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Differences in the rates of gene amplification in nontumorigenic and tumorigenic cell lines as measured by Luria–Delbrück fluctuation analysis

(genetic fluidity/mutagenesis/mutation rate/rat liver epithelial cells)

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ABSTRACT **It has been hypothesized that genomic fluidity is an important component of tumorigenesis. Previous studies described the relationship between tumorigenicity and one marker for genomic fluidity, gene amplification. In this report, these studies are extended with the rat liver epithelial cell lines to show that: (*i*) the amplification in these cells arises in a spontaneous fashion in the population (i.e., the variants detected are not preexisting in the population), and (*ii*) the rate of spontaneous amplification (mutation), as measured by Luria–Delbrück fluctuation analysis, is significantly lower in the nontumorigenic cells than in the tumorigenic cells. The rate was estimated by using the P_0 method and the method of means. The rate of spontaneous amplification of the gene encoding the multifunctional protein CAD (containing the enzymatic activities carbamoyl-phosphate synthase, aspartate transcarbamylase, and dihydroorotase) in the highly tumorigenic cells was significantly greater than that for the nontumorigenic cells, reaching almost 1×10^{-4} events per cell per generation. The rate of this mutagenic event is high compared to the rate of point mutations usually reported in mammalian cells, and its potential contribution to the tumorigenic process will be discussed.**

Nowell (1) has hypothesized that the acquisition of genetic instability or lability may be an initiating step in the process of producing malignant cells. The acquired genetic variability allows for the production of a heterogeneous population and the subsequent selection of cells with increased malignant potential. Key to this hypothesis is the proposition that tumor cells are more genetically unstable than nontumorigenic cells. To address this point, several previous studies have examined the rate of point mutations as an indicator of genetic fluidity and found them to be elevated in tumorigenic cells (2–4). In contrast, three studies have found that the rate of point mutation was equivalent in nontransformed and transformed lines as well as between metastatic and nonmetastatic murine tumor cells (5–7). Taken together, these reports do not establish a relationship between "genomic fluidity," mutability, and tumorigenicity. Because the establishment of such a relationship between genomic fluidity and tumorigenicity could provide a basis for elucidating the cause(s) of tumor cell heterogeneity, it remains an important question.

To understand the process better, we have used the detection of amplification of the gene encoding the multifunctional protein CAD containing the enzymatic activities carbamoyl-phosphate synthase, aspartate transcarbamylase, and dihydroorotase as a molecular tool for studying genetic fluidity of neoplastic cells. Amplification of the CAD gene occurs when cells are exposed to the cytotoxic drug, *N*-(phosphonoacetyl)-L-aspartate (PALA), which inhibits aspartate transcarbamylase activity (8). Unlike resistance to methotrexate, which may occur through multiple mechanisms (9), resistance to PALA has only been reported to occur through amplification of the CAD gene (10). Correlations between amplification and tumorigenicity also have been investigated at the dihydrofolate reductase locus. An earlier report by Sager *et al.* (11) observed that methotrexate-resistant cells emerged more rapidly from a tumorigenic population than from a nontumorigenic cell population. While both Otto *et al.* (12) and Sager *et al.* (11) have shown an increased emergence of drug-resistant colonies in tumorigenic cells, to date it has not been shown that the rate of amplification is different between tumorigenic and nontumorigenic cells.

In the present study we utilized the Luria–Delbrück fluctuation analysis (13) for two purposes: to investigate the nature of the amplification events and, subsequently, to determine the rate of spontaneous gene amplification in each of the cell lines. The Luria–Delbrück fluctuation analysis is a combined experimental and statistical method that allows one to distinguish between variant cells arising by rare spontaneous mutations and variant cells arising through adaptation to an environmental selection (13). The analysis is based on the variation that is seen in the emergence of colonies from parallel cultures (14).

