transcription in the class I cell line V2D1 was taken as 1.0.

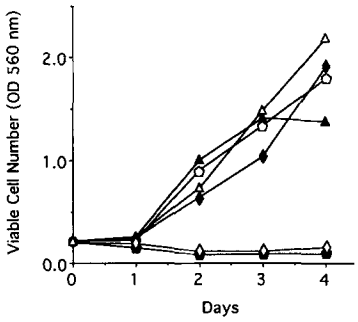

Figure 7. IL-3–independent proliferation of tumor and cell hybrids. The increase in the number of viable cells in the absence of exogenous IL-3 were compared in an MTT assay (31) (see Materials and Methods). The indicated cells were washed three times and resuspended in IL-3–free IMDM/10% FCS at 2 × 10^4 cells/ml as triplicates in microtiter plates. At the indicated days, the MTT assay was performed. Class I tumor V2D1 (*open triangles*); class I hybrid PB3c/V4D6 (*open diamonds*); class II tumor V4D6 (*closed triangles*); class II hybrid PB3c/V4D6 (*closed diamonds*); and PB-3c as control with IL-3 (*open penungle*), or without IL-3 (*closed pentangle*).

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Table 1. *Tumor Formation and Latency of Class I and II Somatic Cell Hybrids*

Cell line	Cells injected	2	4	6	8	10	12	17	26	42
						wk				
PB-3cH	10^5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	10^6	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
V2D1	10^5	0/5	2/5	5/5						
	10^6	0/5	3/5	5/5						
PB3c/V2D1	10^5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	10^6	0/5	0/5	0/5	0/5	2/5	3/5	5/5		
V4D6	10^5	0/5	5/5							
	10^6	0/5	5/5							
PB3c/V4D6	10^5	0/5	0/5	5/5						
	10^6	0/4	3/4	4/4						

Results given as number of tumors formed/number of animals injected.

inant and recessive lesions (11). Using this tool, we provide evidence that v-H-*ras*–mediated tumor formation of IL-3–dependent PB-3c cells can occur by two different mechanisms, both of which allow the establishment of an autocrine IL-3 loop. Class I tumors arise frequently, and show no detectable alteration in the vicinity of the IL-3 locus (Fig. 1). Although we cannot exclude a more distant chromosomal alteration in class I tumors, the cell fusion experiments with the non-

Table 2. *Effect of Somatic Cell Fusion on IL-3 Expression and Viability*

Cell line		Viability w/o IL-3	IL-3 mRNA	IL-3 secretion	IL-3 gene insertion
		%			
Parental	PB3c	0*	–‡	–§	–‖
	V2	1	(+)	–	–
	R56V	0	–	–	–
Class I	V2D1	92	+ + +	+ + +	–
	PB3c/V2D1	0	–	–	–
	15V-T2	80	+	–	–
	PB3c/15VT2	5	ND	–	ND
	8V4-T12	93	+	–	–
	PB3c/8V4T12	1	ND	–	ND
Class II	V4D6	94	+ + +	+ + +	+
	PB3c/V4D6	88	+ +	+ +	+
	R56VT	93	+ +	+	+
	PB3c/R56VT	81	ND	+	ND
	22V4-T1	92	(+)	–	+
	PB3c/22V4T1	93	ND	–	ND

* By trypan blue color exclusion after 18 h in IL-3 free IMDM/10% FCS.
‡ by (polyA)+ Northern blot or when indicated (+) by reverse PCR.
§ Mitogenic activity in supernatants.
‖ By Southern blot hybridization.

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tumorigenic parental PB-3c cells provide support for the existence of two different routes. Class I tumors appear to carry a recessive lesion that can be complemented by the precursor cell as the resulting cell hybrids showed loss of IL-3 expression, IL-3 dependence in vitro, and partial tumor suppression in vivo. Class II tumors, however, arise rarely, and show an about 5-kb insertion in the IL-3 gene (Figs. 3 and 4 and Tables 1 and 2). The insertion is linked to a dominant alteration, since IL-3 expression is maintained in the class II hybrids, and rapid tumor formation is observed upon injection into syngeneic mice (Tables 1 and 2). In the class II tumor V4D6, cloning and sequencing identified the insertion of an IAP, an endogenous retroviral element, at position −252 from the transcription start of IL-3 (Fig. 2). Preliminary restriction analysis indicates the presence of IAP insertions also in the other class II tumors (data not shown). Nuclear run-on data show that this insertion is associated with an increased rate of transcription as compared with class I tumor cells or the IL-3–dependent precursor cells (Figs. 3 and 6). In agreement with the enhancer-type orientation, the major form of the IL-3 mRNA expressed is of normal size. The larger IL-3 transcripts detected in the class II tumor V4D6 do not represent IAP–IL-3 chimera (Fig. 3, lane *5*), but normal unspliced IL-3 mRNA precursor (data not shown). Somatic cell fusion of class II tumors appeared to reduce the IL-3 transcription rate approximately twofold (Fig. 6) and the cytoplasmic IL-3 mRNA level by about fourfold (Fig. 4). The reason for this is unknown, but a certain reduction appears compatible with changing the ratio of rearranged to unaltered IL-3 alleles from 1:1 to 1:3 upon cell hybridization.

