Increased Incidence of CAD Gene Amplification in Tumorigenic Rat Lines as an Indicator of Genomic Instability of Neoplastic Cells*

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It has been hypothesized that genomic instability is an important component of tumorigenesis. In an attempt to establish this relationship, we determined the frequencies with which two nontumorigenic and four tumorigenic rat liver epithelial cell lines underwent a particular type of genetic instability, gene amplification. By exposing cells to N-(phosphonomethyl)-L-aspartate (PALA), a drug which specifically inhibits the aspartate transcarbamylase activity of the multifunctional CAD enzyme and selects for amplification of the CAD gene, we observed a striking parallel between the ability of these cell lines to become resistant to this drug and the ability of these same cells to form tumors after injection into day-old syngeneic rats. Cells of one highly tumorigenic line became resistant to PALA greater than 70 times more often than those of a nontumorigenic line. Molecular analyses of eight independent PALA-resistant subclones confirmed that, in each case, this resistance was due to amplification of the CAD gene. Thus, our results demonstrate the relationship between tumorigenicity and at least one measure of genomic instability, CAD gene amplification. The method developed in this study provides a quantitative, rapid indicator of tumorigenicity and should prove useful in trying to elucidate the underlying basis of genomic instability in neoplastic cells.

Tumor cell heterogeneity is a universal property of neoplastic tissue (1–3). As early as the late 1800s, morphologically distinct subpopulations of cells could be identified within a single tumor (1). Such heterogeneity now includes variation in antigenicity, enzymatic activities, drug resistance, growth characteristics, and most importantly, metastatic ability (1). Genotypic variation within tumor cell populations has also been revealed by karyotypic and molecular analyses; chromosomal abnormalities including aneuploidy, translocations, deletions, and amplifications have been observed in a variety of tumor cells (4, 5). Recent molecular studies have shown that these rearrangements, as well as single base mutation, can alter the expression of oncogenes (6).

Clinical investigations have documented that this phenotypic and genotypic variation within neoplastic populations is not static: multiple and independent changes accumulate and result in increased variability within the population during tumor progression. This genomic instability has been observed in a wide variety of tumor types and thus appears to also be a universal feature of neoplastic tissue (1, 7, 8). Based on these observations, it has been postulated that it is the acquisition of this genetic instability which generates the heterogeneous population of the tumor and provides the material for secondary changes that result in the malignant phenotype (9). Mutation rates of normal and malignant cells have been compared to try and understand the relationship between genetic instability and tumorigenicity. Chinese hamster embry fibroblasts show a higher spontaneous mutation rate to thioguanine resistance and ouabain resistance after the cells are doubly transformed with SV40 and polyoma virus (10). Singly transformed fibroblasts (polyoma only) show no increase in mutation rate. Similarly, murine fibrosarcoma cells with high metastatic ability have a higher mutation rate (at these same two loci) than cells with a low metastatic ability (11). Recent studies with human lymphocytes show that spontaneously malignant tissue mutants to thioguanine resistance at a much higher rate than normal lymphocytes (12). In contrast, a nontransformed human fibroblastic line was equivalent to a transformed human line in the rate of mutation to thioguanine and ouabain resistance (13). Taken together, these reports suggest a relationship between gene mutation rate and tumorigenicity. We have chosen to look at the incidence of a different endpoint of genomic instability, gene amplification, in an attempt to see if this also relates to tumorigenicity. Based on recent oncogene studies, one would expect the frequency of point mutations, amplification, and rearrangements to correlate with tumorigenicity.

We chose to study genetic instability using gene amplification as a marker for several reasons. For several decades, the manifestations of gene amplification, homogeneously staining regions and double minute chromosomes, have been observed in neoplastic tissue, although at the time the molecular basis of these chromosomal abnormalities was not known (14–16). In the 1970s, studies by Beidler and co-workers (17) associated these chromosomal abnormalities with drug resistance; Schimke and co-workers (18–21) found that the basis of the resistance in this instance was an increase in gene copy number (gene amplification). These observations prompted investigations into the identity of the sequences which are carried on homogeneously staining regions and double minute chromosomes in human tumor biopsy materials and led to the discovery that oncogenes are often amplified, especially in certain tumor types (22–25). Finally, gene amplification is an example of a genomic instability or rearrangement which is easy to measure on a molecular level. Unlike random chro-

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mosomal breaks or translocations, amplification of a targeted sequence can be measured using colony formation and easily verified by determination of gene copy number.

