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Bromodeoxyuridine Dependence—A New Mutation in Mammalian Cells

(hamster cells/drug dependence/DNA)

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ABSTRACT When cells of a Syrian hamster melanoma were grown in increasing concentrations of bromodeoxyuridine (BrdU), lines of mutant cells able to grow well at high concentrations of BrdU were isolated. The mutant cells were characterized by BrdU dependence. In the absence of BrdU, the cells grew very poorly. The requirement for BrdU was specific for both the bromine and the deoxy sugar. The mutant cells incorporated BrdU into the DNA, replacing about 50% of the thymine residues with bromouracil. The reason for the BrdU dependence is not known.

The drug 5-bromodeoxyuridine (BrdU), which can be incorporated into DNA in place of thymidine, has many types of effects on cells. It has been shown to act as a mutagen (1), to suppress differentiated functions (2), to induce latent viruses (3), and to affect the strength of binding of a bacterial repressor to its operator (4). Mammalian cells resistant to the drug have been isolated (5). A search for additional cell lines able to grow in BrdU has led to the discovery of a new type of mutation in mammalian cells, BrdU dependence. The isolation and properties of the mutant cells are described in this paper.

MATERIALS AND METHODS

Isolation of Cells in BrdU. The cells were derived from the pigmented Syrian hamster melanoma line RPMI 3460 (6). This line has been maintained in culture for several years, and its derivatives have been used extensively in cell hybridization studies (7). A pigmented clone called 3460 W1 (referred to below as W1) was used as the parental line for the isolation of the mutant cells. The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal-calf serum (E medium).

For the isolation of cells able to grow in BrdU, 60-mm (Falcon) plastic tissue culture dishes were inoculated each with 2×10^5 W1 cells in E medium containing 3 μg/ml BrdU (Sigma Chemical Co.). After 3 weeks in this medium, about 20 large colonies of cells were present in each dish. The colonies from two dishes were pooled, and the cells were passed two more times in medium with 3 μg of BrdU per ml, and then passed two times in medium containing 30 μg of BrdU per ml. (E medium containing 30 μg of BrdU per ml will be referred to as E–B medium.) During these passages, the cells were transferred at 2- to 3-week intervals. 2 Weeks after the second passage in E–B medium, large colonies of cells were observed. Several colonies were picked and subcultured individually in E–B medium. The lines obtained were called 3460 B1, B2, B3, etc., and as a group will be referred to as 3460 B. Because of the repeated transfers in BrdU before the isolation of colonies, it is not certain that all of the colonies are of independent origin.

Pyrimidine Requirements. The 3460 B cells were tested for the ability to grow in E medium alone or supplemented with one of the following: BrdU, thymidine, uridine, deoxyuridine, bromouracil, bromouridine, and iododeoxyuridine. Since cells resistant to BrdU often lack the enzyme thymidine kinase (EC 2.7.1.21), and are therefore unable to grow in HAT medium [E medium supplemented with 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (8)], the 3460 B cells were also tested for their ability to grow in HAT medium. For the growth tests, 60-mm dishes were inoculated with 10^4 cells (maintained in E–B medium until harvested for the tests) in E medium containing different additives. After 1 week the dishes were renewed with fresh medium; after 2 weeks, the cells (from two dishes with each medium) were counted.

Labeling of Cells and DNA Isolation. Cells were grown in E or E–B medium to which was added the appropriate radiolabel at an activity of 10 μCi/ml for 2–3 population doublings before harvesting the cells. Cells were labeled with either [^{3}H]thymidine (6.7 Ci/mmol), [^{3}H]BrdU (26.4 Ci/mmol), or $H_3{}^{32}PO_4$. (All three compounds were purchased from New England Nuclear Corp.)

