Morphological Transformation, DNA Damage, and Chromosomal Aberrations Induced by a Direct DNA Perturbation of Synchronized Syrian Hamster Embryo Cells

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ABSTRACT

The cellular effects of a direct perturbation of DNA during various portions of the DNA synthetic period (S phase) were examined. Early-passage Syrian hamster embryo cells were synchronized by growth in medium containing 1% serum, followed by hydroxyurea treatment. This method resulted in about 80% of the cells entering S phase synchronously and did not induce any detectable chromosomal abnormalities. Cells at different periods in the S phase were treated for 1 hr with 5-bromodeoxyuridine, followed by irradiation with near UV. This treatment induced chromosomal aberrations and DNA damage, as measured by changes in sedimentation profile in alkaline sucrose gradients. No specific period during S phase was significantly more sensitive to treatment with respect to cell survival, chromosomal aberrations, or DNA damage and its repair. The induction of morphological transformation was also cell phase dependent, occurring only in cells synthesizing DNA. However, in contrast to the results with the above parameters, the occurrence of morphological transformation was dependent on the portion of the S phase during which treatment was administered. The highest frequency of morphological transformation was observed for treatment in early to middle S phase, particularly in the second hr of S phase, whereas no transformation was observed in late S phase, G1-S boundary, and G2 phase. 5-Bromodeoxyuridine treatment or irradiation alone induced no changes. These results suggest that certain region(s) in the DNA of Syrian hamster embryo cells, as designated by their specific temporal relationship in the S phase, may be the more sensitive targets to the perturbation by 5-bromodeoxyuridine treatment plus near-UV irradiation for the initiation of morphological transformation.

INTRODUCTION

We have demonstrated previously that a direct perturbation of DNA by combined BrdUrd4 and near-UV irradiation treatment is sufficient to initiate neoplastic transformation of asynchronous Syrian hamster embryo cells in vitro (8, 42). BrdUrd is incorporated into only DNA of cells in place of its analog dThd, and irradiation with near UV produces a significantly higher number of photochemical lesions in BrdUrd-substituted DNA than in nonsubstituted DNA. The major photoproduct is uracil, which is formed by the dissociation of bromouracil in the radical formation, and these radicals may extract an electron from the furanose backbone. This ultimately leads to a break in the DNA, particularly when it is subjected to alkaline conditions (22, 35).

Several studies (3, 12, 25, 29, 30, 38, 39) have indicated that the DNA of mammalian chromosomes is replicated in a given chronology and pattern during the S phase. Furthermore, it has been suggested that the time at which individual genes are replicated during the DNA synthetic period is specific and constant from one cell generation to the next (2, 4, 19, 21, 23, 36). The period of S phase during which a given gene is replicated can be determined by treatment of synchronized cells with mutagens which are selective for replicating DNA, to produce mutations in the gene (1, 13, 24, 31, 34, 37). One such specific mutagenic treatment for replicating DNA is the incorporation of BrdUrd, followed by illumination with near UV (1, 8, 14, 34, 37, 40–43).

To probe further the involvement of DNA perturbation in the initiation of neoplastic transformation, we have conducted BrdUrd pulse experiments with synchronized Syrian hamster embryo cells. Experiments with synchronized cells allow us to determine whether DNA synthesis is required for the transformation of cells in culture induced by BrdUrd treatment plus near-UV irradiation. Furthermore, when synchronized cultures are treated with 1-hr pulses of BrdUrd during different hr of S phase, irradiation damage can be directed to the specific portion of DNA replicated during a pulse period. It is thus possible to demonstrate whether damage to specific regions of cellular DNA is involved in neoplastic transformation.

In this paper, we report the effects of BrdUrd treatment and near-UV irradiation upon DNA replicated during different periods of S phase in synchronized Syrian hamster embryo cells, as well as the role of specific DNA damage in the induction of morphological transformation.

MATERIALS AND METHODS

Cells and Culture Conditions. Syrian hamster embryo cell cultures were established from 13-day gestation fetuses collected aseptically by cesarean section from inbred Syrian hamsters, strain LSH/ss LAK (Lakeview Hamster Colony, Newfield, N. J.). Pools of primary cultures from littermates were stored in liquid nitrogen (5). Secondary cultures were initiated from the frozen stocks, checked for chromosome number, and pre-tested for their ability to undergo morphological transformation by treatment with BrdUrd plus near-UV irradiation without synchrony. Secondary cultures which exhibited both over 90% of

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4 The abbreviations used are: BrdUrd, 5-bromodeoxyuridine; dThd, thymidine; dCyd, deoxythymidine; HU, hydroxyurea; PBS, phosphate-buffered saline [0.14 M NaCl:3 mM KCl:8 mM Na2HPO4:1 mM KH2PO4 (pH 7.4)].

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diploidy and a suitable response to the pretesting were restored in liquid nitrogen. Ampuls from the same pool of the cryopreserved secondary cultures were used for all subsequent experiments.

