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Amplification and rearrangement of the Kirsten *ras* oncogene in virus-transformed BALB/c 3T3 cells during malignant tumor progression

(genotypic alterations/metastasis/lung colonization and growth ability)

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ABSTRACT **Analyses of the cellular and viral Kirsten *ras* genes (c-Ki-*ras* and v-Ki-*ras*, respectively) during malignant tumor progression were performed by using Kirsten murine sarcoma virus-transformed BALB/c 3T3 cells that harbor a replication-defective provirus. After injection into athymic nude mice by four different routes, primary tumors and secondary lung metastases were isolated, adapted to *in vitro* growth, and analyzed for DNA levels and mRNA expression of both genes for comparison with the originally injected transformed cells and untransformed 3T3 cells. For all tumors (primary or secondary), the v-Ki-*ras* gene was amplified and v-Ki-*ras* mRNA expression was highly elevated above that observed in the original transformed cell population. In two of five lung metastases from the i.v. and footpad injection routes, rearranged Ki-*ras* DNA sequences were observed. Micrometastases from the s.c. route of injection did not display these alterations. Injection of footpad lung tumor cells with rearrangements into a second group of animals led to multiple lung metastases with even further rearrangements correlating with more effective lung colonization/growth ability (overt lung tumors in five of eight animals <20 days after injection). However, reinjection of an i.v. lung tumor with rearranged Ki-*ras* led to no further rearrangements in the lung microfoci tumors isolated >40 days after injection. These data suggest (*i*) the significance of amplification and elevated expression of v-Ki-*ras* in tumor formation, (*ii*) correlation of this amplification with more effective tumor progression, and (*iii*) the selective advantage that cells with Ki-*ras* DNA sequence additions have in the formation of overt lung tumors.**

During tumor progression many changes may occur genotypically (1) and phenotypically (2), including increases in gene dosage and expression of oncogenes (3). The *ras* oncogene has special importance in select tumors. Harvey and Kirsten *ras* oncogenes were discovered as the transforming component of murine sarcoma retroviruses (4, 5), and N-*ras* was found in human DNA (3). DNA transfection experiments and isolation of *ras* genes from numerous tumors established that activation of the cellular *ras* protooncogene's transforming and tumorigenic potential occurs either by a point mutation replacing amino acids at position 12 or 61 in the encoded 21-kDa membrane protein (6) or by increasing the amount of the *ras* gene and hence its product (7). *ras* oncogenes can enhance transformation and tumorigenicity in established cell lines, but in primary cells a second oncogene is required with some exceptions (8).

Although *ras* has been analyzed in primary tumor cells, much less is known about its state in metastatic populations. *ras* is of particular interest because of recent evidence pointing to its involvement in malignant tumor progression. Transfection with tumor DNA (9, 10) or activated *ras* oncogenes (11–13) demonstrated that the metastatic phenotype can be transferred between cells (14). *ras* DNA and RNA levels in NIH 3T3 transfectants and *N*-nitroso-*N*-methylurea-induced tumors containing an activated cellular Harvey *ras* protooncogene (c-Ha-*ras*) showed gene amplification and increased expression in primary tumors with comparable levels in the metastatic lesions (14). These data suggest a role for *ras* in malignant tumor progression.

These data prompted analyses of the Kirsten *ras* oncogene during tumor progression using a Kirsten murine sarcoma virus (KiMSV)-transformed BALB/c 3T3 cell line that harbors a replication-defective viral Kirsten *ras* oncogene (v-Ki-*ras*)-containing provirus (15). Cells were injected into athymic nude mice in four anatomical locations. The resulting primary tumors and lung metastases were isolated, adapted to *in vitro* growth, and analyzed for DNA levels and expression of v-Ki-*ras* and the cellular Kirsten *ras* protooncogene (c-Ki-*ras*) relative to the untransformed BALB/c 3T3 and KiMSV (parental) transformed cells. Analyses of lung metastases undergoing a second round of *in vivo* selection using specific tumor populations from the first round of injections were also performed.

