Clonal Diversity of the Kirsten-ras Oncogene during Tumor Progression in Athymic Nude Mice: Mechanisms of Amplification and Rearrangement

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ABSTRACT

Single-cell clones from primary and lung metastatic tumors have been evaluated for the state of the viral-Kirsten-ras oncogene (v-Ki-ras) by Southern blot analysis after injection of Kirsten sarcoma virus-transformed BALB/c 3T3 cells (KiMSV, with a replication-defective provirus) into athymic nude mice by four different injection routes. While all clones of early-passage KiMSV cells contained an EcoRI-generated 5.3-kilobase DNA fragment at high dosage level, most clones of late-passage cells had lost this v-Ki-ras fragment or had greatly diminished levels. However, all clones of all tumors (>90 tested) obtained after injection of these late-passage cells contained a dosage of the 5.3-kilobase v-Ki-ras band similar to that of the early-passage KiMSV cells, suggesting either a very strong selection for v-Ki-ras-bearing cells of the early-passage type in tumor formation and/or the ability of a subset of late-passage cells to amplify this gene to some minimal dosage level. Both flow cytometric analyses for DNA content and quantitation of chromosomes showed that all primary and lung metastatic tumors had more than twice the number of chromosomes as the late-passage KiMSV cells; however, four of 80 late-passage cells had a chromosome count in the range of tumors, consistent with their importance in tumor generation and possibly amplification of the v-Ki-ras-bearing chromosome. Clonal analyses of lung micrometastases (CANCER RESEARCH 48, 4941-4953, September 1, 1988) could induce some metastatic competence in recipient cells by itself in the nude mice, that led to rescue/reinfection of tumor cells by a basal level of ras, showing a second mechanism of activation by increasing the level of the ras proto-oncogene's product, p21, resulting in tumorigenic conversion of NIH 3T3 cells. Transfection experiments using DNA from naturally occurring tumors have revealed "activated" c-Ki-ras in numerous carcinomas, sarcomas, and solid tumors (17); "activated" c-Ha-ras has also been found in T24 bladder carcinoma cells (18). Isolated ras oncogenes were found to induce transformation and tumorigenicity in established cell lines but in primary cells a second oncogene was required (19). These data indicate that the tumorigenic phenotype can be transferred to cells using specific DNA sequences such as ras.

INTRODUCTION

Tumor progression may require activation, mutation, or loss of one or more classes of genes (1, 2). At least three categories of genes are postulated to play roles in neoplastic processes—oncogenes (3, 4), tumor suppressor (5), and modulator genes (6). Oncogenes act dominantly and transform cells in culture. One oncogene family, ras, is involved in transformation, tumorigenicity, and malignancy in certain cell types, including the BALB/c 3T3 cells used here. At least four types of ras genes exist in mammals. v-Ha-ras and v-Ki-ras were first discovered in the replication-defective genomes of the Harvey and Kirsten murine sarcoma viruses (7, 8), with cellular analogs discovered in uninfected mammalian cells (9). N-ras was found in human tumor DNA as a participant in transformation (4, 10); however, N-ras is not associated with a known retrovirus. The fourth, R-ras, was isolated from mammals by its homology to c-Ha-ras DNA sequences (11). Cellular and viral analogs of ras genes encode a similar M, 21,000 protein (p21), identified as an inner surface membrane protein that binds guanine nucleotides and that has GTPase activity (9).

Activation of ras genes to ones with transforming and tumor-inducing potential occurs by either qualitative or quantitative mechanisms. Point mutations resulting in amino acid substitutions at positions 12 or 61 in the p21 protein have been observed (12). This mechanism was confirmed by site-directed and random mutagenesis studies (13, 14). In addition, transgenic mice developed tumors in the pancreas when "activated" ras genes, not normal proto-oncogenes, were under the control of the rat I elastase regulatory element (15). Chang et al. (16) showed a second mechanism of activation by increasing the level of the ras proto-oncogene's product, p21, resulting in tumorigenic conversion of NIH 3T3 cells. Transfection experiments using DNA from naturally occurring tumors have revealed "activated" c-Ki-ras in numerous carcinomas, sarcomas, and solid tumors (17); "activated" c-Ha-ras has also been found in T24 bladder carcinoma cells (18). Isolated ras oncogenes were found to induce transformation and tumorigenicity in established cell lines but in primary cells a second oncogene was required (19). These data indicate that the tumorigenic phenotype can be transferred to cells using specific DNA sequences such as ras.

Studies have also suggested that the metastatic phenotype can be transferred to nonneoplastic cells through insertion of ras oncogenes (20-27), kinase-encoding oncogenes (28), or tumor DNA (29). There was no clear correlation between ras gene expression and metastatic competence when metastatic lesions were compared to primary tumor (30) or to the originally injected cells (23, 31). Therefore, a basal level of ras expression may be necessary to induce the metastatic phenotype but is certainly not sufficient. Some caveats should be noted. "Preneoplastic" cell lines such as NIH 3T3 became tumorigenic and neoplastic 20 weeks after injection without insertion of a ras oncogene (21). Kerbel et al. (32) observed that transfection of a plasmid containing the neomycin-selectable gene (without ras) could induce some metastatic competence in recipient cells and calcium phosphate treatment of cells, for transfection procedures, has weak transforming ability. Nevertheless, the ras proto-oncogene may be necessary for the induction of some metastatic properties in recipient cells.
gene is a strong signal for affecting metastatic capability. These results also indicate that ras is needed for the induction and/or establishment of the metastatic phenotype but may not be needed for independent growth at the secondary site (23).

In light of these studies, our laboratory showed that injection of KiMSV-transformed BALB/c 3T3 cells into athymic nude mice by four anatomical routes resulted in rapidly growing primary tumors that metastasized to lungs; in most cases, micrometastases were observed while in several cases overt lung nodules were observed (33). Southern and mRNA blot analyses of the cellular and viral Kirsten-ras genes (c-Ki-ras and v-Ki-ras, respectively) in tissue culture-adapted primary and lung metastatic tumor cells showed v-Ki-ras gene amplification (4- to 8-fold) and increased mRNA expression (4- to 60-fold) when compared with the originally injected KiMSV cells. Amplification of v-Ki-ras and increased mRNA expression were comparable in many of the primary and secondary tumor cell lines. However, in two of five lung tumors from the i.v. and footpad injection routes, Ki-ras rearrangements (extra Ki-ras hybridizable bands) were observed in addition to the v-Ki-ras at its original integration site. Injection of footpad lung tumor cells with Ki-ras rearrangements into a second group of animals led to multiple and rapidly growing lung metastases with even more Ki-ras rearrangements. In contrast, injection of an i.v. lung micrometastatic tumor cell with Ki-ras rearrangements led to no further rearrangements in the lung micrometastases subsequently isolated. These results were consistent with, but did not prove, the importance of amplification and elevated expression of v-Ki-ras in tumor progression and correlation of the Ki-ras rearrangements with formation of rapidly growing metastatic lung tumors in this system (33).