The cell culture medium (complete medium) used was IBR modified Dulbecco's Eagle's reinforced medium (Biolabs, Northbrook, Ill.) supplemented with 0.22 g NaHCO₃ per 100 ml and 10% fetal bovine serum (Reheis Chemical Co., Kankakee, Ill.) without antimicrobial drugs. Cells were transferred by gentle trypsinization with 0.1% trypsin solution (1:250; Grand Island Biological Co., Grand Island, N.Y.) for 5 min at 37°C. All cells were tested periodically by Microbiological Associates (Walkersville, Md.) and found free of Mycoplasma contamination. dCyd at a concentration of 0.2 mM decreased the cytotoxicity of BrdUrd (6, 28). For this reason, 0.2 mM dCyd was included in all experiments involving BrdUrd treatment.

Chemicals and Isotopes. BrdUrd, dCyd, and HU were purchased from Sigma Chemical Co. (St. Louis, Mo.), Chemicals were dissolved in medium without serum, filter sterilized, and diluted with complete medium to the desired concentration immediately before use. All BrdUrd-containing solutions, media, and treated cells were exposed only to red-filtered light. [³H]dThd (62 Ci/mmol) and [³H]BrdUrd (23.7 Ci/mmol) were purchased from Schwarz/Mann (Orangeburg, N.Y.) and New England Nuclear (Boston, Mass.), respectively.

Cell Synchronization. Cells (2 x 10⁵) on logarithmic growth were plated in triplicate on 15-mm-diameter glass coverslips in 16-mm tissue culture cluster dishes (Costar, Cambridge, Mass.) in complete medium. After an overnight incubation at 37°C, complete medium was replaced with medium containing 1% serum, (or, in certain experiments, 2% serum) and the cultures incubated for ~36 hr. At this time, the cultures were treated with 0.32 mM HU in medium containing 10% serum for an additional 12 hr, the cultures were washed once with complete medium, and prewarmed medium was added. This was the 0-hr time point. At 0.5, 1.5, 2.5, . . . 16.5, and 17.5 hr after release from HU block, [³H]dThd was added at a final concentration of 1 μCi/ml for a 30-min pulse label. The uptake of [³H]dThd was stopped by rinsing the coverslips twice with cold 0.15 M NaCl and immersing twice in 5% cold trichloroacetic acid for 15 min. After washing with deionized water and drying, the coverslips were transferred to scintillation vials and counted in a Beckman liquid scintillation spectrometer for determination of tritium incorporation. After counting, the coverslips were removed from the vials, washed with acetone, dried, dipped in Kodak NTB-2 liquid emulsion, and exposed for 2 days for autoradiography. Following development and fixation, they were stained with hematoxylin.

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Chart 1. Experimental scheme of BrdUrd (BrdU) treatment and near-UV irradiation. As indicated in Chart 2, the length of S phase is 5 hr after the removal of HU.

Calif.). After irradiation, PBS was replaced with complete medium. Untreated cultures, cultures receiving irradiation alone, or cultures treated with BrdUrd alone were used as controls.

Cytotoxicity and Morphological Transformation. Cells (10⁴) were inoculated into each of five 100-mm dishes/group, synchronized, and treated with BrdUrd and irradiation as described above. After treatment, the cells were incubated for 7 days to allow colony formation. The colonies were fixed with methanol and stained with Giemsa, and both the number of colonies and the number of morphologically transformed colonies were scored. The cloning efficiency of untreated cells was 1 to 2%. Colonies in which cells exhibited 3-dimensional growth with random orientation and extensive crisscrossing at the periphery of the colony (5, 10, 17) were scored as morphologically transformed. Figure 1 is a representative example of a colony transformed by treatment with BrdUrd plus near-UV irradiation. The morphology of the normal colony or colonies transformed by benzo(a)pyrene was shown in our previous publication (5).

Chromosomal Aberrations. Cells were plated in 100-mm dishes at a density of 2 x 10⁵ cells/dish and synchronized. After removal of HU, the cultures were treated with 1 and 10 μM BrdUrd for 1 hr during different periods of S phase and subsequently irradiated for 5, 10, 20, and 30 min, as shown in Chart 1. Cultures were then incubated with complete medium containing 10 μM dThd. Colcemid (0.2 μg/ml) was added at the fifth hr after release from HU treatment, and the metaphase-blocked cells were harvested for chromosome preparation after 4 hr. Over 100 metaphases were scored for chromosomal aberrations.

DNA Damage Assayed by Alkaline Sucrose Gradients. Cells were plated in 100-mm dishes at a density of 2 x 10⁵ cells/dish, synchronized, treated with medium containing both 1 or 10 μM BrdUrd and 2 μCi [³H]dThd per ml for 1 hr during different periods of the S phase, and incubated for 1 hr with medium containing 10 μM dThd to allow short pieces of newly synthesized DNA to elongate. Cultures were then washed twice with PBS and irradiated for 5 to 30 min. Following irradiation, some cultures were reincubated with medium containing 10 μM dThd for 1 hr at 37°C to examine repair of DNA breakage. After harvesting by treatment with 0.5% trypsin for 3 min at 4°C, 10° cells in 0.02 ml of PBS were gently placed on a 0.25-mI lysing solution on top of a 5 to 20% alkaline sucrose gradient prepared in a 5-mI cellulose nitrate centrifuge tube. The lysing solution consisted of 0.55 M NaCl, 0.45 M NaOH, and 0.01 M disodium EDTA. The gradient contained 0.55 M NaCl, 0.45 M
NaOH, and 0.003 mM disodium EDTA. After 1 hr lysis at 37°, tubes were centrifuged in a Beckman L2-65B ultracentrifuge at 106,000 x g (34,000 rpm; SW 50.1 rotor) for 90 min at 12°C as described previously (43, 44). The tubes were pierced at the bottom and approximately thirty-nine-drop fractions were collected onto Whatman GF/C filters. The radioactivity on the filters was counted by a liquid scintillation counter. It has been demonstrated that the assay system results in sedimentation profiles with DNA dissociated from lipoprotein (45) and that the average molecular weight of DNA from untreated cultures was 2 to 3 x 10^6 (44, 45).

