HYDROXYUREA DOES NOT PREVENT SYNCHRONIZED G_1 CHINESE HAMSTER CELLS FROM ENTERING THE DNA SYNTHETIC PERIOD

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SUMMARY

Using very high concentrations of radioactively labeled thymidine, we show that synchronized G_1 cells treated with hydroxyurea entered the DNA synthetic period at a time and rate indistinguishable from that of untreated cells, although the rate of DNA synthesis was greatly reduced in the drug-treated cultures. The DNA synthesized in the presence of hydroxyurea was $\leq 1 \times 10^7$ daltons, all of which could be chased into bulk DNA of $\sim 3.5 \times 10^8$ daltons within 3 hr after removal of hydroxyurea. Hydroxyurea synchronized cells are apparently not blocked at the G_1/S boundary but in the S phase itself.

INTRODUCTION

Mammalian cell populations synchronized at various stages within the cell cycle have proven to be very useful in studying the biochemistry of the cell cycle and the cellular response to external agents at various stages of the cell cycle. As a result of the high rate of synchrony decay during the G_1 period of the cell cycle (1), it is often necessary in the study of late interphase events to resynchronize cells initially synchronized by mitotic selection. Although a number of agents which inhibit DNA synthesis can be used for this purpose, many have been found to allow significant amounts of DNA synthesis (2,3). However, when hydroxyurea is used in combination with mitotic selection, it provides a highly synchronized, viable cell population (4) with the point of synchronization usually presumed to be at the G_1 side of the G_1/S boundary, as suggested by several lines of evidence (5). The results presented below indicate that hydroxyurea does not block cells at the G_1/S boundary but, rather, permits G_1 cells to enter the S phase at the same time and rate as the untreated controls.

METHODS

Chinese hamster cells (line CHO) were grown exponentially in suspension culture as previously described (6). Cells were synchronized by selective detachment of mitotic cells from monolayer cultures on glass and treated with

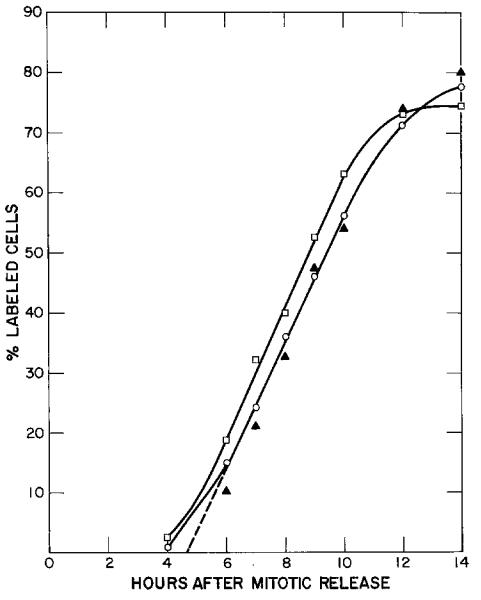

Fig. 1. Entry of mitotically synchronized G_1 cells into the DNA synthetic period assayed by autoradiography. (-□-) Control cells pulse-labeled 15 min with 2.0 μCi/ml ^{3}H-thymidine; (-▲-) control cells continuously labeled with 0.1 μCi/ml ^{3}H-thymidine beginning 1 hr post-synchronization; and (-○-) cells treated with hydroxyurea and continuously labeled with 100 μCi/ml ^{3}H-thymidine beginning 1 hr post-synchronization.

hydroxyurea (1 mM) according to the protocol described by Tobey and Crissman (5). The fraction of cells in S phase was determined by autoradiography after labeling the cells with (methyl-^{3}H)-thymidine (55 Ci/mmole, New England Nuclear), as previously described (7). Alkaline sucrose density gradient centrifugation of DNA was performed under conditions described by Walters and Hildebrand (8). Gradients were fractionated as described earlier (9).