We chose to study genetic instability using PALA1 resistance and the ensuing amplification of the CAD gene, because unlike methotrexate resistance which may occur through multiple mechanisms, the only reported mechanism of resistance to PALA is through amplification of the CAD gene (26, 27).

The CAD gene codes for the multifunctional CAD enzyme, and PALA inhibits the aspartate transcarbamylase activity of this enzyme. Thus, in asking whether tumorigenic cells amplify DNA sequences more often than nontumorigenic cells, we used the incidence of resistance to PALA as an indirect indicator of a cell's ability to amplify DNA.

The cell lines used in this report are cloned lines of rat liver epithelial cells which were developed to study the relationship between selected paratumorigenic phenotypes (such as growth in soft agar and differential isozyme expression) and the ability of these cells to form tumors when injected into newborn isogenic rats (28, 29). The studies, investigations, performed with clonal subpopulations which varied in their tumorigenicity (28), have shown conclusively that these phenotypic markers do not co-segregate precisely with tumorigenicity (30). The extensive characterization of this in vitro system qualifies these cells as ideal candidates for the further investigation of the relationship between gene amplification and tumorigenicity.

WB20 is a nontransformed cell line derived from a normal adult rat liver which was predominantly diploid (31). Transformation of this cell line with N-methyl-N'-nitro-N-nitrosoguanidine produced a phenotypically heterogeneous population from which was isolated many subclones with altered properties resembling those seen in hepatocarcinogenesis in vivo (32). The cell lines designated GP and GN were selected from this population on the basis of whether they were histochemically γ-glutamyl transpeptidase-negative (GN) or γ-glutamyl transpeptidase-positive (GP). Cloned lines of GN and GP cells were propagated and extensively characterized for growth conditions, DNA content, isozyme profiles, and tumorigenicity (28, 29). Some of these cloned lines were used in this study and in parallel studies reconfirming tumorigenicity.

In this study, we have examined the relationship between genetic instability and tumorigenicity using gene amplification as a marker for the genetic instability. Specifically, we have asked whether the ability of a cell to amplify DNA sequences correlates with its ability to form tumors. Our results demonstrate a strong correlation between tumorigenicity and at least one measure of genomic instability, CAD gene amplification.

**EXPERIMENTAL PROCEDURES**

**Cells and Culture Conditions**—Rat liver epithelial cell lines were developed by Tsao et al. (32) and provided to us by J. W. Grisham. All cell lines were grown in complete medium consisting of α-minimal essential medium (α-MEM) supplemented with 10% dialyzed fetal bovine serum (GIBCO), 100 units/ml penicillin, and 100 μg/ml streptomycin. Stock cell lines were used for a maximum period of 3 months, at which time frozen aliquots of cells were thawed for use in subsequent experiments to minimize changes resulting from extended propagation. As an additional control, dialyzed serum from a single lot was used in all experiments. The aliquots of frozen cells used in these experiments were the same as those used for previous and ongoing tumorigenicity studies. At the conclusion of these experiments, the aliquots were reconfirmed in their respective tumorigenicities.

**Plating Efficiencies and Cell Cycle Times**—To determine the plating efficiencies of unselected lines, 100 cells of each were seeded into 100-mm diameter dishes containing complete medium. After 8–10 h, this medium was replaced with fresh medium to remove any unattached cells. Colonies appeared within 5–7 days; they were fixed with 3:1 methanol:acetic acid, stained with 2% Giemsa (Gurr's), and those with greater than 50 cells were counted. The cell cycle time for each line was determined by plating cells into 100-mm diameter dishes at 2 × 10⁵ cells/dish. Each day, plates were trypsinized and the total number of cells determined. The cell cycle time presented in Table I is the time required for cells to double during exponential growth in complete medium.

