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Late S phase cells (Chinese hamster ovary) induce early S phase DNA labeling patterns in G1 phase nuclei*

(order of S phase/S phase inducers/heterokaryons/cell cycle)

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ABSTRACT **Cells (Chinese hamster ovary) in G1 phase were fused with cells in late S phase to determine if a cell in late S phase can induce DNA synthesis in the nucleus of a G1 cell and, if so, to determine if the DNA synthesis so induced in a G1 phase nucleus has an autoradiographic pattern characteristic of early or of late S phase synthesis. The results indicate (*i*) that 89% of the G1 nuclei in late-S/G1 binucleates synthesized DNA, while only 2% of the control unfused G1 cells synthesized DNA, and (*ii*) that in all late-S/G1 binucleates the G1 nucleus was induced to synthesize early S phase DNA. These results are compatible with the idea that a cytoplasmically transmissible factor initiates DNA synthesis but that an intranuclear mechanism defines the temporal order of replication.**

Two important problems in the study of cell reproduction are the mechanism governing the initiation of DNA replication and the mechanism responsible for the temporal ordering of DNA synthesis within the S phase. Evidence indicates that a cytoplasmically transmissible inducer of DNA synthesis is present in early S phase HeLa cells; when a G1 phase cell was fused with an early S phase cell 1 hr after its release from a double thymidine block, DNA synthesis was induced prematurely in the G1 phase nucleus (1). In addition, fusion of erythrocytes (2) or macrophages (3) with S phase cells that had been selected at various times after mitosis has suggested that a cytoplasmically transmissible inducer is present throughout the S phase. However, the difficulty in positively identifying late S phase cells in these types of experiments leaves open the question of whether such an inducer of DNA synthesis is present in late S phase. If such an inducer is present in late S phase, does it induce the synthesis of early or late S phase DNA in a G1 nucleus; i.e., is DNA synthesis induced in the normal temporal order or are there specific late S phase initiators that can alter the normal replication sequence?

To study these problems we fused Chinese hamster ovary (CHO) cells in late S phase with G1 phase cells and determined by autoradiography the pattern of [^{3}H]thymidine ([^{3}H]dThd) incorporation in the heterophasic homodikaryons. The experiments were based on the existence of different topographical patterns of DNA synthesis for early and late S phase cells, as determined by electron microscope autoradiography (4). In general, euchromatin replicates early in S phase; cells in early S phase show evenly distributed [^{3}H]dThd labeling of the nuclei. The bulk of heterochromatin replicates late in S phase; label is distributed primarily at the periphery of the nuclei and in the nucleoli. Thus, at least three possible outcomes may be predicted for the fusion of a late S phase cell to a G1 phase cell. In a late-S/G1 binucleate the nucleus in G1 phase at the time of fusion may be (*i*) unlabeled, if a late S phase cell does not contain a cytoplasmically transmissible inducer of DNA synthesis, (*ii*) labeled evenly throughout its nucleus if early S phase DNA synthesis is induced, or (*iii*) labeled at the periphery of its nucleus and in the nucleolus if late S phase DNA synthesis is induced.

MATERIALS AND METHODS

Cells. Chinese hamster ovary (CHO) cells were grown in monolayer culture in Ham's F12 medium supplemented with 6% fetal calf serum. Under our culture conditions, the generation time of the CHO cells was approximately 15 hr.

Cell Synchrony. CHO cells were grown in the presence of 2 mM deoxythymidine (dThd) for 9 hr to synchronize them coarsely in S phase (5–7). The medium was replaced, and 5–7 hr later mitotic cells were collected by the shake-synchrony procedure (8, 9). With this procedure generally 95–99% of the detached cells were in mitosis. The mitotic cells were grown in conditioned medium for 1 hr to obtain G1 phase cells. Alternatively, the mitotic cells were grown in conditioned medium containing 1 mM hydroxyurea for 9 hr to obtain early S phase cells (10, 11). The hydroxyurea was removed and the cells were allowed to proceed to late S phase.

