Proc. Nat. Acad. Sci. USA
Vol. 71, No. 5, pp. 2082–2086, May 1974

Total Substitution of Bromodeoxyuridine for Thymidine in the DNA of a Bromodeoxyuridine-Dependent Cell Line

(mammalian cells)

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Communicated by Boris Ephrussi, March 4, 1974

ABSTRACT **In an attempt to isolate cells that could survive with total replacement of thymidine by bromodeoxyuridine in nuclear DNA, cells of a bromodeoxyuridine-dependent Syrian hamster line were cultured in medium containing aminopterin and bromodeoxyuridine but no thymidine. A line of cells, called HAB, was isolated. The HAB cells have been maintained in continuous cultivation for over nine months and have undergone more than 125 population doublings. Direct base analysis showed that the level of substitution of bromodeoxyuridine for thymidine in nuclear DNA was at least 99.8%, and possibly 100%. The existence of such cells raises many questions. The expected high frequency of bromodeoxyuridine-induced base transitions, including errors in both replication and transcription, would seem to be incompatible with the apparently stable transmission and expression of the genetic information in these cells.**

We have previously reported the isolation of a bromodeoxyuridine-dependent cell line that was derived from a Syrian hamster melanoma line (1). The dependent cells were characterized by the requirement for high concentrations of the drug 5-bromodeoxyuridine (BrdU) for optimal growth. The cells incorporated BrdU into their nuclear DNA, replacing approximately 50% of the thymidine residues with BrdU.

The properties of the BrdU-dependent cells are of particular interest in light of the wide range of disruptive effects that BrdU is known to have on biological systems. For example, BrdU has been shown to act as a mutagen (2), to alter patterns of transcription (3, 4), to enhance the binding of the *lac* repressor to its operator (5), to increase the affinity of chromosomal proteins for BrdU-containing DNA (6), to generally inhibit the expression of differentiated functions (7), and to bring about alterations in membrane properties (8).

The ability of the BrdU-dependent cells to survive for long periods of time with approximately 50% replacement of thymidine by BrdU led to attempts to isolate cells that could survive with total substitution of thymidine by BrdU. To obtain total substitution, we exposed BrdU-dependent cells to culture medium containing aminopterin, a powerful inhibitor of thymidine biosynthesis, and BrdU in the absence of added thymidine. Previous attempts by others to isolate cells under such conditions have been unsuccessful (9, 10). However, we report here the isolation of a cell line in which at least 99.8% of the thymidine in the nuclear DNA has been replaced by BrdU.

Abbreviation: E medium, Eagle's medium containing 10% fetal-calf serum; E-HAB medium, E medium containing 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 10 μM BrdU; E-B medium, E medium containing 10 μM BrdU; E-FUBAHD medium, E medium containing 10 μM FdU, 0.1 mM uridine, 0.1 mM BrdU, 2 μM aminopterin, 0.1 mM hypoxanthine, and 10% dialyzed fetal-calf serum in place of fetal-calf serum.

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MATERIALS AND METHODS

Isolation of Cell Lines. The isolation and properties of the BrdU-dependent cell line, B4, were described (1). The basic growth medium for these cells was Dulbecco's modified Eagle's medium supplemented with 10% fetal-calf serum (E medium). The B4 cells were maintained in E medium containing 0.1 mM BrdU. In an attempt to isolate cells with totally substituted DNA, 100-mm Falcon plastic tissue culture dishes were inoculated with 10^6 B4 cells in E medium containing 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 10 μM BrdU (E-HAB medium). Over the course of the next 6 weeks the cells were passaged three times at high density (5×10^5 cells per 100-mm dish) in E-HAB medium. During this period there was an overall increase in cell number corresponding to approximately 5 population doublings. The cells were then plated at low density (1000 cells per 60-mm dish) in E-HAB medium. Three weeks later, approximately 10 large colonies were observed in each dish. The cells from one dish were harvested and maintained as a new cell line called HAB. Because of the expected photosensitivity of BrdU-containing cells, the HAB cells were at all times protected from environmental lighting.

After 100 cell generations in E-HAB medium, an aliquot of HAB cells was transferred back to E medium. A new subline of cells, called HAB-E, was isolated and maintained in E medium.

In one set of experiments, the HAB cells were grown in E medium containing 10 μM fluorodeoxyuridine (FdU), 0.1 mM uridine, 0.1 mM BrdU, 2 μM aminopterin, 0.1 mM hypoxanthine, and 10% dialyzed fetal-calf serum (Gibco) in place of fetal-calf serum (E-FUBAHD medium). Cells grown in this medium were termed FUBAHD.

