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Genetics

Phenotypic evolution of cells resistant to bromodeoxyuridine

(drug resistance/somatic cell genetics/thymidine kinase)

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ABSTRACT **Variants resistant to bromodeoxyuridine (BrdUrd) and deficient in thymidine kinase (ATP:thymidine 5′-phosphotransferase; EC 2.7.1.21) have been obtained from V79 Chinese hamster cells by a combination of spontaneous and drug-induced change. Initial mutations take place in wild-type populations as a facilitating step to give partially resistant clones that can be isolated by one-step selection in BrdUrd. When these tolerant populations are maintained for extended periods in BrdUrd-containing medium, a gradual phenotypic transition occurs in which BrdUrd appears to act as an inductive as well as selective agent. Thymidine kinase activity declines logarithmically over an interval of 8–10 weeks as the growth rate rises and the cells become completely resistant to BrdUrd. Relative plating efficiency in hypoxanthine/aminopterin/thymidine medium also decreases, but the decrease is not coordinate with shifts in thymidine kinase activity. The potential for colony formation in hypoxanthine/aminopterin/thymidine continues to decrease exponentially for at least 18 weeks after thymidine kinase deficiency and complete resistance to BrdUrd have been established. These phenotypic modifications are continuous or multistep in character; by clonal analysis they are found to occur in most, if not all, cells maintained in the presence of BrdUrd. Populations in transition thus come to be complex mosaics of different phenotypes that are comparatively stable if isolated in drug-free medium. The progressive evolution of cells resistant to BrdUrd will require new models for an underlying explanation.**

Resistance to bromodeoxyuridine (BrdUrd) is a well-known marker in animal cells and is usually linked to a deficiency in thymidine kinase (TK) (ATP:thymidine 5′-phosphotransferase; EC 2.7.1.21) activity (1, 2). TK-deficient cells do not incorporate thymidine or BrdUrd and, accordingly, fail to survive in hypoxanthine/aminopterin/thymidine (HAT) medium (3, 4) which contains aminopterin to block *de novo* synthesis of nucleotides. Mapping studies show that TK is coded for by a nuclear locus that is autosomal in human cells and probably in other cell types as well (5). On the basis of these facts, a simple genetic model has been proposed to account for BrdUrd resistance and TK deficiency (2, 6). Because fully resistant variants are rarely if ever obtained by one-step selection in BrdUrd (1, 6, 7), it is assumed that conversion proceeds by a sequence of two recessive mutations at the *TK* locus. Although the putative heterozygote has not been isolated directly from wild-type populations, cells with similar properties can be obtained from BrdUrd-resistant variants by back-selection in HAT medium. Such revertants regain partial TK activity and can then be used for studies of rereversion in the presence of BrdUrd. This process has been characterized as a simple one-step mutation from $TK^+/-$ to $TK^-/-$ and viewed as a general model of mutagenesis in somatic cells (8–10).

Other concepts of BrdUrd resistance and TK deficiency need to be considered, however, because evidence for a double mutation at the *TK* locus is indirect and no detailed study has been made of the phenotypic transition from wild-type to fully resistant cells. A two-stage model also fails to account for the prolonged period of growth in BrdUrd that is nearly always required for the emergence of fully resistant strains (1, 6, 7). The significance of this observation could be that resistance is a multistep rather than a two-stage process, taking place gradually in the presence of BrdUrd by a cascade of genetic or even epigenetic (11, 12) events. To test the multistep concept we have monitored the acquisition of BrdUrd resistance by V79 Chinese hamster cells during long-term culture in BrdUrd. Particular attention has been given to the kinetics of transition; the time course for the two models should be predictably different. Our data show that conversion is indeed a multistep process and that it proceeds by a combination of spontaneous mutation and drug-induced change, a paradigm that may apply to other types of variation in somatic cells as well.

