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Bromodeoxyuridine mutagenesis in mammalian cells: Mutagenesis is independent of the amount of bromouracil in DNA

(Syrian hamster/ouabain/thioguanine/aminopterin/fluorodeoxyuridine)

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ABSTRACT **Studies were undertaken to determine how a line of mutant Syrian hamster melanoma cells (HAB-2E) that displays unlimited growth potential when all of the thymine residues in nuclear DNA are replaced by bromouracil (BrUra) could avoid the deleterious effects of bromodeoxyuridine (BrdUrd) mutagenicity. It was found that BrdUrd could be mutagenic to these cells. However, there was a nonlinear relationship between mutagenicity and the amount of BrUra in the DNA of the HAB-2E cells. With these cells, mutagenicity apparently is determined by the concentration of BrdUrd to which the cells are exposed rather than the amount of BrUra in DNA. These results were obtained with both the induction of ouabain resistance and thioguanine resistance as markers for mutagenesis. The dependence of BrdUrd mutagenicity on BrdUrd concentration was also observed for the parental melanoma cells.**

The thymidine (dThd) analog 5-bromodeoxyuridine (BrdUrd) has various effects on biological systems, including the induction of mutations and the photosensitization of DNA. Although the mutagenicity of BrdUrd was among the first of its effects to be observed (1), the mechanism by which BrdUrd induces mutations remains to be elucidated. One of the earliest, and still most widely accepted, explanations (based on experiments with prokaryotic systems) is that BrdUrd-induced mutations occur as the result of occasional mispairing of the analog during DNA replication (2, 3). However, the results of some of the early studies also suggested that BrdUrd mutagenesis is not simply due to incorporation of the analog into DNA (4).

We have isolated lines of mutant mammalian cells (5–9) whose properties raise many questions about the proposed mechanisms of BrdUrd action, including the mechanisms of mutagenesis. Some of these cell lines are able to grow with essentially 100% of the thymine (Thy) residues in their nuclear DNA replaced by bromouracil (BrUra) (6, 8). If BrdUrd were highly mutagenic for these cells, it would be expected that the ability of the cells to proliferate would be impaired. However, the proliferative capacity of the cells with BrUra totally replacing Thy is apparently unlimited (8).

In most of the experiments, the occurrence of ouabain (OB) resistance was used as the marker for mutagenesis. It has been shown in other systems that OB resistance behaves as a codominant mutation, is determined by nuclear genes, and involves alterations in the properties of the Na^+,K^+-activated plasma membrane ATPase (10–12). In some experiments the occurrence of 6-thioguanine (Sgu) resistance was monitored. Sgu resistance generally is due to hypoxanthine phosphoribosyltransferase (HPRT) deficiency and has been shown to be a recessive mutation.

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MATERIALS AND METHODS

Cell Lines. The characteristics of the cell lines in this study have been described. RPMI 3460 is a pigmented Syrian hamster melanoma line (13). HAB-2 is a line derived from RPMI 3460 by selection for viability with all the Thy residues in nuclear DNA replaced by BrUra (8). To avoid detection of preexisting drug resistant mutants, HAB-2 cells were cloned in the absence of BrdUrd, and a clone called HAB-2E was isolated and used in all the mutagenesis experiments.

Media. RPMI 3460 and HAB-2E cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (E medium). For the exposure of cells to BrdUrd, five different types of modified E media were used: E-B medium contains BrdUrd at 1–40 μM, as indicated below; HAB medium (6) contains 0.1 mM hypoxanthine, 0.4 μM aminopterin (Apt, an inhibitor of *de novo* dThd biosynthesis), and 10 μM BrdUrd; HABT medium (14) contains 0.1 mM hypoxanthine and 0.4 μM Apt, plus BrdUrd and dThd at varying ratios but with a total concentration of 10 μM; FB and FBT media are similar to HAB and HABT media, respectively, but with 10 μM fluorodeoxyuridine (FdUrd, another inhibitor of dThd biosynthesis) instead of Apt and with no hypoxanthine. After the growth of cells in any BrdUrd-containing medium, the cells were passaged in E medium containing 0.1 mM hypoxanthine and 16 μM dThd (HT medium).