In some of the experiments on the growth of cells in different media, the HAB cells were pregrown for a short period (see table legends) in E medium containing 10 μM BrdU but no aminopterin (E-B medium).

Labeling of Cells and DNA Isolation. Cells were grown in their respective media (described above) to which was added

$H_3{}^{32}PO_4$ (New England Nuclear Corp.) at an activity of 2 μCi/ml for 2–3 population doublings prior to harvesting. DNA was isolated as described (1) except that after the first phenol extraction, DNA preparations were routinely treated with RNase A at a concentration of 50 μg/ml for 60 min at 37°. After a second phenol extraction, the DNA was extensively dialyzed against 1.0 M NaCl, 10 mM Tris·HCl pH 7.6, 1 mM EDTA, and then finally dialyzed against 10 mM Tris·HCl pH 7.6, 1 mM EDTA.

Base Analysis. DNA preparations were hydrolyzed to nucleoside 5′-monophosphates by the sequential action of DNase I and phosphodiesterase I. Reaction mixtures (1 ml) contained 20 mM Tris·HCl pH 7.6, 10 mM $MgCl_2$, approximately 0.1–0.2 mg of DNA, and 50 μg of DNase I. After incubation at 37° for 60 min, an additional 10 μg of DNase was added for 30 min. The solution was then made 100 mM with glycine buffer pH 9.1, and venom phosphodiesterase was added to a concentration of 50 μg/ml. Incubation was continued for 60 min, at which time another 10 μg of phosphodiesterase was added for an additional 30 min. In some cases nucleoside 3′-monophosphates were produced by the sequential action of micrococcal nuclease II and bovine spleen phosphodiesterase II. All enzymes were purchased from Worthington Biochemical Corp.

The 3′- or 5′-deoxymononucleotides of adenine, cytosine, guanine, thymine, and 5-bromouracil were separated by thin-layer chromatography on plates of polyethyleneimine (PEI)–cellulose, as described by Couch and Hanawalt (11). Digestion products were concentrated under a stream of nitrogen gas and suspended in absolute methanol. Samples (10–50 μl) were applied to a PEI–cellulose plate along with 10 μl of a solution contining 10 mM each of unlabeled 5′-dAMP, 5′-dCMP, 5′-dGMP, and 5′-dTMP. Chromatography was carried out in solvent systems of 0.3 M LiCl and 1 M acetic acid as described (11). Plates were air dried and then overlaid with Kodak NS-2T x-ray film for 12–18 hr. The positions of the deoxymononucleotides were determined from both the resultant autoradiograms and the detection of markers under UV light. Spots were scraped from the plates and counted in a toluene-based scintillation fluid.

Some samples were recovered from PEI–cellulose plates and subsequently subjected to electrophoresis on Whatman 3 MM paper as described by Richardson (12). UV markers were also included. After visualization of the standards under UV light, the electrophoresis strips were cut into 0.5-cm pieces and counted as described above.

RESULTS

Growth Characteristics of HAB Cells. The HAB cells have been maintained in continuous cultivation in E-HAB medium for more than 9 months, undergoing more than 125 population doublings. During this period the cells have shown little variation in growth rate, growing with a generation time of approximately 36 hr. (For comparison, the B4 cells from which the HAB cells were derived have a generation time of approximately 30 hr.)

Only a small fraction of the B4 cells survived when they were initially cultured in E-HAB medium. Tests were therefore performed to determine whether the cells that were selected in E-HAB medium represented a stable variant population specifically adapted to survive in the presence of aminopterin and BrdU, or whether any cell in the B4 population had a small chance of surviving in E-HAB medium. HAB cells were grown for 2 weeks in E-B medium containing no aminopterin. After this period, the ability of the cells to grow in E-HAB medium was determined in comparison with HAB cells that had been continuously maintained in E-HAB medium and B4 cells that had never been exposed to E-HAB medium. The results are shown in Table 1. It can be seen that the HAB cells maintained for 2 weeks in the absence of aminopterin retain the ability to grow well in E-HAB medium. These cells, therefore, seem to represent a variant in which there is a stable alteration allowing the cells to grow in the presence of aminopterin and BrdU.

