Tyrosine Aminotransferase Sensitivity to Bromodeoxyuridine during Restricted Intervals of S Phase in Hepatoma Cells

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ABSTRACT Synchronized hepatoma tissue culture (HTC) cells, accumulated at the G_1/S boundary with aminopterin, were released into S phase with either thymidine or 5-bromodeoxyuridine (BUdR). Tyrosine aminotransferase (TAT) activity was found to be unaffected by BUdR over the initial 3 h of S phase, but then to rapidly decline to a new basal level of 40% of control by 9 h. There was no corresponding response in the activities of alcohol dehydrogenase, malate dehydrogenase, acid phosphatase, and alkaline phosphatase, or in the rate of protein and RNA synthesis. If BUdR incorporation was restricted to limited periods of S phase, TAT was found to be maximally suppressed by incorporation into the initial 40% of the DNA. Incorporation of the analogue into the latter 60% of DNA synthesized during S phase had no effect on TAT. This is the first report that the effect of BUdR on TAT in HTC cells is associated with incorporation of the analog into DNA synthesized during a specific interval of S phase.

The thymidine (dThd) analogue 5-bromodeoxyuridine (BUdR) has been found to be a unique modulator of eukaryotic gene expression. Its incorporation into DNA is associated with blocked differentiation, the induction of viral particles, and either the suppression or stimulation of specific functions in terminally differentiated cell types (for review, see Goz [7]).

In attempts to understand the mechanism(s) of action of BUdR, one of the most extensively studied systems is tyrosine aminotransferase (TAT) in hepatoma tissue culture (HTC) cells. Stellwagen and Tomkins (19, 20) reported that growth of HTC cells in BUdR led to a rapid decrease in TAT activity that could not be attributed to a soluble inhibitor, a defective enzyme, or a change in enzyme degradation. Within the time period studied, there was no effect on other enzymes, cell proliferation, or protein and RNA synthesis. The analogue affected TAT only if present during S phase, and O'Brien and Stellwagen (16) found the TAT decrease was proportional to the percent of dThd residues replaced by BUdR in each new strand of DNA. Stellwagen and Tomkins (19) proposed that BUdR blocked transcription of the TAT structural gene.

The S phase of the eukaryotic cell is envisioned as being a synchronous period within itself during which genes replicate in repetitive sequence from one S phase to another (for review, see Hand [8]). If the TAT gene exists as a unique, single copy of DNA, and BUdR exerts its effect only when in the structural gene, then the effect should be limited to the incorporation of BUdR during a specific interval of S phase. That interval should be the time during which the effected gene replicates.

The plating efficiency of HeLa cells (9), various enzymes in L cells (10), viral antigen production in rat embryo cultures (17), and mutagenesis in various hamster cell lines (4, 22) have been found to respond to the incorporation of BUdR into specific intervals of S phase. In HTC cells, however, TAT decreased whenever BUdR was incorporated into DNA during S phase (19). If the model of Stellwagen and Tomkins (19) is correct, either the TAT gene replicates nonsynchronously throughout S phase, exists in multiple copies, or is sensitive to BUdR incorporation into DNA other than the TAT structural gene.

Another consideration, and one advanced by Tomkins and Stellwagen (19), is that the level of synchrony was low. Because this is a critical point in understanding the mechanism of BUdR action, the present study reevaluates the effect of the analogue using highly synchronized HTC cells.

MATERIALS AND METHODS

Cell Synchrony

HTC cell stocks were maintained in spinner in the absence of antibiotics, as previously described (18). Cell synchrony was achieved by initially adjusting a logarithmically growing suspension culture to 40×10^4 cells/ml, and transferring 25-ml aliquots into 150-cm^2 flasks (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.). The following morning, each flask was made to 2×10^{-7} M with Colcemid. After a 3-h incubation, the flasks were gently rocked, the old medium and unattached cells decanted off, and fresh medium containing Colcemid was carefully added. The unattached mitotic cells were harvested 8 h later, pooled, and resuspended in medium containing 0.1 mM hypoxanthine and 1.0 μM aminopterin (HA medium). From this point on, all media contained 25 μg/ml Garamycin. The resulting suspension culture was adjusted to 40×10^4 cells/ml. If monolayer conditions were to be used, 4.0-ml aliquots were transferred to

60-cm² petri dishes. The cells were maintained in HA medium for 13 h and released into S phase by the addition of either dThd or BUdR (10 μM). The mitotic index was determined by suspending a cell pellet in methanol–acetic acid (3:1), air-drying on a slide, and staining with acetoorcein.