The cells were protected at all times from exposure to light with wavelengths below 550 nm, as described (9).

Mutagenesis. Cells growing in E medium were collected by trypsinization; Falcon plastic tissue culture flasks (75 cm^2) were inoculated with 5×10^5 cells in 10 ml of BrdUrd-containing (or control) medium. At 3 and 6 days after inoculation, the medium was replaced with 50 ml of fresh medium containing the same additives. After 1 week in medium with BrdUrd, the cells were harvested, and flasks (75 cm^2) were reinoculated with 5×10^5 cells in 10 ml of HT medium (15). The cells were cultured in HT medium for 1 week and then collected and tested for drug resistance. In order to determine the frequency of OB-resistant mutants, five tissue culture dishes (150 mm diameter) were inoculated with 10^6 cells in E medium containing 0.9 mM OB. In addition, three dishes (60 mm diameter) were inoculated with 250 or 500 cells in E medium alone. At 7–10 days after inoculation, the dishes were fixed with methanol and stained with Giemsa for colony counting. Only colonies with more than 50 cells were counted. The frequencies of mutant cells are presented below as the number of OB-resistant colonies per 10^6 cells (mean of five dishes), after correction for the plating efficiency of the cells in the absence of OB. In most cases, the plating efficiencies in medium without OB varied between 15 and 40%.

Abbreviations: BrUra, bromouracil; BrdUrd, bromodeoxyuridine; Thy, thymine; dThd, thymidine; OB, ouabain; Sgu, thioguanine; Apt, aminopterin; FdUrd, fluorodeoxyuridine; HPRT, hypoxanthine phosphoribosyltransferase; dCyd, deoxycytidine.

The induction of Sgu-resistant mutants was measured exactly as described above, except that the selection dishes were inoculated with 2×10^5 cells in E medium containing 18 μM Sgu.

DNA Base Composition. At the same time that the cells in an experiment were first exposed to BrdUrd, two dishes (150 mm diameter) were inoculated with 10^6 cells in 30 ml of the same BrdUrd-containing medium. Three days later, the medium was replaced with 50 ml of fresh medium with the same additives plus 2 μCi of $H_3{}^{32}PO_4$ per ml. After 2 more days, the cells were harvested by scraping and the nuclear DNA was purified (9). The DNA preparations were hydrolyzed to deoxynucleoside 5'-monophosphates, which were separated and quantitated as described (6, 16). The results are expressed as "BrUra substitution", defined as the percentage of Thy residues in nuclear DNA replaced by BrUra. The results represent the mean of four determinations per sample; the SEM was less than ±0.5% in all cases.

RESULTS

The apparently unlimited proliferative capacity of the mutant cells that replace all their Thy residues in nuclear DNA with BrUra (6, 8) raises many questions about the mutagenicity of BrdUrd. Because the cells exposed to BrdUrd in our laboratory are shielded at all times from wavelengths of light below 550 nm, the possibility was tested that BrUra in the nuclear DNA of these cells is mutagenic only when the cells are exposed to wavelengths of light (around 300 nm) that cause breakage of BrUra-substituted DNA.

HAB-2E cells were cultured for 1 week in E medium containing either 30 μM BrdUrd or 30 μM dThd. Throughout the experiment, the cells were shielded from wavelengths of light below 550 nm. After 1 week of expression time in the absence of BrdUrd (in HT medium) the frequency of OB-resistant HAB-2E cells was determined (Table 1). The growth of the cells in E-B medium containing 30 μM BrdUrd (under which condition the cells replaced approximately 75% of their Thy residues in nuclear DNA with BrUra) resulted in an increase of at least 200-fold in the frequency of OB-resistant cells. Similar results were observed in a series of six independent experiments (with frequencies of mutants induced by 30 μM BrdUrd of 0.4, 0.8, 1.2, 1.6, 1.9, and 1.9×10^{-4}).