TABLE 1. *Cloning of cells in E-HAB medium*

Cell line*	Cloning efficiency in E-HAB medium†
B4	0.01
HAB	0.67
HAB grown in E-B medium	0.35

* HAB cells were grown in E-B medium for 2 weeks. HAB cells that had been maintained in E-HAB medium and B4 cells that had never been exposed to E-HAB medium were also tested. Dishes (60-mm) were inoculated with 10^3 cells in E-HAB medium or E-B medium.

† The cloning efficiency is the number of colonies per dish (average of two dishes) at 10 days in E-HAB medium relative to the number of colonies in E-B medium. The absolute cloning efficiency for all three lines in E-B medium ranged from 15 to 25%.

The HAB cells were also tested for BrdU-dependence by comparing their rates of growth in medium containing either 10 μM BrdU or 10 μM thymidine. As shown in Table 2, the HAB cells grow considerably faster in medium with BrdU than in medium with thymidine, indicating that they have retained the characteristic of BrdU dependence.

The ability of HAB cells to grow at very high concentrations of BrdU was examined (see Table 3). When the concentration of BrdU in the E-HAB medium is increased 100-fold (to 1 mM), the HAB cells are unable to grow. Since the HAB cells grown in E-HAB medium have essentially all of the thymidine in DNA replaced by BrdU (as will be shown below), the sensitivity of HAB cells to increased amounts of BrdU is presumably not linked to incorporation into DNA but is due to effects of BrdU at other levels.

To decrease the possibility that HAB cells grown in E-HAB medium might be able to synthesize, or obtain from the me-

TABLE 2. *BrdU dependence of HAB cells*

Medium additive*	Cell number†
BrdU	3.3×10^5
Thymidine	8.0×10^4

* Dishes (60-mm) were inoculated with 10^4 HAB cells in E medium containing 10 μM BrdU or 10 μM thymidine. Prior to the inoculation of the dishes, the HAB cells were grown for four days in E-B medium.

† The cell number is the total number of cells per dish (average of two dishes) at 8 days after inoculation.

TABLE 3. *Sensitivity of HAB cells to excess BrdU*

Medium additive*	Cell number†
HAB	22×10^4
HAB + 0.1 mM BrdU	12×10^4
HAB + 0.3 mM BrdU	2×10^4
HAB + 1.0 mM BrdU	1×10^4

* Dishes (60-mm) were inoculated with 10^4 HAB cells in E-HAB medium to which was added BrdU at the indicated concentrations. Recall that E-HAB medium contain 10 μM BrdU.

† See footnote to Table 2.

dium, small amounts of thymidine, the cells were grown in E-FUBAHD medium. In this medium FdU has been added as a second inhibitor of thymidine biosynthesis; uridine has been added to reduce effects of FdU on RNA and protein biosynthesis; the BrdU concentration has been increased 10-fold over that in E-HAB medium; the aminopterin concentration has been increased 5-fold; and fetal-calf serum, which could contain small amounts of thymidine, has been replaced by dialyzed fetal-calf serum. Despite these very stringent conditions used to eliminate all sources of thymidine, the HAB cells continue to grow when shifted from E-HAB medium to E-FUBAHD medium. In E-FUBAHD medium, the generation time of the cells was approximately 50 hr.

Base Composition of DNA. We have previously shown by isopycnic banding in CsCl that the DNA from B4 cells has approximately 50% of its thymidine replaced by BrdU (1). Preliminary experiments in which the DNA of HAB cells was banded in CsCl showed it to have a bouyant density of approximately 1.800 g/ml, corresponding to at least 90% substitution of BrdU for thymidine. To more accurately determine the extent of BrdU substitution in these cells, a direct analysis of base composition was performed. DNA samples were digested to nucleoside 5′-monophosphates by the sequential action of DNase I and venom phosphodiesterase and then subjected to chromatography on PEI–cellulose plates. Fig. 1 is a typical autoradiogram of 5′-deoxymononucleotides on PEI–cellulose. This sample was taken from B4 cells and, therefore, contains all five of the deoxymononucleotides.

The base composition of the various cell lines is given in Table 4. In terms of the *total* base composition, the thymidine content of HAB DNA is seen to be only 0.7%, whereas the BrdU content is 29.9%. Thus, more than 97% of the thymidine in HAB DNA is replaced by BrdU. At the time of these analyses, the HAB cells had been growing in E-HAB medium for approximately 100 generations. Similar results were found for FUBAHD DNA. When HAB cells were grown for several generations in E medium containing 0.1 mM BrdU but no aminopterin (the same medium as for the growth of B4 cells), the BrdU substitution dropped back down to 68% (data not shown), the same as that for B4.