Assays

Monolayer cultures were initially dislodged with a rubber policeman, and cells harvested by centrifugation at 1,700 *g*. The cell pellets were washed twice with 0.14 M NaCl, 0.01 M potassium phosphate buffer (PBS) at 0°C, and frozen at −20°C. Enzyme assays were performed on the 1,700-*g* supernate after the frozen cells were thawed in 0.05 M potassium phosphate buffer, pH 7.6, containing 0.2 mM pyridoxal phosphate and 0.5 mM α-ketoglutarate. TAT was assayed by the method of Diamondstone (6), malate dehydrogenase by that of Thorne et al. (21), and alcohol dehydrogenase by the method of Bonnichsen and Brink (1). Uncentrifuged lysate was used for acid phosphatase (19). A unit of enzyme activity is defined as the production of 1 mmol of product minute per milligram of protein. The temperature was 37°C for the TAT and acid phosphatase assays and room temperature for the others. Protein concentration was determined by the method of Lowry et al. (11), using bovine serum albumin as a standard.

Nucleic acids and proteins were isolated and measured by the method of Munro and Fleck (14), as described by Stellwagen and Tomkins (19). Radioactive compounds were [*methyl*-^{3}H]dThd (20 Ci/mmol), [2-^{14}C]BUdR (55.3 mCi/mmol), [6-^{3}H]uridine (24.2 Ci/mmol), and a ^{14}C-amino acid mixture (298 mCi/mmol), all purchased from New England Nuclear, Boston, Mass. Radioactive samples were assayed as described by O'Brien and Stellwagen (16), using an L5-200B liquid scintillation spectrometer (Beckman Instruments, Inc., Schiller Park, Ill.).

To determine the percent of cells actively involved in DNA synthesis at 0, 1, and 2 h after release into S phase with 10 μM dThd, separate cultures were given a 30-min pulse of [*methyl*-^{3}H]dThd (0.5 μCi/ml). At 9 h, after the initiation of S phase, aliquots were collected, washed two times with PBS, suspended in methanol–acetic acid (3:1), and air-dried on slides. Radioautographic analysis was done with Ilford Nuclear Research L4 emulsion (Ilford Limited, Basildon, England), the slides being stained with Giemsa. The percent of nuclei with silver grains was determined on a count of 400 cells.

Buoyant density analysis of DNA was done by initially extracting the DNA by the method of Marmur (12). The ethanol-precipitated DNA was solubilized in 0.15 M NaCl, 0.015 M Na citrate, pH 7.0, and adjusted to an optical density of 0.1 at 260 nm. A 200-μl aliquot was mixed with 4.0 ml of a cesium chloride solution with a density of 1.745 in the citrate buffer above. This was overlaid with 1.0 ml of mineral oil and centrifuged 72 h at 27,000 rpm in a SW50.1 rotor at 20°C. The resulting gradient was analyzed with a flow-through cuvette in a 2400 Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 260 nm.

RESULTS

Cell Synchrony

Within 2 h of suspending Colcemid-arrested mitotic cells in HA medium, the mitotic index fell from >95 to <5%, while the cell concentration nearly doubled. When 13 h later the suspension cultures were supplemented with either labeled dThd or BUdR, to initiate S phase, DNA specific activity rose over a 7–8 h period (Fig. 1). This is similar to the length of S phase reported in both synchronized (13) and unsynchronized (23) HTC cells. 1 h after completion of DNA synthesis, the mitotic index began to rise, reaching a peak of 22% before declining as the cells divided and passed into G_1.

The cultures were held for 13 h in HA medium, because this gave the maximum increase in DNA specific activity after addition of nucleoside. At longer times, even by 14 h, there was a dramatic decrease in DNA synthesis. At 10 μM the concentration of nucleoside was not limiting for DNA synthesis, as results similar to those in Fig. 1 were found with 5 and 100 μM dThd and BUdR.

The percentage of cells involved in DNA synthesis was determined by radioautography at 0, 1, and 2 h after the addition of dThd and found to be 70, 81, and 90%, respectively. Buoyant density analysis of DNA isolated 9 h after initiation of synthesis with BUdR showed 87% of the DNA to be bifilar and 13% to be unsubstituted (data not shown).

