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Metastatic variants are generated spontaneously at a high rate in mouse KHT tumor

(cancer/experimental metastasis/tumor heterogeneity/tumor progression)

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ABSTRACT Using the Luria–Delbrück fluctuation analysis, we have examined the lung tumor-forming ability of a series of parallel clones derived from the KHT tumor, grown to small defined sizes. From these studies, we conclude that metastatic variants arise spontaneously in the clonal lines during their growth, at an apparent rate of $\approx 10^{-5}$ per cell per generation. This rapid rate has implications for our understanding of tumor heterogeneity and the process of tumor progression. Previous results have suggested that heterogeneity observed in cloning experiments reflects stable subpopulations of cells in the original tumor. We propose here an alternative "dynamic heterogeneity" model, in which metastatic variants arise at a high rate (as detected in the cloning experiments) but need not be stable mutations in order to effectively produce metastases.

Recent studies have suggested that tumor populations are heterogeneous when assayed for properties thought to be relevant to the metastatic process. Clonal populations derived from a number of tumors, including melanomas, fibrosarcomas, and a lymphosarcoma, have all shown wide diversity in their ability to form secondary, usually lung, tumors (1–10). Such results are consistent with the current concept of tumor progression—that mutation-like events spontaneously occur in tumors, generating heterogeneity that, in combination with the selectivity of the host environment, results in the emergence of subpopulations with increased malignancy and metastatic potential (11–15). In our previous study of clonal heterogeneity in KHT mouse sarcoma (10), we observed that the apparent frequency of metastatic cells (i.e., cells with the ability to form lung tumors when injected intravenously into mice) increased significantly with time in culture in certain clonal lines. This raised the possibility that we were observing a *de novo* generation of metastatic variants. In the present report, we examine the validity of this hypothesis by formally measuring the rate of generation of such presumptive metastatic variants in KHT clonal lines by using the Luria–Delbrück fluctuation analysis.

MATERIALS AND METHODS

The cells were derived originally from the mouse KHT sarcoma line (16) and were grown and handled as reported (10). Clonal populations were prepared *in vitro* and *in vivo* from KHT tumor cells, and stocks were maintained frozen at the population size indicated. Cells were recovered from the frozen stocks in plastic tissue culture flasks in α-minimal essential medium (17) with 10% fetal calf serum and then cloned *in vitro* by plating at limiting dilution in 24-well Linbro trays. Subclones of clones 24 and 35 were transferred by trypsinization to 75-cm^2 tissue culture flasks after $\approx$10 days and grown to $\approx 5 \times 10^6$ cells per clone before injection into mice. Subclones from clones 3 and 13 were dispersed with trypsin on days 11–13 and injected into mice when they were counted *in situ* to have $(1.0\text{–}1.7) \times 10^5$ cells per well. To allow the testing of essentially the whole of these cell populations in mice, the cell number in Linbro monolayers was determined by counting the number of cells in three microscope fields (×320) chosen at random. The number of cells recovered after trypsinization was found to be linearly related to the number of cells counted by this procedure and the standard curves so produced were used to determine the total number of cells per Linbro well.

The metastatic ability of the KHT cells was assessed by counting lung tumors from cells injected intravenously as described (10). Briefly, cells from each clonal population were trypsinized and prepared at 5×10^4 cells per ml in α-minimal essential medium with 10% fetal calf serum. They were maintained on ice and injected intravenously (10^4 cells per mouse in 0.2 ml) into the tail vein of each of 7–15 male C3H/He/Dub mice age 8–16 wk. Fetal calf serum was included in the cell suspension medium to maintain cell viability. In control experiments, the number of lung tumors formed with cells suspended in saline or in medium containing serum was similar. The mice were killed 18–20 days after injection, and the number of lung tumors was counted. In this assay, KHT cells rarely form metastases at sites other than the lung.