Fig. 1 shows a schematic diagram of a fluctuation experiment and the hypothetical results one could obtain when analyzing a spontaneous mutation. The first set (indicated by the downward arrow) contains replicate samples in which aliquots of cells from the parent population are plated directly into selection medium and analyzed for the number of resistant colonies that emerge. The colonies on these plates will represent rare, resistant, preexisting variants. The number of colonies on each plate should exhibit a Poisson distribution, and the mean number of colonies per plate will reflect the prevalence of resistant variants in the parental population. Replicate platings from the same parent population should show variation due only to random sampling; the variance from these replicate samples should equal the mean.

For the second set, a small number of cells (small enough to assure no preexisting variants are present) is plated and allowed to propagate under nonselective conditions for a given amount of time. When the individual populations have reached the same cell density plated in set 1, they are transferred to fresh plates for an even distribution of cells and placed under selection (in this case PALA). If the drug-resistant cells are the result of exposure to PALA (i.e.,

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Abbreviations: PALA, *N*-(phosphonoacetyl)-L-aspartate; CAD, the multifunctional protein containing the enzymatic activities carbamoyl-phosphate synthase, aspartate transcarbamylase, and dihydroorotase.

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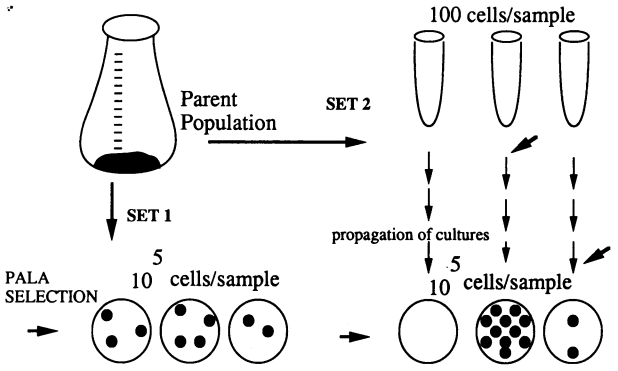

FIG. 1. Schematic diagram of a Luria–Delbrück fluctuation analysis of a spontaneous mutation event. Set 1 samples the variance of the parent population; the distribution should be Poisson, and the variance should equal the mean. Set 2 measures the variance of small aliquots that have been propagated under nonselective conditions. In this example a spontaneous mutation event has given rise to the colonies depicted in each plate. The horizontal arrows in samples 2 and 3 of set 2 indicate mutational events occurring early and late during the propagation of these cultures, respectively. For further explanation, see the Introduction.

adaptive), each cell should have a similar probability for survival in selective media, and the appearance of resistant variants should be similar in all plates. The variation from plate to plate would be consistent with the Poisson model. If the events that lead to resistance are spontaneous, as each parallel culture expands it will have a given probability for generating resistant variants with each cell division. In some cultures the event (amplification) will occur early and many of the progeny of the resistant cell will be present to form drug-resistant colonies. In others, the event will occur during one of the last cell divisions, and few drug-resistant progeny will result. The appearance of variants will be random, and the contribution to the surviving colonies when the cells are placed in selection will vary greatly depending on when in the propagation of the population the mutation occurred. Statistical analysis of this variation allows one to calculate the rate of appearance of spontaneous variants.

MATERIALS AND METHODS

Cells and Culture Conditions. Rat liver epithelial cell lines were derived and grown as described (12, 15, 16). The aliquots of frozen cells used in these experiments were the same as those used for previous and ongoing tumorigenicity studies (16). WB_{20}, GN_5, and GP_9 cells exhibited 0%, 11–50%, and 100% tumorigenicity upon injection into isogenic newborn rats (16) and plating efficiencies of 0.78, 0.84, and 0.55, respectively. At the conclusion of these experiments, several of these cell lines were reconfirmed in their respective tumorigenicities.

Drug Selections. Selection experiments were as described (12). The incidence of PALA resistance is the proportion of attached cells that gave rise to resistant colonies and, thus, is relative to the plating efficiencies of the cells in medium without drug (100% survival). LD_{50} values represent the concentration of PALA that allows 50% survival. LD_{50} values for the WB_{20}, GN_5, and GP_9 cells are 12.5, 8, and 9 μM PALA, respectively.