Effect of Continuous BUdR Incorporation during S Phase

After release into S phase with BUdR, TAT activity remained unchanged for 3 h, then rapidly declined to a new basal level of 40% of control by 9 h (Fig. 2). TAT activity in the control showed a gradual increase in activity of ~15% during progression through S phase, as also reported by Martin et al. (13). To keep the cells as synchronous as possible, we

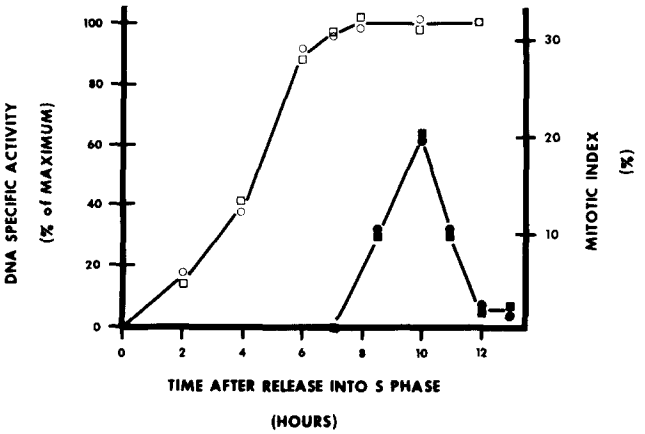

FIGURE 1 DNA synthesis and mitotic index after release of synchronized HTC cells into S phase. Cultures were synchronized as described in Materials and Methods and released into S phase with 10 μM [^{3}H]dThd (0.50 μCi/ml) (circles) or [^{14}C]BUdR (0.50 μCi/ml) (squares). DNA specific activity (open symbols) and mitotic index (solid symbols) were determined at the indicated times. Each point represents the average of duplicate determinations on duplicate samples. The maximum specific activity of the DNA labeled with dThd was 1,880 cpm/μg DNA and with BUdR it was 3,595 cpm/μg DNA.

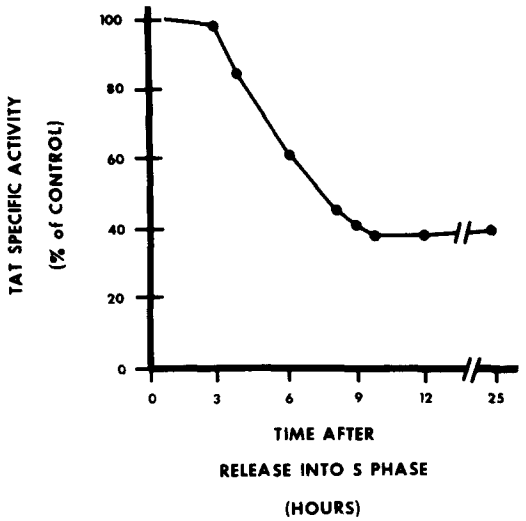

FIGURE 2 Effect of BUdR on TAT after release into S phase. Parallel spinner cultues, synchronized as described in Materials and Methods, were released into S phase with either 10 μM dThd (control) or BUdR. At 12 h, each culture was resuspended in HA medium and allowed to continue through the cell cycle. Duplicate aliquots were collected at various times and assayed in duplicate for TAT activity. Results are expressed relative to values for the dThd control. The initial value of TAT was 9,700 units/mg protein.

resuspended the cultures in HA medium at 12 h to prevent later entry into a second S phase. Under these conditions, the activity of TAT relative to the control remained constant from the level at 9 h out to 25 h. These same results were observed when the TAT assay was done on cell lysates prepared by sonication as opposed to the routine freeze-thaw method.

In the synchronized system employed here, after a single S phase in BUdR there was little change in the activities of malate dehydrogenase, alcohol dehydrogenase, alkaline phosphatase, or acid phosphatease (Table I). Although in a preliminary study (15) RNA synthesis was found to be significantly lowered, further evaluation revealed this to be in error. Protein and RNA synthesis were only slightly affected by BUdR (Table I).