The goal in this study was to analyze the state of the c-Ki-ras and v-Ki-ras genes in multiple single-cell clones of these tumor populations to gain insight into the mechanisms for v-Ki-ras amplification and the Ki-ras rearrangements. Tumor populations were dilution-cloned immediately after culture outgrowth and single cell clones analyzed by Southern analyses using several specific probes. Chromosome quantitation and DNA content analyses were performed on these tumor cell populations and on the originally injected KiMSV cell population. Mechanisms for both v-Ki-ras amplification and for Ki-ras rearrangements in overt-growing metastatic lung tumors are suggested from these studies.

MATERIALS AND METHODS

Cell Lines. BALB/c 3T3 (clone A31), Kirsten murine sarcoma virus-transformed BALB/c 3T3 (nonproducer clone k-234, referred to as KiMSV) (34-36), and C3H10T1/2 cells (37) were grown Mycoplasma-free in DMEM with 250 units/ml penicillin, 250 μg/ml streptomycin sulfate, and 10% fetal bovine serum (33). Primary tumor and lung metastatic cell lines were established as described (33). Briefly, 10⁶ KiMSV cells were inoculated s.c., i.v., i.m., or into the hind footpad of BALB/c (nu/nu) athymic mice (for description of nude mouse colony, see Reference 38). Lethargic animals were sacrificed and at autopsy the major organs examined grossly and histologically for visually overt or several specific probes. Chromosome quantitation and DNA content analyses were performed on these tumor cell populations and on the originally injected KiMSV cell population. Mechanisms for both v-Ki-ras amplification and for Ki-ras rearrangements in overt-growing metastatic lung tumors are suggested from these studies.

Viral RNA Dot Blot Assay. Tissue culture media (20 ml) was harvested at two consecutive 24-h intervals from subconfluent cells in 175-cm² flasks. Cell debris was removed by centrifugation (10,000 x g for 0.25 h) and virus particles pelleted by ultracentrifugation through a 1-m1 sucrose cushion [20% (w/v), 20 mm Tris (pH 7.4), 0.1 μg/ml NaCl] at 25,000 x g for 2 h at 4°C. RNA was extracted from the pellet in buffer containing 5% (w/v) NaDodSO₄, 0.2 mg/ml proteinase K, and 10 μg/ml tRNA added as carrier. After 30 min at 50°C, the homogenate was extracted with phenol/chloroform and RNA precipitated with ethanol. RNA was resuspended in H₂O/formaldehyde (3:1 v/v), heated 15 min at 65°C, and diluted 1:4 and 1:16 with 16x SSC [1x SSC = 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0)]. RNA was dot blotted onto GeneScreen and hybridized with the designated radioactively labeled DNA probes.

Cellular Transformation Assay. Using cellular focus formation to assay murine sarcoma virus for its ability to morphologically transform indicator cells (43), monolayers of either BALB/c 3T3 or C3H10T1/2 cells were incubated with filtered (0.45-μm pores) culture media harvested from subconfluent tumor cells (as described above) for 2 h. Medium was replaced with fresh medium every 3 days. At Day 14, these monolayers were photographed by phase contrast microscopy, fixed with 95% methanol, stained with 10% (v/v) Giemsa's stain: H₂O, and photographed a second time by bright field microscopy. Molecularly cloned MoMuLV was purified from the medium of NIH Swiss 3T3 cells (21-15/NIH) transfected with pBR322 containing the MoMuLV genome (44) obtained through the courtesy of Drs. W. D. Hanks and E. M. Scolnick of the NIH. The supernatant was found to contain approximately 1 x 10⁶ XC plaque-forming units/ml (45) and reverse transcriptase activity of 1.6 x 10⁶ counts per minute/ml (46). As a positive control, the Kirsten murine sarcoma virus was rescued from subconfluent KiMSV cells by adding MoMuLV (at dilu-
tions of 1:10 or 1:20 of stock titer) to the medium of a culture for 0.75 h; media was replaced with fresh medium and the cells grown to confluence. Supernatants from these cells contain both the MoMuLV and rescued KiMSV viruses as described below.

DNA Content Analysis. For DNA content, whole cells were fixed in phosphate-buffered saline/ethanol and stained in 100 μg/ml mithramycin in Tris buffer (pH 7.3), 20 mM MgCl₂ (47). Cells were analyzed on the flow cytometer at the Los Alamos National Laboratories (48). CHO cells were used as an internal control, directly relating DNA content to chromosome number. Single parameter data were displayed in 256 channels as a histogram. Mithramycin-stained cells were also examined by fluorescence microscopy for confirmation that a single cell suspension existed prior to flow analysis.

Chromosome Analysis. Chromosomes were prepared by standard procedures (38). Briefly, KiMSV and tumor cell lines were colcemid-blocked for 2 h, harvested by trypsinization, and pelleted by centrifugation. The pellet was resuspended in 1 ml of medium made hypotonic by adding 7 ml of 0.075 M KCl, cells pelleted again by centrifugation, and finally cells fixed twice in a freshly prepared 3:1 (v/v) methanol-acetic acid solution for 10 min. The final pellet was resuspended in fixative, spread on cold wet slides, and air dried. Slides were stained with 10% (v/v) Giemsa's stain:H₂O (pH 6.8) for 10 min and photomicrographs made of >72 metaphase spreads for each cell line for chromosome quantitation.

Materials. Cluster dishes and 100-mm Petri dishes were obtained from Costar, Cambridge, MA; tissue culture flasks from Becton Dickinson Labware, Oxnard, CA; DME and colcemid from GIBCO, Grand Island, NY; fetal bovine serum from Biologics, Inc., Napherville, IL; methylcellulose (400cps), methanol, chloroform, formaldehyde, and microscope slides from Fisher Scientific Co., Fairlawn, NJ; Diffco-Bacto agar from Difco Laboratories, Detroit, MI; protease K and restriction enzymes from Boehringer Mannheim Biochemicals, Indianapolis, IN; [α-³²P]deoxyribonucleotide triphosphates from Amer sham Corp., Arlington Heights, IL; EDTA, RNAase A, and trRNA from Sigma Chemical Co., St. Louis, MO; Pronase from Calbiochem, San Diego, CA; Genescreen from Du Pont-New England Nuclear, Boston, MA; agarose (electrophoresis grade) from Bethesda Research Laboratories, Gaithersburg, MD; nitrocellulose (0.45 μm) from Schleicher & Schuell, Inc., Keene, NH; technical pan film 2415 and Kodak diagnostic film were from Eastman Kodak Co., Rochester, NY; mithramycin was from Pfizer, Inc., Groton, CT.