**Drug Selections**—For all selection experiments, cells were seeded into complete medium at the appropriate density, allowed to attach for 8–10 h, and then exposed to PALA by replacing this medium with medium supplemented with the drug; PALA was obtained from the Drug Evaluation Branch of the National Cancer Institute and Drs. Von Hoff and Needham-VanDewerker. Except for additional replacement with fresh selection medium each week, cells grew undisturbed until colonies were visible: colonies selected in low PALA concentrations (3 × LD₅₀) were visible within 6–9 days; colonies resistant to higher concentrations required 2–4 weeks of growth. Colonies were then fixed, stained, and counted as described above. The incidence of PALA resistance (percent survival, Table II) is the proportion of cells that grew resistant colonies after treatment. PALA has no effect on plating efficiencies of the cells in medium without drug (100% survival). To minimize variation between experiments, these plating efficiencies were determined independently and in triplicate for each drug-selection experiment. LD₅₀ values (Table I) represent the concentration of PALA that allows 50% survival and were estimated by interpolation from values of percent survival in 5, 10, and 20 μM PALA; these values were determined in triplicate at both the beginning and end of our experiments.

**Subcloning PALA-resistant Cells**—Cells from individual PALA-resistant colonies were carefully scraped from the bottom of 100-mm Petri dishes using a sterile micropipette tip and were collected in a 25-μl drop of PALA medium. These cells were transferred to a 24-well cloning dish (Falcon No. 3047) and expanded in the same concentration of PALA-containing medium (3 or 9 × LD₅₀) until a total of 4 × 10⁵ cells had been obtained. A small portion of these cells (10%) was frozen in 90% dialyzed serum, 10% dimethyl sulfoxide; genomic DNA was isolated from the remainder (see below).

**DNA Cytologrammetric Analysis**—Cells were prepared for analysis by the procedure of Gray and Coffino (33) with the following modifications: fixed cells were treated with 5 mg/ml RNase A for 30 min at 37°C, then stained with 0.5 μg/ml propidium iodide for 30 min at room temperature. Cell suspensions (2–5 × 10⁴ cells/ml) were kept on ice and analyzed at a flow rate of approximately 500 cells/s using a Becton-Dickinson FACS IV with an argon laser (488 nm). The average cellular DNA content presented in Table I are relative to karyotypically determined diploid (2n) rat cell standard, WB-F344 (31).

**Molecular Analyses**—Genomic DNA was isolated as described in Brown et al. (34). Briefly, cells were lysed by the addition of 0.1 M Tris-HCl (pH 8), 0.1 M EDTA, 0.01 M sodium chloride, 0.02% sodium dodecyl sulfate, treated with proteinase K, extracted with phenol-chloroform (1:1), and treated with RNase A. DNA was quantitated spectrophotometrically and the indicated amounts electroforesed on 0.7% agarose gels after digestion with EcoRI. DNA was transferred to nitrocellulose (35) and hybridized to probes at 65°C under standard conditions: 10–20 μg/ml probe DNA, 0.45 M sodium chloride, 0.045 M sodium citrate, 1% SDS, 0.5 X Denhardt's solution, and 50 μg/ml salmon sperm DNA. Radioactive probes were prepared by either nick translation or randomly primed DNA synthesis (multipipette system, Amersham Corp.) using [³²P]dCTP and had average specific activities of 10⁶ cpm/μg and 10⁹ counts/min/μg, respectively.

**RESULTS**

**Characteristics of Rat Liver Epithelial Cell Lines**—The cell lines used in this report are cloned lines of rat liver epithelial cells which differ in their ability to form tumors when injected into newborn syngeneic rats. The parental cell line, WB20, shows a fairly homogeneous morphology, exhibiting small polygonal cells of uniform size which grow in a monolayer. In
Morphology

C, Southern and a androgen binding protein fragment; each ratio hybridized each lane and contrast, (lane typically under identical conditions are mined lines amplification ability GN subpopulations DNA encoding a rat androgen binding protein (53); the other (lane d) was hybridized to only the CAD probe. The number at the bottom of each lane reflects the ratio between the densities of hybridization of the 3.4-kilobase (kb) CAD fragment and the 6-kb androgen binding protein fragment; each ratio has been normalized to that obtained for WB20 (lane a). Values for the intensity of hybridization were determined by desitometry and were within the range showing a linear relationship between the amount of DNA in each lane and the amount of hybridization (not shown).