Labeling with Latex Beads. To identify fusion products between cells from S and G1 phase populations we used latex beads (Dow Chemical Co., Indianapolis, IN) of two different sizes to prelabel the cytoplasms of the cells (approximately 1 μm diameter beads for the S phase cells and approximately 2 μm diameter beads for the G1 phase cells; ref. 12). Cells were allowed to incorporate latex beads during the period after the removal of 2 mM dThd and before the mitotic cells were collected. Because CHO cells do not incorporate latex beads well, 10 μg of DEAE-Dextran (Sigma) per ml was added simultaneously with the latex beads to stimulate phagocytosis.

Cell Fusion. The method described by Rao and Johnson (13) was used with minor modifications. In brief, 1×10^6 cells of each type were placed in a total volume of 1 ml of fusion medium (14), pH 8, containing 200 hemagglutinating units of ultraviolet-inactivated Sendai virus. The virus/cell mixture was kept at 4° for 15 min and then at 37° for 30 min. The resulting fused cells were grown on carbon-coated coverslips. At 2 and 3 hr after fusion the cells were pulse labeled with [^{3}H]dThd to determine labeling patterns.

Percent [^{3}H]dThd-Labeled Nuclei. Cells were labeled with [^{3}H]dThd (5 μCi/ml and 20 Ci/mmol, or 10 μCi/ml and 50 Ci/mmol) for 15 min. The cells were fixed in methanol/acetic acid (3:1 vol/vol), and the slides were dipped in Kodak NTB3 liquid emulsion and exposed for 7 days. After autoradiographic processing, the cells were stained with Giemsa stain (15).

[^{3}H]dThd Labeling Patterns. Cells grown on carbon-coated

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Abbreviation: CHO, Chinese hamster ovary.

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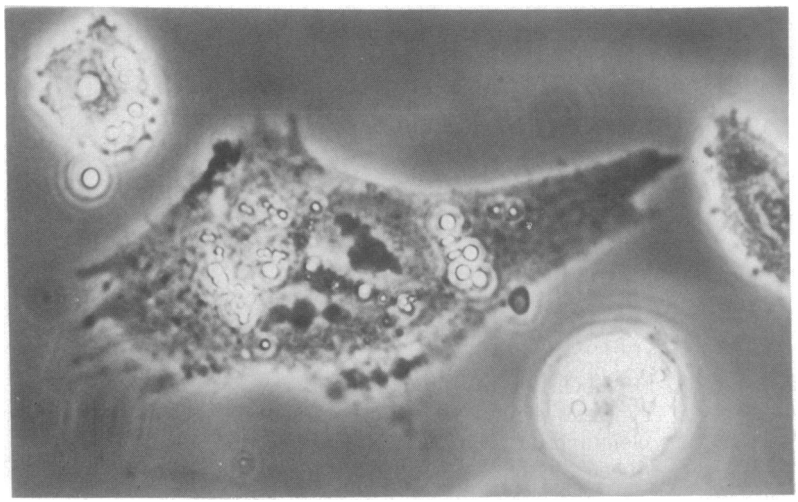

FIG. 1. Phase photomicrograph of a binucleated CHO cell containing 1-μm and 2-μm latex beads.

coverslips were labeled with [^{3}H]dThd (5 μCi/ml and 20 Ci/mmol, or 10 μCi/ml and 50 Ci/mmol) for 15 min. The cells were fixed in 1.5% glutaraldehyde, dehydrated, and embedded by placing the coverslips cell-side down onto Epon/Araldite-filled caps made of polyethylene. Serial sections of the monolayer were cut with a glass knife and mounted on microscope slides. The slides were dipped in NTB3 emulsion, and exposed for 30 days for 0.5-μm thick sections and for 21 days for 1-μm thick sections. After autoradiography, the cells were stained at room temperature for 20 min with a 1:25 dilution of a crystal violet stock solution (which contained 20 g of crystal violet in 95 ml of 95% ethanol and 8 g of ammonium oxalate in 800 ml of water), or they were stained at approximately 50° for 10 sec–2 min with 1% basic fuchsin in 50% acetone.