The apparent rate of formation of metastatic variants was determined from an analysis of the number of lung tumors formed by a series of parallel, clonal populations. The rate estimation was based upon Luria–Delbrück analysis that uses the formula $\mu \ln(3.46\mu NC) - (M/N)\ln 2 = 0$, in which μ is the apparent mutation rate (per cell per generation), N is the number of cells per parallel clonal population, C is the number of parallel clonal populations, and M is the mean number of lung tumors per parallel clonal population (18–20). The number (M/N) may be identified as the mean frequency of lung tumor formation in a series of parallel clonal populations. This equation slightly overestimates the apparent mutation rate due to the skewness of the distribution (19).

When essentially all cells of the parallel culture were injected into mice, an independent estimate of the apparent mutation rate was obtained by using the Poisson method (18, 21). The fraction of cultures with no events, P_o, is related to the mutation rate, μ, and the number of cells per parallel clonal population, N, by $\mu = -(\ln P_o)/N$. This method may underestimate the apparent mutation rate if there is phenotypic lag (19).

The spontaneous mutation rate to ouabain resistance (Oua^R) for KHT cells was measured by using an experimental design based upon that described by Baker *et al.* (22). KHT cells de-

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rived from clone 3 were plated in 15 replicate T150 flasks at a density of $\approx 10^2$ cells per replicate. The cells were dispersed uniformly in the flask by trypsinization on day 12. The replicate cultures were grown to a final cell number of $(2.3 \pm 0.09) \times 10^7$ cells per replicate. The time of growth varied from 19–26 days. To select for Oua^R colonies, all of the cells from the replicate cultures were plated in 100-mm tissue culture plates in growth medium supplemented with 2 mM ouabain (Sigma). The plating efficiency of the parental cells in growth medium was 0.76 and in selective (ouabain-containing) medium was $<10^{-6}$. The mutation rate was estimated by using the P_o method (18, 21).

RESULTS

The concepts of Luria and Delbrück established a theoretical analysis for dealing with a heritable property that is acquired by stochastic mechanisms (18). When a series of clonal, parallel populations is assessed for metastatic ability, the theory of Luria and Delbrück predicts that the frequency of altered cells in such parallel cultures is subject to large variance. Thus, a series of parallel cultures originating from single cells (i.e., clonal populations) will have large variance among populations in the number of altered cells arising after cloning. As described in a footnote to Table 2, our observations of the original *in vitro* and *in vivo* clones of KHT sarcoma (10) are consistent with a stochastic generation of metastatic variants as defined by Luria and Delbrück (18). Moreover, a Kruskal–Wallis analysis (23) of the data indicated that the clonal lines were significantly different from each other. This raises the question of the basis for this difference. Two models are possible. (*i*) Each line is a homogeneous clonal population with respect to the properties that contribute to formation of lung tumors. (*ii*) Each line, although initially clonal, has in fact become a heterogeneous population by the time the experimental metastasis assay is performed, and differences between the lines reflect differences in the frequency of a subpopulation of metastatic variants that arise spontaneously at a relatively rapid rate during the expansion of the clone by a stochastic process.

These models can be distinguished by analyzing parallel newly subcloned populations. Model *i* predicts that the metastatic ability of the subclones would be similar to that of the parental line because subcloning would be sampling cells that make up the majority of the population. By a similar argument, model *ii* predicts that, because the presumptive metastatic variants present in the parental line only represent a minor subpopulation, their probability of being represented at early times in a set of parallel subclones would be low, so that the subclones would display apparent metastatic abilities different from the parental line.

The result of such an experiment is shown in Fig. 1*a*, with the data presented in detail in Table 1. Parallel subclones were derived from KHT-35 and KHT-24, lines previously established from clones (10). These subclones were grown to $(1–2) \times 10^7$ cells prior to analysis for metastatic potential. KHT-35 forms an average of 16 tumors per 10^4 cells injected per mouse. The eight subclones derived from KHT-35 all possess lung tumor-forming ability lower than the parental KHT-35 line, and the range of variation between the subclones of KHT-35 was large. Results from the subclones of KHT-24 were equally remarkable. Two of the seven subclones showed metastatic potential considerably higher than that of the parental KHT-24 line, and the range in lung tumor-forming ability was similar for the subclones of KHT-35 and KHT-24. These results clearly are not consistent with model *i* but are completely consistent with model *ii*, suggesting that within each clonal population there exists only a