MATERIALS AND METHODS

All experiments were performed with clone V79-5, a near-diploid line of Chinese hamster cells (13). Stock populations were maintained as monolayers in prescription bottles (Brockway Glass, Brockway, PA) in basal medium containing 10% (vol/vol) fetal calf serum (Microbiological Associates, Bethesda, MD, or Flow Laboratories, McLean, VA) and 90% (vol/vol) Dulbecco's modification of Eagle's medium (containing 4.5 mg of glucose per ml with pyruvate omitted). This basal medium was supplemented before use with sodium penicillin G (60 μg/ml), streptomycin (50 μg/ml), and L-glutamine (100 μg/ml). HAT medium (3, 4) was prepared by adding 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine to basal medium. Stock solutions of BrdUrd and iododeoxyuridine (IdUrd) were kept in opaque bottles. However, no attempt was made to protect cultures containing these inhibitors from nominal exposure to light, although none received intense illumination. Parent V79-5 cells and derivatives were occasionally screened by culture methods for mycoplasma; none was found. Plating experiments were performed in 60-mm petri dishes that were maintained at 37°C in a humidified CO_2 incubator with fluid changes once or twice a week. When colonies were well formed, experiments were terminated by staining the cultures for 30 min in a saturated solution of crystal violet in 0.85% NaCl, after which the dishes were washed in water and air dried. Fluctuation tests and measurements of TK were carried out as described (14, 15).

RESULTS

Isolation of One-Step Variants in BrdUrd. We found initially that stable variants with partial resistance could readily be obtained by plating wild-type V79-5 cells into basal medium

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Abbreviations: TK, thymidine kinase; HAT, hypoxanthine/aminopterin/thymidine; basal medium, 10% (vol/vol) fetal calf serum/90% (vol/vol) Dulbecco's modification of Eagles's medium without pyruvate and containing 4.5 mg of glucose per ml.

containing BrdUrd (10 μg/ml). Mass populations were destroyed in 2–4 weeks, but a few cells survived and gave rise to discrete, progressively growing colonies. When isolated, such variants retained the ability to grow in BrdUrd at 10 μg/ml, even after subculture in drug-free medium for two passages or more. The colony-forming fraction (10^{-4}–10^{-5}) varied with the fetal calf serum used, presumably owing to variation in serum thymidine content. Colony formation was proportional to the size of the inoculum except at high population densities, at which the assay was obscured by persisting masses of sensitive cells.

Table 1 summarizes the properties of these first-stage variants. Each subline shown originated from a single colony in BrdUrd and was grown in drug-free medium for 15 generations or more before testing. Resistance to BrdUrd was stably enhanced over the V79-5 population in each case but fell far short of the response typical of fully resistant cells, which grow freely in BrdUrd at concentrations of 1000 μg/ml or more (15). All of the variant clones retained the ability to grow in HAT medium and showed reduced, but still appreciable, levels of TK activity. Freshly isolated clones showed some variation in chromosome number. Two populations (94-6 and 204-2) were initially bimodal but became near-diploid in continuing culture; others (204-3 and 204-6) stabilized at near-tetraploid levels (Table 1). No obvious correlation was observed between chromosome number and marker characteristics.

We performed fluctuation tests (14, 16) with V79 cells to see whether the partial resistance noted in one-step variants was an adaptive response to the selecting drug or if variation had occurred independently by a spontaneous and random process. Background studies with replicate platings of V79-5 cells in BrdUrd (10 μg/ml) showed that variance in the colony counts was approximately equal to the mean, thus falling within the limits of Poisson sampling error (data not shown). The variance was then determined for similar assays between independent sublines, with groups of 15 clones that had been derived in each experiment from single cells of a small, common wild-type colony. These cultures were grown in parallel to mass populations and assayed by plating graded numbers of cells (10^4–10^5) from each subline into petri dishes containing BrdUrd (10 μg/ml) in basal medium.

Table 2 gives the results of these studies. In all three experiments the variance in average number of colonies per subline definitely exceeded the mean and thus was greater than Poisson sampling error. χ^2 tests were also strongly positive ($P < 0.001$). These results make clear that first-step variants with partial resistance to BrdUrd arise spontaneously and at random, with the characteristics of a mutational process. The frequency of mutation, as estimated by two methods, was approximately 4.7 × 10^{-6}/cell per generation, and showed little variation from one experiment to the next.

Table 1. Characteristics of clones isolated from V79-5 cells by one-step selection in BrdUrd (10 μg/ml)*

Clone	Resistance index†	Plating efficiency in HAT, %	TK activity, cpm/μg protein	Chromosome no.‡
V79-5	0.95	100	5721	22.5 ± 0.17
94-6	15.0	97	1155	21.6 ± 0.14
204-1	17.0	112	501	21.3 ± 0.14
204-2	17.0	97	601	21.9 ± 0.08
204-3	10.5	77	3244	38.7 ± 0.40
204-5	14.0	81	673	22.0 ± 0.19
204-6	14.5	99	817	42.8 ± 0.37
204-7	9.0	82	649	22.5 ± 0.19
204-8	14.5	90	772	21.4 ± 0.29

* All lines were maintained in drug-free medium for 15 generations or more before being assayed.
† Concentration of BrdUrd (μg/ml) that reduces relative plating efficiency to 10%.
‡ Modal value ± SEM.