In the fusion experiments, we had to determine that a cell contained two nuclei and both 1-μm and 2-μm latex beads before sectioning, in order to identify unequivocably homodikaryons from the G1 and S phase populations (Fig. 1). Therefore, after the cells were fixed, the coverslips were placed cell-side down onto microscope slides containing coverslip fragments as spacers. The coverslips were affixed to slides with a 1:1:1 mixture of vasoline/lanolin/paraffin (wt/wt) (16). Binucleates containing small and large latex beads were located with a phase microscope, encircled by an objective scribe, and photographed for future identification. The coverslips were removed from the slide, dehydrated, and embedded. Serial sections of the monolayer were cut, and the sections were dipped in emulsion.

RESULTS

Labeling patterns in CHO cells in early and late S phase

To confirm that CHO cells exhibit easily recognizable patterns of labeling with [^{3}H]dThd in relation to temporal position in S phase, i.e., that cells in early S phase are labeled evenly throughout the nucleus and that cells in late S phase are labeled only at their periphery of the nucleus and in nucleoli, we did the following: CHO cells selected in mitosis were grown in the presence of 1 mM hydroxyurea to synchronize them in early S phase (see *Materials and Methods*). Fig. 2 shows the percentage of nuclei labeled with [^{3}H]dThd at various times after removal of hydroxyurea. At 5 hr after removal of hydroxyurea, 91% of the nuclei were labeled. Labeling fell sharply from 83% at 7 hr to 15% at 9 hr and then to 10% at 10 hr. The data show that S cells were well synchronized with respect to transit through the S phase.

Fig. 3 shows the labeling pattern in sectioned cells at 1 hr (Fig. 3*A*) and 5 hr (Fig. 3*B*) after removal of hydroxyurea. For cells in early S phase (Fig. 3*A*) the labeling is evenly distributed throughout the nucleus. Fig. 3*B* contains sections of cells in late S phase; in most of the cells the incorporated [^{3}H]dThd is restricted to the periphery of the nuclei and to the nucleoli, a pattern characteristic of late S phase. Two cells in Fig. 3*B* show a more even distribution of grains. In addition, approximately 3% of the cells (not illustrated) exhibited a labeling pattern intermediate between an even distribution and a peripheral distribution. These labeling patterns were presumably due either to cells that had made the transition from early to late S phase during the labeling period or to sections that were some distance away from the middle of the nucleus. Nuclei with such patterns were not included in the quantitative data (Table 1).

Table 1 shows the percentage of labeled nuclei and the percentage of cells with a peripheral labeling pattern in unsynchronized, logarithmic phase cells and for synchronized cells

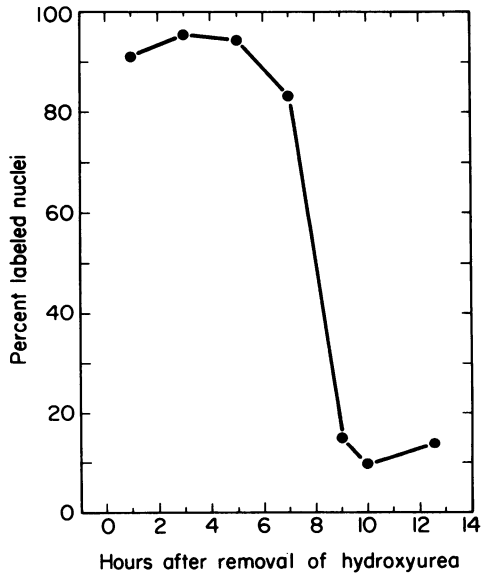

FIG. 2. Percentage of cells synchronized in S phase. CHO cells were grown in the presence of 2 mM dThd for 9 hr. The dThd was removed; after 5 hr mitotic cells were selected by the shake-synchrony procedure. Then the cells were grown in the presence of 1 mM hydroxyurea for 9 hr. The hydroxyurea was removed and the cells were labeled with 5 μCi of [^{3}H]dThd per ml for 15 min.