BrdUrd Substitution for dThd. The extent of BrdUrd substitution for dThd in newly synthesized DNA during each 1-hr period of the S phase was assayed by CsCl gradient equilibrium centrifugation according to the method of Chen et al. (15) and calculated by using a formula described by Luk and Bick (27). Cells were plated at a density of 2 x 10^6 cells/100-mm dish, synchronized, and labeled with 2 µCi [3H]BrdUrd per ml for 1 hr of S phase at a final concentration of 1 or 10 µM BrdUrd. Control cultures were labeled with 2 µCi [3H]dThd. Following the labeling period, cells were rinsed with PBS and harvested by treatment with trypsin. Cells were collected by centrifugation; resuspended at a density of ~10^6 cells/ml in a solution which contained 0.015 M NaCl: 1.5 mM sodium citrate buffer (pH 7.3; NaCl:citrate), 20 mM EDTA, 0.1% Sarkosyl NL-30 (Geigy Chemical Corp., Ardsley, N. Y.), and 100 µg proteinase K (EM Laboratories, Inc., Elmsford, N. Y.) per ml; and digested for 3 hr at 37°C. After digestion, 0.2 ml of sample was added to 4.0 ml of NaCl: citrate-buffered CsCl (Schwarz/Mann) at a density of 1.745 g/ml, overlaid with 0.4 ml of mineral oil, and centrifuged in the SW 50.1 rotor of a Beckman L2-65B ultracentrifuge at 33,000 rpm for 72 hr at 21°C. Nine-drop fractions centrifuged in the SW 50.1 rotor of a Beckman L2-65B ultracentrifuge at 33,000 rpm for 72 hr at 21°C. Nine-drop fractions were collected from the bottom of each gradient onto Whatman GF/C glass fiber filters. The radioactivity on the filters was counted by a liquid scintillation counter. It has been demonstrated that the assay system results in sedimentation profiles with DNA dissociated from lipoprotein (45) and that the average molecular weight of DNA from untreated cultures was 2 to 3 x 10^6 (44, 45).

RESULTS

Cell Synchronization. Syrian hamster embryo cells were synchronized by a modification of the method of Chang and Baserga (14), which combines a growth period in medium containing 1% serum with a subsequent blockade of the cells at the G1-S boundary by 0.32 mM HU. The progression through the cell cycle of cells released from HU blockage by the addition of complete medium is shown in Chart 2. The cells entered S phase immediately after HU release; [3H]dThd incorporation and the labeling index reached a maximum after 2 to 3 hr; S phase lasted 5 hr. A second peak of [3H]dThd incorporation and labeling index was observed 15 hr after HU release. To determine the percentage of cells capable of DNA synthesis after the HU treatment, a continuous labeling experiment was performed. Cells (2 x 10^6) were plated on 15-mm glass coverslips and synchronized. Following removal of HU, complete medium containing 1 µCi [3H]dThd per ml with 1 µM dThd was added to the cultures. After 2, 4, 5, and 6 hr, the cultures were rinsed with cold 0.15 M NaCl followed by washing with 5% cold trichloroacetic acid. The coverslips were autoradiographed to determine the labeling index. As shown in Chart 3a, the labeling index reached a maximum of 80%. With asynchronous cells in logarithmic phase, the continuous labeling index reached 100% after 24 hr (Chart 3b). These results indicate that after the synchronization process, 20% of the cells are unable to enter S phase. Together, the data indicate that Syrian hamster embryo cells which are capable of entering the S phase exhibited about 80% synchrony within the first S phase following release from HU blockages. Chart 2 also shows that the labeling index of SHE cells between the fifth

![Chart 2](chart2.png)

**Chart 2.** Progression of Syrian hamster embryo cells through the cell cycle following release from HU by the addition of complete medium. 
0, no BrdUrd treatment; •, BrdUrd (10 µM) treatment.