Table 2. Fluctuation tests for colony formation by V79-5 cells under one-step selection in 10 μg/ml BrdUrd

Variable	Experiment I	Experiment II	Experiment III
No. of independent sublines	15	15	15
Initial cell no.	1	1	1
Mean final cell no. × 10^{-6}	9.52	9.59	26.1
Total cells tested per subline × 10^{-5}	3.3	4.9	5.4
No. of resistant colonies in sublines* × 10^5			
Mean	5.0	2.8	3.6
Median	1.3	2.8	2.1
Variance	143.6	8.8	18.6
Ratio of variance to mean	28.7	3.1	5.2
χ^2	399.6	44.6	72.4
P	<0.001	<0.001	<0.001
Mutation rate per cell per generation × 10^6			
From ref. 17	2.9	5.4	3.6
From ref. 32	7.3	4.4	4.8
Average	5.1	4.9	4.2

* Colony counts were based on 6–12 assayed cultures per subline.

Transitional Changes in Serial Culture. Because spontaneous mutants isolated by one-step selection were only partially resistant to BrdUrd, we maintained the clonal populations in medium containing inhibitor and looked for second-level variants with a well-established TK^- HAT^- phenotype. Fully resistant cells with these characteristics could eventually be obtained if partially resistant clones were subcultured serially for long intervals in BrdUrd (10 μg/ml). However, such variants did not emerge by abrupt, stepwise change. Conversion was effected by a gradual shift in phenotypic pattern that took place over a period of weeks or months and at rates that were different for the loss of TK activity and potential for growth in HAT medium. Under these conditions fluctuation analysis was precluded, but rates of change could be established by following the time course for each variable separately.

Studies made with 94-6 cells serve to illustrate this process. Clone 94-6 was first isolated in BrdUrd (10 μg/ml) (see Table 1) and grew progressively in this medium, although growth rate and plating efficiency in the presence of inhibitor were initially low. During serial passage for 7–8 weeks in the same medium the growth rate gradually increased, and cells were assayed at regular intervals for resistance by plating into BrdUrd at 10 μg/ml and 1000 μg/ml. In both media the plating efficiency rose progressively to 100%, and the cells at later passages were found to be completely resistant to both BrdUrd and IdUrd at 100 μg/ml (Fig. 1).

Periodic assays were also made during subculture of 94-6 cells for TK activity and for colony formation in HAT medium (Fig. 2). The frequency of HAT^+ cells in the 94-6 population declined logarithmically and at a remarkably constant rate (31% per week) throughout the 28-week observation period. If these events represent a probability phenomenon, the $HAT^+ \rightarrow HAT^-$ conversion can be expressed by the relationship $Q = P^n$ (18). In this context, P is the probability per generation that any particular cell will remain HAT^+, Q is the proportion of HAT^+ cells in the population after n generations, and $1 - P$ is the probability of conversion to the HAT^- state. From the data

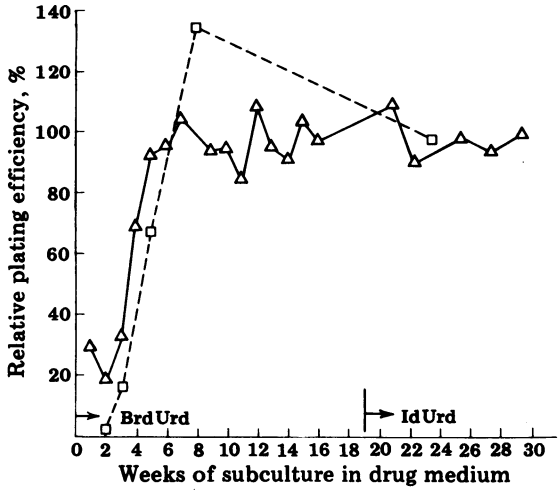

FIG. 1. Relative plating efficiency of 94-6 cells in 10 μg (△—△) and 1000 μg (□---□) of BrdUrd per ml during serial subculture of mass population in 10 μg of BrdUrd per ml (weeks 0–19) and in 100 μg of IdUrd per ml (weeks 19–29). Each point shown represents the average colony count for three to six petri dish cultures.

shown in Fig. 2 for the interval between 6 and 27.5 weeks, $Q = 0.001$ after an estimated 224 generations, and a calculation gives $1 - P = 3.0 \times 10^{-2}$ per generation. This result agrees with the slope of the regression line (−31% per week) when the average number of generations per week (10.4) is taken into account.