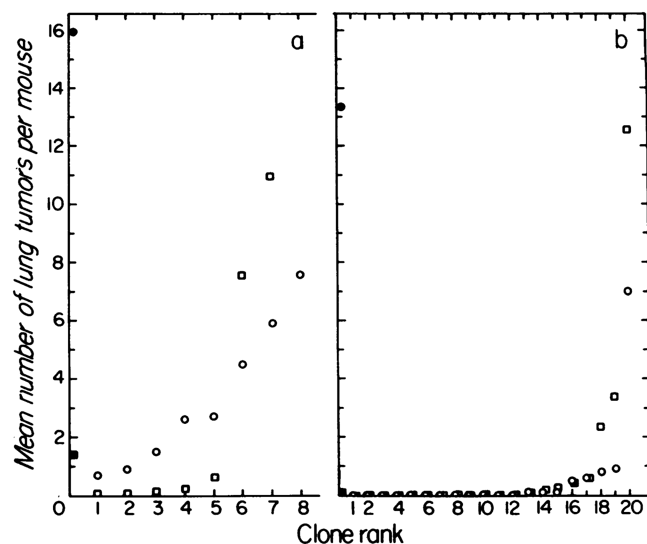

FIG. 1. Heterogeneity of metastatic ability of subclones of KHT clones. Clone rank is based on the mean number of lung tumors. (*a*) Eight subclones of KHT-35 (○) and seven subclones of KHT-24 (□) were grown to $\approx 10^7$ cells per subclone and were assayed for metastatic ability by injection into mice at 10^4 cells per mouse. The metastatic abilities of the parental clones KHT-35 (●) and KHT-24 (■) were assayed at the same time. The data for these experiments are presented in full in Table 1. (*b*) Twenty subclones of KHT-3 (□) and 20 subclones of KHT-13 (○) were grown to $\approx 10^5$ cells per subclone, as determined by *in situ* counting, and were assayed for metastatic ability. The parental lines KHT-3 (■) and KHT-13 (●) were tested at the same time. The data for these experiments are presented in full in Table 3.

small subpopulation of metastatic variants. Thus, assuming that the large variation between the subclones results from spontaneous events (mutations?) which give rise to heritable changes, we proceeded to measure the apparent mutation rate

Table 1. Metastatic ability of subclones of KHT-35 and KHT-24

Clones	Lung tumors: Number per mouse	Lung tumors: Mean
KHT-35		
(parent)	3, 5, 7, 7, 10, 11, 11, 13, 16, 18, 23, 25, 26, 48	15.9
KHT-35 subclones		
35-S3	0, 0, 0, 0, 0, 0, 0, 1, 1, 1, 1, 2, 2, 2	0.7
35-S24	0, 0, 0, 0, 0, 0, 0, 0, 1, 1, 2, 2, 3, 4	0.9
35-S9	0, 0, 0, 0, 0, 1, 1, 1, 2, 2, 2, 2, 4, 6	1.5
35-S19	0, 0, 0, 0, 0, 0, 0, 0, 0, 1, 2, 2, 8, 24	2.6
35-S20	0, 0, 0, 0, 0, 1, 1, 2, 3, 6, 7, 7, 8	2.7
35-S23	0, 0, 0, 1, 1, 2, 3, 3, 3, 3, 8, 8, 26	4.5
35-S13	0, 0, 0, 1, 2, 2, 2, 7, 7, 8, 11, 11, 12, 12, 13	5.9
35-S11	1, 1, 2, 2, 5, 5, 6, 6, 8, 9, 14, 15, 16, 17	7.6
KHT-24		
(parent)	0, 0, 0, 0, 0, 1, 1, 1, 1, 1, 2, 2, 5, 6	1.4
KHT-24 subclones		
24-S12	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0
24-S22	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0
24-S3	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 1	0.1
24-S21	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 1, 1, 1	0.2
24-S19	0, 0, 0, 0, 0, 0, 1, 1, 1, 1, 1, 1, 1, 2	0.6
24-S1	0, 2, 4, 5, 6, 7, 8, 9, 10, 11, 14, 15	7.6
24-S10	0, 2, 4, 7, 7, 7, 8, 9, 14, 14, 15, 18, 21, 28	11.0

