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Cell Biology

Most of the G_1 period in hamster cells is eliminated by lengthening the S period

(growth cycle/chromosome cycle/hydroxyurea)

GEORGE M. STANCEL*†, DAVID M. PRESCOTT*, AND R. MICHAEL LISKAY‡

*Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado 80309; and ‡Department of Therapeutic Radiology, Yale University School of Medicine, New Haven, Connecticut 06510

Contributed by David M. Prescott, July 6, 1981

ABSTRACT **Two Chinese hamster cell lines, G_1^+-1 and CHO, have been grown in the presence of low concentrations of hydroxyurea to determine how a slowing of DNA synthesis (i.e., a lengthening of the S period) affects the length of the G_1 period. Hydroxyurea concentrations of ≈10 μM do not alter the generation times of these cell lines but do cause increases in S with corresponding decreases in G_1. In both cell lines, 10 μM hydroxyurea reduces G_1 to an absolute value of 1 hr, which represents decreases of 70% (G_1^+-1) and 60% (CHO) from control values. Higher concentrations of hydroxyurea increase the generation times and lengths of S for both cell lines but do not reduce G_1 below the minimum value of 1 hr. These observations indicate that the majority of G_1 is expendable and most of G_1 therefore cannot contain specific events required for the initiation of DNA synthesis. This result supports the hypothesis that G_1 is a portion of the cell growth cycle but not of the chromosome cycle.**

The cell life cycle contains two interacting cycles, a chromosome cycle and a growth cycle, as first suggested by Mitchison (1). The chromosome cycle consists of replication and distribution of chromosomes. The growth cycle accomplishes a doubling in the size of the cell, with increases in all of the functional and structural components of the cell (1, 2). We know relatively little about these increases in cell constituents and the mechanisms that coordinate them.

Clearly, the chromosome cycle spans the S, G_2, and M periods; what is less clear is the relationship of the G_1 period to the chromosome cycle. Traditionally, G_1 has been regarded as a period of preparation for initiation of DNA synthesis, but no specific events have been discovered. The best evidence for specific G_1 events is provided by isolation of cells having conditional mutations that arrest in G_1 at the restrictive temperature (3–5). The G_1 period must contain at least one specific event—i.e., the event that terminates G_1 by triggering the initiation of DNA synthesis. But this event presumably occurs extremely rapidly and does not occupy enough time to account for a measurable part of G_1. This assumption is consistent with the observation that the cells in cleavage stages of embryos of a wide range of animal species have cycles lacking a G_1 period (G_1^- cells) (refs. 6–8; for review, see ref. 2). At least one normal, adult cell type—one of the bone marrow cells in the erythrocyte series—has been reported to lack a G_1 period (9), and two cultured cell lines normally proliferate without a G_1 period (10–12).

The existence of cell types that initiate DNA synthesis without a G_1 period suggests that G_1, when it does exist, has no role in preparations for DNA synthesis. According to this view, G_1 is not part of the chromosome cycle but rather an interruption between the completion of one chromosome cycle and the start of the next.

This interruption may result from failure of the cell growth to keep pace with the chromosome cycle. We know that cell growth and the chromosome cycle are somehow interconnected. Considerable evidence indicates that the interconnection occurs at the beginning of the chromosome cycle—i.e., initiation of DNA synthesis is triggered by attainment of a critical cell size (or some derivative of cell size). If growth is inhibited, for example, by limitation of an essential amino acid or lack of sufficient serum or specific growth factors, DNA synthesis is not initiated. Similarly, we have previously shown that slowing growth by partial inhibition of protein synthesis induces a G_1 period in a cell that is normally G_1^- (13).

We propose that the G_1 period in cultured cells is not part of the chromosome cycle but belongs to the growth cycle. If doubling in cell size is completed as rapidly as the chromosome cycle (equal to S + G_2 + M), the cell cycle will lack a G_1 period. When growth is slower, the initiation of DNA synthesis is delayed (a G_1 period is introduced) until growth is completed.

The work described in this paper is a test of the view that the G_1 period is a period of growth belonging to the growth cycle but is not part of the chromosome cycle. The basic plan was to lengthen the chromosome cycle by slowing DNA synthesis with a low level of hydroxyurea without changing the cell growth rate. By this manipulation, we expected to equalize the growth and chromosome cycles and eliminate the G_1 period in cell types that normally have cell cycles with G_1 periods. The experiments confirm the hypothesis that at least most of G_1 is a period of growth and not an essential part of the chromosome cycle.