Effect of BUdR Incorporation during Restricted Intervals of S Phase

In the experiment depicted in Fig. 3, parallel cultures were released into S phase with either dTHd or BUdR, and at various times aliquots were removed, resuspended in HA medium containing the other nucleoside, and allowed to continue through the cell cycle. A chase of equal molarity (10 μm) of either nucleoside by the other was found to be completely effective in blocking the further incorporations of the previous nucleoside (data not shown). Thus, the time of media change (or initiation of chase) represents the end of incorporation of the initial nucleoside and the beginning of incorporation of the second nucleoside. TAT activity was assayed on the resulting cultures 12 h after the initial release into S phase.

As seen in Fig. 3, BUdR incorporation over the initial 4 h of S phase was sufficient to achieve a maximal suppression of TAT, equivalent to that seen with incorporation of the analogue over the entire S period. On the other hand, when BUdR was used to chase dThd, the analogue had no effect on TAT if the chase began after 4 h. Attempts to limit the effective period of BUdR action to shorter increments such as 1- and 2-h pulses confirmed what is apparent from Fig. 3. The response of TAT is proportional to the net incorporation of BUdR over the initial 4-h period.

TABLE I

Effect of BUdR on Various Parameters

Parameter	Nucleoside	
	dThd	BUdR
	units/mg protein	
Malate dehydrogenase	190,000	205,000
Alcohol dehydrogenase	73,000	68,000
Alkaline phosphatase	4,610	4,200
Acid phosphatase	23,000	22,300
TAT	12,000	5,400
	cpm/mg	
Protein synthesis	10,200	9,800
	cpm/μg	
RNA synthesis	174	184

Synchronized spinner cultures were released into S phase with either 10 μM dThd or BUdR, and 4.0-ml aliquots were transferred to 60-cm² petri dishes. At 12 h, the media was replaced with HA medium to maintain synchrony; at 25 h, triplicate cultures were harvested for each enzyme to be assayed. The units and assays are described in Materials and Methods. RNA and protein synthesis were determined by a 60-min incorporation of either 90 μM [6-^{3}H]uridine (0.1 μCi/μmol) or a ^{14}C-amino acid mixture (0.5 μCi/ml) and assayed as described in Materials and Methods. All values are the averages of duplicate determinations on triplicate cultures.

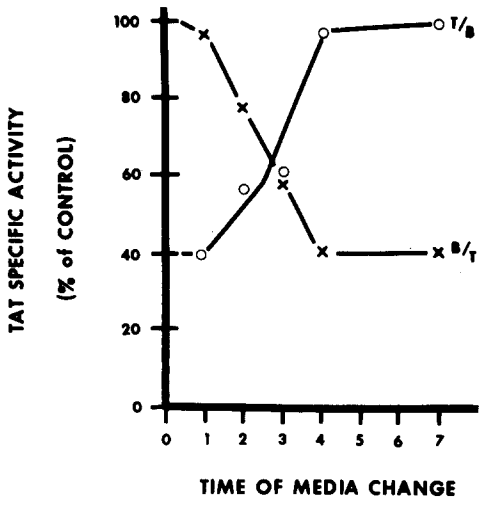

FIGURE 3 TAT response to the incorporation of BUdR during restricted intervals of S phase. Parallel synchronized cultures were released into S phase with either 10 μM dThd or BUdR. At various times, aliquots were collected and resuspended in medium containing 10 μM of the other nucleoside. The resulting cultures were all assayed 12 h after the initiation of S phase. Each value is an average of triplicate determinations and is expressed relative to a dThd control culture. BUdR chased with dThd, *B/T* (×); dThd chased with BUdR, *T/B* (○).

In many separate experiments it was confirmed that BUdR incorporation during the initial 4 h of S phase accounted for the entire effect of BUdR on TAT, whereas incorporation of the analogue after 4 h had no effect on TAT. When the cultures were synchronized at the G_1/S boundary with hydroxyurea instead of aminopterin, these same results were obtained.

DISCUSSION

It was critical to these studies that a high level of S phase synchrony be achieved. Martin et al. (13) previously showed that when HTC cells were released from Colcemid-arrested mitosis, there was a rapid loss of synchrony, with S phase beginning as a nondistinct event 10–12 h later. In the method employed here, the initially synchronized mitotic cells were released from mitosis and accumulated at the G_1/S boundary for 13 h with HA medium. The folic acid analogue, aminopterin, has been shown to be an effective inhibitor of *de novo* thymidylate synthesis in HTC cells (16), blocking DNA synthesis and allowing for the initiation of S phase by simply supplementing the medium with either dThd or BUdR. Because DNA synthesis under these conditions must use the exogenous nucleosides, the incorporation of those nucleosides into DNA is a direct measure of net DNA synthesis.