RESULTS

Tumor Formation in Athymic Nude Mice. Injection of KiMSV-transformed BALB/c 3T3 cells into athymic nude mice produced rapidly growing tumors by the s.c., i.m., and footpad routes (33). These viral Kirsten ras (v-Ki-ras)-induced primary tumors were progressive and formed lung metastases 3 to 4 weeks postinjection. Histology of primary tumors revealed irregularly shaped cells indicative of fibrosarcomas (Fig. 1B) quite different from lung tissue (Fig. 1A). Examination of lung fields demonstrated either overt (pea size) or microfoci metastases and 3 to 4 weeks postinjection. Histology of primary tumors revealed irregularly shaped cells indicative of fibrosarcomas (Fig. 1B) quite different from lung tissue (Fig. 1A). Examination of lung fields demonstrated either overt (pea size) or microfoci metastases (Fig. 1C and D). Microfoci were confirmed as being tumor derived by adapting these lungs to in vitro cell culture with resultant tumor cell growth over 1 to 2 weeks (33; see below). Specific tumor cells were also injected into a second group of nude mice (round 11), and the resulting lung metastases isolated for in vitro growth (33). Histology of second round lungs revealed either overt or microfoci metastases (data not shown).

Amplification and Rearrangement of the Viral-Kirsten-ras Oncogene. It has previously been shown that the v-Ki-ras oncogene may be amplified in these tumors and Ki-ras gene sequences rearranged in select cases (33). Using DNA blot analysis, the untransformed BALB/c 3T3 cells contain only the endogenous c-Ki-ras proto-oncogene (11.5-, 8.5-, 1.5-, and 0.7-kilobase EcoRI restriction fragments in Fig. 2, lane A). In addition, the KiMSV parental cells used for injection (passage 22) contained a 5.3-kilobase EcoRI DNA restriction fragment indicative of the unique KiMSV (v-Ki-ras containing) provirus (Fig. 2, lane B) (33). All tumor cell populations (>30 examined) contained this 5.3-kilobase EcoRI restriction fragment providing direct evidence that tumors induced in vivo and adapted to in vitro growth were derived from the originally injected KiMSV population (Fig. 2, lanes F–K). Densitometric quantitation of a ratio of the intensities of the 5.3-kilobase v-Ki-ras and the 1.5-kilobase c-Ki-ras bands from primary or secondary tumor cells revealed a 4- to 8-fold amplification of v-Ki-ras when compared with the originally injected KiMSV cells (Fig. 2, compare lanes F–K with lane B) (33). Ki-ras rearrangements (additional Ki-ras hybridizable bands) were observed in lung metastatic cell populations from the i.v. and footpad injection routes (Fig. 2, arrows, lanes H and J, respectively), and upon a second round of tumor formation even further rearrangements were observed for lung metastatic tumor cells originally from the footpad injection route (Fig. 2, arrows, lane K) (33). Molecular bases for v-Ki-ras amplification and Ki-ras rearrangements were investigated further by analyzing many clones from these tumor populations for the state of the c-Ki-ras and v-Ki-ras genes.

DNA blot analysis compared the injected KiMSV cell population (passage 22) with the same cells at passage 6, soon after their original virus transformation and cloning. In high passage cells, a substantial decrease in the intensity of the 5.3-kilobase v-Ki-ras DNA fragment (using the 1.5-kilobase c-Ki-ras DNA fragment as an internal control for DNA concentration) was observed relative to the early-passage cells (Fig. 2, compare lanes B and D). This result indicates that during long-term in vitro growth dosage of the v-Ki-ras gene is reduced. Experiments have shown that when passage 22 KiMSV cells are injected into athymic nude mice, the intensity of the 5.3-kilobase v-Ki-ras DNA fragment in all primary tumors and lung metastatic tumors is comparable to passage 6 cells and not with the low level of intensity in passage 22 cells (33).

Further study of Ki-ras genes in KiMSV cells (passage 6 or 22) was undertaken by dilution cloning and subsequent Southern blot analyses. Ten single-cell clones at passage 6 yielded an identical DNA restriction enzyme fragment pattern and intensity of the 5.3-kilobase v-Ki-ras DNA fragment when compared to the total uncloned population (Fig. 2, compare lanes D and E); this intensity was comparable to the same band in the primary tumors and lung metastatic cells (>30 analyzed) (Fig. 2, compare lane E with lanes F–K). In contrast, most single-cell clones from passage 22 cells showed the absence (or severely reduced intensity) of the 5.3-kilobase v-Ki-ras fragment as compared with the total population or with passage 6 clones (Fig. 2, compare lanes C with B and E). Therefore, only a small subpopulation of passage 22 cells have retained the 5.3-kilobase-characterized v-Ki-ras copy found in all passage 6 clones, revealing the significance of v-Ki-ras dosage in the formation of tumors in this system. There are two possible origins of the 5.3-kilobase v-Ki-ras dosage in these tumors: (a) very strong selection for a minor subpopulation of passage 22 cells that have retained the level of v-Ki-ras observed in passage 6 cells; (b) alternatively, a subpopulation of cells upon in vitro pressure may be able to amplify the v-Ki-ras gene to some minimum level required for tumor progression.

Chromosome/DNA Content Analyses of Tumor Cell Populations. That the v-Ki-ras gene is "amplified" from 2- to 8-fold in both primary and lung metastatic tumor cells compared with uncloned passage 22 cells is consistent with a mechanism in which duplications of a specific chromosome containing the...
Fig. 1. Histological analysis of primary tumors and tumor-bearing lungs. KiMSV cells were inoculated into athymic nude mice by the indicated routes, and at 3 to 4 weeks postinoculation the animals were sacrificed for histology of primary tumor and lung tissue. Primary tumor and lung tissue were thin sectioned, hematoxylin and eosin stained, and histologically examined. A, normal mouse lung field (×175); B, KiMSV cell-induced primary tumor resulting from an s.c. route of injection (×115); C, KiMSV cell-induced lung metastasis resulting from an i.v. route of injection (×175); D, KiMSV cell-induced lung micrometastasis resulting from the s.c. route of injection (×175). Magnifications are given in parentheses.