Contrast, the GP and GN cell lines are typified by varying amounts of heterogeneity in size, adhesion, and growth properties. Fig. 1 shows the morphology of WB20, GN5, and GP9, three of the six cell lines used in this study. WB20 and GP3 cells do not produce tumors when transplanted into newborn syngeneic rats (Fischer 344), whereas the other four lines used in this study produced tumors in 11–100% of the recipients under identical conditions (see Table I and Ref. 29). Table I also shows growth parameters which are important in the determination of drug resistance. Although all of these lines are quite similar in their cell cycle time, plating efficiency, and initial sensitivity to PALA, any differences evident in these determinations were accounted for in the assay for amplification ability (see below).

Previous characterizations of the DNA content of these cell lines have shown that cells of the GP subpopulations are typically hyperdiploid or hypotetraploid, whereas cells of the GN subpopulations are pseudodiploid (28). We have reinterpreted the DNA content of the cell lines used in this study and confirmed the original observation (see Table I). The CAD copy number of the initial unselected population in three cell lines used in this study has been quantitated using Southern hybridization. Fig. 2 shows that the WB20, GN5, and GP9 cell populations unchallenged by PALA each contain one genome equivalent of the CAD gene.

**Table 1** Characteristics of rat epithelial cell lines

|                | WB20 | GP3 | GN5 | GP2 | GP9 | GN6 |
|----------------|------|-----|-----|-----|-----|-----|
| Tumorigenicity (%) | 0.0  | 11  | 12.5 | 16  | 100 | 100 |
| Cell cycle time (h)  | 13   | 14  | 12  | 16  | 14  |      |
| Plating efficiency (%) | 78   | 26.5  | 84  | 48  | 57  | 50  |
| LD50 (PALA) (μM)    | 12.5 | 6   | 5   | 9   | 11  |      |
| DNA content/cell     | 2.3n | 2.8n| 2.2n| 3.6n| 3.6n| 1.9n|

Tumorigenicity and Drug Resistance—Using the cell lines described above, the relationship between tumorigenicity and genetic instability was studied by determining the ability of each line to generate PALA-resistant colonies. To obtain initial estimates of the incidence of PALA resistance in these rat epithelial lines, cells from each line were seeded into culture dishes at several different densities and then selected using medium with a PALA concentration equivalent to nine times the LD50 (9 × LD50) of each cell line; similar levels of this drug have previously been shown to provide selective pressure that is sufficient to allow only the growth of hamster cells having amplified CAD genes (36). Results of this experiment indicated that tumorigenic cells give rise to colonies resistant to this drug levels more often than not tumorigenic cells; representative culture dishes illustrating the difference between one of the most and least tumorigenic lines (GP9 and WB20, respectively) are shown in Fig. 3. To more accurately define these differences, cells from each line were plated at densities that yielded between 10 and 100 colonies/dish upon selection. The incidences of PALA resistance, calculated from the results of three independent experiments, are presented in Table II (percent survival at 9 × LD50). These results verified the striking differences observed earlier, with the most tumorigenic cells forming resistant colonies 73 times more often than do cells that do not form tumors. Similar results were obtained when the WB20, GN5, and GP9 cell populations were placed in the identical concentration of PALA (60 μM); the correlation with tumorigenicity held. The relative abilities to generate PALA-resistant colonies was 1 × for the 0% tumorigenic WB20 line, 7 × for the 11% tumorigenic GN5 line, and 65 × for the 100% tumorigenic GP9 line. In addition, both these results revealed a direct correlation between the degree of tumorigenicity of each cell line and its ability to form resistant colonies.