The cells were collected by trypsinization, centrifuged, resuspended in 1 ml of 0.01 M Tris·HCl–0.01 M KCl–1.5 mM $MgCl_2$ (pH 8.5), containing 5% NP-40 detergent, and lysed by vigorous agitation with a Vortex mixer. The nuclei were collected and resuspended in 0.1 M NaCl, 0.03 M Tris·HCl (pH 7.5) 0.01 M EDTA, and 2% Sarkosyl. Previously digested Pronase (Calbiochem) was added to a concentration of 1 mg/ml, and the mixture was gently rocked at 37° for 1 hr, extracted twice with phenol, then extracted once with chloroform, and finally dialyzed overnight against 0.01 M Tris·HCl–1 mM EDTA (pH 7.5). When ^{32}P was used as the label, the DNA was subjected to a 30-min treatment with RNase A (previously boiled for 10 min) before dialysis.

CsCl Gradient Centrifugation. Neutral gradients were prepared by the addition of CsCl to an initial density of either 1.700 g/ml (for 3460 and W1) or 1.750 g/ml (for 3460 B) in a total volume of 5 ml. Gradients were run for 72 hr in a Beckman L2-65B ultracentrifuge at 35,000 rpm. For alkaline CsCl gradients, the DNA sample was denatured and the gradient was prepared as described (9), at an initial density of either 1.760 g/ml (for 3460 and W1) or 1.790 g/ml (for 3460 B). The tubes were punctured and 10-drop fractions were collected. Samples were taken for scintillation counting and determination of refractive indices.

Radiation Sensitivity. 0.2-ml DNA samples were irradiated in small, open petri dishes with a UV Germicidal Lamp (Syl-

vania G15T8) at a distance of 10 cm for 10 min at room temperature (24°). After irradiation, samples were denatured in 0.3 N NaOH and analyzed for single-chain scissions on alkaline sucrose gradients. The gradients were run on 5–25% sucrose in 0.3 N NaOH–0.7 M NaCl for 90 min at 49,000 rpm in an SW50.1 rotor. Denatured bacteriophage T7 DNA was used as a marker.

Cells were irradiated with light from a fluorescent source (ITT F40, Cool White) at a distance of 10 cm at room temperature. The cells were growing in (Falcon) plastic tissue culture dishes, and the medium was left in the dishes with the top on during irradiation.

RESULTS

The 3460 B sublines were maintained in E–B medium after their isolation. All of the lines were maintained for at least 6 weeks, during which time they were subcultured at least four times. One of the sublines, 3460 B4 (referred to below as B4), was maintained in continuous multiplication in BrdU for more than 6 months, undergoing more than 75 cell divisions with no decrease in growth rate. Cells of the B4 subline had as much thymidine kinase activity as the 3460 cells. (Thymidine kinase assays were kindly performed by Dr. S. J. Adelstein.) Cells of all of the 3460 B sublines were unpigmented, consistent with previous observations of the effect of BrdU on pigment cells (10).

Soon after their isolation, it became apparent that the 3460 B cells were not simply BrdU resistant but were actually BrdU dependent. When transferred to E medium, the cells stopped growing. The cell morphology was also altered in the absence of BrdU, with the cells becoming flattened and enlarged. [This is the reverse of the effect of BrdU on the morphology of mouse melanoma cells, in which case the cells exposed to BrdU became more flattened (11).] Seven of the 3460 B sublines were tested for their ability to grow in medium without BrdU, and it can be seen from Table 1 that all of the sublines grew very poorly in the absence of the drug. On the average, the increase in cell number over the 2-week period of the test was 20-fold greater in E–B medium than in E medium.

Cloning experiments were performed in an effort to determine the significance of the small increase in the number of 3460 B cells in the absence of BrdU. The B4 line was subcloned

TABLE 1. *BrdU dependence of 3460 B sublines*

Cell line*	Cell number† (×10⁻⁴) +BrdU	−BrdU	Cell line*	Cell number† (×10⁻⁴) +BrdU	−BrdU
3460 B4	150	4	3460 B4(A)	330	12
B8	110	3	B4(B)	380	15
B9	130	6	B4(C)	430	8
B10	72	8	B4(D)	580	10
B11	24	2			
B12	36	5			
B13	28	2			

* 3460 B4, B8, B9, etc. are independent clones of cells isolated in E–B medium. 3460 B4(A), B4(B), etc. are subclones of 3460 B4.