RESULTS

The effect of hydroxyurea on the ability of G_1 cells to enter the DNA synthetic phase (S) was examined in the following manner. At 1 hr after mitotic synchronization, hydroxyurea (1 mM) and ^{3}H-thymidine (100 μCi/ml) were added. Samples were taken for autoradiographic analysis at intervals thereafter. Untreated control cultures were also assayed for entry into S by either (a) pulse labeling 15 min with 2 μCi/ml ^{3}H-thymidine at intervals after mitotic synchronization or (b) continuous labeling with 0.1 μCi/ml ^{3}H-thymidine beginning 1 hr after mitotic synchronization. The photographic emulsion was exposed for 1 day in the case of untreated control cells and 3 days in the case of hydroxyurea-treated cells. The results are shown in Fig. 1. It is clear that hydroxyurea did not affect either the time or rate of entry of G_1 cells into S. It should be noted that either a brief exposure

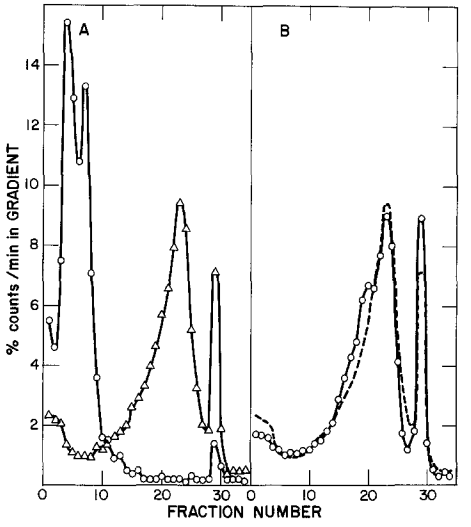

Fig. 2. Alkaline sucrose density gradients of DNA from 2 x 10^5 cells. (A) DNA from mitotically synchronized cells grown in hydroxyurea and 100 μCi/ml ^{3}H-thymidine from 1.0 to 9.5 hr post-synchronization (-O-); and DNA from exponentially growing cells labeled for 36 hr with 0.05 μCi/ml ^{14}C-thymidine, followed by a 3-hr chase (-Δ-). (B) DNA from cells grown in hydroxyurea and 100 μCi/ml ^{3}H-thymidine for 1.0 to 9.5 hr post-synchronization, followed by a 3-hr chase period after removal of hydroxyurea and ^{3}H-thymidine (-O-). The dashed line in (B) represents bulk DNA and was taken from panel A. DNA recovery from the gradients was ~80%. Direction of sedimentation was left to right.

(e.g., 15-min pulse label) or continuous exposure to radioactivity of untreated control cells yielded very similar estimates of the time and rate of entry of cells into S. The ~ 0.5-hr difference in time of entry into S obtained between pulse labeling and continuous labeling (Fig. 1) is within the variation normally seen among different cultures. This fact, together with the observation that a 1000-fold increase in concentration of radioactivity during continuous labeling of hydroxyurea-treated cells yielded identical estimates of the time and rate of entry into S as that of untreated cells (Fig. 1), suggests that exposure to radioactivity did not significantly perturb the cells. The earlier failure of a number of different measurements to detect entry of hydroxyurea-treated cells into S was likely a problem of sensitivity (5).

To examine the DNA synthesized during hydroxyurea treatment, hydroxyurea (1 mM) and ^{3}H-thymidine (100 μCi/ml) were added 1 hr after mitotic synchronization. Cells were harvested 9.5 hr after mitotic synchronization when 50-55% had entered S (Fig. 1). The DNA was analyzed on alkaline sucrose density gradients with the results shown in Fig. 2A. The DNA synthesized

during hydroxyurea treatment was quite small, yielding a bimodal distribution with molecular weights at the peaks of $\sim 2 \times 10^6$ and $\sim 1 \times 10^7$ daltons, respectively. For comparison, we also show in Fig. 2A the distribution of bulk DNA obtained from exponentially growing cells labeled for 36 hr with 0.05 μCi/ml (methyl-^{14}C)-thymidine (55.3 mCi/mmole, Schwarz/Mann), followed by a 3-hr chase. The bulk DNA sedimented at $\sim 3.5 \times 10^8$ daltons under these conditions.

We also examined the fate of radioactive DNA synthesized during the 1.0 to 9.5 post-mitotic synchronization hydroxyurea treatment. At 9.5 hr after mitotic synchronization, cells were removed from hydroxyurea and ^{3}H-thymidine by low-speed centrifugation, washed twice with cold F-10 culture medium, and resuspended in warm, fresh culture medium. The cells were allowed to grow for 3 hr, then harvested for DNA analysis on alkaline sucrose gradients. It is clear from the data in Fig. 2B that virtually all of the radioactivity from small DNA synthesized in the presence of hydroxyurea chased into bulk DNA within 3 hr after removal of radioactive label and hydroxyurea. A more detailed analysis of chase kinetics will be presented elsewhere (manuscript submitted).