To determine the minimum concentration of PALA that would show a similar relationship between these two variables, three lines were selected with two lower drug concentrations: 3 × and 6 × LD50 (Table II). Selection with the latter concentration showed a relationship similar to that obtained for 9 × LD50, although the differences between the lines were not as great. This relationship was not observed after selection at the lower concentration, 3 × LD50; although both tumorigenic lines formed resistant colonies more often that the WB20 line, the incidences of resistance of these lines was no longer directly correlated with their degree of tumorigenicity.

**Fig. 1.** Morphology of rat liver epithelial cells. Phase contrast view of unselected cells: A, WB20; B, GN5; and C, GP9. Magnification 100 x.

**Fig. 2.** Quantitation of CAD genes in unselected cell lines. Southern analysis of rat genomic DNA isolated from WB20 (lane a), GN5 (lane b), and GP9 cells (lanes c and d). The DNA in each lane was digested with EcoRI. One portion of the blot (lanes a–c) was hybridized to probes derived from both a hamster CAD cDNA (52) and a cDNA encoding a rat androgen binding protein (53); the other (lane d) was hybridized to only the CAD probe. The number at the bottom of each lane reflects the ratio between the densities of hybridization of the 3.4-kilobase (kb) CAD fragment and the 6-kb androgen binding protein fragment; each ratio has been normalized to that obtained for WB20 (lane a). Values for the intensity of hybridization were determined by desitometry and were within the range showing a linear relationship between the amount of DNA in each lane and the amount of hybridization (not shown).
Gene Amplification and Tumorigenicity

**Fig. 3. Incidence of PALA resistance at 9 × LD_{50}.** PALA-resistant colonies obtained after selection of 10^6 WB20 cells in 112.5 μM PALA (A) or 5 × 10^4 GP9 cells in 81 μM PALA (B); LD_{50} for PALA is 12.5 and 9 μM for WB20 and GP9, respectively (see Table I). For each line, cells were seeded into 100-mm diameter dishes containing complete medium. After 9 h, this medium was removed and replaced by medium supplemented with PALA. Except for additional replacements with fresh medium each week, cells were allowed to grow undisturbed in the drug for approximately 4 weeks, fixed, and stained. For details see “Experimental Procedures.”

**Table II.**

| Cell line | Tumorogenicity | 3 × LD_{50} | 6 × LD_{50} | 9 × LD_{50} |
|-----------|---------------|-------------|-------------|-------------|
| WB20      | 0             | 2.1 ± 0.1^* (1 ×) | 0.011 ± 0.001 (1 ×) | 0.0013 ± 0.0005 (1 ×) |
| GN5       | 11            | 45.3 ± 1.0 (22 ×) | 0.100 ± 0.007 (9 ×) | 0.0242 ± 0.0055 (18 ×) |
| GP2       | 12.5          | ND^a         | ND           | ND          |
| GP9       | 100           | 16.4 ± 0.8 (8 ×) | 0.253 ± 0.023 (23 ×) | 0.0950 ± 0.0642 (73 ×) |
| GN6       | 100           | ND           | ND           | ND          |

^a Tumorogenicity assayed in cells incubated in trace amounts of serum (see Ref. 29).

^b Mean ± S.D. of three independent determinations, each performed in triplicate.

^c For each drug concentration, the values within the parentheses represent the relative incidences of resistance to PALA normalized to the WB20 value.

^d ND, not determined.

**Fig. 4. CAD gene amplification in PALA-resistant subclones.** A, eight independent subclones were expanded from individual PALA-resistant colonies selected from the WB20 line; four were resistant to PALA concentrations equivalent to 3 × LD_{50} (lanes a–d), and four were resistant to 9 × LD_{50} (lanes e–h). Genomic DNA from each subclone was isolated, digested with EcoRI, and 7.5 μg electrophoresed on a lane of 0.7% agarose gel. DNA from this gel was transferred to nitrocellulose and hybridized to the CAD probe; the 3.4-kb fragment revealed by this probe is shown (top). This probe was then removed and the filter rehybridized to probes derived from rat cDNAs encoding transforming growth factor-α (TGFα) (54) and androgren binding protein (ABP); the 12-kb transforming growth factor-α and 16-kb androgen binding protein fragments revealed by these probes are shown (middle). B, Southern analysis of DNA from PALA-resistant subclones of the GP9 cell line; analysis is the same as that performed in A.