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![Chart 3](chart3.png)

**Chart 3.** a, continuous labeling index of synchronized Syrian hamster embryo cells obtained by autoradiography after release from HU. Cells were continuously labeled for 2, 4, 5, and 6 hr with medium containing 1 µCi [3H]dThd per ml and 1 µM dThd. b, continuous labeling index obtained by autoradiography of asynchronous Syrian hamster embryo cells in logarithmic growth. Cells were continuously labeled for 6, 24, and 48 hr with medium containing 0.1 µCi [3H]dThd per ml and 0.1 µM dThd. c, effects of serum concentration and HU dose during the synchronization procedure on DNA synthesis of Syrian hamster embryo cells. 
0, 1% serum medium for 36 hr and 0.32 mM HU for 12 hr; ○, 2% serum medium for 36 hr and 0.32 mM HU for 12 hr; ●, 1% serum medium for 36 hr and 1 mM HU for 12 hr; ▲, effect of 20% BrdUrd treatment (during the first hr of S phase) on DNA synthesis. Cells (2 x 10^6) were inoculated into 25-cm flasks and synchronized. Immediately after removal of HU, cells were treated with 10 µM BrdUrd for 1 hr. The cells were then washed once with complete medium and cultured in medium containing 10 µM dThd. At 0.5, 1.5, 2.5, 4, and 5.5 hr after BrdUrd treatment, the cells were labeled with [3H]dThd (1 µCi/ml) for 30 min. In control cultures, the cells were labeled with 1 µCi [3H]dThd per ml for 30 min at 0.5, 1.5, 2.5, 4, and 5.5 hr after HU release by replacement with medium containing 10 µM dThd. ○, no BrdUrd treatment; ●, BrdUrd (10 µM) treatment.

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and 12th hr after HU release was approximately 15%, representing the subpopulation not in synchrony. The population entering into second S phase was small due to contact inhibition of cell replication as the cells reached saturation density. The degree of synchrony was not improved by the application of 2% serum medium or 1 mm HU (Chart 3c).

The synchronization by treatment with medium containing 1% serum and subsequent 0.32 mm HU resulted in 20% of the cells grown at high cell density not entering S phase. Additionally, the synchronization process reduced the cloning efficiency of cells plated at a low cell density to 50% of asynchronous cells. Increasing the serum concentration from 1 to 2% during the synchronization process improved the cloning efficiency to 70% that of untreated cells. The 2% serum:0.32 mm HU synchronization yielded an identical pattern of [3H]dThd uptake (Chart 3c) (45). Chromosome analysis of cells in the first mitosis after release from HU block demonstrated that the synchronization procedures induced no change in chromosome number and no gross chromosomal abnormalities.

**Effect of BrdUrd Treatment on Cycling Progression of Cells in S Phase.** The treatment of cells with 10 μM BrdUrd for the first hr of S phase did not disturb the normal cycling progression of synchronized cells since identical patterns of [3H]dThd incorporation were observed for the BrdUrd-treated and control cultures in the S phase (Chart 3d).

**Effect of BrdUrd plus Irradiation Treatment on Mitotic Delay.** The treatment of cells with 1 μM BrdUrd during the first, third, and fifth hr of S phase, followed by irradiation for 30 min, did not cause mitotic delay. The peaks of mitotic indices under these conditions occurred at the same time as untreated cultures, and the level of mitotic indices was similar to that of the control (Chart 4). This suggests that there is no difference between treated and control cultures in the progression of the cell cycle prior to the first metaphase.

**BrdUrd Substitution for dThd.** The extent of BrdUrd substitution for dThd in newly synthesized DNA during each 1-hr period of S phase was determined by CsCl buoyant density analysis. In this experiment, [3H]dThd and [3H]BrdUrd were used to monitor the newly synthesized DNA from the control cells or from the BrdUrd-treated cells respectively, in the CsCl gradient sedimentation. The percentage of BrdUrd substitution is calculated from the difference in density of the [3H]dThd peak and the [3H]BrdUrd peak according to Luk and Bick (27). Therefore, the percentage of BrdUrd substitution is the % of BrdUrd substituted for dThd in the newly synthesized DNA, not the total DNA. As shown in Table 1, the extent of BrdUrd substitution in DNA replicated was found to be similar during each hr of the S phase. When synchronized cells were labeled with 0.1, 1.0, and 10 μM BrdUrd during the entire S phase, the extent of BrdUrd substitution for dThd in newly synthesized DNA occurred in a linear manner with the logarithm of BrdUrd concentration (7.6, 16.5, and 25.5%, respectively) (Chart 5). In the case of Chinese hamster cells, the logarithm of the % of dThd replacement was found to be linear with the logarithm of BrdUrd in the medium (9).

**Cytotoxicity Induced by BrdUrd plus Irradiation.** BrdUrd dose and irradiation time response curves of cell survival after treatment with BrdUrd during the first hr of S phase followed by irradiation are shown in Chart 5. Cytotoxicity increased with increasing BrdUrd dose and irradiation time.

**Effect of BrdUrd plus Irradiation Treatment on Chromosomal Aberrations.** To examine possible damage to the genetic apparatus at the chromosome level caused by BrdUrd plus near-UV treatment, treated cells were analyzed for the incidence of chromosomal aberrations (Table 2). In control cultures, none of the 100 metaphases examined contained chromosomal aberrations. Following 20-min irradiation of BrdUrd-treated cells, only 2% of the metaphases contained chromatid gaps. However, a significant increase of chromosomal aberrations was observed in BrdUrd-treated cells irradiated for 30 min. Chromosomal aberrations were induced at the chromatid level in every period of BrdUrd labeling during S phase. The incidence of chromosomal aberrations increased.

![Chart 4. Effect of BrdUrd plus irradiation treatment on mitotic delay of synchronized cells.](image)

![Chart 5. % of dThd substitution of newly synthesized DNA in hamster cells versus concentration of BrdUrd (BrdU) (in logarithm) added to culture.](image)

**Table 1.** Dependence of percentage of substitution of BrdUrd for dThd on the different periods in S phase during which BrdUrd was incorporated.