KHT-35 and KHT-24 lines were subcloned, and each subclone was grown to $(1–2) \times 10^7$ cells and was tested for metastatic ability by intravenous injection into mice (1×10^4 cells per mouse). Mice were killed 18–20 days later, and the number of lung tumors was counted.

of formation of metastatic variants in KHT cells as described below.

The experiment described in Fig. 1 and Table 1 was analyzed to obtain a preliminary indication of the presumptive mutation rate of generation of metastatic variants. The data are tabulated in Table 2 (experiments C and D). When each of the subclones was compared to the properties of its parental cell population using the Mann–Whitney U test (23), all of the subclones of KHT-35 and six of the seven subclones of KHT-24 were significantly different from their parental populations ($P < 0.05$). An apparent mutation rate towards generation of metastatic variants was calculated with the assumption that the variant cells were 100% efficient in forming lung colonies. Values of $\approx 2 \times 10^{-5}$ and 1×10^{-5} apparent mutations or spontaneous events per cell per generation were obtained for KHT-35 and KHT-24, respectively. These estimates were only approximate because of uncertainties in the total population size at the time of testing and relatively small numbers of parallel subclones examined.

A more carefully controlled analysis was performed for another pair of KHT lines (KHT-3 and KHT-13) also derived from clones (10). Twenty parallel subclones from each line were grown to a population size of about 10^5 cells per subclone prior to analysis. From the preliminary estimates for KHT-35 and KHT-24, only a few metastatic variants would be predicted to occur per culture of this size, in accordance with the Poisson law.

The results of this more extensive subcloning are shown in Fig. 1*b*, with the data presented fully in Table 3. There are only small numbers of lung tumors observed for most of the subclones, with the mean number of lung tumors per mouse injected ranging from 0 to ≈ 13. Even when the subclones are expanded to a total size of only $\approx 10^5$ cells, heterogeneity is still observed although, as predicted by the rate calculation described above, the majority of the subclones contain no or very few observable metastatic variants. In addition, the number of lung tumors arising from the parental populations is similar to that observed in the initial testing. The subclones of KHT-3 and KHT-13 show similar ranges in lung tumor-forming ability and do not appear to reflect the metastatic abilities of their parental lines (Fig. 1*b*). These results are consistent with the stochastic generation of the metastatic variants.

The analysis of the apparent mutation rate of these clones is presented in Table 2 (experiments A and B). Because this experiment was designed to sample the majority of cells (0.7–0.9), an independent estimate of the apparent rate of mutation was obtained by using the Poisson method. The analysis of apparent rate by the Poisson method gives a minimum estimate because less than 100% of the cells were sampled, and any phenotypic lag in the expression of metastatic potential would also underestimate P_o. The results of the two different methods of analysis yield an apparent rate of $(0.6\text{–}0.9) \times 10^{-5}$ mutations per cell per generation and $(0.5\text{–}0.8) \times 10^{-5}$ mutations per cell per generation for KHT-3 and KHT-13, respectively (Table 2). These results are in good agreement with the rates calculated for KHT-24 and KHT-35, when one considers the complexity of the experimental procedure. It should be emphasized that the rate measured is an *effective rate* in that we have made a simplifying assumption that metastatic variants are 100% efficient in forming lung colonies. Because this is unlikely, the real rate of metastatic variant formation could be significantly higher than the rates determined here.