MATERIALS AND METHODS

The V79-8 (10–12) and G_1^+-1 (14) cell lines used in these studies have been described. The CHO line was a gift of R. G. Ham. In all cases, cells were grown at 37°C in Dulbecco's modified Eagle's medium (GIBCO)/10% fetal calf serum (Flow Laboratories, McLean, VA) containing nonessential amino acids (GIBCO), additional glutamine added just prior to use (0.58 mg/ml), and gentamycin sulfate (50 μg/ml, Sigma) under a 96% air/4% CO_2 atmosphere. Hydroxyurea (Sigma) was prepared in water, diluted with medium, filter sterilized, and added to cultures at the start of each experiment.

The procedures used for cell cycle analysis have been described (13, 14) and are briefly noted below. Stock cultures were trypsinized and cells were plated in 25-cm^2 T flasks at 75,000–150,000/flask. After 16–18 hr, generation times were determined by direct cell counts of marked areas on each flask

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† On sabbatical leave from: The University of Texas Health Science Center at Houston, Medical School, Department of Pharmacology, Houston, Texas 77025.

over a 22- to 28-hr period. The experimental points were fitted by linear regression analysis, and the slopes obtained from the linear regression analysis were used to calculate generation times. In most cases, correlation coefficients (r^2) of 0.99 were observed and, in all cases (with or without hydroxyurea), values of 0.95 or more were obtained, indicating that cultures were in logarithmic phase growth over the entire course of each experiment.

The length of S period was determined from the percent labeling index as follows. Cultures were pulsed for 15 min with [^{3}H]thymidine (4 μCi/ml; specific activity, $\approx$50 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), harvested, fixed in methanol/acetic acid (3:1), and dropped on glass slides. Slides were dipped in Kodak NTB2 emulsion and stored at 0–4°C for 3–4 weeks prior to developing and scoring. Both the labeling index and generation times were corrected for any noncycling cells in the population by determining the percentage of cells labeled after exposure to a low concentration of [^{3}H]thymidine (0.4 μCi/ml) for a time equal to the experimentally measured generation time. The corrected generation times and labeling indices, along with the measured values of G_2 + M/2, were then used to calculate the length of S from an age-distribution formula (15).

The length of G_2 + M/2 was determined in all cases by the percent labeled mitoses method as described (12). Since the value of G_2 + M/2 was not affected by hydroxyurea for the cell lines used, M was taken to be 0.5 hr, the value previously measured by direct observation of V79-8 cells (11).

The length of G_1 was then determined by subtraction of (S + G_2 + M) from the corrected generation time.

RESULTS

Hydroxyurea decreases the rate of DNA synthesis by inhibiting the enzyme ribonucleotide diphosphate reductase (16). We initially sought to determine whether a level of this drug could be found that would increase the length of S without increasing the generation time of G_1^+-1 cells. For this purpose, we examined the relationship between hydroxyurea concentration and generation time.

As shown in Fig. 1, concentrations of hydroxyurea up to 25 μM do not alter the generation time of G_1^+-1 cells; above 25 μM, the generation time increased in a dose-dependent fashion. We therefore examined the effect of 25 μM hydroxyurea on the cell cycle of this cell line. The results of these studies are shown in Fig. 2.

We found that 25 μM hydroxyurea does not increase the generation time of the cells but does increase S by 2 hr, with a corresponding decrease in the length of G_1. This level of hydroxyurea had no measurable effect on the length of G_2 + M/2 determined experimentally by the fraction-labeled mitoses method. The length of G_1 in the presence of this level of hydroxyurea was 0.85 hr (29% of the control value of 2.9 hr), a reduction in G_1 length by 71%.

The increase in S corresponding to a decrease in G_1 induced by hydroxyurea is a dose-dependent phenomenon, as shown by the effect of lower levels of the drug. At 5 μM and at 10 μM, hydroxyurea G_1 decreased by 30% and 44%, respectively, with corresponding increases in S (Table 1), with no effect on the generation time (see also Fig. 1).