A functional equivalent to class II tumors has been described in a human B cell leukemia with t(5;14) translocation, which placed the IL-3 gene in the vicinity of the IgH enhancer (35). This translocation was associated with high serum levels of IL-3 which correlated with the number of leukemic cells in the course of the disease. In murine malignancies, the insertion of IAP was reported to be involved in the activation of the IL-3, IL-5, IL-6, GM-CSF, as well as the IL-2R and IL-6R receptor genes (34, 36–42). In some of these tumor lines, activating lesions in the *myb* or *hox2.4* genes are known to be present as well (40, 43). It has been observed that the overall transcriptional activity of IAPs is higher in tumor cells than in normal adult tissues (32). In fact, transactivation of IAP-LTR reporter constructs by the oncogene products such as E1A, SV40 large-T antigen, and *myc* has been described (44). Most notably, the v-K-*ras* oncogene product enhanced the transcription rate of 5′IAP-LTR reporter constructs via a cAMP-responsive element (45). This in vitro observation is of particular interest as it suggests a rationale for increasing IAP transposition events in *ras*-transformed PB-3c which become apparent as class II tumors in vivo. Stocking et al. (39) have observed that insertions into growth factor loci can occur spontaneously in vitro at a frequency of around two in 10^7 murine cells, which could be increased 20-fold by retroviral insertion mutagenesis. It is therefore of importance to note that without v-H-*ras*, PB-3c is not tumorigenic, even when infected with retroviral control vectors conferring neomycin or hygromycin resistance (Table 1; 16, 23). The clonal origin of the class II tumors R56VT and 22V4-T1 from subclones without IL-3 alteration (Fig. 1), and the rarity of these tumors suggest that IL-3 gene rearrangement provides an alternative route to tumor formation in vivo. It remains to be shown that the transcriptional activation is sufficient to explain the IL-3 autocrine loop in class II tumors, or that the v-H-*ras* oncogene is necessary as well by perturbing other regulatory functions.

90% of tumors obtained from v-H-*ras*–expressing PB-3c cells are of the class I type that produce IL-3 without detectable IL-3 gene rearrangement. Clonal precursor cells of class I tumors 8V4 and 15V4 repeatedly showed a high tumor incidence of 80–100% (17). Recent data suggest that v-H-*ras* synergizes efficiently with a premalignant lesion in these cells (23). Comparison of the IL-3 transcription rate in the *ras*-expressing class I precursor line V2 with the respective class I tumor line V2D1 (Fig. 3, *B* and *D*) indicates no increase upon tumor formation, yet, a striking difference in the amount of cytoplasmic IL-3 mRNA is observed (Fig. 4, lanes *2* and *3*). Cell fusion of the class I V2D1 with PB-3c, reduced the IL-3 mRNA expression ~20-fold to a level comparable with the precursor line V2 (Fig. 3, lane *4*), but the rate of IL-3 transcription was basically unaltered in the class I hybrid (Fig. 6). Taken together, these data suggest that class I tumor formation may arise from the loss of a negative regulator of IL-3 expression which is present in untransformed cells and which acts by a posttranscriptional mechanism. Since the tumor cells were hybridized to the parental cell line, effects of tissue-specific extinction do not apply. This is supported by the fact that IL-3 expression could still be induced in class I somatic cell hybrids by treatment with calcium ionophore (Hirsch, H. H., unpublished data) as demonstrated for PB-3c and primary mast cells (18, 21). As shown previously (16), the level of p21 *ras* expression in the cell hybrids was unaltered (data not shown). Work in our laboratory indicated mRNA stabilization as an important posttranscriptional control element for the physiological IL-3 expression in PB-3c mast cells which may be increased upon the expression of *ras* oncogene (22, 23). At this time, there is only one well-documented report that increased cytokine mRNA stability might be an oncogenic principle. Schuler and Cole (46) have described a monocytic tumor line induced by the v-*myc* oncogene in which the lymphokine GM-CSF had an unusually slow decay rate. The mechanism was active in *trans* and involved the 3′ untranslated region of GM-CSF containing the characteristic AUUUA motives also found in IL-3 (29, 47) which are involved in the rapid mRNA decay of cytokines and proto-oncogenes (48). With the precursor cells at hand, the molecular elucidation of the tumorigenic lesion in PB-3c class I tumors should be possible and provide new insights into the posttranscriptional regulation of IL-3 expression. Whereas v-H-*ras* expression appears to be necessary for the progression to either class of autocrine tumors, the role of the oncogene in maintenance of the tumor phenotype is still open.

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