These results suggest that BrdUrd is an effective mutagen in HAB-2E cells in the absence of wavelengths of light that cause breakage of BrUra-substituted DNA. Similar experiments were performed with the parental RPMI 3460 line, except that the cells were exposed to either 10 μM BrdUrd or 10 μM dThd. The growth of RPMI 3460 cells in E-B medium with 10 μM BrdUrd (under which condition the cells replaced approximately 75% of their Thy residues in nuclear DNA with BrUra) resulted in an increase in the frequency of OB-resistant cells of at least 29-fold (Table 1). Comparison of the results obtained with the two cell lines suggests that BrdUrd is (at least) as mutagenic for HAB-2E cells as it is for the parental RPMI 3460 cells.

Table 1. Induction of OB-resistant mutants by BrdUrd

Cell line	Medium additive	BrUra substitution, %	Mutant frequency*
HAB-2E	BrdUrd, 30 μM	74.9	160
	dThd, 30 μM	—	<0.8
RPMI 3460	BrdUrd, 10 μM	74.8	29
	dThd, 10 μM	—	<1

* Frequency of OB-resistant mutants is expressed as the number of OB-resistant colonies per 10^6 cells after correction for the plating efficiencies of the cells in the absence of OB.

The relationship between mutagenicity and the amount of BrUra in DNA was studied in experiments in which HAB-2E cells were grown in E-B medium with BrdUrd at concentrations ranging from 3 to 40 μM and with the level of BrUra substitution ranging from 29 to 83%. There was not a linear relationship between BrUra substitution and mutagenesis (Fig. 1). Below 70% BrUra substitution (a level of substitution attained in E-B medium with 20 μM BrdUrd), there was no significant induction of OB-resistant mutants. Above this level of substitution, however, mutagenesis was observed and BrdUrd became increasingly mutagenic.

Because mutagenesis was observed in the above experiment only at levels of BrUra substitution greater than 70%, the effect of "total" substitution by BrUra (accomplished by growing the cells in HAB medium) was tested. [Although the DNA of cells growing in HAB medium should be 100% BrUra substituted, analysis of the DNA base composition indicated BrUra substitution levels of only 96–99%. However, because this seems to be at least in part an artifact (6), we will refer to the DNA of cells growing in HAB medium as "totally" substituted.] As shown in Table 2 (experiment 1), the frequency of OB-resistant mutants induced when HAB-2E cells were grown in HAB medium was less than 0.9×10^{-6}. In contrast, the frequency of OB-resistant cells induced by growth in E-B medium with 30 μM BrdUrd in the same experiment was at least 40-fold higher. This pattern was observed consistently in a series of six independent experiments.

In other experiments, totally substituted HAB-2E cells were obtained by growing the cells in FB medium. It was seen that the frequency of OB-resistant mutants induced by growth of HAB-2E cells in FB medium was at the same very low level as in HAB medium (data not shown).

Because levels of BrUra substitution around 75% were associated with mutagenesis in HAB-2E cells grown in E-B medium

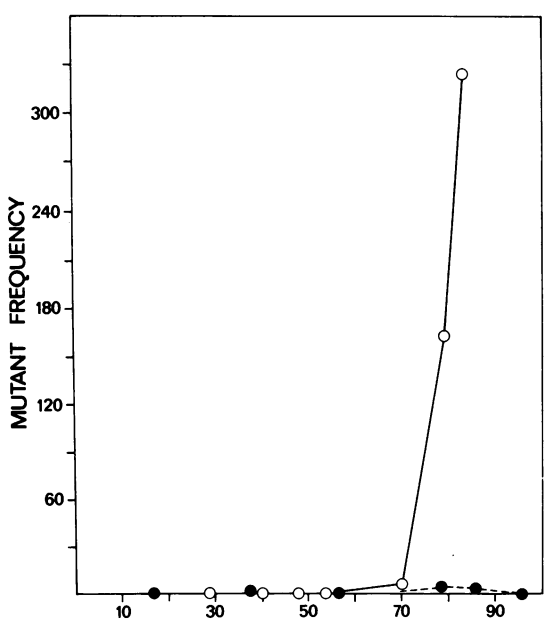

FIG. 1. Mutant frequency (OB resistance) as a function of BrUra substitution. ○, HAB-2E cells grown in E-B medium with BrdUrd concentrations of 3, 5, 7, 10, 20, 30, and 40 μM, respectively; ●, HAB-2E cells grown in HABT medium with ratios of BrdUrd/dThd of 2:8, 4:6, 6:4, 8:2, and 9:1, respectively, with the concentration of BrdUrd plus dThd fixed at 10 μM, and in HAB medium for the highest point.