† The cell number is the total number of cells per 60-mm dish (average of two dishes), counted 14 days after inoculation with 10^4 cells in medium containing either 30 μg of BrdU per ml or no BrdU.

TABLE 2. *BrdU concentration and thymidine competition*

Medium additive*	Cell number† (×10⁻⁴)	Medium additive*	Cell number† (×10⁻⁴)
None	12	BrdU (30)	415
BrdU (0.03)	12	BrdU (30) + Thymidine (3)	402
BrdU (0.3)	75		
BrdU (3)	180	BrdU (30) + Thymidine (30)	365
BrdU (30)	445		
BrdU (90)	2	BrdU (3)	192
Thymidine (3)	10	BrdU (3) + Thymidine (3)	152
Thymidine (30)	12		
		BrdU (3) + Thymidine (30)	26

* 60-mm Dishes were inoculated with 10^4 B4 cells in E medium, supplemented as indicated in the Table. The concentrations of the additive, in μg/ml, are indicated in parentheses.

† See note to Table 1.

in BrdU, and four subclones were tested for their ability to grow in the absence of the drug (see Table 1). It can be seen that all of the subclones were BrdU dependent and, like the parental population, underwent a small increase in cell number in the absence of BrdU.

Cells of the B4 line and of one of the subclones, B4(D), were cloned in E and E–B medium, to determine whether all of the cells in the BrdU-dependent populations could grow slowly in the absence of BrdU or whether a small fraction of the cells could survive and grow rapidly without the drug. The cloning dishes were stained and counted after 2 weeks, and it was seen that the cloning efficiency of the dependent cells was about the same in medium with or without BrdU (8% in E medium and 11% in E–B medium). However, the average colony size in E medium was less than 10 cells, whereas the average colony size in E–B medium was more than 200 cells. These results suggested that all of the cells in the dependent populations could grow very slowly, at least for a few generations, in the absence of BrdU.

Since BrdU-dependent cells had not been observed previously, it was necessary to show that these cells actually required BrdU and not some trace contaminant in the commercially supplied material. Two different lots of the drug were tested and both gave the same results. One sample of BrdU was further purified by paper chromatography [with Whatman 3Mm paper and a solvent of butanol–formic acid–water 770:108:122]. The BrdU spot was detected with UV light, cut out, and eluted. The chromatographed BrdU was tested for its ability to support the growth of B4 cells at two different concentrations (15 μg/ml and 3 μg/ml); the dependent cells grew as well with the chromatographed BrdU as with nonchromatographed material. These results suggested that the cells actually required BrdU itself.

The B4 line was used to study in detail the requirement for BrdU. The rate of growth of the cells was dependent upon the concentration of BrdU (Table 2). Over a thousandfold range of concentrations, from 0.03 μg/ml to 30 μg/ml (about 0.1 μM–100 μM) BrdU, the cells grew faster as the concentration of the drug was increased. At a concentration of 90 μg of BrdU per ml, the cells did not grow at all. It was mentioned earlier that the dependent cells in the absence of drug became very flattened and enlarged. In this experiment, it was observed that the

TABLE 3. *Thymidine requirement of BrdU-dependent cells*

Medium additive*	Cell number† ($\times 10^{-4}$)
None	18
BrdU	425
HAT	20
HA + BrdU	2
HAT + BrdU	127

* BrdU was used at a concentration of 30 μg/ml. H, A, and T represent hypoxanthine, aminopterin, and thymidine at the concentrations indicated in *Methods*.

† See note to Table 1.

extent of enlargement decreased progressively as the concentration of BrdU was increased.

Experiments were performed to determine if thymidine could be substituted for BrdU (Table 2). The results demonstrated that thymidine could not satisfy the requirement for BrdU. The fact that the cells could not survive when thymidine was substituted for BrdU in the medium demonstrated that the BrdU was not being used by the cells to compensate for an inability to synthesize thymidine.