Subcloning PALA-Resistant Lines. Cells from individual PALA-resistant colonies were expanded in 9× LD_{50} PALA-containing medium. Genomic DNA was isolated from a portion of these cells and analyzed for the gene copy number (12, 17).

Determination of Experimental Parameters. In this paper we have used both the P_0 method and the method of means for measuring the mutation rate in mammalian cells. The P_0 estimate was calculated according to Luria and Delbrück (13) as modified by Lea and Coulson (18) as described in the legend to Table 1. Estimates of mutation rate using the mean method were calculated as described by Capizzi and Jameson (19) (described in the legend to Table 1) and verified by direct solution of their transcendental equation 2.

RESULTS

Determining the Nature of the PALA-Resistant Variants by Using the Luria–Delbrück Fluctuation Analysis. A fluctuation analysis was performed on each of the cell lines to determine the nature of the generation of PALA-resistant variants—i.e., to distinguish between a spontaneous mutation event and an adaptive event. Exponentially growing cells were plated as described in Fig. 1 for both sets 1 and 2. In set 1, 2×10^5 cells of each cell line were plated in 100-mm dishes; after 6 hr the medium was replaced by selective medium containing a PALA concentration equivalent to 9× LD_{50} for each respective cell line. Previous studies have shown that the colonies arising under these conditions have amplified the CAD gene (12). The data presented in Table 1 show that the variation in these samples is consistent with Poisson variability, indicating that any large fluctuations that may be seen in set 2 must be due to processes other than sampling error.

At the same time, exponentially growing cells were plated as described for set 2. Two hundred cells of each cell line were plated in replicate cultures and expanded to 2×10^5 cells per plate. After attaining this density, the cells were trypsinized, dispersed, and placed in a 100-mm dish. After 6 hr the medium was replaced with selective medium containing PALA at a final concentration of 9× LD_{50} for each cell line, respectively. Results are presented in Table 1. In each of the three cell lines studied, the variance significantly exceeded the mean (P values $< 10^{-4}$), indicating that amplification of the CAD genes in these cell lines arose spontaneously.

Determination of Spontaneous Amplification Rates Through Luria–Delbrück Fluctuation Analysis. Fluctuation analysis also can be used to estimate the mutation rate of a given event in mammalian cells. Mutation rates were determined for WB_{20}, GN_5, and GP_9. The tumorigenic cells demonstrate a much greater rate of spontaneous CAD gene amplification than the nontumorigenic WB_{20} cells (Table 1). By averaging the rate values from Table 1 and additional determinations (data not shown) and using both types of determinations when possible (P_0 and method of means), the nontumorigenic WB_{20} cells were found to amplify the CAD gene at a rate of 1.07×10^{-6} events per cell per generation. The moderately tumorigenic GN_5 cells and highly tumorigenic GP_9 cells yielded rates of 1.34×10^{-5} and 7.6×10^{-5} events per cell per generation, respectively, 12 and 71 times higher than that of the WB_{20} cells.

Repeated Measurement of Mutation Rate During Exponential Growth. The mutation rate measured above is contingent on several assumptions, one being that the probability of the event is constant throughout exponential growth. To address this point, we determined the mutation rate for GP_9 cells at three different points during exponential growth. The mutation rate remained reasonably constant through early, mid, and late phases of exponential growth, with average values of 6.2, 5.3, and 13.8×10^{-5} mutants per cell per generation, respectively.

Derivation and Characterization of Nontumorigenic and Tumorigenic PALA-Resistant Subclones. The number of PALA-resistant colonies that arise in a population will depend on the rate of mutation (in this case the rate of CAD gene amplification), the time of appearance of the variant cell, and the reproductive capacity of the variant and nonvariant cells.