Table 2. BrUra substitution and the induction of OB-resistant mutants*

Inhibitor†	Additive, μM BrdUrd	Additive, μM dThd	BrUra substitution, %	Mutant frequency‡
		Experiment 1		
None	—	30	—	<0.4
None	30	—	78.0	36
Apt	10	—	96.2	<0.9
		Experiment 2		
None	—	30	—	3.8
None	10	—	45.9	2.6
None	20	—	65.9	36
None	30	—	73.1	122
Apt	6	4	56.4	<0.3
Apt	7	3	65.0	0.5
Apt	8	2	76.2	1.3
FdUrd	6	4	55.3	<0.3
FdUrd	7	3	66.9	<0.4
FdUrd	8	2	75.0	<0.4

* The cell line HAB-2E was used in these experiments.
† Apt and FdUrd were provided in HABT and FBT media, respectively.
‡ See footnote * to Table 1.

but total BrUra substitution appeared to be nonmutagenic in the cells grown in HAB medium, tests were carried out to determine whether some Thy had to be present in the DNA for BrUra to be mutagenic. HAB-2E cells were grown in HABT medium with BrdUrd and dThd present at ratios ranging from 2:8 to 9:1 and with levels of BrUra substitution ranging from approximately 20% to 90%. There was no significant induction of OB-resistant mutants at any level of BrUra substitution when BrdUrd was provided in HABT medium (Fig. 1). This is in marked contrast to the results obtained with cells exposed to BrdUrd in E-B medium.

In another experiment, BrdUrd mutagenicity in HAB-2E cells was studied after growth in E-B, HABT, and FBT media (Table 2, experiment 2). Similar to the results presented in Fig. 1, levels of BrUra substitution around 70% and higher were associated with effective mutagenesis when the cells were grown in E-B medium, whereas the same levels of substitution were nonmutagenic in cells grown in HABT or FBT medium.

The finding that a given amount of BrUra in DNA could be highly mutagenic when the cells were grown in one medium (E-B medium) but not in others (HABT or FBT medium) seemed inconsistent with the hypothesized mechanisms of BrdUrd mutagenesis. Two possibilities were tested: (*i*) BrUra in DNA is not mutagenic in the absence of *de novo* dThd biosynthesis, and (*ii*) it is the concentration of BrdUrd and not the level of BrUra substitution in DNA that determines mutagenicity. The second possibility was considered because BrdUrd in E–B medium is not mutagenic for HAB-2E cells until the concentration reaches 20–30 μM, whereas the concentration of BrdUrd used in HABT or FBT medium to yield comparable levels of BrUra substitution (around 70%) is only 7–8 μM.

HAB-2E cells were grown in a series of Apt-containing HABT-type media in which the BrdUrd/dThd ratio was fixed (8:2) but in which the total amount of BrdUrd plus dThd was varied. Increasing the total amount of BrdUrd plus dThd (while maintaining the same ratio) in the presence of Apt did not significantly affect the level of BrUra substitution in DNA (Table 3, experiment 1). However, there was a striking effect on mutagenicity. BrdUrd at a concentration of 8 μM in the presence of Apt (and 2 μM dThd) was essentially nonmutagenic.

Table 3. BrdUrd concentration and the induction of OB-resistant mutants

Cell line	Apt*	BrdUrd, μM	dThd, μM	BrUra substitution, %	Mutant frequency†
			Experiment 1		
HAB-2E	−	—	30	—	<0.3
	−	30	—	73.2	321
	+	8	2	76.7	5.4
	+	30	7.5	80.3	368
	+	50	12.5	79.8	3100
			Experiment 2		
HAB-2E	+	10	—	ND‡	3.2
	+	30	—	ND	119
			Experiment 3		
HAB-2E	+	10	20	30.6	<0.8
	+	20	40	30.8	148
	+	30	60	29.4	235
			Experiment 4		
RPMI 3460	+	6	4	56.2	1
	+	12	8	60.8	173
	+	18	12	60.1	369

* Apt was provided in HABT medium.
† See footnote * to Table 1.
‡ ND, not determined. The cells in both media in this experiment would be "totally" BrUra substituted.