It was surprising that cells that could replace more than 97% of their thymidine with BrdU did not totally replace thymidine even under the very stringent conditions imposed by E-FUBAHD medium. To test the possibility that the small amounts of radioactivity recovered from the 5′-dTMP spot of HAB cells on PEI–cellulose plates represented something other than thymidine, the material containing these counts was rerun using a different chromatographic system. Digests of HAB DNA were first run on PEI–cellulose as usual. The 5′-dTMP spot, which was localized by a UV marker, was cut out and the deoxymononucleotide was eluted from the PEI–cellulose powder. This was then rerun by electrophoresis on Whatman 3 MM paper. Similarly, the material in the 5′-dTMP spot from B4 DNA was run on PEI-cellulose, eluted, and rerun on Whatman 3MM as a control. The results are shown in Fig. 2. It can be seen that essentially all of the radioactivity eluted from the 5′-dTMP spot on PEI–cellulose in the B4 control reruns as 5′-dTMP on Whatman 3MM. In contrast, when the material in the 5′-dTMP spot eluted from the HAB digest is rerun on Whatman 3 MM, less than 10% of the total radioactivity can be accounted for as 5′-dTMP. This too may be an overestimate, since 5′-BrdUMP runs just between 5′-dGMP and 5′-dTMP on this system and usually overlaps slightly with 5′-dTMP (see Fig. 2). It is likely, therefore, that nearly all of the counts in the 5′-dTMP spot of HAB DNA can be accounted for by 5′-BrdUMP. We conclude from these results that at most 10% of the thymidine content of HAB given in Table 4 is truly thymidine, the remainder being spillover from 5′-BrdUMP on the thin-layer chromatography plate. The degree of substitution in HAB is therefore considered to be greater than 99.8%.

The (G+C) content of the parental cell line 3460 (see Table 4), from which B4 was isolated, is slightly higher than is generally reported for rodent cells (13). The (G+C) content appears to be decreased slightly in the B4 cells, in which 67% of the thymidine has been replaced by BrdU. [This level of substitution is somewhat higher than the 50% substitution initially determined by banding in CsCl (1). It is not yet certain whether this is due to an inaccuracy in determining the level of substitution from density shift or whether it represents an actual increase in the degree of substitution in the B4 cells over the past 12 months.] The cell lines HAB, FUBAHD,

TABLE 4. *Base composition of HAB cells*

Cell line	G	T	A	C	BrdU	G+C	T/G (BrdU/G)	% Substitution*
3460 (7)†	24.0 ± 0.8	26.5 ± 0.6	28.1 ± 0.5	21.3 ± 0.4	—	45.3	1.10	—
B4 (5)	23.1 ± 0.5	8.4 ± 0.9	29.7 ± 0.7	21.6 ± 0.7	17.2 ± 1.4	44.7	1.11	67.2
HAB (11)	22.4 ± 1.2	0.73 ± 0.09	28.1 ± 1.2	19.2 ± 1.5	29.9 ± 1.2	41.6	1.36	97.7 (≥99.8)‡
HAB-E (4)	21.0 ± 1.2	29.5 ± 1.8	29.6 ± 0.8	20.9 ± 0.6	—	41.9	1.40	—
FUBAHD (6)	21.8 ± 1.2	0.42 ± 0.2	26.7 ± 1.3	20.5 ± 0.9	30.5 ± 0.6	42.3	1.42	98.7 (≥99.8)‡

* % substitution of BrdU for thymidine is calculated from the sum of T + BrdU, *not* from the T content of cell line 3460.

† Numbers in parentheses show number of trials, always with at least two different DNA preparations.

‡ See explanation in *text*.

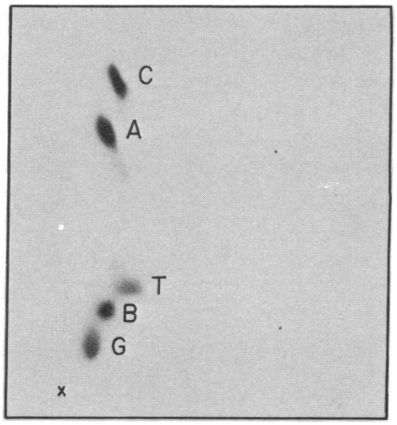

FIG. 1. Typical autoradiogram of 5′-mononucleotides separated on PEI–cellulose. A digest of B4 DNA was prepared and chromatographed as described in *Methods*. The letters *A*, *B*, *C*, *G*, and *T* refer to the 5′-deoxymononucleotides of adenine, bromouracil, cytosine, guanine, and thymine, respectively. The origin is indicated by the *X*.

and HAB-E all show a reduction in (G+C) content when compared to 3460 and B4. This reduction is primarily accounted for by a decrease in deoxyguanosine alone, rather than by a decrease in deoxyguanosine and deoxycytidine together. In addition, it can be seen that the BrdU content in HAB and FUBAHD, and the thymidine content of HAB-E, is greater than the corresponding thymidine content in 3460. Finally, while the deoxyadenosine content of HAB-E appears to be slightly higher than that of 3460, this is not true for HAB and FUBAHD.