MATERIALS AND METHODS

Cells and Tumors. BALB/c 3T3 cells (clone A31) and KiMSV-transformed BALB/c 3T3 cells (nonproducer clone k-234) were grown as described (15, 16). Tumorigenicity and lung metastatic potential were assayed in athymic nude mice (17, 18) by inoculating 10^5 cells into the supraclavicular region (s.c.), thigh muscle (i.m.), tail vein (i.v.), or hind footpad (ftpd.). Primary tumors were monitored every 2–3 days. Lethargic animals were sacrificed ≈3 weeks after injection (s.c. and i.m.); for primary ftpd. tumors (1–2 cm^3), the leg was amputated anesthetically (19) and the animals were sacrificed when moribund. At autopsy major organs were examined grossly and histologically for overt or micrometastases. Tumors were dispersed into culture with 0.25% trypsin/50 mM EDTA and serially propagated no more than six times prior to analyses. To isolate micrometastatic cells, whole lungs were dispersed into culture to allow overgrowth of tumor cells.

DNA and RNA Analyses. Cellular DNA and RNA were extracted from 10^8 cells (20). Restriction enzyme cleavage, Southern blot analyses, and hybridization were performed as described (21). Filters were washed at 68°C with 30 mM NaCl/3 mM sodium citrate, pH 7.2/0.1% $NaDodSO_4$. For

Abbreviations: c-Ha-*ras*, cellular Harvey *ras* protooncogene; c-Ki-*ras*, cellular Kirsten *ras* protooncogene; ftpd., footpad; KiMSV, Kirsten murine sarcoma virus; LTR, long terminal repeat; v-Ki-*ras*, viral Kirsten *ras* oncogene.

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RNA transfer blot analyses, poly(A) RNA was prepared by oligo(dT)-cellulose chromatography, fractionated on 1% denaturing formaldehyde/agarose gels, and electrotransferred at 0.6 A to GeneScreen (New England Nuclear).

Probes for DNA and RNA Sequences. Three DNA probes were used: (*i*) a 1.0-kilobase (kb) *Eco*RI restriction fragment (corresponding to exons 1–4) of the v-Ki-*ras* oncogene from the plasmid pHiHi 3 (22); (*ii*) a 4.8-kb *Bam*HI–*Xba* I restriction fragment from the plasmid pSVc-myc (23), representing exons 2 and 3 of the murine c-*myc* protooncogene; (*iii*) a 1.2-kb Jird actin gene fragment (courtesy of T. Nilsen of this department). All fragments were purified by agarose gel electrophoresis and electroelution prior to radiolabeling. Hybridization probes were synthesized from the isolated DNA fragments by nick-translation, and bands were quantitated densitometrically on a Shimadzu Chromato Scanner.

RESULTS

Multiple-Site Injections: Round I. By injecting KiMSV-transformed BALB/c 3T3 cells into four sites to test the influence of anatomical location on the formation of metastases (19), cells undergoing different *in vivo* selection pressures can be isolated and analyzed for DNA and mRNA expression levels of the v-Ki-*ras* and c-Ki-*ras* genes (Fig. 1). These cells produced rapidly growing tumors in all injection sites. Metastatic lung microfoci were observed histologically in all routes of injection after 3–5 wk (data not shown). At autopsy, if overt lung tumors were not observed upon gross examination, the lungs were adapted to cell culture with tumor cell overgrowth within 1–2 wk. When primary tumor burden was relieved (ftpd. route) (11, 19), thereby increasing the life span of the animal, "pea"-size lung tumor nodules were observed (Fig. 1).

DNA Analysis of Round I Tumor Cell Lines. The premise is that each tumor cell line will contain a defective provirus harboring the v-Ki-*ras* oncogene (Fig. 2*A*). The importance of this is 2-fold: (*i*) primary tumors and lung metastases will originate directly from the injected KiMSV cell population and (*ii*) the state of the v-Ki-*ras* oncogene (as well as its cellular homolog) can be monitored throughout malignant tumor progression. Southern blot analysis using restriction enzyme *Eco*RI or *Xba* I confirmed that all tumor cell lines contained the v-Ki-*ras* oncogene (5.3-kb *Eco*RI band; 1.25-kb