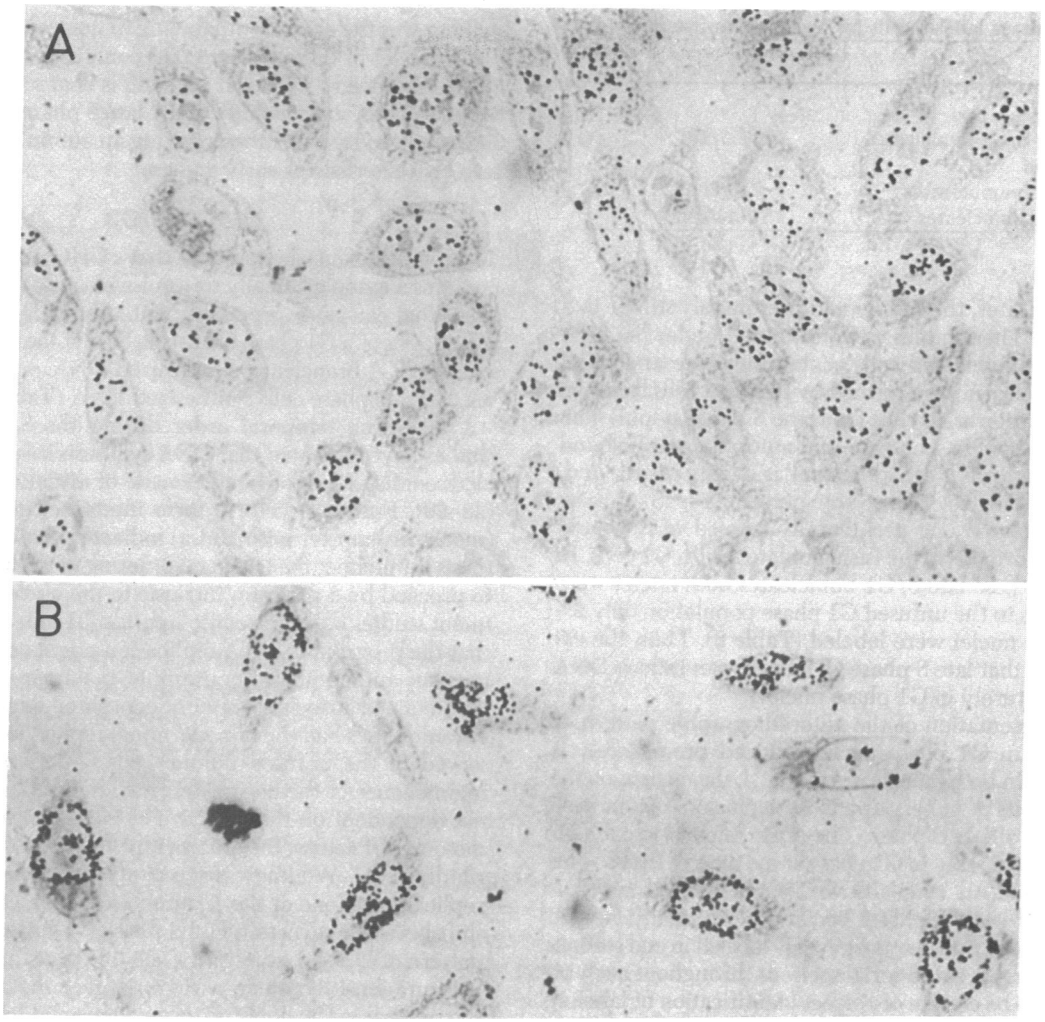

FIG. 3. Light autoradiogram demonstrating the [^{3}H]dThd labeling pattern in early and late S phase CHO cells (0.5 μm thick sections). Cells were synchronized and labeled as described in the legend of Fig. 2. (*A*) Cells at 1 hr after removal of hydroxyurea have an evenly distributed labeling pattern. (*B*) Cells at 5 hr after removal of hydroxyurea. All labeled cells but two have a peripheral labeling pattern.

in S phase (shown in Fig. 2). In logarithmic phase, 65% of the cells were labeled. Of the labeled cells, 29% had a peripheral labeling pattern. Of early S phase cells, at 1 hr after removal of hydroxyurea, 1% of the labeled cells had peripheral labeling patterns. The percentage of labeled cells with peripheral patterns rose to 54% at 5 hr after removal of hydroxyurea and reached a peak of 71% at 7 hr after removal. These results therefore established that CHO cells in early S phase synthesize DNA throughout the nucleus (even distribution of silver grains) and CHO cells in late S phase synthesize DNA only near the nuclear envelope and in the nucleoli (peripheral distribution of grains). These patterns presumably reflect replication of euchromatic DNA in early S phase and of heterochromatic DNA in late S phase (17–19).