<table>
<thead>
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<th>Period of BrdUrd labeling</th>
<th>1 μM BrdUrd</th>
<th>10 μM BrdUrd</th>
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<tr>
<td>1st hr</td>
<td>12.9*</td>
<td>21.2</td>
</tr>
<tr>
<td>2nd hr</td>
<td>15.2</td>
<td>24.1</td>
</tr>
<tr>
<td>3rd hr</td>
<td>15.2</td>
<td>24.1</td>
</tr>
<tr>
<td>4th hr</td>
<td>15.2</td>
<td>24.4</td>
</tr>
<tr>
<td>5th hr</td>
<td>16.4</td>
<td>26.3</td>
</tr>
</tbody>
</table>

* Average of 3 independent experiments.
Incidence of chromosome aberrations in synchronized hamster embryo cells induced by treatment with 1-hr pulse of BrdUrd and subsequent irradiation.

<table>
<thead>
<tr>
<th>Period of BrdUrd labeling during S phase</th>
<th>BrdUrd dose (µM)</th>
<th>Irradiation time (min)</th>
<th>No. of metaphases analyzed</th>
<th>Type of aberrations (%)</th>
<th>Aberrant metaphase (%)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>G*</td>
<td>ICQ</td>
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<tr>
<td></td>
<td>10</td>
<td>30</td>
<td>132</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>2nd hr</td>
<td>1</td>
<td>30</td>
<td>100</td>
<td>19</td>
<td>0</td>
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<tr>
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<td>1</td>
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<td>100</td>
<td>16</td>
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<td>113</td>
<td>18</td>
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<tr>
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<td>30</td>
<td>111</td>
<td>7</td>
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</tbody>
</table>

* G, gap; ICQ, isochromatid gap; B, break; E, exchange; D, Dicentric; O, O-ring; F, fragmentation.

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with increasing BrdUrd dose and irradiation time except for the fifth hr. The highest incidences were observed in the second and third hr of S phase, and the lowest incidences were induced in the first and fifth hr.

Degradation and Repair of Irradiated-Cell BrdUrd DNA. The effect of BrdUrd plus irradiation treatment on sedimentation profiles of DNA on alkaline sucrose gradients was analyzed. This study was divided into 2 phases. The first phase was the effect of BrdUrd dose and irradiation time, by using synchronized cells in the first hr of S phase. The second phase concerned the dependence of the DNA damage on the different periods of S phase during which BrdUrd was incorporated.

In untreated cultures, the sedimentation profile of DNA from synchronized cells was identical to that of unsynchronized cells, indicating that the synchronization procedure itself did not produce any perturbations in the cellular DNA. By using alkaline sucrose gradient analysis, the radiosensitivity to the near UV of DNA from cells labeled was compared with either 2 µCi [3H]dThd per ml or 0.76 µCi [3H]BrdUrd per ml, plus nonradioactive BrdUrd at a final concentration of 1 µM. The same degree of DNA damage under alkaline conditions was observed in both DNA preparations. Moreover, the extent to which BrdUrd substituted for dThd was found to be identical in DNA labeled with either [3H]dThd or [3H]BrdUrd (data not shown) suggested that there was no preferential utilization of either nucleoside for DNA synthesis under these conditions. Therefore, we used [3H]dThd rather than [3H]BrdUrd for labeling newly replicated DNA in synchronized cells.

The solid lines (indicating no subsequent irradiation) in Chart 7A show that the sedimentation profiles of DNA from cells treated with 0.1 or 1 µM BrdUrd (Curves b and c) were similar

Chart 6. Effects of BrdUrd dose and irradiation time on cell survival. Cells (10⁶) were plated in 100-mm dishes, synchronized, and treated with BrdUrd for the first hr of S phase and subsequently irradiated for 0, 10, 20, or 30 min. Complete medium was then added to the dishes, and the cells were incubated to form colonies for 7 days. The colonies were fixed with methanol and stained with Giemsa. The cloning efficiency of untreated cultures was normalized to 1. O, near UV alone; •, 1 µM BrdUrd; △, 10 µM BrdUrd.

Chart 7. Sedimentation profiles of DNA from cells treated with BrdUrd plus near-UV irradiation. Vertical lines, position of the main peak of DNA from the untreated cells in the sedimentation profile. A, Dependence of DNA damage on BrdUrd dose. Cells (2 x 10⁵) were plated on 100-mm dishes, synchronized, and treated with 0(a), 0.1 (b), 1.0 (c), and 10 µM (d) BrdUrd, and 2 µCi [3H]dThd per ml for the first hr of S phase. The cultures were then incubated for 1 hr with complete medium containing 10 µM dThd and irradiated for 5 min with near UV. A, no irradiation; B, 5-min irradiation. B, Dependence of DNA damage on irradiation time. Cells (2 x 10⁵) were plated on 100-mm dishes, synchronized, and treated with 1 µM BrdUrd and 2 µCi [3H]dThd per ml for the first hr of S phase. The cultures were then chased for 1 hr with complete medium containing 10 µM dThd and irradiated for 10 (b), 20 (c), and 30 min (d) with near UV. a, 30-min irradiation alone.
to that of untreated cells (Curve a). The sedimentation profiles of DNA from cells treated with 10 μM BrdUrd without subsequent irradiation (Chart 7A, Curve d) had a lower peak and slightly broader trailing portion than did those of the control. The dotted lines in Chart 7A show the sedimentation profiles of DNA from cells treated with BrdUrd for the first hr of S phase and subsequently irradiated for 5 min. No DNA lesions apparent as single-strand breaks under alkaline conditions were detectable following 5-min irradiation without prior incubation with BrdUrd (Chart 7A, Curve a). In contrast, BrdUrd-substituted DNA was degraded by 5-min irradiation in a BrdUrd dose-dependent fashion (Chart 7A, Curves b, c, and d).