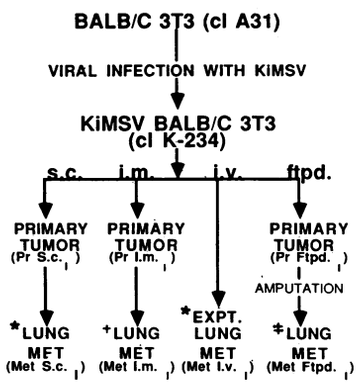

FIG. 1. Round I injection scheme. KiMSV cells (10^5, passage 22) in 0.2 ml of normal saline were injected into ten 8- to 12-wk-old female athymic nude mice per category: s.c., i.m., i.v., and the hind ftpd. Lethargic animals were sacrificed, and the major organs were examined grossly and histologically for evidence of metastases; only lungs yielded tumors. Primary (Pr) and secondary (Met) tumors were isolated and adapted to *in vitro* growth, and each animal was identified for the origin of the secondary tumor. *, Microfoci metastases isolated; +, microfoci observed only histologically; ‡, pea-size lung tumor nodules isolated. EXPT., experimental; cl, clone.

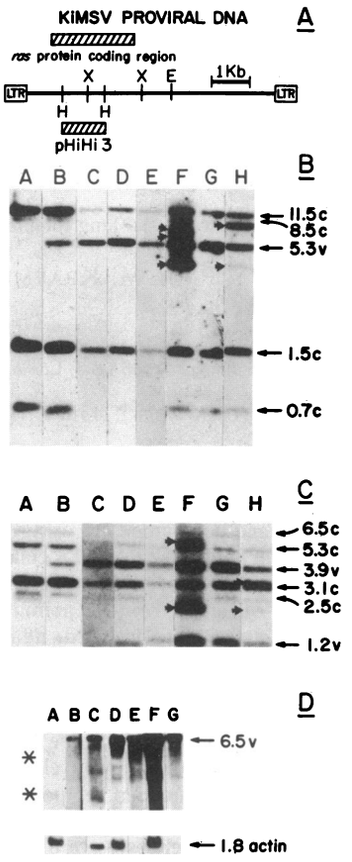

FIG. 2. Southern and RNA transfer blot analyses of round I tumor cell lines. (*A*) A 1-kb fragment of pHiHi 3 was used to identify c-Ki-*ras* and v-Ki-*ras* sequences. Genomic DNA was cut with restriction endonuclease *Eco*RI (E) or *Xba* I (X). *Eco*RI cuts the provirus in half; hence, a vi-Ki-*ras* fragment of >4 kb is expected since the pHiHi 3 probe hybridizes to the 5′ half of the provirus. c-Ki-*ras*-specific bands of 11.5 kb, 8.5 kb, 1.5 kb, and ≈0.5 kb are expected when the mouse DNA is digested with this enzyme and probed with pHiHi 3 (22). *Xba* I cuts at two sites within the provirus (and once within the probed area); therefore, two bands corresponding to 1.25 kb and >2 kb are expected for v-Ki-*ras* sequences. (*B*) Southern blot analysis of *Eco*RI-digested DNA (25 μg per lane) probed with pHiHi 3. DNA fragment sizes were estimated by using *Hin*dIII-digested λ DNA and *Hae* III-digested φX174 phage DNA. Lanes: A, BALB/c 3T3 cells; B, KiMSV (parental) cells; C, Pr (primary) S.c.$_I$-1; D, Met (secondary) S.c.$_I$-1; E, Pr Ftpd.$_I$-3; F, Met Ftpd.$_I$-1; G, Met Ftpd.$_I$-2; H, Met I.v.$_I$-1 (subscript I indicates cells from round I injection). Arrowheads indicate additional Ki-*ras* bands in two cases. (*C*) Southern blot analysis of *Xba* I-digested DNA probed with pHiHi 3. Conditions and lanes are identical to *B*. Arrowheads indicate additional Ki-*ras* bands in two cases. (*D*) RNA transfer blot analysis. Probes used were either (*i*) pHiHi 3, where c-Ki-*ras* transcripts of 5.2 kb and 2.0 kb are expected (asterisks in lane A) (24), and a v-Ki-*ras* transcript of 6.5 kb (25) or (*ii*) a Jird actin 1.2-kb DNA fragment that detects the actin 1.8-kb transcript. Cellular mRNA (4 μg) was used in all cases. Less exposed lanes are shown for clarity to enhance the 6.5-kb v-Ki-*ras* transcript; unfortunately, this decreased the intensity of the actin transcript in some cases. Lanes: A, BALB/c 3T3 cells; B, KiMSV (parental) cells; C, Pr S.c.$_I$-1; D, Met S.c.$_I$-1; E, Pr Ftpd.$_I$-3; F, Met Ftpd.$_I$-1; G, Met I.v.$_I$-1.