In contrast, BrdUrd at 30 μM in the presence of Apt (and 7.5 μM dThd) was highly mutagenic, and BrdUrd at 50 μM in the presence of Apt (and 12.5 μM dThd) was even more so.

These results indicate that BrdUrd in the presence of Apt can be mutagenic, depending on the concentration of BrdUrd plus dThd in the medium. Thus, the first possibility mentioned above is ruled out. Other experiments have shown that mutagenicity is not dependent upon the presence of dThd. HAB-2E cells were grown in different Apt-containing HAB-type media in which the BrdUrd concentration was varied and no dThd at all was provided. Increasing the BrdUrd concentration in HAB medium caused a major increase in mutagenicity, even though there could not have been an associated increase in BrUra substitution (because the cells already were totally substituted at the lowest concentration of BrdUrd) (Table 3, experiment 2).

The cells in both of these last two experiments (experiments 1 and 2, Table 3) had levels of BrUra substitution greater than 70%. The effects of increasing the BrdUrd concentration at low substitution levels also were tested. HAB-2E cells were grown in HABT-type media in which the BrdUrd/dThd ratio was fixed at 1:2 but the total amount of BrdUrd plus dThd was varied. This generated levels of BrUra substitution around 30%. In E-B medium, this level of substitution would be obtained in the presence of 3 μM BrdUrd, and this condition is totally nonmutagenic (see Fig. 1). However, a marked increase in mutagenicity occurred as the concentration of BrdUrd (and dThd) increased (Table, 3 experiment 3).

These results suggest that it is the concentration of BrdUrd rather than the amount of BrUra in nuclear DNA that determines mutagenicity. It seems that the concentration of BrdUrd required for mutagenesis in HAB-2E cells is approximately 20 μM, whether the level of BrUra substitution is 30% or 100%.

Because all of the above experiments were carried out with OB resistance as the marker for mutagenesis, experiments were carried out with Sgu resistance as the marker. HAB-2E cells were cultured in a series of FdUrd-containing FBT-type media in which the BrdUrd/dThd ratio was fixed (8:2) but in which

Table 4. BrdUrd concentration and the induction of Sgu-resistant mutants*

FdUrd†	BrdUrd, μM	dThd, μM	BrUra substitution, %	Mutant frequency‡ Sgu^r	Mutant frequency‡ OB^r
−	—	30	—	26	2.9
+	5	1.25	72.8	65	1.8
+	10	2.5	74.6	200	1.9
+	20	5	75.1	475	52

* The cell line HAB-2E was used for these experiments.
† FdUrd was provided in FBT medium.
‡ Frequencies of Sgu-resistant and OB-resistant mutants are expressed as the number of drug-resistant colonies per 10^6 cells, after correction for the plating efficiencies of the cells in the absence of drug. Sgu^r, Sgu-resistant; OB^r, OB-resistant.

the total amount of BrdUrd plus dThd was varied. (FBT rather than HABT-type media were used in these experiments because, if HPRT-deficient, Sgu-resistant cells could not grow in HABT medium but could grow in FBT medium.) After 1 week of expression time in the absence of BrdUrd, the frequency of cells resistant to Sgu (or OB) was determined. The results for Sgu resistance were basically in agreement with those for OB resistance (Table 4). In the absence of significant changes in the level of BrUra substitution, increasing the concentration of BrdUrd (and dThd) caused a large increase in the frequency of Sgu-resistant cells induced.

The main difference between the results obtained for OB and Sgu resistance is that the locus for Sgu resistance seems to be much more mutable (both with and without BrdUrd treatment) than the locus for OB resistance in these cells. It seems that lower concentrations of BrdUrd (in FBT medium) are required for effective mutagenicity of the locus for Sgu resistance (10 μM BrdUrd) than for OB resistance (20 μM BrdUrd). Despite this difference, the results for Sgu resistance support the conclusions from the experiments on OB resistance.