As can be seen in Fig. 1, DNA synthesis commences immediately upon addition of the nucleosides to the G_1/S-arrested cultures. The slight increase found in the rate of nucleoside incorporation during the initial 2 h is, at least in part, a reflection of a 20% increase in the number of cells entering S phase. Radioautography revealed initially that 70% of the cells took up label, and this increased to 90.1% by 2 h.

The existence of a subpopulation of nonparticipating cells is indicated by the buoyant density analysis of BUdR-substituted DNA, where ~13% of the DNA was unsubstituted upon completion of DNA synthesis. It is unlikely this is attributable to a toxic effect of the BUdR, for the growth rate of interphase

cells has been reported to be unaffected for at least two generations in the presence of BUdR and HA medium (16). Also, as seen in Fig. 1, the mitotic peak after completion of DNA synthesis is the same whether the cultures were released with dThd or BUdR. Because BUdR affects TAT only if present during S phase (19), and the extent of the effect on TAT is proportional to the percent at which BUdR replaces dThd in each new strand of DNA (16), BUdR should affect only those cells involved in DNA synthesis. The nonparticipating cells will only limit the level to which TAT decreases.

O'Brien and Stellwagen (16) reported that when interphase cells were grown in medium containing HA and BUdR, TAT declined to ~35% of control after a single generation (24 h), which is similar in extent to the response reported here for a single S phase (Fig. 2). However, the decrease in enzyme activity reported by O'Brien and Stellwagen (16) was gradual over the entire 24-h period, a reflection of the continuous and random entry of interphase cells into S phase. The data in Fig. 2, obtained using synchronized cells, limits the entire decrease in TAT activity to a 5- to 6-h period. Also, as reported with nonsynchronized HTC cells (19), the effect of BUdR is very specific for TAT (Table I).

One of the more intriguing aspects of the action of BUdR is that within any one cell system the effects of the analogue are very selective. However, when comparing the different enzymes that are affected, irrespective of cell type, we find no apparent pattern as to the enzyme's type, function, metabolic pathway, stability, or response to hormones. The enzymes studied in Table I were chosen for their reported response to BUdR. Acid phosphatase is depressed in L cells (10), whereas alkaline phosphatase is induced in other cell lines (2, 5). In unsynchronized HTC cells, growth for over two generations in BUdR results in a decrease in alcohol dehydrogenase and an increase in malate dehydrogenase (15). As shown in Table I, none of the above-mentioned enzymes were affected in HTC cells after a single S phase in BUdR, nor was there any apparent effect on overall RNA and protein synthesis. Only TAT was dramatically altered.

The primary goal of this study was to determine if the effect of BUdR on TAT could be limited to incorporation of the nucleoside into a specific period of S phase. In Fig. 3, the sensitivity of TAT to BUdR is limited to its incorporation into DNA synthesized during the initial 4 h of S phase. Judging by Fig. 1, this represents ~40% of the DNA, a value that was confirmed in many separate experiments. Incorporation of BUdR into the last 60% of the DNA was without effect on TAT.

These results differ from those reported by Stellwagen and Tomkins (19), who found that TAT decreased whenever during S phase BUdR was incorporated into DNA. It is likely that this discrepancy arises because the method of synchrony employed in these studies was specific for S phase. The method of Stellwagen and Tomkins (19) involved a BUdR pulse to interphase cells in monolayer and the serial collection of the Colcemid-arrested mitotic cells. The primary assumption of that method is a uniform G_2 period. Recently, the G_2 period of interphase HTC cells has been shown to be highly variable (13).

The data presented here are consistent with the model of BUdR action requiring its incorporation into the structural gene (19). The gene for TAT should exist in unique copy DNA, the euchromatin, which is thought to replicate during the first half of S phase. Although in some systems the order in which genes replicate appears to be highly temporal (3, 19), this is not always the case (24, 25). The period of TAT sensitivity to BUdR reported here, though broad, is similar in extent to that reported for the effect of the analogue on other systems (4, 9, 17, 22).

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