and 3.9-kb *Xba* I bands). This is in contrast to the untransformed BALB/c 3T3 cells that only contain bands from the c-Ki-*ras* gene (11.5-kb, 8.5-kb, 1.5-kb, and 0.7-kb *Eco*RI bands; 6.5-kb, 5.3-kb, 3.1-kb, and 2.5-kb *Xba* I bands) (compare lanes b and d–h with lane a, Fig. 2 *B* and *C*). These results confirm that all tumor lines are derived from the injected KiMSV population and indicate that one proviral integration site is present, since only one *Eco*RI >4-kb band

or *Xba* I >2-kb band containing host flanking DNA is present (legend, Fig. 2*A*).

All tumor cell lines (>20 analyzed) contained changes in v-Ki-*ras* as shown by Southern analysis (Fig. 2 *B* and *C*). s.c. tumor lines exhibited a 4- to 6-fold amplification of the v-Ki-*ras* gene (lanes C and D) when quantitation of the relative level of c-Ki-*ras* (*Eco*RI 1.5-kb band) and v-Ki-*ras* (*Eco*RI 5.3-kb band) of primary and secondary tumor cell lines (Fig. 2*B*, lanes C–H) was compared to the level in the KiMSV (parental) cell line (lane B, Fig. 2*B*, and Table 1, column 4). Use of c-Ki-*ras* as a control for differences in the amount of DNA in each lane is valid because c-Ki-*ras* levels are similar in all cell lines relative to untransformed BALB/c 3T3 cells (data not shown). The i.v. metastatic tumor line exhibited a 2.6-fold amplification of the v-Ki-*ras* gene (Table 1, column 4) as well as two additional Ki-*ras* bands: 8.3 kb and 4.2 kb in *Eco*RI-digested DNA and 2.9 kb and 2.0 kb in *Xba* I-digested DNA (arrowheads, lane H, Fig. 2 *B* and *C*). Ftpd. tumor lines (lanes E–G, Fig. 2 *B* and *C*) showed a 6- to 8-fold amplification of the v-Ki-*ras* gene (Table 1, column 4) as compared to the injected population. In one cell line, Met Ftpd.$_I$-1, the addition of 6.3-kb, 5.9-kb, and 4.3-kb bands in Southern blots of *Eco*RI digests was observed (arrowheads, lane F, Fig. 2*B*) as well as 5.3-kb and 2.0-kb bands in Southern blots of *Xba* I digests (arrowheads, lane F, Fig. 2*C*). It appears from these results that the v-Ki-*ras* oncogene is amplified in primary tumor and lung metastatic cells relative to the KiMSV (parental) cell with no substantial increases in the secondary metastatic cells as compared to the primary tumor cells. However, in two of the three injection routes (i.v. and ftpd.) and for two of five independent lung tumors, rearranged sequences (i.e., additional Ki-*ras*-hybridizable bands) that result in further amplification relative to primary tumors were observed (Table 1, column 4, in parentheses). These calculations do not differentiate their origin as c-Ki-*ras* or v-Ki-*ras*. The Ki-*ras* rearrangements seem to be specific for metastatic tumors since they were not observed in 13 independent primary tumor cell lines analyzed.

To test for nonspecific genomic rearrangements, the identical blots were probed for the c-*myc* protooncogene that resides on chromosome 15 versus c-Ki-*ras* on chromosome 6 (26, 27). No alterations in the *Eco*RI fragment pattern for c-*myc* were observed, either in terms of amplification as determined by densitometric comparison with the untransformed 3T3 cells or in terms of rearranged sequences (data not shown). In addition, v-Ki-*ras* amplification was confirmed by using the c-*myc* sequences as a control (Table 1, column 5). The ftpd. metastatic cell line exhibited a 2.5-fold amplification of v-Ki-*ras* over the primary tumor cell line; by including the additional bands, a 6.7-fold increase was observed (Table 1, column 5, and values in parentheses).