The dependence of DNA damage on near-UV irradiation time following BrdUrd treatment was examined in cells treated with 1 μM BrdUrd for 1 hr and subsequently irradiated for 10, 20, and 30 min (Chart 7B). Thirty-min irradiation alone had no effect on DNA damage detectable in alkaline sucrose gradients (Chart 7B, Curve a), whereas the peaks of DNA profiles from cells treated with BrdUrd plus irradiation shifted toward the lower sedimentation coefficient values in a manner dependent on the irradiation time (Chart 7B, Curves b, c, and d).

The dependence of DNA damage on the periods in S phase during which BrdUrd was incorporated was examined. DNA from cells treated with 1 μM BrdUrd during the first hr of S phase followed by a 10-min irradiation sedimented slower and with a broader distribution than did DNA from cells treated with BrdUrd but not exposed to near UV (Chart 8, a and b). Treatment during the second hr of S phase produced a further decrease in size. However, BrdUrd treatment during the third, fourth, or fifth hr of S phase produced sedimentation profiles quite similar to that produced by the BrdUrd treatment during the second hr (Chart 8, c to f).

The DNA damage induced by treatment with 1 μM BrdUrd for the first hr, followed by 10-min irradiation, was repaired almost completely during 1-hr incubation at 37° in medium containing 10 μM dThd. The DNA damage induced in the other periods of S phase was partially repaired at rates which were similar to one another (Chart 8, b to f).

In our alkaline sucrose gradient system, the treatment of synchronized cells with 1 μM BrdUrd for the first hr, followed by 5-min irradiation, caused enough perturbation to allow the detection of DNA damage. Such DNA damage was repaired in a subsequent 1-hr incubation (Chart 8b). In contrast, 30 min of irradiation were required to produce detectable chromosomal aberrations in cells that incorporated BrdUrd (Table 2). To study the relationship between DNA damage and chromosomal aberrations, cells pulsed with 1 μM BrdUrd and 2 μCi [3H]dThd per ml for the first hour of S phase, followed by exposure to near UV for 20 or 30 min, were subjected to repair incubation with medium containing 1 μM dThd. At the ninth hr, following removal of HU block, 10° metaphases (arrested by 0.2 μg Colcemid per ml and collected by shaking) were assayed for repair of DNA damage by alkaline sucrose gradient centrifugation. The DNA damage induced by treatment with 1 μM BrdUrd plus either 20- or 30-min irradiation was not completely repaired (Chart 9). A significant increase in chromosomal aberrations was not observed in cells which were irradiated for 20 min following BrdUrd treatment. Thus, no correlation between DNA damage and chromosomal aberrations was found.

**Morphological Transformation.** To study the relationship of a direct perturbation of DNA to the initiation of neoplastic transformation, we examined morphological transformation induced by BrdUrd plus irradiation treatment as described in "Materials and Methods." Morphologically transformed colonies (Figure 1) were not observed in the control cultures (Table 3). Chart 10 shows both the relative cell survival calculated by using untreated cultures as controls and the frequency of morphologically transformed colonies induced by treatment of synchronized cells with 10 μM BrdUrd for 1 hr of S phase and subsequent irradiation for 5 min. This chart is a compilation of 7 experiments, and the total number of transformed colonies per surviving colonies is presented. The cytotoxicity was minimal (approximately 20%), and there was no specific period in the S phase that was more sensitive to the toxic effect. A 1-hour pulse with 10 μM BrdUrd, followed by 5-min irradiation, induced morphological transformation. The highest incidence of morphological transformation was observed when the pulses were administered in the middle of S phase, particularly in the second hr, whereas no transformation was observed in late S phase, in the G1-S interphase, or in the G2 phase.

As shown in Chart 7A, Curve d, and Chart 7B, Curve b, treatment of cells with 10 μM BrdUrd plus 5-min irradiation induced a similar degree of DNA damage as with 1 μM BrdUrd.
plus 10-min irradiation. In addition, both treatments induced a similar degree of cytotoxicity and frequency of morphological transformation (Charts 10 and 11). In these 2 sets of experiments, change in the dosage of BrdUrd was compensated by change in duration of irradiation.