The B4 cells were grown in mixtures of BrdU and thymidine to test whether there was any competition between them (see Table 2). With equal amounts of BrdU and thymidine in the medium, the presence of thymidine had no effect on cell growth. However, with a 10-fold excess of thymidine over BrdU, cell growth was significantly inhibited. Thus, even though thymidine could not be utilized in place of BrdU, it did compete with BrdU.

Experiments were also performed to determine whether the cells could survive without any thymidine at all. The *de novo* synthesis of thymidine was blocked by aminopterin, forcing the cells to obtain any necessary thymidine from the medium. As shown in Table 3, when aminopterin was added to the medium, the cells could not grow in the presence of BrdU without added thymidine (E medium contains no thymidine), nor could they grow with thymidine in the absence of BrdU. However, if both thymidine and BrdU were present when aminopterin was in the medium, the cells could grow. These results showed that the cells required both thymidine and BrdU for growth and that neither one alone was sufficient. The necessary thymidine could be provided by the cell's own synthetic mechanisms (indicated by the fact that exogenous thymidine was not required for growth in the absence of aminopterin) or, in the presence of aminopterin, thymidine had to be obtained from the medium.

TABLE 4. *Specificity of BrdU requirement*

Medium additive*	Cell number† ($\times 10^{-4}$)	Medium additive*	Cell number† ($\times 10^{-4}$)
None	6	None	18
BrdU	298	BrdU	425
Bromouracil	10	Bromouridine	18
Uridine	8	Iododeoxyuridine	2
Deoxyuridine	10		

* All of the additives were tested at a concentration of 30 μg/ml.

† See note to Table 1.

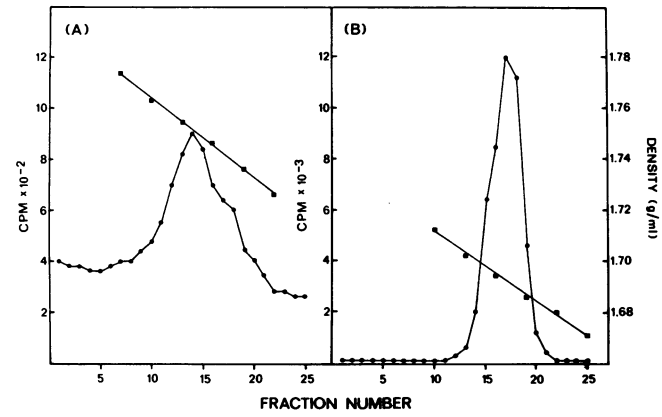

FIG. 1. Incorporation of BrdU into DNA of dependent cells. Neutral CsCl gradients were prepared as described in *Methods*. (*A*) B4 DNA, labeled with ^{32}P; (*B*) 3460 DNA, labeled with [^{3}H]-thymidine. ●——● radioactivity; ■——■, density.

The specificity of the requirement for BrdU was tested by culturing the B4 cells in medium in which BrdU was replaced by uridine, deoxyuridine, bromouracil, bromouridine, or iododeoxyuridine. The results of the tests are shown in Table 4, and it is clear that none of the compounds tested could be substituted for BrdU, indicating that the requirement for BrdU was specific for both the bromine and the deoxy sugar.

The specificity of the requirement for BrdU suggested that the BrdU was being incorporated into the DNA of the dependent cells. Since cells with BrdU-substituted DNA have been shown to be sensitive to visible light (12), the effect of fluorescent light on the B4 cells was determined. It was found that the cells were extremely light-sensitive, with a 10-min exposure reducing the fraction of surviving cells to 0.01%. The same exposure had no effect on cells not growing in the presence of BrdU.