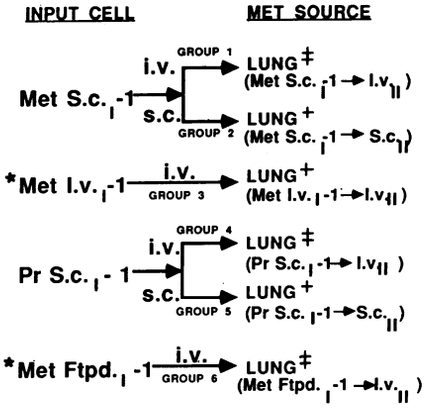

FIG. 3. Round II injections of select round I tumor cell lines. Pr S.c.$_I$-1 and Met S.c.$_I$-1 from round I (Fig. 1) contained no Ki-*ras* rearrangements, whereas Met I.v.$_I$-1 and Met Ftpd.$_I$-1 cell lines did contain Ki-*ras* rearrangements (Fig. 2). Ten mice were injected by the indicated routes with 10^5 cells and analyzed for metastatic (Met) tumors as described in the text. *, Genotypic alteration observed in round I Southern analysis; +, metastatic microfoci isolated; ‡, pea-size lung nodules isolated.

RNA Analysis of Round I Tumor Cell Lines. Analysis of mRNAs was carried out to correlate the abundance of the 6.5-kb v-Ki-*ras* transcript (25) and the 5.2-kb and 2.0-kb c-Ki-*ras* transcripts (24) to the level of *ras* DNA. RNA transfer blots of the KiMSV (parental) cells and round I tumor lines showed the expected v-Ki-*ras* transcript (lanes B and C–G, Fig. 2*D*). To visualize c-Ki-*ras* transcripts in the untransformed BALB/c 3T3 and round I tumor lines, longer exposure of the autoradiogram was required (data not shown). Densitometry of the 6.5-kb v-Ki-*ras* transcript with actin gene transcripts serving as the control (Fig. 2*D*) revealed elevated levels (15- to 60-fold) of v-Ki-*ras* mRNA in *all* tumor populations when compared to the KiMSV (parental) cells (Table 1, column 6). Therefore, v-Ki-*ras* DNA amplification leads to enhanced v-Ki-*ras* mRNA expression. c-Ki-*ras* mRNA expression was similar in all cell lines (data not shown).

Round II Injection of "Select" Round I Tumor Cell Lines. A second round of injections using cells with or without genotypic changes from the round I injections was performed

Table 1. Densitometric quantitation of v-Ki-*ras* sequences in round I cell lines

Round I cell line	Ki-*ras* *Eco*RI additional bands, kb	Type of lung metastasis	DNA amplification (v-Ki-*ras*/c-Ki-*ras*)*	DNA amplification (v-Ki-*ras*/c-*myc*)†	mRNA expression (v-Ki-*ras*/actin)‡
KiMSV (parental)	–	—	1.0	1.0	1.0
Pr S.c.$_I$-1	–	—	6.6	14	ND
Met S.c.$_I$-1	–	Microfoci	4.8	9.1	15–59
Pr Ftpd.$_I$-3	–	—	8.3	7.8	32–58
Met Ftpd.$_I$-1T	6.3, 5.9, 4.3	Nodule	6.5 (+17)	19 (+53)	19–30
Met I.v.$_I$-1	8.3, 4.2	Microfoci	2.6 (+4.9)	5.5 (+10)	38–46

ND, not determined. T indicates pea-size nodule in lung.

*Densitometric quantitation of v-Ki-*ras* DNA amplification calculating a ratio of the areas of the 5.3-kb v-Ki-*ras* band and the 1.5-kb c-Ki-*ras* band from *Eco*RI Southern blots under film subsaturating conditions. The ratio of the band areas is given with the KiMSV parental cells arbitrarily set at 1.0. The areas of the additional Ki-*ras* bands added to the area of the 5.3-kb v-Ki-*ras* band are in parentheses.