We have observed that a series of 40 KHT clones isolated *in vitro* and a series of 26 KHT clones isolated *in vivo* both displayed a wide range of their abilities to form lung tumors (10). In the light of current work implicating a high spontaneous rate of generation of metastatic variants, we tested the possibility that the heterogeneity previously observed in those two series of KHT clones could have resulted during the growth of the

Table 2. KHT sarcoma fluctuation tests to estimate the apparent rate of change to increased metastatic ability

Exp.	Parent of clonal populations*	$C^{\dagger}$	$N^{\ddagger} \times 10^{-5}$	$(M/N)^{\S} \times 10^4$	Apparent mutation rate¶
A	KHT-3	20	1.7 ± 0.05	0.62	0.9 (0.6 ± 0.2)
B	KHT-13	20	1.0 ± 0.03	0.46	0.8 (0.5 ± 0.2)
C	KHT-24	7	100–200	0.84–1.7	0.7–1.4
D	KHT-35	8	100–200	1.6–3.2	1.2–2.4
E	KHT *in vitro*	36	100–500	2–10	1.2–6
F	KHT *in vivo*	22	1,000–5,000	7.1	3.5–4.0

* The subclones in experiments A–D refer to clones derived from the KHT parent *in vitro* (experiment E is from ref. 10). In experiment F, the subclones were isolated and grown *in vivo* (10).

† C, number of parallel clonal populations.

‡ N, number of cells per parallel clonal population. In experiments A and B, the fraction of the replicate sampled was 0.70–0.90, and the number of cells per subclone (±SEM) was measured by using a standard curve. In experiments C–F, the range of number of cell divisions from initial cloning was estimated from available data using conventional Coulter counts. The fraction of the clonal population tested was ≈1/500 per mouse in experiments C, D, and E.

§ The distribution of the number of lung tumors per 10^4 cells injected per mouse is presented in Tables 2 and 4 for experiments A–D and ref. 10 for experiments E and F. The mean number of lung tumors per clonal population, M, was estimated from the number of lung tumors per 10^4 cells injected per mouse and the fraction of the total culture sampled per mouse in experiments C, D, and E.

¶ The apparent mutation rate (or range) was calculated as described and is shown $\times 10^5$ mutations per cell per generation. In experiments A and B, the number in brackets is the apparent rate as determined by the Poisson method from the fraction of replicates with no lung tumors, P_o. In experiments E and F, the total number of tumors observed and the number of parallel cultures examined was large enough to apply the variance/mean ratio test of Luria and Delbrück (18). In experiment E, the variance/mean ratio was 20 and 6.1 ($n = 36$) for the mean of the clonal and replica sampling, respectively (10). In experiment F, the corresponding numbers are 8.5 and 2.1 ($n = 22$). Because the variance/mean ratio is much larger for the clonal sampling as compared to the replica sampling of individual animals, the distribution of lung tumors for these experiments is consistent with the theory of Luria and Delbrück, which describes a stochastic, heritable process.