As Fig. 2 shows, TK activity in 94-6 cells declined even more rapidly than HAT plating during subculture in BrdUrd. The exponential slope represents a decrease of 76.9% per week, twice as great as the fractional reduction in HAT^+ cells. Thus, loss of TK activity and conversion to the HAT^- state are not coordinate events. The divergence is emphasized further by a leveling off of TK activity after 8–10 weeks, whereas the decrease in incidence of HAT^+ cells continued without a break for 28 weeks or more. Residual TK function in these cells (0.2–0.4% of wild type) presumably represents mitochondrial activity (19), although we have not checked this point directly. The chromosome pattern of the 94-6 population remained remarkably uniform throughout these declines in TK activity and HAT plating potential, with a tight mode at 21 chromosomes (data not shown).

To see whether phenotypic shifts observed in the presence of BrdUrd were reversible at the population level, we performed an experiment with 94-6 cells that had been propagated for only 5 weeks in BrdUrd (10 μg/ml). The mass population was subcultured and maintained for 7 days in drug-free medium, then divided into three sublines which were carried by serial subculture in drug-free medium (basal medium), BrdUrd (10 μg/ml), and HAT medium. Cells from each subline were plated out at weekly intervals in HAT and in BrdUrd (10 μg/ml), and the colony counts were expressed as the HAT/BrdUrd plating ratio (Fig. 3). The subline in BrdUrd continued to show a decline in HAT^+ cells, but with counterselection in HAT medium the process was reversed. TK activity also rose for cells maintained in HAT (data not shown) but did not exceed the level recorded for clone 94-6 at its original isolation from BrdUrd. Subline cells maintained in a neutral medium (basal medium) showed no significant change in the HAT/BrdUrd plating ratio. These observations demonstrate that phenotypic shifts at an early stage are potentially reversible for populations maintained in BrdUrd and that selective pressure is required for transition in either direction.

A question at this point was the generality of temporal changes observed in the presence of BrdUrd. Conceivably, such shifts could be potentiated by a unique mutation in 94-6 cells that was not typical of other variants obtained by selection with BrdUrd. A broader study was therefore initiated with nine partially resistant clones that had originated independently from V79-5 cells by spontaneous mutation. These sublines were cultured in parallel for 25 weeks in BrdUrd (10 μg/ml) and the incidence of HAT^+ cells was determined by periodic plating. Fig. 4 shows that the frequency of HAT^+ cells decreased progressively in almost all of these lines, although the time of onset and rate of decline varied widely between individual clones. At least one variant (204-8) declined at approximately the same rate as 94-6 cells (compare with Fig. 2), but the process was initiated at a later time point. Other clones (204-5, 204-6, and 204-7) showed a distinctly higher rate of conversion from the HAT^+ to the HAT^- state, whereas for clones 94-2 and 204-2

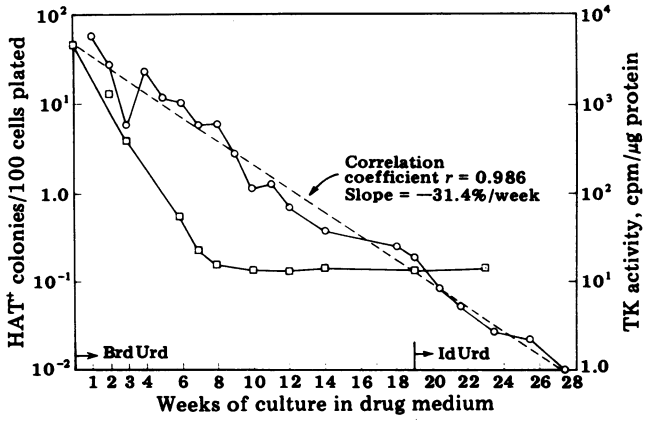

FIG. 2. TK activity (□) and colony formation in HAT medium (○) by 94-6 cells during serial subculture in BrdUrd (10 μg/ml) and IdUrd (100 μg/ml). TK values shown are averages for five samples each. Plating data are based on three to six petri dish cultures per point.