Fig. 2. Southern blot analyses of tumor cell lines. Genomic DNA (25 μg per lane) was cut with restriction endonuclease EcoRI and probed with a 1-kilobase DNA fragment of pHiHi 3 (40). This probe hybridizes with both v-Ki-ras and c-Ki-ras sequences (33). A v-Ki-ras DNA fragment of 5.3-kilobases and c-Ki-ras-specific bands of 11.5, 8.5, 1.5, and 0.7 kilobases are expected when probed with pHiHi 3 (33, 40). DNA fragment sizes were estimated by using HindIII-digested λ DNA and HaeIII-digested φX174 phage DNA. Lanes: A, BALB/c 3T3 cells; B, originally injected KiMSV cells (passage 22); C, KiMSV passage 22 single cell clone (II-1); D, KiMSV cells (passage 6); E, KiMSV passage 6 single cell clone (B-3); F, Pr (primary) s.c.-1; G, Met (secondary) s.c.-1; H, Met i.v.-1; I, Pr Ftpd-1; J, Met Ftpd-1; K, Met Ftpd-1T. Arrows, additional rearranged Ki-ras bands in three cases. The size in kilobases of DNA fragments is shown at the right with c = cellular-Ki-ras and v = viral-Ki-ras.

KiMSV provirus, a chromosomal segment containing the provirus, or a large segment of DNA (>1,000 kilobases) containing v-Ki-ras is disproportionately replicated (i.e., more than once per mitotic event) (49). This possibility was investigated by quantitating chromosome number in metaphase cells from specific tumor populations. Tumor cells contained at least a two-fold increase in chromosome number compared to the originally injected KiMSV cells at passage 22 (Table 1 and Fig. 3). DNA content analysis by flow cytometry confirmed this and showed a twofold increase in modal chromosome number in tumor...
Table 1 Quantitation of chromosome number

<table>
<thead>
<tr>
<th>Tumor cell line</th>
<th>Flow cytometric DNA content analysis*</th>
<th>Metaphase spreads**</th>
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<tbody>
<tr>
<td></td>
<td>G1 mode</td>
<td>Ratio</td>
</tr>
<tr>
<td>KiMSV (parental)</td>
<td>100.13</td>
<td>1.33</td>
</tr>
<tr>
<td>CHO</td>
<td>75.27</td>
<td></td>
</tr>
<tr>
<td>Pr S.c.r-1</td>
<td>184.60</td>
<td>2.80</td>
</tr>
<tr>
<td>CHO</td>
<td>65.79</td>
<td></td>
</tr>
<tr>
<td>Met Ftpd-1T</td>
<td>217.66</td>
<td>2.76</td>
</tr>
<tr>
<td>CHO</td>
<td>78.84</td>
<td></td>
</tr>
<tr>
<td>Met Ftpd-1T → i.v.,-1BT</td>
<td>192.22</td>
<td>2.88</td>
</tr>
<tr>
<td>CHO</td>
<td>66.65</td>
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* Mithramycin-stained cells were analyzed for DNA content using flow cytometry (47, 48). The expected modal chromosome number was calculated from the DNA content data using CHO cells as a control for diploid mammalian DNA content. CHO cells contain 21 chromosomes per cell, nearly normal DNA content. The ratio was calculated by dividing the G1 mode of the test cells by the G1 mode of the CHO cells; this value was then multiplied by 40 (the normal murine chromosome value) resulting in the expected modal chromosome number.

** Cells were colcemid blocked in metaphase. Hypotonic cells were then fixed and spread onto glass coverslips (38). Chromosomes were quantitated from photomicrographs of individual metaphase cells. The average chromosome number and standard deviation are given. n — number of metaphase cells analyzed.

populations as compared to passage 22 KiMSV cells (Table 1). However, four of 80 metaphase cells analyzed from passage 22 KiMSV cells contained elevated chromosome numbers 80–107, comparable with the number observed in tumor cells (Fig. 3, compare A with B–D). A minor number of such cells in a population could easily be missed during Southern blot analysis of ten randomly chosen clones. Comparison among PrS.c.r-1, Met Ftpd-1T, and Met Ftpd-1T → i.v.,-1BT tumor populations showed similar chromosome numbers and DNA content (Table 1), consistent with the importance of elevated chromosome number for competence in tumor progression in this system. Chromosome histograms were also similar, except for metastatic populations containing Ki-ras rearrangements (Fig. 2, lanes J and K); at least two cells were present with three times the chromosome number of the originally injected KiMSV cells (Fig. 3, compare B with C and D). These results are consistent with a chromosome-based mechanism for two-fold v-Ki-ras amplification, involving in vivo selection for cells with increased chromosome number (see discussion); however, such a mechanism may not explain the >8-fold “amplification” unless the v-Ki-ras bearing chromosome or DNA segment were specifically amplified.

Clonal Analysis of Ki-ras in Tumor Populations. These experiments led to analyses of the relative homogeneity of the v-Ki-ras oncogene in the tumor populations from nude mice, using many single cell clones. Southern blot analysis is estimated to be sensitive enough to detect a unique clone at 5% of the total cell population during analysis of the DNA from a heterogeneous population (50). Therefore, a subpopulation of cells with a unique genotypic marker and comprising less than 5% of the total population may be masked. The Ki-ras restriction enzyme fragment pattern (including Ki-ras rearrangements) was used as a genotypic marker to test for microheterogeneity in the state of v-Ki-ras and/or c-Ki-ras in tumor populations when primary and metastatic lung tumors were dilution cloned. In addition, if host cell contamination exists in tumor populations, then clonal DNA blot analysis would identify a sizable level of contamination with host cells, since they would not contain a v-Ki-ras-specific band. Ninety clones (ten single-cell clones from nine different primary or secondary tumor populations, only three sets of which are shown in Fig. 4) showed no evidence of host cell contamination; all clones contained the 5.3-kilobase v-Ki-ras EcoRI generated DNA fragment (Fig. 4, A–C). DNA blot analysis using the restriction enzyme Xbal confirmed these results.

Analysis of single cell clones from primary and lung metastatic tumors may also uncover subsets of cells selected for during tumor progression. Ten clones from an s.c. primary tumor (PrS.c,r-1) showed a DNA blot pattern analogous to the whole population (data not shown). In addition, a lung metastatic population (Met S.c.r-1) derived from this primary s.c. tumor showed eight single-cell clones with the same DNA blot pattern and v-Ki-ras levels as the total population, or PrS.c,r-1 cells from which the tumor was derived (compare Fig. 4A clones with Fig. 2, lanes G and F, respectively). These results indicate
the stability of v-Ki-ras during tumor progression via the s.c. inoculation route.

Clonal analyses of two different lung metastatic tumors with Ki-ras rearrangements demonstrate different results. In the first case, eight clones from an i.v. experimental lung tumor (Met i.v.-1) containing a more complex DNA restriction enzyme fragment pattern (i.e., with extra 8.3-kilobase and 4.2-kilobase Ki-ras bands; Fig. 2, lane H) yielded the same Ki-ras pattern as the total population (compare Fig. 4B with Fig. 2, lane H). However, one of these clones contained even more Ki-ras rearrangements (Fig. 4B, cl 3). No clone was found without the Ki-ras rearrangements found in the original Met i.v.-1 population. Therefore, this lung tumor cell may be derived from a very minor cell type in the KiMSV cell population (passage 22) or genotypic changes are occurring during in vivo selection that confer an advantage to these cells during metastasis.