Table 1. Fluctuation analysis of PALA-resistant ($PALA^R$) variants in rat liver cell lines with differing degrees of tumorigenicity

	WB_{20}		GN_5		GP_9	
	Set 1	Set 2	Set 1	Set 2	Set 1	Set 2
Replicate cultures, no.	79	56	39	51	40	23
No. of plates containing the following no. of $PALA^R$ colonies:						
0	73	38	—	1	—	—
1	6	4	—	3	—	—
2		8	—	6	—	—
3		3	—	8	—	1
4		—	1	6	—	1
5–8†		3	10	16	—	4
9–16		—	28	9	—	4
17–32		—	—	—	—	2
33–64		—	—	1	18	8
65–129		—	—	1	22	3
Mean colonies per replicate (r)	0.076	0.82	10.18	7.98	65.75	34.96
Variance	0.071	2.26	7.36	243.14	66.09	1050.95
Variance/mean	0.94	2.75	0.72	30.45	1.01	30.06
P value‡	0.64	$<10^{-4}$	0.90	$<10^{-4}$	0.46	$<10^{-4}$
Mutation rate						
P_o calculation§	—	1.72×10^{-6}	—	1.62×10^{-5}	—	NA
Mean method¶	—	1.88×10^{-6}	—	1.06×10^{-5}	—	6.27×10^{-5}

Data for plates in set 1 were obtained from aliquots of cells from the indicated parent populations, which were placed in PALA concentrations equivalent to 9× the LD_{50} for each cell line (WB_{20} in 112.5 μM, GN_5 in 72 μM, and GP_9 in 81 μM PALA). Data for plates in set 2 were obtained from aliquots of 200 cells from the original parent populations, propagated until a cell density of 2×10^5 cells was obtained, and then plated for selection in the PALA concentrations indicated above.

†The colonies per plate for each cell line within these intervals are as follows: WB_{20} cells set 2, $5_{(2)}$ and 7. GN_5 cells set 1, $6_{(3)}$, $7_{(3)}$, $8_{(4)}$, $9_{(3)}$, $10_{(7)}$, $11_{(6)}$, $12_{(4)}$, $13_{(4)}$, $14_{(2)}$, 15, and 16; GN_5 cells set 2, $5_{(4)}$, $6_{(6)}$, $7_{(3)}$, $8_{(3)}$, $9_{(3)}$, $10_{(3)}$, 11, 15, 16, 33, and 111; GP_9 cells set 1, 51, 52, 53, 54, 57, 58, $59_{(2)}$, $60_{(4)}$, $61_{(3)}$, $62_{(3)}$, $66_{(3)}$, $68_{(4)}$, $69_{(2)}$, $70_{(4)}$, 71, 72, 74, $75_{(2)}$, 76, 77, 83, and 87; GP_9 cells set 2, 3, 4, 6, $7_{(2)}$, 8, $12_{(2)}$, 13, 15, 21, 24, 37, $38_{(2)}$, 43, 48, 52, 60, 61, 68, 98, and 129. The subscript in parentheses indicates the number of plates containing the indicated number of colonies when the number exceeds 1. For example, for the WB_{20} cell line in set 2, two plates contained 5 colonies and one plate contained 7.

‡Obtained from the dispersion test for Poisson sampling (20).

§Calculated according to Luria and Delbrück (13) as modified by Lea and Coulson (18) from the calculation $\mu = [(\ln 2)\ (-\ln P_o)]/(N_t^* - N_o^*)$ where P_o represents the fraction of cultures with no variants, μ is the rate of amplification per cell per generation, and N_t^* and N_o^* are, respectively, the N_t (final cell number) and N_o (initial cell number) adjusted for plating efficiency.

¶Calculated according to Capizzi and Jameson (19) by the equation $Cr = (C\mu N_t^*) \ln (C\mu N_t^*)$, where C is the number of replicate cultures, r is the average number of variants per culture, μ is the amplification rate per cell per generation, and N_t^* is N_t adjusted for plating efficiency. Given r, N_t^*, C, Cr is obtained, and the corresponding value for $C\mu N_t^*$ is extrapolated from table 3 of ref. 19.