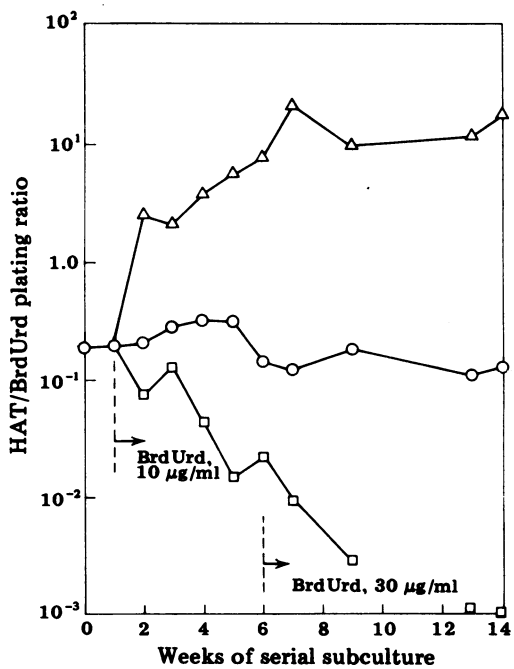

FIG. 3. Selective shifts in subpopulations of 94-6 cells cultivated serially in different media. Each point shown is based on colony counts from six to nine petri dish cultures. Cells subcultured in: △, HAT medium; ○, basal medium; □, BrdUrd (10 μg/ml).

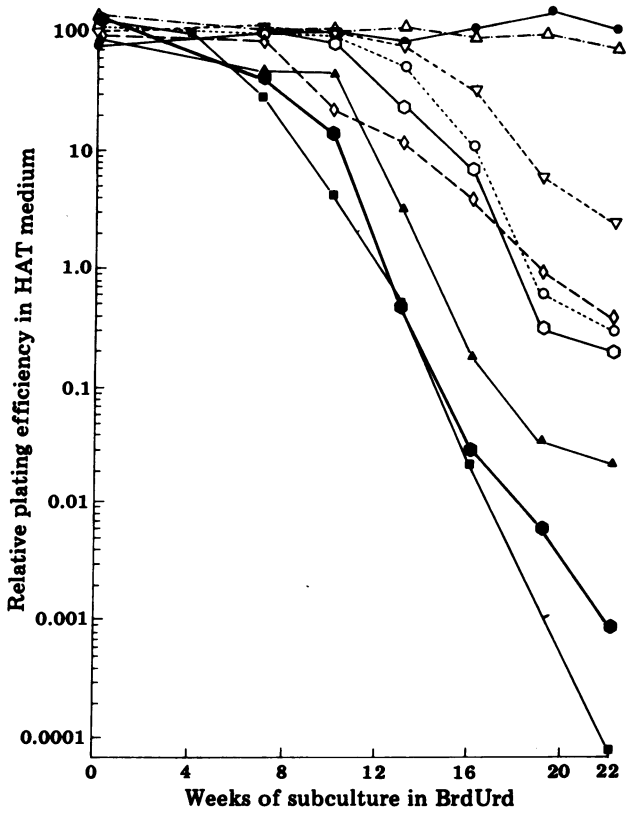

FIG. 4. Evolution of resistant clones isolated independently from V79 cells, under long-term culture in BrdUrd (10 μg/ml). Each point shown is based on colony counts from three to six petri dish cultures. ○, 94-1; △, 94-2; ▽, 101-2; ○, 204-1; ●, 204-2; ▲, 204-5; ■, 204-6; ●, 204-7; ◊, 204-8.

there was little or no decrease in HAT^+ cells even after subculture for 25 weeks in BrdUrd. These data indicate that population changes in the presence of BrdUrd are characteristic of most, if not all, first-stage variants, although the time course follows a pattern that is individually determined.