Essentially identical results with respect to base composition and degree of BrdU substitution (98.4%) were obtained when nucleoside 3′-monophosphates were prepared from HAB DNA rather than 5′-monophosphates as described above. This result rules out the possibility that a minor pathway might be operative in the HAB cells, allowing for the incorporation of non-^{32}P-labeled dTMP into DNA, which would then elude detection.

DISCUSSION

The results reported in this paper demonstrate that it is possible to isolate a mammalian cell line in which there has been essentially total replacement of thymidine by BrdU in nuclear DNA. While approximately 0.7% of the total 5′-mononucleotide radioactivity of HAB cells is routinely found in the 5′-dTMP spot, the results of Fig. 2 suggest that no more than 10% of these counts are actually 5′-dTMP. This may also be an overestimate. Because of the very small number of counts in the 5′-dTMP spot of HAB DNA, it was necessary to apply large amounts of the 5′-mononucleotides to the PEI–cellulose plates in order to detect any activity. Therefore, the low level of spill (approximately 1% of the total 5′-BrdUMP counts) from 5′-BrdUMP into the 5′-dTMP spot is not unexpected. Additionally, the stringent conditions imposed by the E-FUBAHD medium to eliminate all possible sources of thymidine do not significantly alter the detectable thymidine level in FUBAHD DNA. These points strongly suggest that the nuclear DNA of the HAB cells has at least 99.8%, and possibly 100%, of the thymidine residues replaced by BrdU. The HAB cells to date have grown for more than 125 generations.

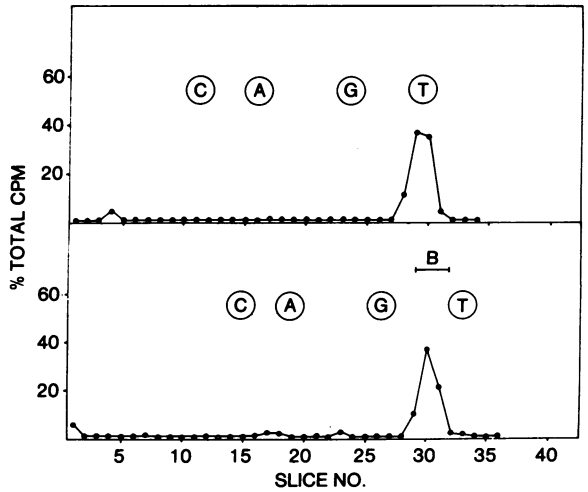

FIG. 2. Electrophoresis of the material in the 5′-dTMP spot removed from PEI–cellulose. The material in the 5′-dTMP spots of B4 DNA (*upper panel*) and HAB DNA (*lower panel*) were recovered from PEI–cellulose plates and subjected to electrophoresis on Whatman 3MM paper as described in *Methods*. The position of 5′-BrdUMP was determined by electrophoresis of ^{3}H-labeled 5′-BrdUMP on a separate paper with UV markers of the other 5′-mononucleotides. The letters *A*, *B*, *C*, *G*, and *T* are as in the legend of Fig. 1.

Given the very unusual feature of BrdU dependence in the HAB cells and the B4 cells from which they were derived, it might be argued that there exists some novel mechanism for the biosynthesis of dTTP in the HAB cells which could somehow escape labeling by ^{32}P. Such a mechanism would lead to the incorporation of unlabeled 5′-dTMP into DNA, which would not be detected by analysis of 5′-mononucleotides. This does not seem to be the case, however, since identical results with respect to extent of BrdU substitution are obtained when 3′-mononucleotides are prepared, in which case the labeled phosphate is donated by the adjacent nucleotide in DNA rather than by dTTP itself.