In order to test more directly whether the dependent cells were incorporating BrdU into DNA, the buoyant density of the DNA was examined. The 3460 and W1 cells were grown in E medium containing [^{3}H]thymidine or $H_3{}^{32}PO_4$, and the B4 cells were grown in E–B medium plus [^{3}H]thymidine, [^{3}H]-BrdU, or $H_3{}^{32}PO_4$. The DNA from the labeled cells was extracted, and the buoyant density of the DNA was determined in neutral CsCl. As shown in Fig. 1, the DNA from the 3460 cells gave a single peak with a density of 1.693 g/ml. The DNA from W1 cells (not shown) gave an identical result. The DNA from the B4 cells also gave a single peak, but the peak was much more disperse, and the mean density had increased to 1.753 g/ml. No labeled DNA with the density of 3460 DNA was detected in the dependent cells. The large increase in the density of the B4 DNA indicated a significant incorporation of BrdU into the DNA of the dependent cells. Table 5 shows the density ranges from five different CsCl gradients with four different DNA preparations and all three labeled precursors. While the peak of radioactivity for the B4 cells was heterogeneous, the mean density, as well as the upper and lower extremes, were always the same, regardless of the labeled precursor used. The fact that the distribution of label showed the same density ranges with both [^{3}H]thymidine and [^{3}H]BrdU suggested that BrUra was evenly distributed throughout the DNA.