Table 1. Percentage S phase nuclei and percentage late S phase nuclei for unsynchronized and synchronized cultures

Cells	% labeled nuclei*	% labeled nuclei with late S phase pattern†
Unsynchronized	65	29 (70/243)
S phase, hr after removal of hydroxyurea		
1	91‡	1 (3/312)
3	95	—
5	94	54 (241/450)
7	83	71 (135/189)
9 & 10	12	50 (83/167)

* Three hundred cells counted.
† Calculated as (nuclei with peripheral pattern)/(nuclei with peripheral and evenly distributed pattern) × 100.
‡ From data in Fig. 2.

It is reported that under extreme conditions hydroxyurea might affect labeling patterns; when CHO cells were exposed to hydroxyurea for long durations, peripheral labeling patterns were produced (20). We have treated cells with hydroxyurea for 9 hr, which does not produce such an effect. One hour after removal of hydroxyurea only 1% of the cells had a peripheral labeling pattern (Table 1). Also, after removal of hydroxyurea, the S phase was 8–9 hr, which is the reported length of S phase in CHO cells (21, 22). Thus, in our hands CHO cells behave normally with respect to labeling pattern and length of S phase after reversal of a 9-hr inhibition by hydroxyurea.

Fusion of late S phase cells with G1 phase cells

CHO cells in late S phase (containing 1-μm latex beads) were fused to CHO cells in G1 phase (containing 2-μm latex beads). For late S phase cells we used cells 5 hr after removal of hydroxyurea so that by the time the cells were fused and samples

Table 2. Percentage labeled cells for fused and unfused cultures

Cells	% labeled
Fused	
Late-S/G1 binucleates	89 (17/19)
Unfused	
G1 mononucleates	2 (6/300)
S mononucleates	87 (435/500)

taken 2 and 3 hr later, the cells would presumably still be in S phase (Table 1). The samples were examined under the light microscope for binucleated cells containing large and small beads; such binucleates were formed by fusion of cells from the G1 phase population with cells from the S phase population. These binucleates were sectioned and autoradiographed and their labeling patterns were identified as evenly distributed, peripheral, or unlabeled. Thirty-nine percent (19/48) of these binucleates contained one peripherally labeled nucleus and therefore were formed by the fusion of late S with G1 cells. In 89% (17/19) of these late-S/G1 binucleates both nuclei were labeled, whereas in the unfused G1 phase population only 2% of the G1 phase nuclei were labeled (Table 2). Thus, the experiment shows that late S phase CHO cells can induce DNA synthesis prematurely in G1 phase nuclei.

Typical representation of the autoradiographic pattern of DNA synthesis in G1 phase nuclei induced prematurely is shown in Fig. 4. In both binucleates in Fig. 4, the nucleus on the left is labeled at its periphery and in its nucleolus. This pattern is characteristic of late S phase. The other nucleus in each binucleate was presumably in G1 phase at the time of fusion. This nucleus produced an even distribution of grains, which is characteristic of early S phase. Because peripherally labeled nuclei (late S phase) would appear evenly labeled in end sections of the nuclei, we prepared serial sections throughout each binucleated cell to be certain of correct identification of labeling patterns. In all 17 late-S/G1 binucleates examined, one nucleus had peripheral label and one had evenly distributed label.

In the late-S/G1 binucleates, if early S phase and late S phase DNA synthesis had both been induced in G1 phase nuclei, we would expect to see nuclei possessing both an evenly distributed labeling pattern (early S phase synthesis) and a more intense labeling pattern at the periphery and in the nucleoli (late S phase synthesis). Such patterns were never seen. Particularly striking was the absence of labeling in nucleoli in induced G1 phase nuclei in those sections that contained identifiable nucleoli. Absence of nucleolar labeling is characteristic of early S phase. Thus, we conclude that a late S phase cell induces in a G1 nucleus DNA synthesis having an autoradiographic pattern characteristic of early S phase.