In designing the fluctuation analysis, Delbrück made several assumptions that must be addressed here if the results are to have any validity (see *Discussion*). To address these assumptions, we have isolated PALA-resistant subclones that have undergone CAD gene amplification and characterized them for their reproductive capacity in the absence of selection pressure. In a previous study (12), cells were placed in PALA concentrations equivalent to 9× LD_{50}. Resistant colonies emerged and were picked, expanded, analyzed for CAD gene copy number, and found to be amplified (2- to 4-fold; ref. 12). For the present study, several of these subclones were characterized for their growth characteristics out of selective medium to closely approximate the conditions that exist as the cells are expanding during the fluctuation analysis. Reproductive capacity of these subclones was measured by using their cell cycling time and plating efficiency in the absence of selection. While the plating efficiencies of the PALA-resistant subclones are similar to that of the PALA-sensitive cells, the cell cycle times may vary from slower to comparable rates (data not shown).

We also propagated four WB_{20} PALA-resistant subclones and four GP_9 PALA-resistant subclones out of selection media for various amounts of time to determine the stability of the initial amplification. The cells were grown for 1–5 months out of selection (out of PALA-containing medium) and then rechallenged at monthly intervals with the original concentration of the drug (9× their LD_{50} values, respectively). All PALA-resistant subclones demonstrated a plating efficiency equivalent to that of the parental population when rechallenged with the drug within 4 weeks after their emergence. This indicates, that in the rat liver epithelial cells, the CAD gene is amplified and retained in a stable state during the initial weeks of growth, and loss of resistant colonies is negligible during the initial expansion of the culture before selection (unpublished data).

DISCUSSION

In this study, we have used the Luria–Delbrück fluctuation analysis to show that: (*i*) amplification of the CAD gene in the rat liver epithelial cells is arising in a spontaneous fashion in the population, and (*ii*) the rate of spontaneous CAD gene amplification is significantly higher in the tumorigenic cell lines compared with the nontumorigenic cell line.

Biological Considerations Relevant to the Estimation of Mutation Rate by Using the Luria–Delbrück Fluctuation Analysis. The variation in colony number seen in Table 1 unequivocally demonstrates the spontaneous nature of the amplification of the CAD gene in these cells. Our data indicate that PALA is not facilitating the emergence of a small subpopulation of cells that is PALA resistant; rather we find that amplification of the CAD locus occurs spontaneously in all rat liver epithelial cell lines studied. These results are consistent with those reported for CAD gene amplification in another rodent, the hamster cell lines studied by Stark and co-workers (21).

Although the fluctuation analysis is adequate for determining the spontaneity or nonspontaneity of a given event as discussed above, the use of this procedure for accurate determination of rate requires attention to a multitude of experimental conditions (22). In the fluctuation-type experiment outlined in Fig. 1, the number of PALA-resistant colonies in a population will depend not only on the rate at which they arise but also on their time of appearance (early or late in the expansion of the culture) and the reproductive/survival capacity of the individual colonies. Assumptions concerning these variables for the variant (PALA-resistant cells) and wild-type cells determine whether the fluctuation analysis can be applied to the estimation of gene amplification. The accuracy and validity of the rate analysis rest on how well these assumptions are met. We have addressed these assumptions in an experimental fashion where possible and discuss them in this section.

The fluctuation analysis, as originally devised, was used to determine the probability of a discrete event, the generation of a phage-resistant bacterium. When the bacterium was resistant, it lived; when it wasn't, it died (13). Drug resistance can be assessed by gene amplification in a similar manner if the conditions for survival are strictly defined. Under our conditions, we require that the surviving colony be resistant to $9\times$ LD_{50} of the drug. At this stringency of selection, we have shown that the cells are truly resistant (they may be propagated in that concentration of the drug without loss of plating efficiency), and when analyzed, the basis of the resistance is amplification of the CAD gene (12). Amplification of this gene is the only reported mechanism of resistance to PALA and in our studies is the only observed mechanism of resistance in rat liver epithelial cells.

The fluctuation analysis requires that the probability of mutation for an individual cell be directly related to its growth rate (13). Our experiments are done under logarithmic-phase growth conditions, where the growth rate is constant and reproducible. We have estimated the mutation (amplification) rate during early, mid, and late logarithmic growth and find that the rate is also constant. This allows us to conclude that the probability of variation in our system is constant with exponential growth and that this assumption of the fluctuation analysis has been met.