In the second case, Met Ftpd-1T tumor cells (a metastatic lung nodule isolated from the footpad route with multiple Ki-ras rearrangements (Fig. 2, lane J)) generated eight single-cell clones with multiple Ki-ras DNA fragment patterns (Fig. 4C). Two clones (Fig. 4C, cl 3 and cl 8) exhibited a pattern similar to the total Met Ftpd-1T cell population (Fig. 2, lane J) and one clone (Fig. 4C, cl 7) had a similar Ki-ras pattern as the originally injected KiMSV cells (Fig. 2, lane B). These results suggest heterogeneity in the state of the Ki-ras genes in Met Ftpd-1T cells. Interestingly, in a footpad primary tumor (PrFtpd-3, Fig. 2, lane J) and in a s.c. tail tumor (Pr i.v.-1), no Ki-ras rearrangements were present from the DNA isolated from the total populations; in contrast, individual clones showed multiple patterns of Ki-ras rearrangements (see Table 2). It should be noted that all clones contained the original v-Ki-ras 5.3-kilobase EcoRI DNA fragment. These data indicate that DNA blot analysis on the total tumor population can mask genotypic diversity in small subpopulations and that the molecular basis for these Ki-ras rearrangements must be deciphered.

Several experiments suggest that these clonal data are a valid reflection of the situation in vivo. Ninety individual clones from tumor populations were found to contain the 5.3-kilobase v-Ki-ras EcoRI DNA fragment; no clone was ever missing this band, indicating that the KiMSV provirus is stably integrated in this site (confirmed using the restriction enzyme XbaI) (Fig. 4, and data not shown). Cloned cell lines, as well as the original tumor populations, were grown longterm in tissue culture to assess the stability of the rearrangements and DNA fragment patterns. DNA blot analyses on early passage (passage 6) or late passage cells (>passage 18) and clones showed no alterations in Ki-ras blot patterns as a consequence of in vitro growth (data not shown); therefore, selection pressures in vivo during tumor progression are very different than any selective pressures in vitro.

To test for genotypic instability in tumor cells and in the originally injected KiMSV cells, various in vitro selection pressures were used. If alterations were due to dilution cloning itself, rearrangements in the PrS.c.r-1 and Met S.c.r-1 cell populations would be expected and none were found (Fig. 4A). Methylcellulose-based cloning (to select for cells with anchorage independent growth) of the original KiMSV cells (both passage 6 and passage 22), Pr Ftpd-3, and Met Ftpd-1T cells showed no differences in Ki-ras fragment pattern as compared with the results derived from the dilution cloning experiments described above (data not shown).

Molecular Bases for Ki-ras Rearrangements. Ki-ras rearrangements in lung tumor populations and their single-cell clones might result from one of several mechanisms. Replication-defective KiMSV provirus may be rescued by a helper retrovirus present in vivo or in the originally injected cells. New Ki-ras hybridizable bands in the DNA blot analysis would then represent new proviral integrations. Experimental evidence proving this mechanism relies on the fact that these tumor cell populations would produce retrovirus. Alternatively, chromosome-based rearrangements could be invoked, possibly involving c-Ki-ras and/or v-Ki-ras. c-Ki-ras rearrangements have been ruled out in this system using a DNA probe specific to the 3' untranslated DNA sequences in the c-Ki-ras gene.Southern blot analyses of round I and II tumor cells as well as multiple single-cell clones showed no alterations as compared to the originally injected KiMSV cells or BALB/c 3T3 cells (data not shown). Therefore, the new insertions reactive with the pHiHi 3 probe must be v-Ki-ras, consistent with a viral rescue/reintegration mechanism.

To test this further, a cellular transformation assay was used to assay for a helper retrovirus. Helper virus would lead to KiMSV virions being produced by tumor cell populations containing Ki-ras rearrangements; hence, these virions should be competent for transforming mouse fibroblasts in vitro. Monolayers of untransformed BALB/c 3T3 or C3H10T1/2 cells were incubated with filtered medium harvested from subconfluent tumor cells (with or without Ki-ras rearrangements) for 2 h; monolayers were subsequently grown for 14 days with a medium change every third day. The extent of morphological transformation was scored from one to four plus as compared to positive and negative controls, using media from BALB/c 3T3 or C3H10T1/2 cells as the negative control (Fig. 5A; data not shown). The positive control was from KiMSV-BALB/c 3T3 (passage 22) cells in which in vitro rescue of the KiMSV with purified Moloney murine retrovirus was used.
leukemia virus was performed (Fig. 5B). MoMuLV virus was unable to transform the BALB/c 3T3 or C3H10T1/2 by itself (data not shown). A direct correlation with the DNA blot analyses was observed—i.e., all tumor cells with Ki-ras rearrangements elicited positive morphological transformation (Fig. 5, D–F), and cells without Ki-ras rearrangements showed results identical to negative controls, as well as to medium taken from the originally injected KiMSV cells (Fig. 5C; data not shown). Culture medium from KiMSV cells at passage 6 or 22 yielded no morphological transformation of either BALB/c 3T3 or C3H10T1/2 cells, indicating that the originally injected cells do not harbor a retrovirus which rescues the v-Ki-ras oncogene. Table 2 (column b) summarizes these results, which support the proposal that rescued infectious KiMSV virions are present in the supernatants of tumor cells with Ki-ras rearrangements but not in cells without rearrangements.

These findings were confirmed using a molecular biological approach. RNA was extracted from putative viral particles isolated from tumor cell culture media for testing with hybridization probes in a dot blot assay. The first probe, pHHiHi 3 specific to Ki-ras, hybridizes with only KiMSV RNA containing the v-Ki-ras oncogene. A second probe, specific to the LTR sequences of retroviruses, hybridizes to both helper and KiMSV retroviruses; however, this probe also yields reactivity with mouse DNA, demonstrating LTR-like sequences as a “background.” The third probe, specific to actin, was used to control for contamination in the viral RNA preparations with cellular DNA or RNA as a result of possible cell lysis; negligible

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Fig. 5. Cellular transformation to detect transforming retrovirus in tissue culture medium from tumor cell lines. A monolayer of BALB/c 3T3 cells was incubated with filtered (0.45-μm pore size) tissue culture medium harvested from subconfluent tumor cells for 2 h. The medium was removed and replaced with fresh medium every three days. Fourteen days later, the cells were fixed, stained with 10% Giemsa, and photographed under bright field microscopy. Tissue culture medium was tested from A, BALB/c 3T3 cells; B, KiMSV cells in which the KiMSV virus was rescued by adding purified MoMuLV; C, Pr s.c.-1 tumor cell line (see Fig. 2, lane F); D, Met i.v.-1 micrometastatic cell line with Ki-ras rearrangements (see Fig. 2, lane H); E, Pr Ftpd-1 tumor cell line (see Fig. 2, lane J); F, Met Ftpd-1T tumor cell line containing Ki-nu rearrangements (Fig. 2, lane J, and Fig. 4C). Irregular cells which are piling in these cultures are diagnostic of retrovirus-induced transformation.
hybridization occurred with this actin-specific probe (data not shown).