The extent to which T residues had been replaced by BrUra

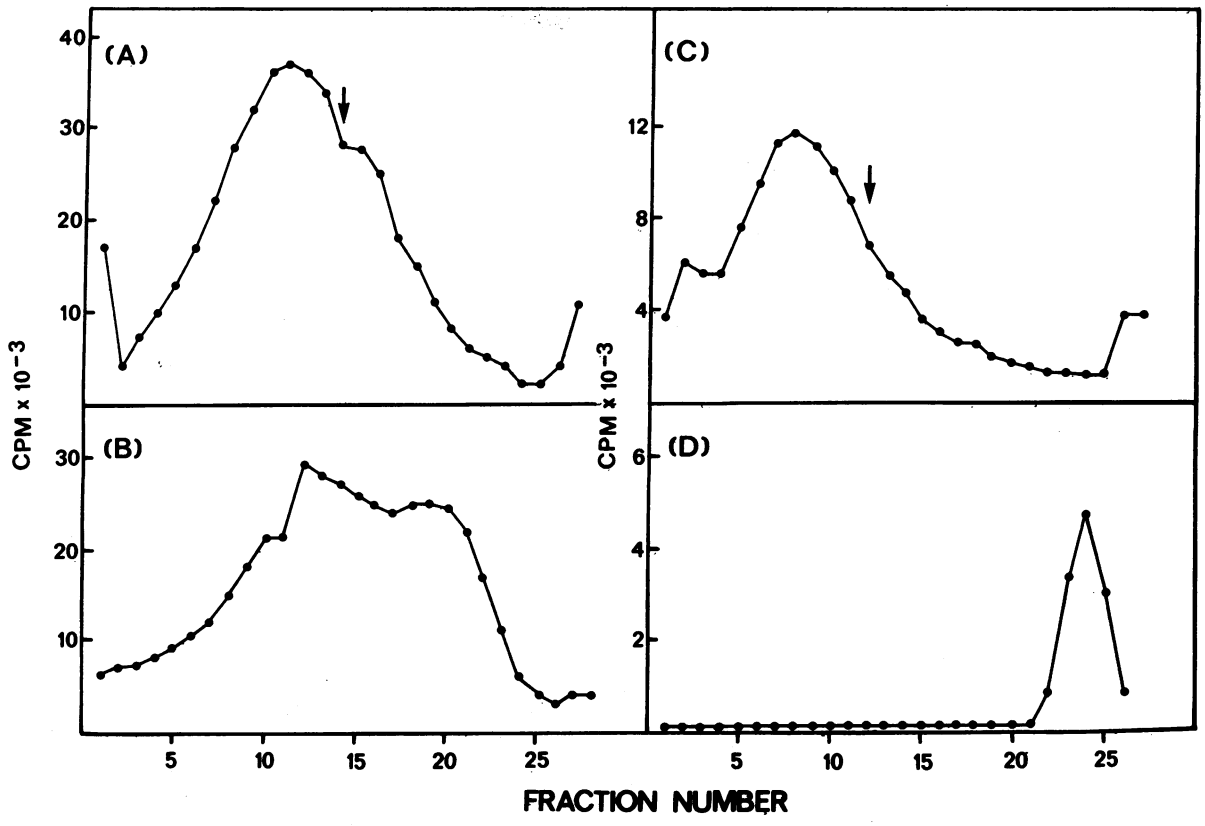

FIG. 2. Effect of UV irradiation on DNA of BrdU-dependent cells. Alkaline sucrose gradients of irradiated and unirradiated DNA, treated and centrifuged as described in *Methods*. (*A*) 3460 DNA, unirradiated; (*B*) 3460 DNA, irradiated; (*C*) B4 DNA, unirradiated; (*D*) B4 DNA, irradiated. DNA of both cell lines was labeled with [^{3}H]thymidine. *Arrow* indicates position of T7 DNA marker.

in the DNA of the dependent cells was calculated on the basis of the shift in density. The density of the 3460 DNA corresponded to a G-C content of 40% or a T content of 30%. In CsCl, the density of poly(dA-dT) is 0.20 g/ml less than that of poly(dA-dBrUra) (13). Thus, the shift in density from 1.693 (for 3460 DNA) to 1.753 (for B4 DNA) indicated a replacement of 50% of the T residues by BrUra in B4. The extremes of density of the B4 DNA corresponded to a range of 33–66% substitution, if we assume that all of the DNA fragments would contain 30% T. The density shifts described above were obtained with DNA at a molecular weight of 10^8. Identical results were obtained with DNA sheared to 6–8 $\times$ 10^6 daltons.

Labeled DNA from B4 cells was run on alkaline CsCl gradients to determine if the BrdU was being incorporated uniformly into both strands of DNA. The DNA of the dependent cells was found to band with a single peak. The fact that the two strands did not separate in alkaline CsCl indicated that approximately equal amounts of BrUra were incorporated into both strands of the DNA.

BrdU-substituted DNA has been shown to be much more light-sensitive than unsubstituted DNA (14). Labeled DNA from 3460 and B4 cells was exposed to UV light and then run on alkaline sucrose gradients to detect single-strand scissions (see Fig. 2). The unirradiated, single-stranded DNA of the 3460 cells had a single broad peak, with a mean molecular weight of about 2 $\times$ 10^7. After irradiation, some DNA of 2 $\times$ 10^7 molecular weight still remained, but the size distribution became more heterogeneous, with an obvious production of some smaller fragments. The unirradiated single-stranded DNA of the dependent cells had a molecular weight of 2 $\times$ 10^7, similar to that of the 3460 cells. However, after irradiation, no label at all was detectable in high molecular weight DNA. All of the label appeared in a single peak, with a molecular weight of less than 2 $\times$ 10^5. The fact that no single-stranded DNA of high molecular weight remained after irradiation suggested again that the BrUra was located in both strands of the DNA, and further suggested that there were very few, if any, long stretches of DNA into which BrdU was not incorporated.

In an effort to further characterize the BrdU-dependence mutation, a population of "revertant" cells able to grow rapidly without BrdU was isolated and studied. The revertant population, called B4-E, was derived from B4 after the cells

TABLE 5. *Density range of B4 DNA*

Label*	Density range†	Peak
[^{3}H]Thymidine	1.736–1.772	1.757
[^{3}H]BrdU	1.733–1.778	1.752
$H_3{}^{32}PO_4$	1.732–1.774	1.752
$H_3{}^{32}PO_4$	1.733–1.766	1.751
$H_3{}^{32}PO_4$	1.731–1.774	1.753
Average	1.733–1.773	1.753

* B4 cells were labeled with [^{3}H]thymidine, [^{3}H]BrdU, or $H_3{}^{32}PO_4$ as described in *Methods*.