†Quantitation of v-Ki-*ras* DNA amplification calculating a ratio of the areas of the 5.3-kb v-Ki-*ras* band and the 22.5-kb c-*myc* band (derived by reprobing the identical blot) from *Eco*RI Southern blots under film subsaturating conditions. The ratio of the band areas is given with the KiMSV parental cells arbitrarily set at 1.0. The areas of the additional bands are added to the area of the 5.3-kb v-Ki-*ras* band and are given in parentheses.

‡Quantitation of v-Ki-*ras* mRNA expression. The ratio of the areas between the 6.5-kb v-Ki-*ras* transcript and 1.8-kb actin transcript was compared with the KiMSV parental cells defined as 1.0. The range of v-Ki-*ras* mRNA expression from the same sample from multiple exposures of different RNA transfer blots is given.

(Fig. 3). The rationale for these experiments is that a second round of *in vivo* selection may further enhance genotypic or phenotypic properties conferring even greater metastatic capability (2). If passage *in vitro* had no effect on metastatic potential, then the type and percentage of lung metastases should be similar to the round I data. In fact, this was observed. A round I s.c. primary tumor line (Pr S.c.$_{I}$-1) with amplified but no rearranged Ki-*ras* sequences was reintroduced either i.v. or s.c. (groups 4 and 5, Fig. 3). Micrometastases resulted from this s.c. injection of the Pr S.c.$_{I}$-1 cell line (group 5, Fig. 3) with no significant divergence from round I injection observations (data not shown). The Met S.c.$_{I}$-1 line with amplified but no rearranged Ki-*ras* sequences acted similarly to the primary tumor (compare groups 2 and 5, Fig. 3), inferring that even a metastatic cell must succumb to similar *in vivo* selection pressures during metastasis. Also, when either of these round I tumor lines was injected i.v., lung nodules were observed in one of eight animals using Pr S.c.$_{I}$-1 and in two of eight animals using Met S.c.$_{I}$-1 (groups 4 and 1, Fig. 3, and Table 2). i.v. reinjection of a round I i.v. metastatic cell line with rearranged Ki-*ras* again formed only microfoci at 30 days (group 3, Fig. 3). In contrast, within 20 days i.v. postinjection of the Met Ftpd.$_{I}$-1 cell line with Ki-*ras* rearrangements, pea-size lung nodules were isolated in five of eight cases (group 6, Fig. 3, and Table 2) as compared to the 60 days it took to form the round I lung nodule.

DNA Analysis of Round II Tumor Cell Lines. Southern analyses of round II metastatic tumor lines were then performed to evaluate further rearrangements of Ki-*ras* and any correlation with increased metastatic competence. Met Ftpd.$_{I}$-1 containing *ras* rearrangements (lane F, Fig. 2 *B* and *C*) when reinjected i.v. (group 6, Fig. 3) generated five lung metastatic cell lines (derived from five individual animals). Southern blot analyses showed even further v-Ki-*ras* rearrangements (arrowheads, lanes I–L, Fig. 4 *A* and *B*, compared to lane F, Fig. 2 *B* and *C*). In all DNA rearrangements, v-Ki-*ras* was amplified 4- to 6-fold over the KiMSV (parental) cell line (Table 2, columns 4 and 5), and the original v-Ki-*ras* or c-Ki-*ras* bands *were not lost*. These genotypic alterations are specific for Ki-*ras* since c-*myc* or actin gene sequences showed no alterations (data not shown). Rescue of the KiMSV replication-defective provirus by helper virus could explain the derivation of the extra Ki-*ras* bands. Therefore, reverse transcriptase activity was assayed from the supernatant of round I and II *in vitro* cultured tumor cell lines as described (28). No reverse transcriptase activity above background (i.e., the BALB/c 3T3 cell) was present in the rearranged tumor populations.

i.v. reinjection of Met I.v.$_{I}$-1 (group 3, Fig. 3) generated lung tumor cells with the same Ki-*ras* fragment pattern (compare lane F, Fig. 4 *A* and *B* with lane H, Fig. 2 *B* and *C*), indicating that rearrangements of Ki-*ras* do not lead inevitably to further rearrangements during tumor progression. The premise of inevitable *ras* rearrangements resulting during a second round of lung metastasis was tested further in metastatic cell lines isolated from groups 1, 2, and 4 (lanes C–E and G and H, Fig. 4 *A* and *B*); rearrangements were not observed.