**DISCUSSION**

It is difficult to obtain early passage, embryonic cell populations which proceed through DNA-synthetic period (S phase) with a high degree of synchrony. Partial synchrony of Syrian hamster embryo cells has been reported by Kuroki and Sato (26), who obtained 50 to 60% synchrony of the cells by excess dThd or HU treatment, and by Popescu et al. (33), who obtained 50 to 60% synchrony of the cells by excess BrdUrd and 2 μCi (3H)dThd per ml for 1 hr, followed by exposure to near UV for 20 and 30 min. The cells were then subjected to repair incubation in complete medium containing 1 μM dThd. At the ninth hr after removal of HU block, 10^4 metaphases, which were arrested by treatment with 0.2 μg Colcemid per ml for 4 hr and collected by shaking, were assayed by alkaline sucrose gradient centrifugation. •, no BrdUrd and no irradiation; O, 1 μM BrdUrd plus irradiation for 20 min; x, 1 μM BrdUrd plus irradiation for 30 min.

**Chart 9.** Effect on DNA damage of repair incubation for 8 hr after BrdUrd plus near-UV treatment. Cells (2 x 10^5) were plated on 100-mm dishes and synchronized. Immediately after release from HU block, the cells were pulsed with 1 μM BrdUrd and 2 μCi (3H)dThd per ml for 1 hr, followed by exposure to near UV for 20 and 30 min. The cells were then subjected to repair incubation in complete medium containing 1 μM dThd. At the ninth hr after removal of HU block, 10^4 metaphases, which were arrested by treatment with 0.2 μg Colcemid per ml for 4 hr and collected by shaking, were assayed by alkaline sucrose gradient centrifugation. •, no BrdUrd and no irradiation; O, 1 μM BrdUrd plus irradiation for 20 min; x, 1 μM BrdUrd plus irradiation for 30 min.

**Table 3**

Summary of morphological transformation of synchronous Syrian hamster embryo cells in control experiments

<table>
<thead>
<tr>
<th>Period of S phase</th>
<th>No BrdUrd and no UV</th>
<th>BrdUrd only</th>
<th>UV only</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st hr</td>
<td>0/372</td>
<td>0/342</td>
<td>0/378</td>
</tr>
<tr>
<td>2nd hr</td>
<td>0/287</td>
<td>0/320</td>
<td>0/297</td>
</tr>
<tr>
<td>3rd hr</td>
<td>0/417</td>
<td>0/306</td>
<td>0/361</td>
</tr>
<tr>
<td>4th hr</td>
<td>0/302</td>
<td>0/283</td>
<td>0/317</td>
</tr>
<tr>
<td>5th hr</td>
<td>0/372</td>
<td>0/402</td>
<td>0/372</td>
</tr>
</tbody>
</table>

not appear to affect the cell cycle or induce any chromosome aberrations. Hamlin and Pardee (20) and Cress and Germer (16) observed that HU treatment similar to that used here did not alter the length of S phase relative to cells selected in mitosis from untreated cultures. Since it is possible, however, that synchronizing cells by chemical(s) produces an artificial cell response, we cannot neglect the possible effect of HU on the temporal order of DNA replication.

Previously, we demonstrated that a direct perturbation of DNA by treatment with BrdUrd and near-UV irradiation results in neoplastic transformation of Syrian hamster embryo cells (8). The present results strongly support the conclusion that DNA is a critical target in these transformation experiments. Transformation occurred only when cells were treated with BrdUrd plus near-UV irradiation during DNA replication and not
Corrected frequency of morphological transformation induced by pulse of BrdUrd treatment plus near-UV irradiation during different periods in S phase (correction from labeling index)

<table>
<thead>
<tr>
<th>Periods in S phase</th>
<th>Labeling index* (%)</th>
<th>Corrected labeling index* (%)</th>
<th>Corrected morphological transformation frequency (%)</th>
<th>Experiment (Chart 10)</th>
<th>Experiment (Chart 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before release</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0–1 hr</td>
<td>47</td>
<td>59</td>
<td>1.1</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1–2 hr</td>
<td>58</td>
<td>73</td>
<td>1.8</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>2–3 hr</td>
<td>64</td>
<td>80</td>
<td>0.96</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>3–4 hr</td>
<td>50</td>
<td>63</td>
<td>0.76</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>4–5 hr</td>
<td>18</td>
<td>23</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>After 5 hr</td>
<td>15</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Data from Chart 1.
* The continuous labeling index during the entire S phase is 80% (Chart 3a).

Therefore, the corrected labeling index = labeling index/0.8.

Corrected morphological transformation frequency (%) = (Total no. of scored survival colonies × Corrected labeling index) × 100

(see text).

Effects of DNA Perturbation on Synchronized Hamster Cells

when cells were treated similarly during the G1 or G2 period, a period at which DNA synthesis does not take place. These findings indicate that BrdUrd incorporation into DNA is required for the neoplastic effect of BrdUrd plus near-UV irradiation.