Table 3. Metastatic ability of subclones of KHT-3 and KHT-13

Clones	Lung tumors	
	Number per mouse	Mean
KHT-3 (parent)	0, 0, 0, 0, 0, 0, 1	0.1
KHT-3 subclones		
3-S14	0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0
3-S16	0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0
3-S15	0, 0, 0, 0, 0, 0, 0, 0	0
3-S9	0, 0, 0, 0, 0, 0, 0, 0	0
3-S11	0, 0, 0, 0, 0, 0, 0, 0	0
3-S12	0, 0, 0, 0, 0, 0, 0, 0	0
3-S3	0, 0, 0, 0, 0, 0, 0	0
3-S17	0, 0, 0, 0, 0, 0, 0, 0, 1	0.1
3-S8	0, 0, 0, 0, 0, 0, 0, 0, 1	0.1
3-S13	0, 0, 0, 0, 0, 0, 0, 1	0.1
3-S19	0, 0, 0, 0, 0, 0, 0, 1	0.1
3-S20	0, 0, 0, 0, 0, 0, 1	0.1
3-S1	0, 0, 0, 0, 0, 1	0.2
3-S10	0, 0, 0, 0, 0, 0, 0, 0, 1, 1	0.2
3-S18	0, 0, 0, 0, 0, 0, 1, 1	0.3
3-S5	0, 0, 0, 0, 0, 1, 2	0.4
3-S6	0, 0, 0, 0, 1, 1, 2	0.6
3-S4	0, 0, 2, 3, 4, 4, 4	2.4
3-S7	0, 0, 0, 0, 0, 0, 0, 2, 29	3.4
3-S2	5, 6, 8, 9, 13, 18, 29	12.6
KHT-13 (parent)	3, 4, 9, 11, 11, 15, 15, 16, 17, 22, 24	13.4
KHT-13 subclones		
13-S9	0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0
13-S11	0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0
13-S5	0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0
13-S10	0, 0, 0, 0, 0, 0, 0, 0, 0	0
13-S12	0, 0, 0, 0, 0, 0, 0, 0, 0	0
13-S2	0, 0, 0, 0, 0, 0, 0, 0	0
13-S4	0, 0, 0, 0, 0, 0, 0, 0	0
13-S7	0, 0, 0, 0, 0, 0, 0, 0	0
13-S8	0, 0, 0, 0, 0, 0, 0, 0	0
13-S19	0, 0, 0, 0, 0, 0, 0, 0	0
13-S13	0, 0, 0, 0, 0, 0, 0	0
13-S17	0, 0, 0, 0, 0, 0, 0	0
13-S6	0, 0, 0, 0, 0, 0, 0, 0, 0, 1	0.1
13-S15	0, 0, 0, 0, 0, 0, 0, 1	0.1
13-S20	0, 0, 0, 0, 0, 0, 1	0.1
13-S14	0, 0, 0, 0, 0, 0, 1, 3	0.5
13-S3	0, 0, 0, 0, 0, 0, 0, 2, 3	0.6
13-S1	0, 0, 0, 0, 0, 1, 2, 3	0.8
13-S18	0, 0, 0, 0, 1, 2, 3	0.9
13-S16	0, 0, 1, 10, 11, 13, 14	7.0

KHT-3 and KHT-13 were subcloned, and each subclone was grown to a population size of only $\approx 1 \times 10^5$ cells. Cell number was determined as described by the nondestructive *in situ* counting procedure. These subclones were then tested for metastatic ability. Due to a calculation error, $\approx 1.4 \times 10^4$ cells were injected per mouse for the subclones of KHT-3.

clonal populations to mass cultures prior to testing. Applying the Luria–Delbrück analysis to the data published previously, we estimated an effective spontaneous rate of generation of metastatic variants $(1.2–6) \times 10^{-5}$ mutations per cell per generation for the series of *in vitro* clones and a rate of $(3.5–4) \times 10^{-5}$ mutations per cell per generation for the *in vivo* clones (Table 2, experiments E and F). We made the assumptions that the *in vitro* clones were grown to $(1–5) \times 10^7$ cells and the *in vivo* clones, to $(1–5) \times 10^8$ cells prior to testing. These estimated rates are consistent with the high rates ($\approx 10^{-5}$ mutations per cell per generation) measured in the better controlled experiments described above, even though the number of cells per clonal population was 100- to 5,000-fold larger. They indicate the likelihood that the clonal heterogeneity initially observed both *in vitro* and *in vivo* (10) was not due to sampling of stable variant subpopulations but rather due to stochastic generation of metastatic variants during the growth of the clones. Whatever mechanism underlies the generation of such variants, it appears not to be greatly affected by the *in vitro* or *in vivo* growth conditions.

We investigated next the question of whether the KHT lines were genetically unstable for mutations towards drug resistance. The mutation rate towards the dominant ouabain-resistance marker has been reported to be $\approx 5 \times 10^{-8}$ per cell per generation for a number of cell lines (22, 24). Using the same protocol, we observed a similar mutation rate for the KHT-3 line of $(3 \pm 1) \times 10^{-8}$ mutations per cell per generation. Therefore, we conclude that the KHT lines are not particularly genetically unstable by this criterion.

DISCUSSION

In this study we used the experimental metastasis assay of injecting KHT sarcoma cells intravenously into syngeneic mice and counting lung tumors 3 wk later. We present data which show that the generation of lung tumor-forming cells (metastatic variants) occurred spontaneously at an apparent rate of $\approx 10^{-5}$ mutations or spontaneous events per cell per generation. No correction to this rate was made for the efficiency with which variant cells form lung tumors. If the process of lung tumor formation after intravenous injection is inefficient, the real rate of metastatic variant formation may be considerably higher than the measured apparent rate.