The mechanisms underlying the results presented above remain to be elucidated. However, tests on the induction of OB resistance in the parental melanoma cells RPMI 3460 suggest that the results are not due to the use of a mutant line (HAB-2) selected for viability with totally BrUra-substituted DNA. RPMI 3460 cells were grown in a series of Apt-containing HABT-type media with a fixed BrdUrd/dThd ratio (6:4) but with varying total amounts of BrdUrd plus dThd (see Table 3, experiment 4). The higher the concentration of BrdUrd in the presence of Apt, the greater the mutagenic effect, even though the level of BrUra substitution was essentially the same in all cases (because of the corresponding increases in dThd concentration). These results are similar to those described above with HAB-2E cells. The main difference between the results with the two cell lines seems to be the minimal concentration at which BrdUrd in the presence of Apt becomes mutagenic. The critical BrdUrd concentration in the presence of Apt is apparently lower for RPMI 3460 cells (around 10 μM) than for HAB-2E cells (around 20 μM).

DISCUSSION

These studies were undertaken to determine how mutant lines of Syrian hamster melanoma cells could grow indefinitely with all of the Thy residues in nuclear DNA replaced by BrUra (6, 8), apparently avoiding proliferative difficulties that would arise from an excessively high frequency of mutations. The results demonstrated that BrdUrd can be highly mutagenic to these cells (HAB-2E), even when they are shielded from wavelengths of light that cause breakage of BrUra-substituted DNA. In fact, at the same level of BrUra substitution, BrdUrd (in E-B medium) was as mutagenic for HAB-2E cells as for the parental melanoma line RPMI 3460. Experiments on the relationship between mutagenicity and the amount of BrUra in the DNA of HAB-2E cells revealed a nonlinear relationship and suggested a possible threshold effect (Fig. 1). A similar nonlinear relationship between BrUra substitution and mutagenicity was observed recently in experiments using a different cell line and with a different marker to detect mutations (17). An explanation for these results is proposed below.

It is recognized that the mutagenesis protocol used in this study (1 week of exposure to BrdUrd and 1 week of expression time) could make it difficult to assess accurately the frequency of induction of mutants. (During a 1-week period in medium with 30 μM BrdUrd, HAB-2E cells undergo approximately six population doublings, with a similar number occurring during the 1-week expression period.) However, experiments have been performed in which HAB-2E cells were exposed to 30 μM BrdUrd for only 24 hr and effective mutagenesis still occurred (though the frequency of mutants was severalfold lower than with a 1-week exposure to BrdUrd). In addition, reconstruction tests involving artificial mixtures of HAB-2E cells and their OB-resistant mutants showed that the yield of mutants after the standard 2-week mutagenesis procedure (with the first week in medium with 30 μM BrdUrd) corresponded closely to the initial input (e.g., input ratio of mutant to wild-type cells of 0.011, final ratio of 0.016). Thus, the mutagenesis protocol used seems to provide an accurate reflection of the frequency of induction of mutant cells.

An extensive series of experiments was performed in which the level of BrUra substitution in HAB-2E cells was held constant (by a fixed ratio of exogenous BrdUrd/dThd in the presence of inhibitors of *de novo* dThd biosynthesis) while the concentration of BrdUrd was varied. The results of these experiments strongly suggest that it is the concentration of BrdUrd to which the cells are exposed and not the level of BrUra substitution in DNA that determines mutagenicity.

The above conclusion was supported by the results of experiments on the induction of Sgu-resistant, as well as OB-resistant, mutants. In addition, it was shown that the dependence of BrdUrd mutagenicity on the concentration of exogenous BrdUrd rather than on the level of BrUra substitution in DNA occurred in the parental RPMI 3460 cells as well as in HAB-2E cells. Thus, these findings are not unique to cells that had been selected for the ability to grow with all the Thy residues in nuclear DNA replaced by BrUra.

The conclusion that the mutagenicity of BrdUrd is determined by the concentration of exogenous BrdUrd suggests an explanation for the nonlinear relationship between BrUra substitution and mutagenicity mentioned above. It appears that HAB-2E cells require a concentration of approximately 20 μM BrdUrd in the medium for mutagenesis (as measured by OB resistance) to occur, whether the BrdUrd is provided in E-B, HABT, or FBT medium, and independent of the amount of BrUra in DNA. Above this threshold concentration, and independent of changes in BrUra substitution, BrdUrd becomes increasingly mutagenic. (In some experiments, including the one shown in Fig. 1, there was a roughly linear increase of approximately 150 mutants per 10^6 cells for every 10 μM increase in the BrdUrd concentration above the threshold. However, a linear increase above the threshold has not been seen in all the experiments.)