As shown in Fig. 6, BALB/c 3T3 (negative control) cells were negative with both Ki-ras and LTR-specific probes (Fig. 6, A and B, lane A) and the rescued KiMSV cells (positive control) exhibited extensive hybridization with both probes (Fig. 6, A and B, lane B). KiMSV cells at passages 26 or 6 showed minimal hybridization with either probe (Fig. 6, A and B, lanes C and D), except for some reactivity with the LTR probe (Fig. 6A, lane C) reflecting cell lysis of high passage cells and liberation of LTR cross-reactive sequences. s.c. primary or metastatic cell populations with no Ki-ras rearrangements (Fig. 2, lanes F and G) also showed negligible reactivity (Fig. 6, lanes E and F, respectively). In contrast, tumor cells with Ki-ras rearrangements—Met i.v.i-1, Met Ftpdi-lT-1, and Met Ftpdi-lT-1T—showed extensive hybridity with both probes (Fig. 6, A and B, lanes G, I, and K, respectively), indicating a direct correlation with Ki-ras rearrangements and retrovirus production by these cells (Table 2, column a) and confirming the cellular transformation assay. These data are consistent with the premise that the KiMSV-provirus is rescued by a helper retrovirus in vivo and not from an in vitro contaminant virus, but only via the footpad and i.v. routes of inoculation.

The origin of this helper virus could be the originally injected KiMSV cells or the athymic nude mouse itself. Cellular transformation and viral RNA dot blot assays rule out the first possibility. Further evidence against helper virus in the originally inoculated population is evident from the s.c. route of inoculation where no Ki-ras rearrangements and no reactivity in the two viral assays were found.

The second possibility, i.e., the athymic nude mouse harboring an endogenous retrovirus, was experimentally tested. Heterozygous breeders (BALB/c nu) in the nude mouse colony (38) are analyzed semiannually for 11 types of viruses; however, retroviruses are not included in this survey. Therefore, a viral RNA dot blot experiment was performed to determine whether nude mouse organs and tissues replicate retrovirus or whether the originally injected KiMSV cells "activate" production of a latent retrovirus in nude mouse tissues. Cells from lung, spleen, and muscle tissues were incubated in culture media alone or added to subconfluent cultures of KiMSV passage 29 or passage 10 cells. If helper virus is present in tissues, it should be detected with hybridization probes and it might rescue the replication-defective v-Ki-ras provirus from these cells. After exposure to mouse tissues, cell lines were passaged in vitro three times to increase viral titer. The media were then collected and analyzed as described above. The positive control (in vitro rescue of the KiMSV using purified MoMuLV) showed hybridization with both probes (Fig. 7, A and B, lane B). Nude mouse spleen tissue from two individual mice showed positive hybridization with the LTR-specific DNA probe (Fig. 7, lanes H and J) and minimal hybridization with the Ki-ras specific probe, as expected, since nude mouse tissue should not normally contain a transforming retrovirus harboring the v-Ki-ras oncogene (Fig. 7B, lanes H and J). Lung tissue isolated from two individual animals showed no hybridization with either probe (Fig. 7, A and B, lanes I and K, respectively). Analysis of tissue culture medium from the originally injected KiMSV cell line at passage 22 cocultured with spleen or lung tissue showed some hybridization with the LTR-specific DNA probe, but no hybridization with the Ki-ras-specific probe (Fig. 7, A and B, lanes E and F).

Similarly, medium from KiMSV-BALB/c 3T3 passage 10 cells incubated with these same tissues showed a very small amount

Fig. 6. RNA dot blot analyses of tumor cell line culture media. Forty milliliters of cell culture medium was harvested from subconfluent cell lines. Cell debris was removed by centrifugation, viral particles pelleted by ultracentrifugation, and RNA extracted ("Materials and Methods"). The viral RNA was resuspended in H2O:formaldehyde (3:1), denatured at 65°C, and diluted 1:4 and 1:16 as indicated. The RNA was dot blotted on GeneScreen and analyzed using two DNA fragments as probes. A, a DNA fragment (0.964 kilobase) specific to the LTR sequences of retroviruses (41) was utilized to detect both helper and KiMSV retroviruses. B, pHHiHI 3 (40), a 1-kilobase probe specific to Ki-ras sequences, was utilized because it will only hybridize to KiMSV retroviral RNA. Cell lines from which culture medium was analyzed are indicated in lanes: A, BALB/c 3T3; B, KiMSV-BALB/c 3T3 cells in which KiMSV virus was rescued using purified MoMuLV as a positive control; C, originally injected KiMSV cells (passage 26); D, KiMSV cells (passage 6); E, Pr s.c.:1; F, Met s.c.:1; G, Met i.v.:1 (Ki-ras rearrangements observed, Fig. 2, lane H); H, Pr Ftpdi-3 (Fig. 2, lane I); I, Met Ftpdi-lT with Ki-ras rearrangements (Fig. 2, lane J, and Fig. 4C); J, Met Ftpdi-2T; K, Met Ftpdi-lT → i.v.:1 BT (contains further Ki-ras rearrangements, Fig. 2, lane K).
Table 2 Summary of analyses on the tumor cell populations and their single-cell clones