The existence of cells with totally substituted DNA is highly unusual, and previous attempts to isolate such cells in other laboratories have been unsuccessful (9, 10). The successful isolation of totally substituted cells in the present study may be in some way related to the BrdU dependence of the parental (B4) cells. Perhaps the most unusual feature of the complete BrdU substitution in these cells is the apparently stable transmission and expression of the genetic information, as indicated by the viability of the cells. In this context, however, it should be noted that the deoxyguanosine content in the totally substituted cells seems to be slightly lower than in the original melanoma (3460) cells, while the BrdU content in the totally substituted cells seems to be higher than the thymidine content in the 3460 cells (see Table 4). The changes in base composition are most noticeable when the T:G ratio of 3460 (1.1) is compared to the BrdU:G ratio of HAB (1.4). The difference is approximately 25%.

The mutagenic effect of BrdU in biological system is thought to be primarily through the production of base transitions. In a study of misincorporation during DNA replication *in vitro*, it was demonstrated that deoxyguanosine is misincorporated at a level of 1 per 2000 to 1 per 25,000 nucleotides polymerized in dA-BrdU-primed reactions (14). (There was no detectable misincorporation in dA-dT-primed reactions.)

Presumably such mispairing between deoxyguanosine and BrdU occurs only when BrdU is in the rare *enol* state. Errors could be of two types. Errors of incorporation would occur when BrdU paired with deoxyguanosine preexisting in the DNA chain; BrdU would pair with deoxyadenosine in the next round of replication, resulting in the transition of a G-C pair to a A-BrdU pair. Errors in replication would occur when BrdU was incorporated correctly in place of thymidine and subsequently mispaired with deoxyguanosine; in the next round of replication deoxycytidine would be incorporated, resulting in an A-T to G-C transition. In a bacteriophage, it has been shown that the major transition caused by BrdU was from G-C to A-T pairs (15). The apparent decrease in deoxyguanosine content and increase in BrdU content in the totally substituted cells are consistent with this. (It should be noted that the thymidine content of HAB-E remains as high as the BrdU content in HAB.)

Assuming that the DNA of a single cell contains approximately 10^9 base pairs, then there are about 5×10^8 thymidine residues per cell. The increase in thymidine (or BrdU) content from 3460 to HAB and its derivatives, an increase amounting to 11–17% of the original thymidine content, corresponds to a minimum change of about 6×10^7 bases, spread out over a period of less than 200 cell generations. This indicates a frequency of approximately 3×10^5 base changes per cell per generation for thymidine alone. This calculation is obviously very crude. It is surprising, nevertheless, that the cells remain viable with a frequency of base changes of such a magnitude.

In addition to base transitions at the level of DNA replication transitions at the level of RNA synthesis would also be expected. It was recently shown that as little as 15% substitution of BrdU for thymidine in mouse-fibroblast DNA results in a significantly higher incorporation of guanosine and a correspondingly lower incorporation of adenosine into RNA transcripts (3). When mouse-fibroblast chromatin in which 15% of the thymidine was replaced by BrdU was transcribed *in vitro* with a homologous RNA polymerase, there was a 35% reduction in the incorporation of adenosine into RNA and an equivalent increase in the incorporation of guanosine. Since the cells described in the present paper are essentially 100% substituted, it seems highly improbable that corresponding changes in the base composition of their RNA would be compatible with cell viability.

Another level at which BrdU substitution has previously been found to have an effect is the interaction between proteins and DNA (5, 6). It has been suggested that BrdU alters the binding of regulator molecules to DNA, and there is direct evidence for this in the case of the *lac* repressor (5). Such altered binding would be expected to pose a significant problem for cells with totally substituted DNA.

Despite all the disruptive effects that BrdU has been reported to have on cells, it is clear that cells can be isolated that survive with at least 99.8% and possibly 100% replacement of thymidine by BrdU. The viability of these cells suggests that they have developed mechanisms to avoid, compensate for, or survive with the disruptive effects of BrdU. Since BrdU has effects on many of the systems that are basic to cell survival, the elucidation of the mechanisms that permit these cells to grow with totally substituted DNA may lead to a better understanding of the ways in which these systems normally function.

We acknowledge the excellent technical assistance of Kathleen O'Malley, Thomas Wheeler, and Sarah Liberman, and the helpful suggestions of Dr. Samuel A. Latt. This work was supported in part by NICHD Grants HD04807 and HD06276. Part of the work was performed in the laboratory of Dr. Charles A. Thomas, Jr., and was supported by NSF Grant GB3118X; we thank him for support and encouragement. M.D.B. is a fellow of the Medical Foundation, Inc.

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