Clonal Analysis of Population Shifts. One possible explanation for phenotypic remodeling at the population level might be that partially resistant clones become composite mixtures of HAT^+ and HAT^- cells as two contrasting components. The ratio of these stable cell types would then decline under selective pressure in BrdUrd and rise in HAT medium. To test this model, we isolated groups of 30 or more subclones from 94-6

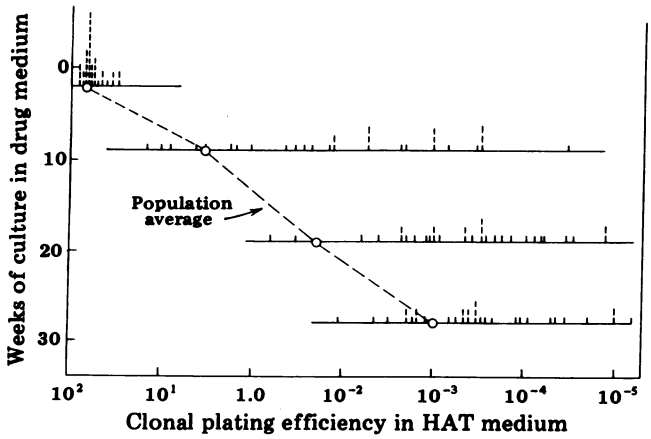

FIG. 5. Plating efficiency in HAT for groups of subclones isolated from mass population of 94-6 cells after 2, 9, 19, and 28 weeks of serial subculture in BrdUrd (10 μg/ml). Values shown for individual clones are based on six petri dish cultures each.

mass populations at each of several passage levels in BrdUrd. The relative plating efficiency in HAT was determined for each subclone soon after isolation in drug-free medium; the results are summarized in Fig. 5. Our data show that subclones from the 94-6 population were comparatively uniform at an early stage, when the parent cells had been subcultured for only 2 weeks in BrdUrd. However, after 9 weeks the 94-6 population had diverged into a broad spectrum of phenotypes, with the frequency of HAT^+ cells varying as much as 10^4 between individual subclones. The incidence of HAT^+ cells in any given subclone proved to be reproducible in successive assays, within the limits of experimental error. With continuing subculture of the 94-6 population in BrdUrd, the range of plating frequencies in HAT among subclones shifted to lower levels, in correlation with the trend in average values observed with the uncloned parent line. However, the latter determinations represent arithmetic means and thus fall to one side of the logarithmic clonal distributions. Overall, our study shows plainly that the 94-6 line is not a simple mixture of two contrasting cell types that shift in frequency as selection proceeds. Instead, the population on subculture in BrdUrd becomes a composite mixture of many different cell types that then retain their individuality in drug-free medium.

Heterogeneity in this system might still represent variation in a minority cell type if these cells were capable of rapid and pleiotropic alteration in the presence of BrdUrd. However, the same result would be obtained if all cells were subject to inductive change on a probability basis. To distinguish between these alternatives, we set up a clonal comparison test, using 94-6 cells that had been growing for only 2 weeks in BrdUrd. From a culture plated out in basal medium we isolated a small colony of 100–200 cells which were used to initiate 10 clonal sublines that were grown to populations of approximately 2×10^5 cells in basal medium. Subcultures from each line were then transferred to medium containing BrdUrd (10 μg/ml) and continued by subculture for 8 weeks. Plating frequencies in HAT were determined at biweekly intervals; the results are shown in Fig. 6. All 10 sublines underwent $HAT^+ \rightarrow HAT^-$ conversion and over much the same time span, although there were clonal differences in rate. Thus, conversion takes place reproducibly in sublines derived from any cell of the original population, and the possibility that phenotypic remodeling can be traced to changes in a minority cell type seems to be ruled out.

DISCUSSION

Our studies reveal a novel mechanism for the evolution of phenotype in cells exposed to BrdUrd. The process begins with one or more spontaneous mutations that establish partial resistance to BrdUrd and confer a unique potential for further change in the presence of the inhibitor. Conversion to the TK^- HAT^- state then takes place by a continuous or multistep process that can be followed by changes in TK activity and plating efficiency in HAT medium. Both variables decline logarithmically over an extended period, but the time course and rate of change for the two processes are distinctively different. Clonal analysis shows that graded shifts occur in most, if not all, cells exposed to BrdUrd, so that populations in transition come to be complex mosaics at the phenotypic level. In every important respect, these changes parallel the segregation of BrdUrd resistance in hybrid cell populations, which we have described previously (15).