DNA from 16 independent, PALA-resistant lines (subclones) was analyzed for increases in gene copy number; each of these subclones was expanded from individual PALA-resistant colonies that had been chosen at random. Half of these subclones, four selected from the WB20 line and four from the GP9 line, were capable of growing in medium containing drug concentrations equivalent to 3 × LD_{50}; the other half were capable of growing in media containing drug concentrations equivalent to 9 × LD_{50}. DNA from each subclone grown in a drug concentration equivalent to its respective 3 × LD_{50} was hybridized to the CAD probe. Fig. 4 (lanes a–d) reveals that each had the same number of CAD genes and that this number is the same as the number quantitated in cells of the unselected population (data not shown). These results indicate that selection using low drug levels provides insufficient se// selective pressure for amplification; thus, the incidence of drug resistance for these lines at this concentration (Table II, percent survival 3 × LD_{50}) does not accurately reflect the ability of these lines to amplify the CAD gene. In contrast to the results obtained for 3 × LD_{50}, similar analyses at 9 × LD_{50} of four WB20 and four GP9 PALA-resistant subclones revealed that amplification of the CAD gene had occurred in each (Fig. 4, compare lanes e–h with lanes a–d); thus, growth of these cells in higher concentrations of PALA required
amplification of the CAD gene. The extent of CAD gene amplification in the WB20 subclones appeared to be twice as great as that observed in the GP9 subclones; dilution analysis showed that the former had a 4-fold increase in CAD sequences, whereas the latter showed a duplication of this gene (Fig. 5). Taken together, our results not only indicate that there is a striking parallel between the tumorigenic potential of these lines and their ability to form PALA-resistant colonies but also show that the basis of this relationship lies in their abilities to amplify the CAD gene.

DISCUSSION

This report formally establishes the relationship between genomic instability, as measured by CAD gene amplification, and tumorigenicity. Our results show that the ability of these rat liver epithelial cell lines to amplify the CAD gene is strongly correlated with their ability to form tumors after injection into day-old syngeneic rats. GP9 cells, a highly tumorigenic line, became resistant to PALA greater than 70 times more often than those of the nontumorigenic WB20 line; the incidences of CAD gene amplification in the other lines were also correlated with their degree of tumorigenicity and were thus intermediate to the values obtained for GP9 and WB20. Hybridization analyses of various probes to DNA from independent PALA-resistant subclones showed that in each case, resistance was due to CAD gene amplification; this result corroborates previous reports that amplification of this gene appears to be the only mechanism of resistance to PALA. We believe that measurement of CAD gene amplification should provide a useful tool for examining the role that genomic instability plays in the progression of normal cells to neoplastic cells.

Recent studies of neoplastic cells have revealed that alterations in cellular proto-oncogenes can be created by point mutations, translocations, and amplification. Amplification of oncogenes has been extensively studied and found to be a common mechanism for increased expression of these genes. Seeger and co-workers (37), for example, found that 23 out of 63 primary tumors of human neuroblastomas exhibited N-myc amplification and that amplification of this gene was most frequent in the tumors that were the most malignant. However, most studies to date do not suggest a mechanistic relationship between neoplastic transformation and amplification of oncogenes. Their studies have shown conclusively that variation in the expression of any one oncogene is not a universal property of all tumor types (8, 37). Thus, in this study, we are not suggesting that amplification of any particular gene is the cause of tumorigenicity but we suggest that gene amplification reflects the underlying genetic instability of a tumorigenic cell which allows it to generate phenotypic diversity and progress to malignancy.