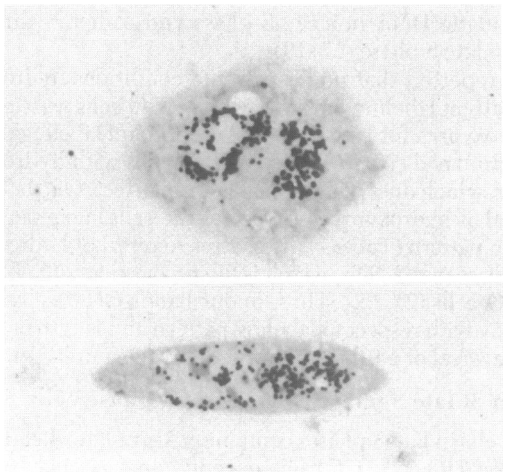

FIG. 4. Light autoradiogram of two late-S/G1 CHO cell binucleates (1 μm thick sections). The fused cells were labeled with 10 μCi of [^{3}H]dThd per ml for 15 min.

DISCUSSION

These experiments demonstrate that a CHO cell in late S phase contains a cytoplasmically transmissible inducer of DNA synthesis that can cause premature initiation of DNA synthesis in the nucleus of a G1 phase CHO cell; 89% of the G1 phase nuclei in late-S/G1 binucleates synthesized DNA, while only 2% of unfused G1 phase cells synthesized DNA (Table 2).

Concerning temporal order during the S phase, several studies have indicated that DNA synthesis in a eukaryotic nucleus consists of an ordered cascade of initiations of replicons (23–29). Fusion of cells to form interspecific heterokaryons (mouse or hamster with HeLa) indicates that once DNA synthesis is initiated, the temporal ordering of replication appears to proceed by a program intrinsic to the nucleus (30). Subsequent studies of interspecific hybrids (31–34) have suggested that the program of DNA replication might actually be chromosome autonomous. For example, in human–mouse hybrids that retained an average of 11 human chromosomes, the human chromosomes had the same terminal replication sequence observed in the unfused human cells (34). Furthermore, the maintenance of the normal terminal replication sequence was not dependent on the presence of any specific human chromosome. Of course, the order with which DNA replicates is not rigidly fixed. We know, for example, that the timing of the replication of one of the X chromosomes in a female cell can shift between early and late S phase (18). Also, the temporal pattern of DNA synthesis in a cell line transformed by a temperature-sensitive simian virus 40 *A* gene mutant differs from the pattern in the nontransformed parental line (35).

Wille and Kauffman (36) have claimed to have demonstrated that fusion of late S phase plasmodia of *Physarum* to early S phase plasmodia induces the premature synthesis of late S phase DNA in the early S phase nuclei. However, the plasmodia they describe as being in early S phase had already synthesized 30–60% of late S phase DNA, and this was increased by 18% by fusion to a late S phase plasmodium. The experiment therefore at best shows some increase in synthesis of late S phase DNA in nuclei presumably already well into synthesis of regularly scheduled, late S phase DNA. Wille and Kauffman acknowledge that these data may be interpreted as a more rapid progression through the normal sequence of replication events rather than a permutation of the normal temporal order of replication.

The experiments reported here indicate that a late S phase cell induces the synthesis of early S phase DNA in a G1 nucleus, i.e., for all late-S/G1 binucleates the nucleus in G1 phase at the time of fusion had an evenly distributed labeling pattern (Fig. 4). We interpret this to mean that the cytoplasmically transmissible inducer present in a late S phase cell cannot alter the temporal order of DNA synthesis in a G1 phase nucleus induced to enter S phase prematurely. Rather, the inducer present in late S phase cytoplasm sets in motion a program of DNA synthesis that follows its normal temporal order. However, more refined techniques will be needed to tell us whether synthesis induced in G1 phase nuclei proceeds throughout the S phase in a completely normal temporal order at a replicon-by-replicon level. Also, the question remains whether the inducer present in late S phase cytoplasm functions only to initiate DNA syn-

thesis at the start of S phase or also has a role in maintaining the progression of DNA synthesis throughout the S phase.

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