The high rate of random change observed for the metastatic ability of KHT sarcoma cells has some important consequences for cloning studies. In clonal populations grown to small size (less than the apparent mutation rate), the number of variant cells depends primarily on the apparent mutation rate. However, it would be expected that selective forces on the variant subpopulation would be an important factor in determining an equilibrium frequency for variant cells in the population after long periods of growth (i.e., $>10^7$ cells). Thus, if the size to which the clonal populations are expanded before testing is too great, what is studied is the variation in the equilibrium levels of metastatic variants in the clones (i.e., the selective fitness of the variants). This may have little to do with the apparent mutation rate.

Whereas the stochastic nature of the process generating metastatic variants in our system is compatible with a mutational origin for these cells, the rate observed is much higher than would normally be anticipated for such a mechanism. Stable mutations to drug resistance are observed at a rate of 10^{-6}–10^{-8} per cell per generation (25). Thus, by this criterion, the metastatic variants probably arise by a mechanism(s) different from those generally thought to be associated with point mutations and deletions. In this context it may be significant that some of the lines isolated from lung tumors are apparently unstable, losing their lung tumor-forming ability after a few weeks in culture (unpublished observation). However, in KHT cells, the mutation rate to ouabain resistance is $\approx 3 \times 10^{-8}$ mutations per cell per generation, similar to that observed for a variety of cell types, including primary human diploid fibroblasts (22, 24), suggesting that, overall, the genome of KHT cells is not especially unstable. Therefore, we conclude that the metastatic variants in KHT cells are probably generated by epigenetic mechanisms or by specific genetic mechanisms that operate at high rates. Genetic mechanisms with high rates have been observed in eukaryotic systems, and such mechanisms have been pos-

tulated to play important roles in malignant transformation (26).

We and other investigators (1–10) have interpreted findings of tumor heterogeneity to reflect clonal sampling of subpopulations of metastatic variants with different lung tumor-forming efficiencies. Such presumptive subpopulations would have to make up the bulk of the tumor because, in an analysis involving only a few decades of clones, they are apparently represented. Thus, it was concluded that most tumors are heterogeneous, being made up of a number of relatively *stable* subpopulations of metastatic variants. The results from our present study are consistent with the concept of tumors being heterogeneous, but we conclude that effective metastatic variants (as measured by the experimental metastasis assay) are present in KHT tumor lines at a relatively low frequency. Thus, in an analysis involving a limited series of clones, they have a low probability of being represented. When isolating a small number of clones, it is the cells that make up the majority of the population that are likely to be picked (i.e., cells with low metastatic ability). During the growth of these cells, heterogeneity is regenerated through stochastic processes to produce a population primarily composed of nonmetastatic cells with a variable number of effectively metastatic cells. This dynamic heterogeneity in the lung tumor-forming ability of these clones results from the high rate and stochastic nature of generation of metastatic variants.

It should be emphasized that our *dynamic heterogeneity* model does not exclude the importance of stable mutational changes in the process of malignant progression. Rather, we wish to focus attention on the possibility that certain cellular properties necessary for the successful establishment of secondary tumors by metastatic variants may occur at a high rate (and need not necessarily be expressed in a stable manner, as their equilibrium frequency in some of the KHT lines tested is apparently low). It is conceivable that, once the variant cells have completed successfully some series of steps in the metastatic process and established themselves at secondary sites, the original selection process may have ended, and daughter cells need not retain the phenotype of the original variants. Indeed, an entirely different complement of properties may be required to favor growth in the new environments.

Although the experimental metastasis assay measures only a part of the complete metastatic process (27) and our model is based on the results from a single tumor line, we believe that this dynamic heterogeneity model can provide a conceptual framework for further understanding of the heterogeneity and clonal instability of tumor cell populations observed in a number of different systems (27–31).

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