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Viral RNA dot blot assay</th>
<th>Cellular transformation assay</th>
<th>Southern blot on total cell population</th>
<th>Southern blot on single cell clonal populations</th>
</tr>
</thead>
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<tr>
<td>BALB/c 3T3</td>
<td>+</td>
<td>-</td>
<td>Cellular</td>
<td>ND*</td>
</tr>
<tr>
<td>BALB/c 3T3</td>
<td>-</td>
<td>-</td>
<td>Cellular</td>
<td>ND</td>
</tr>
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<td>-</td>
<td>Parental</td>
<td>See “Results”</td>
</tr>
<tr>
<td>KiMSV p22 parental cells</td>
<td>±</td>
<td>-</td>
<td>Parental</td>
<td>See “Results”</td>
</tr>
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<td>-</td>
<td>+</td>
<td>Parental</td>
<td>Clonal</td>
</tr>
<tr>
<td>Met s.c.-1</td>
<td>-</td>
<td>-</td>
<td>Parental</td>
<td>Clonal</td>
</tr>
<tr>
<td>KiMSV p30°:1:20</td>
<td>+++</td>
<td>+++</td>
<td>Rearrangements</td>
<td>ND</td>
</tr>
<tr>
<td>KiMSV p30°:1:10</td>
<td>+++</td>
<td>+++</td>
<td>Rearrangements</td>
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</tr>
<tr>
<td>KiMSV p29°:1:10</td>
<td>+++</td>
<td>+++</td>
<td>Rearrangements</td>
<td>ND</td>
</tr>
<tr>
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<td>-</td>
<td>+</td>
<td>Parental</td>
<td>Multiclonal</td>
</tr>
<tr>
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<td>++</td>
<td>Rearrangements</td>
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<tr>
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<td>++</td>
<td>Rearrangements</td>
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<tr>
<td>Met Ftpd-1 → l.v.ar1AT</td>
<td>++</td>
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<td>Rearrangements</td>
<td>ND</td>
</tr>
<tr>
<td>Met Ftpd-1 → l.v.ar3BT</td>
<td>-</td>
<td>+</td>
<td>Rearrangements</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Viral RNA dot blot assay using either the LTR sequence-specific probe or the Ki-ras-specific probe. See Fig. 6 for details. Intensity of hybridization was scored from 1+ = least positive to 4+ = most positive, as compared to negative and positive controls. -, no hybridization.

A. LTR

B. Ki-ras

**DISCUSSION**

Injection of KiMSV-transformed BALB/c 3T3 cells into athymic nude mice via four routes showed rapidly growing primary tumors with subsequent overt lung metastases in a few cases or lung micrometastases in most cases (33). Southern and Northern blot analyses showed that these tumor cells had
amplified the v-Ki-ras DNA sequence from two to 8-fold and increased mRNA expression from 4- to 60-fold (33). Furthermore, in two of five lung metastases from the i.v. and footpad routes, Ki-ras rearrangements were observed in the DNA blots (Fig. 2) (33). Their origin as v-Ki-ras, rather than as c-Ki-ras, have now been established. The mechanisms of the v-Ki-ras “amplification” and rearrangements have been further elucidated in the analyses of cloned populations.

That the injected KiMSV cells at passage 22 exhibited a reduced intensity of the 5.3-kilobase v-Ki-ras band relative to c-Ki-ras when compared with the passage 6 population suggested that under tissue culture conditions v-Ki-ras gene dosage was reduced. Southern blot analyses of single cell clones from these populations indicated that all clones from the passage 6 population were homogeneous (identical to the total population); whereas, most clones from passage 22 population were either missing v-Ki-ras or had a drastically reduced level. All tumors studied here (>30 independent isolates) had a level of the 5.3-kilobase v-Ki-ras DNA fragment comparable to that observed in passage 6 cells. This evidence is consistent with a low-frequency cell in the passage 22 population, retaining the dosage of the 5.3-kilobase v-Ki-ras band from passage 6 cells and having selective advantage for forming tumors by all four injection routes. However, it cannot be excluded that amplification of v-Ki-ras is occurring by multiple duplications of the entire chromosome or segments of the chromosome carrying this gene. In this regard, in situ hybridization to determine the number of v-Ki-ras-bearing chromosomes should be informative. Also, transfection of BALB/c 3T3 cells with varying dosages of Ki-ras utilizing different promoters should be informative in tumor progression studies.

Heterogeneity in cell populations after growth on tissue culture substrata with respect to chromosome properties (51) or in agar suspension with respect to morphology, colony formation, tumor formation potential in nude mice (52), drug sensitivity, metastatic potential, and type IV collagenolytic activity (53) has been well documented. In other retroviral systems, prolonged growth in culture of cells containing a single copy of provirus yielded a high proportion of subclones exhibiting 10-100-fold reduction in viral RNA and protein without a demonstrable change in structure at the DNA level of the provirus (54). Therefore, under relatively nonselective conditions in culture phenotypic and genotypic drift occur in tumor populations, as confirmed in studies described here comparing passage 6 or passage 22 KiMSV cells with the tumor populations. Amplified DNA as a HSR or as double minute bodies causes a cell to grow more slowly—i.e., a mixed population of cells containing an amplification as an HSR may be overgrown by wild-type cells, even if the amplification is quite stable in subclones (49). This mechanism may explain the loss of v-Ki-ras dosage in the passage 22 KiMSV cells because the single cell clones without v-Ki-ras or very low levels of it overgrew cells with the amplification.

These results stand in direct contrast to the results found after in vivo selection. All tumor populations and >90 single-cell clones contained increased v-Ki-ras gene dosage and mRNA expression at a level characteristic of passage 6 KiMSV cells and not the injected passage 22 cells. The rate of tumor progression in this system may depend on a minimum level of v-Ki-ras as necessary/sufficient for these complex processes to occur in the animal (1). At least two possibilities exist for selection of a cell subpopulation from the KiMSV passage 22 cells with the required level of v-Ki-ras: (a) ability to easily amplify this gene under in vivo pressure; or (b) selection for a cell subpopulation with similar v-Ki-ras dosage as found in the passage 6 KiMSV cell population or their single-cell clones. Evidence presented in this study is more consistent with the latter mechanism, although the former cannot be rigorously ruled out. It is intriguing as to which phenotypic changes are conferred on the cell via the activity of the p21 protein coded for by the essential level of the v-Ki-ras gene.

That malignant competence can be established through a mechanism of expanded gene dosage has been reviewed (55) and, therefore, selection for cells with the ability to easily amplify v-Ki-ras is conceivable. Gene selective amplification for methotrexate or other drug resistance is known (49). Such a mechanism for v-Ki-ras amplification was analyzed to a limited extent in our study. Flow cytometric DNA analyses and quantitation of chromosome number of the injected KiMSV cells, as well as of various tumors, revealed at least a twofold increase in modal chromosome number for all tumors (Table 1). Increased aneuploidy and DNA content correlate with greater malignancy (1, 55, 56). Four of eighty metaphase cells from the injected KiMSV population contained a twofold increase in chromosome number and may provide the cell subpopulation that yields all tumors in this system; perhaps, this same subpopulation also bears the passage 6 level of v-Ki-ras (which could not be easily observed in Southern blot analyses of such a low frequency subpopulation).

Precedence exists for selection of cells with a high chromosome level. In vitro, the emergence of permanent Chinese hamster cell lines from a large number of nascent stem cells showed the permanent stem lines to contain excess 3q chromosomal material, providing them with a competitive growth advantage early on (57). In vivo, mutant β-actin gene transfectant strains generated cell subpopulations with an ability to form tumors based on their elevated rate of mutant β-actin synthesis (58). Selection for cells having alterations in modal chromosome number or pattern has been observed when metastatic cell populations were compared with the primary tumor from which they were derived (59-61). Increased chromosome number and possibly increased v-Ki-ras gene dosage may confer a competitive invasion/growth advantage in tumor formation in this system, as reviewed extensively (1, 2, 62).