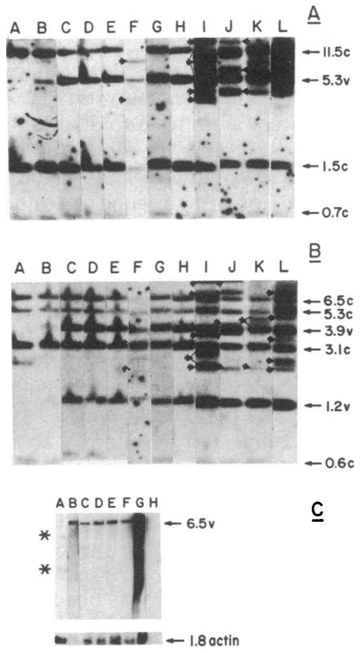

FIG. 4. Southern and RNA transfer blot analyses of round II lung metastatic cell lines. (*A*) Southern blot analysis of *Eco*RI-digested DNA probed with pHiHi 3. Conditions were identical to Fig. 2 *A* and *B*. Lanes: A, BALB/c 3T3 cells; B, KiMSV (parental) cells; C, Met S.c.$_{I}$-1 → I.v.$_{II}$-2T; D, Met S.c.$_{I}$-1 → I.v.$_{II}$-3; E, Met S.c.$_{I}$-1 → S.c.$_{II}$-1; F, Met I.v.$_{I}$-1 → I.v.$_{II}$-2; G, Pr S.c.$_{I}$-1 → I.v.$_{II}$-4B; H, Pr S.c.$_{I}$-1 → I.v.$_{II}$-1B; I, Met Ftpd.$_{I}$-1 → I.v.$_{II}$-1AT; J, Met Ftpd.$_{I}$-1 → I.v.$_{II}$-1BT; K, Met Ftpd.$_{I}$-1 → I.v.$_{II}$-2BT; L, Met Ftpd.$_{I}$-1 → I.v.$_{II}$-4BT. Arrowheads identify additional Ki-*ras* bands for some cases. (*B*) Southern blot analysis of *Xba* I-digested DNA probed with pHiHi 3. Conditions and lanes are identical to *A*. Arrowheads identify additional Ki-*ras* bands for some cases. (*C*) RNA transfer blot of round II tumor lines. The probes used were pHiHi 3 and a Jird actin DNA fragment as described in Fig. 2*D*. Lanes: A, BALB/c 3T3 cells; B, KiMSV (parental) cells; C, Met S.c.$_{I}$-1 → I.v.$_{II}$-2T; D, Met S.c.$_{I}$-1 → I.v.$_{II}$-3; E, Met S.c.$_{I}$-1 → S.c.$_{II}$-1; F, Pr S.c.$_{I}$-1 → I.v.$_{II}$-4B; G, Met Ftpd.$_{I}$-1 → I.v.$_{II}$-1AT; H, Met Ftpd.$_{I}$-1 → I.v.$_{II}$-1BT.

RNA Analysis of Round II Tumor Cell Lines. Analyses of mRNAs were performed on round II metastatic tumor lines. RNA transfer blots showed at least a 4-fold increase in the 6.5-kb v-Ki-*ras* transcript over the KiMSV (parental) cell line (Fig. 4*C* and Table 2, column 6). c-Ki-*ras* transcripts were not increased over the untransformed BALB/c 3T3 cell line (data not shown). Therefore, a second round of *in vivo* selection does not lead to further increases in v-Ki-*ras* expression (compare Table 1, column 6, and Table 2, column 6),