The demonstration that the mutagenicity of BrdUrd can be altered by changing the concentration of exogenous BrdUrd without changing the level of BrUra substitution in DNA seems to be inconsistent with the widely accepted "mispairing" hypothesis (2, 3) for BrdUrd-induced mutations (based on ex-

periments in prokaryotic systems). If BrdUrd mutagenesis in the cells used in the present study were occurring via a mechanism involving replication errors due to base mispairing, the level of mutagenesis should be proportional to the amount of BrUra in the DNA. The results presented in this paper, however, clearly indicate the independence of mutagenicity from the level of BrUra substitution. The possibility of a lack of correlation between mutagenicity and BrUra in DNA was raised previously in early studies with a prokaryotic system (4) and in recent studies with mammalian cells (17). However, only in the present case has it been possible to demonstrate that the mutagenicity of BrdUrd can be experimentally manipulated without changing the amount of BrUra in DNA.

The independence of BrdUrd mutagenicity from the level of BrUra substitution suggests that factors outside DNA are involved in BrdUrd mutagenesis. Our results raise the possibility that BrdUrd mutagenicity might involve starvation for deoxycytidine (dCyd) nucleotides or perturbation of dCyd metabolism. It has been suggested recently (18) that BrdUrd toxicity for mammalian cells might involve dCyd starvation, with the triphosphate of BrdUrd inhibiting the reduction of cytidine diphosphate to dCyd disphosphate by ribonucleotide reductase. A mechanism of BrdUrd mutagenicity involving dCyd metabolism could explain the results presented in this paper, because the size of the intracellular pool of BrdUrd nucleotides responsible for perturbation of dCyd metabolism could reflect the exogenous BrdUrd concentration and vary independently from the level of BrUra substitution. Consistent with this proposal, we have obtained preliminary evidence that the mutagenicity of BrdUrd can be suppressed by exogenous dCyd. For example, when 0.2 mM dCyd was added to cells in E-B medium with 30 μM BrdUrd (from experiment 1 of Table 3), the frequency of OB-resistant mutants induced was decreased to $^1/_{10}$th (from 3.2×10^{-4} to 3.1×10^{-5}). The mechanism by which BrdUrd-induced dCyd starvation could cause mutations is not apparent, but it could conceivably involve the induction of an error-prone DNA repair system (19) or even an increased error frequency by DNA polymerase itself (cf. 17).

It should be pointed out that, even though the results presented in this paper show that even total replacement of Thy by BrUra in DNA is not sufficient for mutagenesis, they do not indicate whether incorporation of the analogue into DNA is necessary for BrdUrd mutagenesis. The results of preliminary experiments suggest, however, that if BrUra in DNA is necessary for mutagenesis, only very low levels of substitution are sufficient.

Because the results presented above indicate that BrdUrd mutagenicity can be experimentally manipulated without changing the amount of BrUra in DNA, it is of interest to note that we have recently demonstrated that the suppression of pigmentation caused by BrdUrd can be reversed (by the addition of dCyd) without altering the amount of BrUra in DNA (20). In that respect, the effects of BrdUrd on mutagenesis and differentiation appear comparable, although it seems unlikely that the same mechanisms are involved. It is possible, however, that the effect of BrdUrd on differentiation, like mutagenesis, might be determined more by the concentration of BrdUrd than by the amount of BrUra in DNA.

The question that initiated the present investigations—How can cells survive with all the Thy residues in nuclear DNA replaced by BrUra if BrdUrd is a mutagen?—appears to have a simple answer based on the results in this paper. Although the HAB-2 cells were grown for hundreds of generations in HAB medium (8) (which contains 10 μM BrdUrd), with their nuclear DNA totally substituted with BrUra, they had never been exposed to a concentration of BrdUrd high enough (around 20 μM) to be mutagenic, at least for one marker (OB resistance).

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