Little study has been devoted to the state of oncogenes in single-cell clones of various tumors and such analyses have been particularly rewarding in this system. Nowell, Fidler, and Nicolson have stated that tumors and their metastatic derivatives may be initiated with single cells but that genetic homogeneity diverges into a heterogeneous population (1, 63). Southern blot analysis has been used to study the clonal composition of tumors containing distinct genetic markers, such as restriction fragment length polymorphisms of X-chromosome genes (64), methylation patterns (65), and transfected plasmids containing bacterial genes (50). Such analyses could detect a unique subpopulation present at approximately 5% of the total population (50). Therefore, we chose to study the clonal state of v-Ki-ras in several primary and lung metastatic tumors because Southern analysis of the total population could mask small subpopulations present at <5% of the total population. Using the Ki-ras EcoRI restriction enzyme fragment pattern (including Ki-ras rearrangements) as a genotypic marker, a stable clonal pattern for s.c. primary tumor and lung metastatic cell populations was observed. Single cell clones from an i.v. experimental lung metastatic population showed nine of ten clones to contain the identical Ki-ras DNA fragment pattern as contained in the whole population. Southern blot analyses of clones from a primary footpad tumor (Pr Ftpd-3, Table 2) or a footpad overt
lung metastasis showed a multilocal pattern (i.e., different Ki-ras patterns with many new integration sites for v-Ki-ras). These cell subsets were masked upon Southern analysis of the total population. In no case were the c-Ki-ras or 5.3-kilobase v-Ki-ras DNA fragments missing. We may postulate that overt tumors were more heterogeneous in their v-Ki-ras patterns as a result of many more cell divisions and hence diverged into multiple subpopulations; in contrast, the micrometastases had few cell divisions and hence were limited in genotypic heterogeneity.

The source of the Ki-ras rearrangements from either cellular or viral Kirsten-ras genes was differentiated, using a DNA probe specific to 3′-untranslated material of the murine c-Ki-ras gene. In all cases new integrations of ras used the viral gene and not the host cell gene. The origin of these v-Ki-ras rearrangements from a helper retrovirus was also tested using cellular transformation and viral RNA dot blot assays. These two assays are complementary; the cellular assay detects a small number of transforming viral virions while the molecular biological assay distinguishes “helper” retrovirus from KiMSV virus. Morphological transformation and positive hybridization with Ki-ras and LTR-specific probes correlated directly with tumor populations containing Ki-ras rearrangements and multiclonal patterns of Ki-ras in Southern blots (see Table 2 for summary). Therefore, the Ki-ras rearrangements represent new proviral insertions into genomic DNA. Evidence consistent with this mechanism was obtained by adding purified Moloney murine leukemia virus to KiMSV cells, thereby rescuing the KiMSV replication-defective virus. Southern analysis of these cells showed additional unique Ki-ras hybridizable bands (data not shown).

Several possibilities exist for the source of the “helper” retrovirus. First, murine retrovirus could be present in the injected KiMSV cells. This mechanism was ruled out using both viral RNA dot blot and cellular transformation assays and from evidence that Ki-ras rearrangements were not observed in the s.c. primary tumor or lung metastatic cell populations or their single cell clones. Xenotropic and ecotropic murine leukemia viruses, endogenous to athymic nude mice, can productively infect transplanted tumor cells (66–68). Reports on the induction of these endogenous murine leukemia viruses by various human tumors, including oat cell carcinoma, have attributed viral inductive qualities to a specific hormone or factor (69). Chemical carcinogens, hormones, and physical agents can activate endogenous mouse mammary tumor provirus replication (70). Therefore, in our system injection of KiMSV cells into nude mice harboring an endogenous retrovirus may “activate” production of the helper retrovirus in select tissues. Spleen tissue (unlike lung tissue) cultured alone hybridized extensively only with the LTR-specific probe (and not the actin probe to discount cell lysis), indicating the presence of such a retrovirus that could potentially superinfect KiMSV cells. Culturing nude mouse tissue alone or coculture with KiMSV cells yielded data consistent with either mechanism. A helper virus would permit replication of the defective KiMSV in these cells and lead to multiple infections with new integration sites of v-Ki-ras. As far as we are aware, this is the first evidence for such a synergistic relationship in a tumor progression system.

These data also imply a special role for v-Ki-ras in tumor progression in this system and are consistent with studies showing direct correlation between ras and tumorigenesis (9). In addition to the essential dosage of v-Ki-ras in all tumors in this system, amplification of the c-Ki-ras gene in normal rat fibroblasts correlated with increased tumorigenicity; further-more, this mutant c-Ki-ras gene in specific cultured subpopulations at low copy number underwent amplification in resultant tumors (26). Increased levels of Harvey-ras were observed in primary tumors but analyses of metastatic foci found variation in ras expression (23). Chang et al. (16) found that linkage of the normal c-Ha-ras gene to an LTR increased the levels of the proto-oncogene product and tumorigenic transformation of NIH 3T3 cells. In human cells the amount of mutant ras gene product (p21) determined the state of transformation (10). These observations suggest that a minimum level of ras gene product is critical for early events in the tumorigenic process (23, 27) and the data described here for the Kirsten-ras/BALB/c 3T3 system support this argument.

Some studies have demonstrated that the metastatic phenotype can be conferred to nonneoplastic cells by transfection with tumor DNA (29), “activated” ras oncogenes (21, 23–25, 27) with exceptions in certain cell types (22), or kinase-encoding oncogenes (28). A basal level of ras expression is needed for establishment of the metastatic phenotype, since in the majority of studies no clear increases in ras expression were observed in the metastatic lesions as compared with the primary lesions (30) or with the originally injected cells (23, 31, 33). In some cases, ras may be acting synergistically with other genes in conferring the metastatic phenotype (31). These data are consistent with a model that ras is required for the induction and/or establishment of the metastatic phenotype in certain tumor classes but may not be needed for subsequent growth of cells at secondary sites.

The data described here are in agreement with the above findings and suggest additionally that Ki-ras rearrangements correlate with enhanced metastasis (33). However, different mechanisms relating new proviral insertions to growth at the secondary site might be invoked. Viral promoter insertion with subsequent activation of specific host cell genes that regulate growth and differentiation remains a possibility in this system for more aggressive invasion and growth in the lung. Alternatively, a strong retroviral promoter or enhancer may facilitate genotypic instability leading to the formation of new variants (71). More detailed molecular biological and virological analyses of the tumor populations described here should resolve these possibilities.

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CLONAL ANALYSES OF Ki-ROS AND TUMOR PROGRESSION


Clonal Diversity of the Kirsten-ras Oncogene during Tumor Progression in Athymic Nude Mice: Mechanisms of Amplification and Rearrangement


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