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 G1/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 analogue 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 affected 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 × 10⁶ cells/ml and transferring 25-ml aliquots into 150-cm² flasks (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.). The following morning, each flask was made to 2 × 10⁻⁶ 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 × 10⁶ cells/ml. If monolayer conditions were to be used, 40-ml aliquots were transferred to
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 G1.

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

\[ \text{DNA synthesis and mitotic index after release of synchronized HTC cells into S phase.} \]

\[ \text{Cultures were synchronized as described in Materials and Methods and released into S phase with} \]

\[ \text{10 μM } [3H] \text{dThd (0.5 μCi/ml) (circles) or } [14C] \text{BUdR (0.5 μCi/ml)} \]

\[ \text{(squares). DNA specific activity (open symbols) and mitotic index} \]

\[ \text{are expressed relative to values for the dThd control.} \]

\[ \text{The results are plotted as}} \]

\[ \text{Figure 2. Effect of BUdR on TAT after release into S phase. Parallel spinner cultures, synchronized as described in Materials and Methods, were released into S phase with either 10 μM dThd (control) or BUdR.} \]

\[ \text{At 12 h, each culture was resuspended in HA medium} \]

\[ \text{and allowed to continue through the cell cycle. Duplicate aliquots} \]

\[ \text{were collected at various times and assayed in duplicate for TAT activity.} \]

\[ \text{Results are expressed relative to values for the dThd control. The} \]

\[ \text{initial value of TAT was 9,700 units/mg protein.} \]
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 phosphatase (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

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>dThd</th>
<th>BUdR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td>units/mg protein</td>
<td>cpm/mg</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>190,000</td>
<td>10,200</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>73,000</td>
<td>12,000</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>4,610</td>
<td>23,000</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>23,000</td>
<td>12,000</td>
</tr>
<tr>
<td>TAT</td>
<td>12,000</td>
<td>-</td>
</tr>
</tbody>
</table>

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 [3H]uridine (0.1 μCi/μmol) or a 14C-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.

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 G1/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 non-distinct event 10-12 h later. In the method employed here, the initially synchronized mitotic cells were released from mitosis and accumulated at the G1/S boundary for 13 h with HA medium. The folic acid analogue, aminopterin, has been shown to be an effective inhibitor of *de novo* thymidylylate 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 G1/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
The data presented here are consistent with the model of BrdU 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 BrdU 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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REFERENCES


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