† Extremes of density of B4 DNA, run on neutral CsCl gradients prepared as described in *Methods*.

had undergone 25 generations in the presence of BrdU, and was obtained after several passages of B4 in the absence of BrdU. B4-E cells were cloned in E and E–B medium; the cloning efficiency of the cells was about 60% in E medium and 5% in E–B medium, with the colonies in both media being about the same size suggesting that about 10% of the cells in the B4-E population were again able to grow rapidly in the presence of BrdU. A new population of cells growing in BrdU, called B4-E-B, was isolated from B4-E; after 15 generations in BrdU, the B4-E-B cells were again tested for BrdU incorporation and dependence. The density of B4-E-B DNA was determined, and it was found that B4-E-B cells incorporated as much BrdU into DNA as B4 cells. However, in contrast to B4, B4-E-B cells were able to grow as well in E medium as in E–B medium. The B4-E-B cells were thus unlike the B4 cells from which they had been derived, even though both populations grew rapidly at high concentrations of BrdU and incorporated BrdU into the DNA. The reason for this difference is not understood.

The mutagenic effect of BrdU is thought to be due to pairing errors during DNA replication, resulting in the replacement of A-T pairs by G-C pairs (15). A progressive increase in G-C content in cells grown in BrdU might, therefore, be expected. The buoyant density of the DNA of the revertant B4-E cells, which had undergone 25 generations in the presence of BrdU before being reselected in E medium, was determined after the cells had grown 30 generations in the absence of the drug. The DNA of these cells was found to have a buoyant density of 1.693, identical to that of 3460 cells. The absence of a detectable shift in buoyant density indicated that the presence of BrUra in the DNA during 25 generations did not result in a large-scale increase in G-C content.

DISCUSSION

This paper has described the characteristics of a new mutation in mammalian cells, BrdU dependence. (The term "mutation" is used to indicate that the variant phenotype is heritable, and it is not meant to imply that a change at the genetic level has been demonstrated.) The requirement for BrdU was specific for both the bromine and the deoxy sugar. Thymidine could not satisfy the BrdU requirement, but the cells needed thymidine in addition to BrdU for survival. The mutation was leaky, in the sense that some growth occurred when the dependent cells were removed from BrdU.

Tests of the physical properties of the DNA of the dependent cells demonstrated that the BrdU was being incorporated into the DNA of the cells. About 50% of the T residues were replaced by BrUra in the dependent cells, and the BrUra seemed to be evenly distributed throughout the DNA. Some of the dependent cells have undergone more than 75 cell divisions in the presence of BrdU, indicating that cells can survive for long periods of time with highly substituted DNA.

The isolation of these BrdU-dependent cells recalls the effect of BrdU on the growth of infectious bovine rhinotracheitis virus. When the virus was grown for one cycle in the presence of BrdU, it was found that BrdU was required for the growth of the virus in the subsequent cycle (16). The reason that BrdU was necessary for viral growth was not determined.

Cells able to grow in media with high concentrations of BrdU have been isolated previously from many different mammalian cell lines. Two classes of BrdU-resistant mutants have been described, one class in which the cells lacked the enzyme thymidine kinase (5) and the other class in which the cells had thymidine kinase activity, but were deficient in the thymidine uptake system (17). In both cases resistance to the drug was associated with the absence of incorporation of BrdU into the DNA. The mutant cells described in this paper differ from those previously isolated in that the 3460 B cells have both thymidine kinase activity and an active thymidine uptake system, incorporate BrdU into the DNA, and, most strikingly, are BrdU dependent.

The existence and properties of the BrdU-dependent cells raise many questions, especially the question of why do the cells require BrdU. Some of the possibilities that are being considered are based on the many effects that BrdU has been shown to have in other systems, e.g., as a mutagen, as a suppressor of differentiated functions, and as an inducer of latent viruses. Another possibility is that the BrdU dependence is not based on the action of the drug itself or on the effect of its incorporation into the DNA, but is due instead to an alteration in the DNA-synthesizing system of these cells.

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