Comparative plating efficiency of the cells is an important variable in two aspects of this analysis. At the initiation of our experiments to apply the fluctuation analysis to this biological process, the cultures of the different cell lines are trypsinized and dispersed into individual culture dishes (100-mm dishes). The different cell lines may vary in their plating efficiency. For our experiments the plating efficiency of each cell population has been determined and is taken into account in the subsequent calculations for the analysis. Second, during the course of our expansion of the cultures and their subsequent placement into selection conditions, the cells must be trypsinized and replated. If the variant (amplified) cells had a lower (or higher) plating efficiency than nonvariant cells, an artifactual depletion (or elevation) of their number would result and be scored as an aberrant rate. To test for this possibility, we determined the plating efficiency of variant and nonvariant cells under nonselective (expansion) conditions for both the WB_{20} and the GP_9 cell lines. The two populations for each cell line studied (WB_{20} and GP_9; variant and wild type) had similar plating efficiencies under these conditions; hence, a differential plating efficiency cannot account for the rate difference we observe for the spontaneous amplification event.

If the generation and detection of PALA-resistant colonies is to represent the rate of gene amplification, both the growth of variant (amplified) and wild-type (nonamplified) cells must be exponential and have equal rates. Unequal growth rates of these two populations would skew the final number of colonies that were tabulated and misrepresent the rate determination (23). To address this question we propagated variant and nonvariant cells separately under conditions that were used for the expansion of the culture (i.e., complete medium not containing any drug). Their growth rates were similar. The plating efficiencies of the PALA-resistant subclones plated in nonselective media are comparable to that of the PALA-sensitive (wild-type) cells. The cell cycling time varies for the different subclones. In both cases, that of the WB_{20} cells and the GP_9 cells, the subclones exhibited, on average, $\approx$15% greater cell cycle time than the corresponding parental populations. The values range from similar cycle times to slightly longer cycling times. This indicates that the PALA-resistant colonies have no growth advantage as the culture is being propagated under nonselective conditions. The slightly slower cycling times observed with some of the subclones would eventually alter the composition of the population but the minimal differences observed would not be a major consideration during the short propagation time during the fluctuation analysis ($\approx$4–6 days). These results suggest that the estimation of the rate of gene amplification in these cells is a minimal value and that the number of PALA-resistant colonies detected when the cells are placed under conditions of selection cannot be attributed to a differential growth rate of one subpopulation *vis* à *vis* the other during the expansion of the culture.

A further requirement for the analysis is that, during selection, the cells be plated under conditions to allow for the growth of independent colonies; no cross-feeding may occur. Selection of PALA-resistant colonies may be circumvented by the transfer of nutrients from one cell to another to overcome the aspartate transcarbamylase inhibition. In our experiments the cells are plated at a maximum of 2×10^5 per 100-mm dish, a density that allows for the growth of well-separated, individual colonies. In support of this claim, we have tested the resistance of numerous of these colonies and have found them to breed true when rechallenged with the drug (12).

The fluctuation analysis requires that in order for the rate to be accurately assessed, no preexisting variant cells can be seeded at the initiation of the expansion of the cultures. The variants that are scored must arise during the expansion of the parallel cultures. Our cultures begin with $\approx$100 viable cells. If a preexisting variant were present in the initial 100 cells, and if it had a doubling time equivalent to that of the wild-type cell population (as shown previously), its contribution to the number of colonies scored as the cells are placed in selection would be great; >1000 colonies per 10^5 cells would be visualized. Since this was not found in any of our analyses, we conclude that the number of preexisting variants in the population is small or nonexistent and that our rate determination is not affected. We are indeed measuring the spontaneous appearance of drug-resistant/CAD-gene-amplified cells.