Somatic variegation in these systems requires the presence of BrdUrd, which appears to act both as an inductive and selective agent. An inductive role is supported by observations that show that BrdUrd is mutagenic for several markers in mammalian cells (20–22) and that mutagenic action is con-

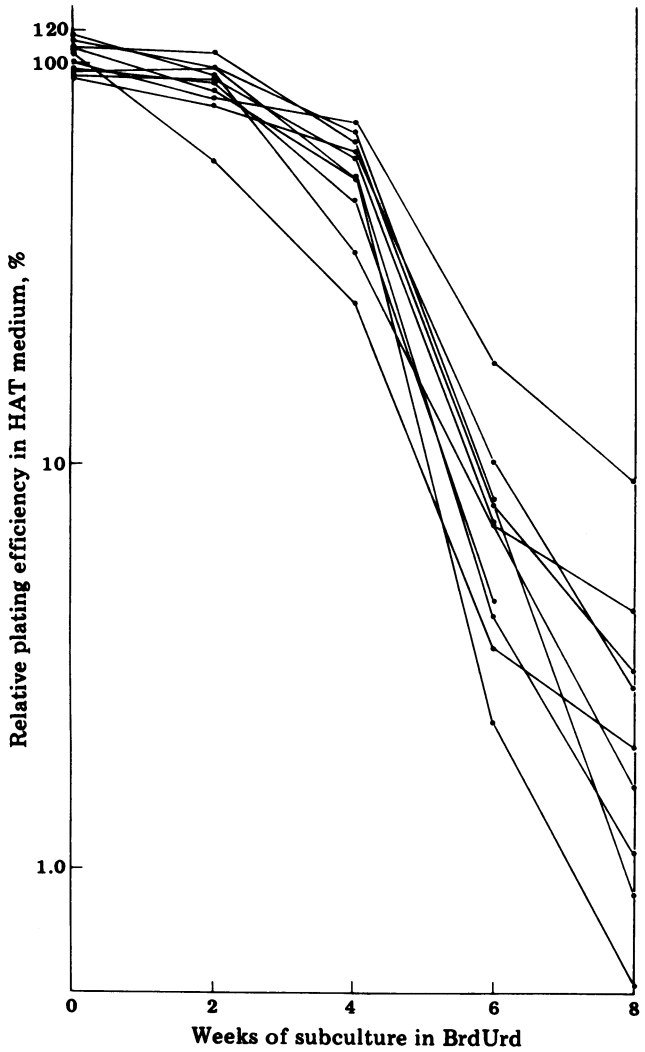

FIG. 6. Progression in plating properties of nine subclones isolated from a single colony of 94-6 cells during serial subculture in BrdUrd (10 μg/ml). Each point shown is based on colony counts from three to six petri dish cultures.

centration dependent (22). Thus, the significance of spontaneous mutations that confer partial resistance may be to render target cells tolerant to a high enough concentration of BrdUrd for changes in TK activity and HAT plating potential to occur at high frequency. The nature of the underlying process is not clear. If the effect of BrdUrd is site specific, it is difficult to account for a graded response because there is no evidence that the *TK* locus is a multisite complex. The possibility of genetic alteration outside the *TK* locus must be kept in mind because our data show a strong discordance in the kinetics of decline for TK activity and growth in HAT medium. Conceivably, suppressors for the *TK* locus may exist and could be amplified in the presence of BrdUrd. Precedent for this model exists in the selective multiplication of dihydrofolate reductase genes in mouse cells, shown by Alt *et al.* (23) to occur during long-term exposure to methotrexate. It is also known that resistance to BrdUrd can be conferred by alterations at the ribonucleotide reductase locus, but in this case TK activity remains intact (24). In any event, the process of suppression, if it occurs, must be confined to the resident *TK* locus because TK^- cells can be readily transformed with TK^+ DNA from viral or cellular sources (25, 26).

The door should perhaps be left open also to epigenetic explanations because differential suppression of gene action by BrdUrd takes place in a number of cell systems (27, 28). The basis for these effects has not been determined, but one possibility is that DNA modification occurs. As a mechanism for altering gene expression, DNA modification has often been discussed (29, 30); more recently, experimental evidence has been provided that site-specific methylation can control expression of the chicken β-globin gene (31). It is therefore conceivable that BrdUrd may induce or select for variants with changes at the *TK* locus that restrict expression but do not affect the coding sequence. As a working hypothesis this concept warrants further study.

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