Table 2. Densitometric quantitation of v-Ki-*ras* in round II cell lines

Round II lung metastatic cell lines	Ki-*ras* *Eco*RI additional bands in round II	Type of lung metastasis	DNA amplification (v-Ki-*ras*/c-Ki-*ras*)*	DNA amplification (v-Ki-*ras*/c-*myc*)†	mRNA expression (v-Ki-*ras*/actin)‡
Met S.c.$_{I}$-1 → I.v.$_{II}$-2T	−	25% nodule	3.2	5.7	5.6
Met S.c.$_{I}$-1 → S.c.$_{II}$-1	−	100% microfoci	ND	ND	8.8
Met I.v.$_{I}$-1 → I.v.$_{II}$-2	−	100% microfoci	5.6 (+9.9)	ND	ND
Pr S.c.$_{I}$-1 → I.v.$_{II}$-4	−	12.5% nodule	ND	ND	12
Pr S.c.$_{I}$-1 → I.v.$_{II}$-1A	−		3.4	11	ND
Met Ftpd.$_{I}$-1 → I.v.$_{II}$-1AT	+	63% nodule	ND	ND	19–23
Met Ftpd.$_{I}$-1 → I.v.$_{II}$-1BT	+		4.6 (+10)	7.2 (+17)	4.7
Met Ftpd.$_{I}$-1 → I.v.$_{II}$-2BT	+		6.1 (+8.3)	10 (+14)	ND

ND, not determined. See Table 1 footnotes *, †, and ‡ for the above measurements.

particularly after reinjection of Met Ftpd.$_1$-1 (group 6, Fig. 3). These results are consistent with the DNA analyses.

DISCUSSION

Primary tumor and lung secondary tumor cell lines after round I injections revealed amplification and increased expression of v-Ki-*ras* in all cases. This was true for 13 primary tumors examined. In contrast, 2 of 5 lung tumors revealed multiple rearrangements of Ki-*ras* by the i.v. and ftpd. routes of injection. A second round of *in vivo* selection showed no further amplification in v-Ki-*ras* on the DNA or mRNA levels. However, in reinjection studies using lung tumor cells with rearrangements, two differences were noted. In the ftpd. case (group 6, Fig. 3) *even more* Ki-*ras* bands were observed and correlated with increased lung colonization/growth ability with overt lung tumors in 5 of 8 of the animals occurring <20 days after injection. In contrast, the i.v. case (group 3, Fig. 3) exhibited no further rearrangements and only microfoci were isolated >40 days after injection. These data suggest that amplification *and* rearrangement of the Ki-*ras* gene leads to a selective advantage for a subpopulation of cells in colonization/growth in the lung. The importance of amplification/rearrangement is also confirmed by clonal analysis of the Ki-*ras* genes in these various tumor populations (unpublished data).

The mechanism of acquisition of these additional Ki-*ras* sequences in the secondary lung tumors is unknown. These additions could occur by chromosome-based rearrangements and/or as a result of viral reinfection. Some evidence (but not conclusive) exists that is inconsistent with rescue of the replication-defective provirus by helper virus. First, reverse transcriptase was not detected above background levels in the supernatants of the rearranged cell lines. Second, if reinfection did occur, an intact Kirsten provirus would be expected to reintegrate, thereby increasing the expression of v-Ki-*ras* as compared to the unrearranged tumor cell lines; higher levels of v-Ki-*ras* mRNA were not observed in rearranged cell lines. Additional and more rigorous approaches must be used to resolve these possibilities.

The results reported here for the Ki-*ras* gene support findings of Ha-*ras* amplification in primary and secondary tumors (14). No large increases in *ras* levels in the secondary tumors as compared to the primary tumors were observed. It was postulated that *ras* may play a role early in developing the fully malignant phenotype (14) or enhance instability by incorporating oncogenes or sequences containing strong promoters and/or enhancers (2). Evidence supporting the former hypothesis strongly suggests a direct role of Ha-*ras* expression at some minimal level in the regulation and maintenance of the metastatic phenotype (29). The analyses herein suggest that Ki-*ras* may play a role early through amplification, and further evidence exists that specific selection for cells with either already amplified *ras* or the capability to easily amplify *ras* occurs during the initial steps of tumorigenesis (unpublished data). In addition, this study noted not only *ras* amplification and increased expression but also rearrangements/additions of Ki-*ras* sequences *only* in the metastatic lesions, correlating with their enhanced lung colonization/growth ability. This would suggest further involvement of *ras* in a later step of malignant tumor progression, possibly in the growth of the secondary lesion itself. Additional experiments can now test this possibility.

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