DISCUSSION

Our results indicate that the DNA synthesized during hydroxyurea treatment cannot be attributed to either cytoplasmic DNA synthesis or repair synthesis. We did not detect significant cytoplasmic DNA synthesis by the following criteria: (a) microscopic examination of the autoradiographs showed that radioactive labeling was almost exclusively nuclear; (b) $\leq$ 2% of the cells could be identified by autoradiography as incorporating ^{3}H-thymidine within 4 hr post-mitotic synchronization (e.g., during G_1) (see Fig. 1); and (c) the small DNA synthesized could be chased into bulk DNA of $\sim 3.5 \times 10^8$ daltons (Fig. 2B), a molecular weight much larger than the alkali-sensitive (10) mitochondrial DNA (11).

Repair synthesis in response to radioactive thymidine exposure during hydroxyurea treatment is unlikely for the following reasons: (a) autoradiographic scoring of cells labeled both in the presence and absence of hydroxyurea showed the same low, early G_1 background and the same time and rate of entry of G_1 cells into S; (b) although repair synthesis of this type is hydroxyurea-resistant (12), virtually none of the DNA synthesized during hydroxyurea treatment sediments with bulk DNA (Fig. 2A); (c) if, in the event the DNA synthesized during hydroxyurea treatment were unintegrated pieces of DNA, part or all of which were repair patches, these pieces would have to be much larger than repair patches known to be inserted after exposure

to ionizing radiation (12); and (d) hydroxyurea does not induce DNA degradation for exposure times < 24 hr (13).

The data presented here show that hydroxyurea does not prevent, or even slow down, the rate of entry of synchronized G_1 cells into S. While only a small quantity of DNA is synthesized during hydroxyurea treatment [1-4% of the total DNA (unpublished observations and 3)], it appears to represent true semi-conservative replication of DNA which is smaller than the average replicon (14). Although Coyle and Strauss (13) and Fujiwara (15) have reported the synthesis of small DNA in the presence of hydroxyurea, their experiments were performed with cells actively synthesizing DNA at the time of hydroxyurea addition and would represent synthesis in which many replicons had already initiated DNA replication. On the other hand, the results reported here indicate that at least some of the replicons can actually initiate DNA synthesis in the presence of hydroxyurea.

We have previously reported that hydroxyurea treatment of synchronized G_1 cells did not prevent the scheduled increase in size of the acid-soluble pools of dTTP, dCTP, and dGTP (16), the increase of which is associated with the entry into S of untreated G_1 cells. However, we were unable to detect any increase in size of the dATP pool (16). We suggested at that time that hydroxyurea prevented DNA synthesis as a consequence of a failure to synthesize dATP. It is clear now that a re-interpretation of those results is in order. From the results presented in this communication, it appears that the normally scheduled increase in the acid-soluble pools of dTTP, dCTP, and dGTP of hydroxyurea-treated G_1 cells occurred not because cells achieved some S functions in the absence of DNA synthesis but because hydroxyurea did not inhibit the entry of G_1 cells into S. It is likely that the absence of an increase in the dATP pool level of hydroxyurea-treated cells is a result of utilization of dATP for DNA synthesis. However, we still feel that, *under our growth conditions*, dATP availability is rate-limiting for DNA synthesis, as opposed to depletion of both dATP and dGTP reported for other cell lines (17,18). F-10 medium in which the cells are grown contains 3 μM thymidine, the transport and phosphorylation of which are not inhibited by hydroxyurea (16). Since these cells obtain $\geq$ 50% of the dTTP pool from exogenous thymidine (6), we might expect to see a sparing effect on dGTP in hydroxyurea-treated cells as a combination of both allosteric stimulation by dTTP of GDP reduction and amelioration of inhibition of GDP reduction as a consequence of the very low levels of dATP.

Sinclair (19) has shown that hydroxyurea is lethal to S-phase Chinese hamster cells. However, we have found that the protocol of hydroxyurea treatment described above for our cells did not reduce the plating efficiency for

cell colony formation below that seen for untreated cells (4). Since it appears that hydroxyurea does not prevent entry of G_1 cells into S phase, killing of S-phase cells may be a time-dependent response, as suggested by Coyle and Strauss (13), and/or may be a consequence of as yet unrecognized hydroxyurea effects on DNA metabolism.

In view of these findings, it is important to recognize that use of hydroxyurea as a synchronizing agent produces a cell population that is both biochemically different from untreated cells (16) and will contain varying numbers of S-phase cells, depending upon the length of hydroxyurea treatment. This does not limit its usefulness, but it does require that these facts be considered.

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