Not only was morphological transformation induced by BrdUrd and near-UV treatment only when this treatment was administered during the S phase, but also certain times during the synthetic period appeared more susceptible to this induction, as shown in Charts 10 and 11. The results in both charts suggest that the induced morphological transformation frequency is highest during the 1- to 2-hr period and lowest during the 4 to 5 hr in the S phase. However, due to imperfection in the synchronization, the labeling indices of all these periods in S phase are not exactly the same. To have the quantitative answer concerning the propensity to morphological transformation of cells having their DNA perturbed at a different period of S phase, this cell number in a population must be correctly identified by the labeling index. Such an analysis was performed in Table 4. The labeling indices in Table 4 were obtained from experiments reported in Chart 2. These indices were further corrected by dividing them with 0.80, the fraction of cells in the population capable of entering into S phase, indicated in the experiment of continuously labeling (Chart 3a). Therefore, the number of the corrected labeling index identified the fraction of cells in each population which had incorporated BrdUrd into their DNA and therefore was perturbed by this treatment in the experiment. For instance, in the experiments reported in Chart 10, the observed number of surviving colonies in each period of the S phase (in parentheses) are as follows: 2023 (0 to 1 hr); 1821 (1 to 2 hr); 1948 (2 to 3 hr); 1872 (3 to 4 hr); and 1869 (4 to 5 hr). The number of surviving colonies in these populations, originating from cells which had incorporated BrdUrd and therefore received the perturbation in their DNA, should be the observed number multiplied by the corresponding, corrected labeling indices and are listed as follows: 1194 (0 to 1 hr); 1329 (1 to 2 hr); 1558 (2 to 3 hr); 1179 (3 to 4 hr); 1179 (3 to 4 hr); and 430 (4 to 5 hr). The corrected morphological transformation frequency (%) shown in Table 4 was obtained by dividing the number of morphologically transformed colonies with the number of the surviving colonies which had been perturbed (i.e., the observed number of surviving colonies multiplied by the appropriate corrected labeling index). For example, the corrected morphological transformation frequency for the 1- to 2-hr period reported in Chart 10 was 24/1329 = 1.8%.

From examining the corrected morphological transformation frequency in Table 4, the cells having their DNA perturbed at the 1- to 2-hr period during the early S phase have twice the propensity to become transformed morphologically compared to cells having their DNA perturbed at the periods of 0 to 1 hr, 2 to 3 hr, and 3 to 4 hr. On the other hand, the cells having their DNA perturbed at the 4- to 5-hr period during the late S phase do not have any tendency to become transformed. These findings suggest that certain region(s) in the DNA of Syrian hamster embryo cells, as designated by their specific temporal relationship in the S phase, are the more sensitive targets to the perturbation by BrdUrd treatment plus near-UV irradiation for the initiation of morphological transformation. These "transformation-sensitive genes" may be replicated more preferentially at the 1- to 2-hr period and least preferentially at the 4- to 5-hr period of the S phase.

In contrast to the results with morphological transformation, no specific period during S phase of synchronized Syrian hamster embryo cells was markedly more sensitive to treatment with BrdUrd plus near-UV irradiation with respect to cell survival, chromosomal aberrations, or DNA damage and repair. The degree of cytotoxicity, incidence of chromosomal aberrations, extent of DNA damage and repair, and the level of BrdUrd substitution showed no correspondence with the frequency of morphological transformation. Although this does not rule out a role for these general toxic effects of BrdUrd plus near-UV treatment in the transformation of the cells, it does indicate that a specific portion of the genome may be important and that a nonspecific action of the treatment, e.g., cytotoxicity, is probably not sufficient to cause transformation.

Although only morphological transformation was studied in this report, many reports (11, 17, 18) have suggested that in vitro morphological transformation of Syrian hamster embryo cells correlate with tumorigenicity. Pienta et al. (32) recently demonstrated a very high positive correlation (90.8%) between morphological transformation and the reported carcinogenic activity of several chemicals. We have presented evidence that morphological transformation is an early event in a progressive development of neoplastic transformation of these cells in culture (6, 7). In further experiments, we have observed that synchronized mass cultures treated with a 1-hr pulse of 10 μM BrdUrd within the first 4 hr of S phase and subsequent irradiation for 5 min exhibit neoplastic transformation following successive passages in culture. Cells treated with BrdUrd plus near-UV irradiation in the absence of DNA synthesis or in late S phase were not induced to undergo neoplastic transformation. Control cultures treated with BrdUrd alone or near-UV irradiation alone in either nonreplicating or S phase cells were not transformed to the tumorigenic state.

In conclusion, this report strongly supports our observations that a direct perturbation of DNA is sufficient to induce neoplastic transformation. This work demonstrates that DNA can be one critical target in chemical-physical carcinogenesis. Fur-
thermore, these studies suggest the intriguing possibility that discrete regions of DNA replicated during different periods of S phase have varying degrees of sensitivity toward this perturbation capable of initiating neoplastic transformation. Since this perturbation also leads to somatic mutation, it is plausible that somatic mutation(s) in this region of genome is the cause of neoplastic transformation. It should be noted that this investigation does not exclude the possibility that other macromolecules inside the cell, such as RNA and proteins, can also be critical targets for other perturbations which might lead to neoplastic transformation or the possibility that neoplastic transformation can be initiated by nonmutational events.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. Morphologically transformed colony of Syrian hamster embryo cells 7 days after treatment with 10 μM BrdUrd during the first hr of S phase plus near-UV irradiation for 5 min. Aqueous Giemsa (3%). × 40.
Morphological Transformation, DNA Damage, and Chromosomal Aberrations Induced by a Direct DNA Perturbation of Synchronized Syrian Hamster Embryo Cells

Takeki Tsutsui, J. Carl Barrett and Paul O. P. Ts'o