Measuring the Incidence of Gene Amplification—The advantages of using the method described in this report are several. First, this assay is rapid. It consists of determination of the plating efficiency and LD$_{50}$ followed by subsequent determination of the incidence of drug resistance (percent survival) at 9 × LD$_{50}$. Although the time required for these measurements is dependent on the doubling times of the cells, determinations for the lines used in this study required only approximately 2 months. Second, this assay is direct. Since amplification is the only reported mechanism for resistance to PALA, complications in interpreting the underlying basis of resistance appear to be avoided by using this drug, thus allowing direct assessment of amplification ability from the incidence of drug resistance. Third, this assay is quantitative; it allows the comparison of cell lines with differing growth properties or sensitivity to the drug. Finally, this assay determines the incidence of gene amplification in these cells in contrast to other studies (38, 39) which measure rate. Both values are determined by 1) the rate at which the cells are generated, 2) their intrinsic stability, and 3) their growth rates relative to the rest of the cell population. This assay, which measures incidence, accounts for these variables and provides a sensitive and direct measurement of gene amplification.

Rate measurements, however, have an additional component of time and the preceding three variables should be taken into account at each step in the selection (rate determination).

In designing this assay to measure amplification ability in these lines, we wanted to develop as rapid an assay as possible. To this end, we chose to use a single step selection protocol and determine the minimal concentration of the selective drug that allowed the correlation of tumorigenicity with amplification ability. Cells placed in a low stringency of selection (3 × LD$_{50}$) produced colonies within 1 week. Rechallenging these colonies with the same drug concentration during clonal propagation revealed that a large proportion of the cells were killed, with the remainder (those used for molecular analyses) growing very slowly (data not shown). This result indicates that these cells were not completely resistant to PALA but were merely capable of tolerating the low drug concentrations. In contrast, cells placed in a higher stringency of selection (9 × LD$_{50}$) take 2–4 weeks to emerge as colonies. Cells from virtually all of these colonies were resistant to killing and grew in the drug at a rate similar to that of unselected cells during clonal propagation (data not shown). These observations are consistent with our results showing that subclones resistant to concentrations of PALA equivalent to 3 × LD$_{50}$ were not amplified, whereas those grown at 9 × LD$_{50}$ were amplified in each case (Fig. 4). It is important to note that at drug concentrations greater than 9 × LD$_{50}$, greater amplification results and the relationship between the incidence of drug resistance and tumorigenicity remains (data not shown).

Two variables are especially important in the utilization of our method to determine amplification ability: the plating efficiency and LD$_{50}$ of each line. The plating efficiency, the base line number of colonies obtained without selection (defined as 100% survival), is the value to which the number of colonies obtained after drug selection is compared. Overesti-
mating or underestimating this number would lead to an incorrect determination of the incidence of drug resistance. Likewise, accurate determination of the LD<sub>50</sub>, the pivotal value for the final stringency of selection, is required. Table I shows that the plating efficiencies and especially the LD<sub>50</sub> values for the cell lines used in this study are similar and only small adjustments were necessary to compare the data between cell lines. Even without adjustments (normalization to these values), the relationship between the ability to form tumors and the ability to amplify held.<sup>2</sup>

**Gene Amplification Correlates with Tumorigenicity**—The correlation we find between PALA resistance and tumorigenicity in these cell lines is striking. Although amplification of the CAD gene is the only reported mechanism of PALA resistance, we wanted to confirm that the resistance to 9×LD<sub>50</sub> of the different tumorigenic lines was due to amplification and did not result from a novel mechanism. Fig. 4 demonstrates this point. Not only confirming that the basis of the frequency of PALA resistance is due solely to amplification but also providing information on the extent of gene amplification. We show that the extent of amplification is identical for each of the four subclones isolated from each line selected in 9×LD<sub>50</sub>. We also note that the extent of CAD gene amplification measured in the PALA-resistant W220 subclones is greater than that seen in the PALA-resistant GP9 subclones. Dilution studies demonstrate that the W220 subclones are increased 4-fold in CAD gene copy number, whereas GP9 subclones are increased 2-fold in CAD gene copy number (Fig. 5). A trivial explanation for the increase in CAD copy number, in all of these subclones, could be polyploidy of the cells. This explanation has been eliminated by the quantitation studies done in Fig. 4 showing an increase in CAD gene copy number relative to two internal standards: the gene coding for the rat androgen binding protein and that coding for transforming growth factor α. Polyploid cells would show no relative increase in the CAD gene copy number. In addition, we have performed flow cytometry on the PALA-resistant subclones to determine their DNA contents. Each subclone shows a DNA content identical to that seen in the unselected parent population as indicated in Table I. Thus, all available evidence points to gene amplification as the basis for the increase in CAD copy number and the resultant resistance to PALA. Although it is clear that we are not looking at polyploidy, the increase in gene copy number could be due to aneuploidy or a duplication of the chromosomal arm which contains the CAD gene. Karyotyping of the resistant cell lines is now underway. Irrespective of how it arises, the correlation between the ability to increase the CAD copy number and the ability to form tumors is strong.