Finally, for this analysis it is assumed that the rate of back mutation (deamplification) is negligible. The effect of reverse mutation on the estimated rate of amplification depends on the absolute number of cells available for the event. Given the

relatively rare number of amplification events, the reverse rate would have to be extraordinarily high to have any effect. We have analyzed the stability of our PALA-resistant colonies, both at the earliest time possible and after expansion, and find that in the WB_{20} and GP_9 rat cell lines the PALA resistance is stable at early times after emergence. We have grown the cells in the absence of selection for 1–5 months and find that during the first month of propagation, they have a similar plating efficiency to the drug-resistant parental population when rechallenged with the selecting concentration of the drug. This determination gives us an indication of the stability of the amplification unit after it has emerged as a colony.

Data from the fluctuation analysis and subsequent controls have allowed us to estimate the rate of spontaneous gene amplification. Mutation rates were estimated via a fluctuation analysis using the P_o method and the method of means (13, 19). The P_o method was modified according to the work of Lea and Coulson (18), Newcombe (24), and Armitage (25). Li *et al.* (26, 27) have reported that there is an optimal value for P_o (between the range of 0.15 and 0.8), which will maximize the sensitivity of the fluctuation analysis. They have developed guidelines for the optimization of the values P_o, N_t, and C to improve the reliability of the estimation of μ. We have followed these guidelines in generating data for the estimation of μ in these studies. The method of means measures the rate from an alternative perspective—namely, equating the observed average rate of variants to its expectation. In our hands, with these cells, both methods are in agreement.

In this study we show quantitatively that individual rat liver epithelial cell lines, which differ in their ability to form tumors, have an inherently different rate of amplifying the CAD gene. The nontumorigenic WB_{20} cell line amplifies at a frequency that is somewhat high for a mutational event in mammalian cells, an average of 1.07×10^{-6} events per cell per generation. The GN_5 cell line, which will form tumors in some but not all animals into which it is injected, spontaneously amplifies the CAD gene at a rate that is more than an order of magnitude higher than that measured for the WB_{20} cells (on average, 1.34×10^{-5} events per cell per generation). The highly tumorigenic GP_9 cell line demonstrates a rate of CAD gene amplification (on average, 7.6×10^{-5} events per cell per generation) that is >70 times higher than that measured in the WB_{20} cell line. It is this inherent ability to amplify the CAD gene and the difference between these cell lines that we would like to understand on a molecular level.

Biological Significance. The rate of gene amplification reported for the tumorigenic cell lines in this study ($1–7 \times 10^{-5}$ events per cell per generation) is quite high compared to the rate of point mutations usually observed in mammalian cells (10^{-7}–10^{-8} events per cell per generation) (14, 28, 29). A frequency of CAD gene amplification has been reported for a Syrian hamster line ($\approx 2 \times 10^{-5}$) (21). If our estimated rate is indicative of amplification rates in tumorigenic cells in general, it is expected that the types of phenotypic changes that result from gene amplification would make a significantly greater contribution to the mutagenic burden of tumorigenic cells than that produced by point mutations. Recent studies on the frequency of large spontaneous deletions suggest that these types of rearrangements are also occurring quite often (30). The observation of deletions and amplifications in tumorigenic cells is well documented and may provide the mechanisms underlying the phenotypic and genotypic heterogeneity seen in neoplastic cells.

Several studies have linked the frequency and extent of amplification of specific oncogenes with the malignancy of the disease (31–33). In neuroblastomas, the frequency and extent of N-*myc* amplification is reported to be a better prognostic indicator than any other determinant for that disease (31). While in some cases amplification of specific oncogenes is correlated with disease progression, amplification of other oncogenes, often in the same cells, is not (33). Our results provide a framework for understanding these clinical data. The more tumorigenic a cell, the higher the probability that it will amplify a segment of its genome. This is consistent with the multiple reports of amplification in neoplastic tissue. We also know that the amplified copies are eventually lost unless selection pressure is exerted upon that population (9, 10). The oncogene products that correlate with disease progression must be conferring a growth advantage on that cell population in that tumor. Studies on gene amplification in these tumors could provide us with a tool to identify signals important in the progression of neoplasia.

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