Could a greater cellular DNA content be the basis of the increased incidence of PALA resistance in the highly tumorigenic GP9 cell line? We reject this interpretation because we find the ploidy of the cell line does not predict its ability to amplify the CAD gene, whereas the tumorigenicity of the cell line does. We have extended our studies to a GN cell line which is comparable in tumorigenicity to GP9 (100%), but is pseudodiploid (GN6), and an additional GP cell line which is comparable to the GN5 in tumorigenicity (11<sup>versus</sup>12.5%) but is hyperdiploid (GP2). In each instance, the incidence of PALA resistance co-segregates with tumorigenicity and not with the pseudodiploid of hyperdiploid DNA content (see Table II). Thus, although the extent of CAD gene copy number may be related to the DNA content per cell, the ability to amplify the gene (the incidence of amplification) is not.

A previous study has reported accelerated evolution of drug resistance in a tumorigenic cell population based on a measurement of rate of dihydrofolate reductase gene amplification (38). In both that study and this one, similar conclusions about the role of genetic instability, as it relates to tumorigenicity, were reached. Sager and co-workers (38) reported that the rate of the evolution of methotrexate resistance was greater in one tumorigenic cell line than in its nontumorigenic counterpart. We attempted such a rate analysis with our cell lines for both methotrexate and PALA resistance (data not shown) and found no correlation between rate of amplification and tumorigenicity. We believe that this lack of correlation may be due to variables, such as cell cycle time, plating efficiency, or drug sensitivity (see above), which may change at each selection step in the previously published method and are not accounted for. A further basis for the lack of correlation may be due to our examination of a greater number of cell lines. The single step selection method used in this study provides a better approach because it eliminates differences in cycle time, plating efficiency, and LD<sub>50</sub> which would have to be recharacterized at each step in the multiplicative step method.

The studies described herein, as well as earlier observations, have reinforced the suggestion that genetic instability underlies the neoplastic transformation of a cell. Our previous studies have shown several parallels between the evolution of a drug-resistant population and the evolution of a tumorigenic population. As they emerge, both generate a spectrum of chromosomal abnormalities which include translocations, chromosomal breakage, and the production of extrachromosomal DNA (4, 40–42).<sup>2</sup> Both occur through step-wise processes and produce widely heterogeneous populations which then progress through several step-wise selections to the terminal phenotype (43–45). Both can be enhanced by treatment with carcinogens or tumor promoters (46–49). In addition, both the enhancement of dihydrofolate reductase amplification (50) and chemical transformation of cultured cells (51) are cell cycle-dependent. The emergence of drug resistance is of major clinical importance; this study establishes that the populations of cells that are most tumorigenic are the very populations of cells which develop drug resistance most readily. If we can gain an understanding of the molecular mechanisms which generate phenotypic diversity in tumor cells, it may be possible to modulate the frequency to the benefit of therapeutic efficiency. The basis of the transition of a cell population from a regulated stable genome to that of instability and heterogeneity typical of neoplastic and drug-resistant populations is unknown. Studies which contribute to our understanding of the cellular and biochemical control of gene amplification and how they relate to tumorigenicity may provide insights into the molecular basis of this transition.

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