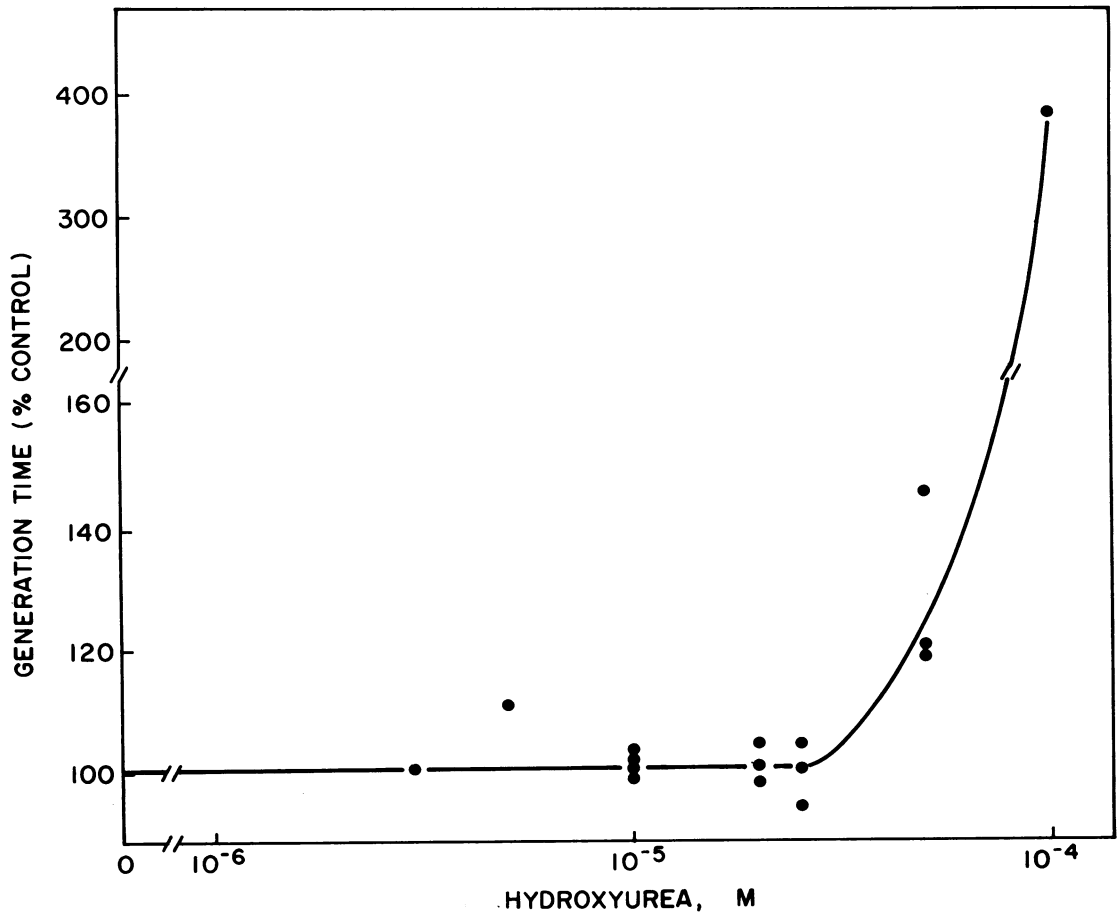

FIG. 1. Effect of hydroxyurea concentration on generation time of G_1^+-1 cells. The range of control values was 12.8–14.1 hr.

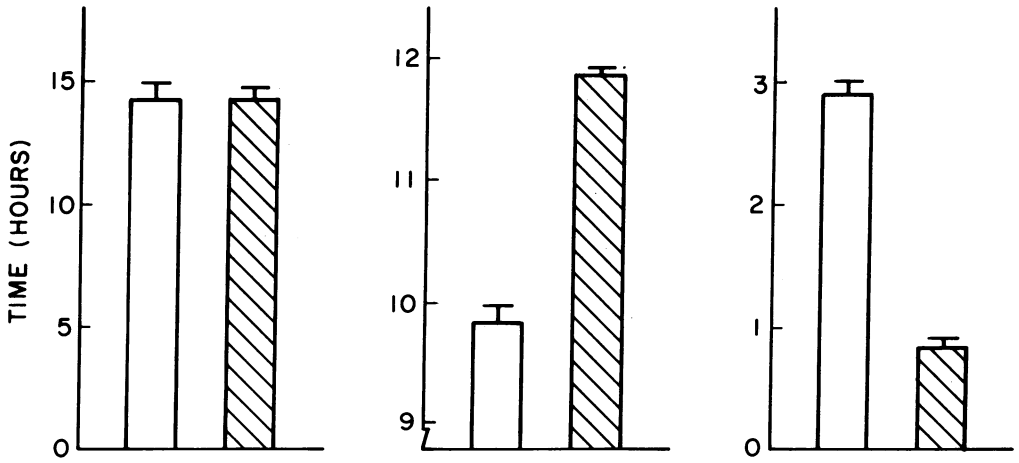

FIG. 2. Effect of 25 μM hydroxyurea on the cell cycle of G_1^+-1 cells. (*Left*) Generation time. (*Middle*) S period. (*Right*) G_2 period. □, Control; ▨, presence of hydroxyurea. Values represent mean ± SEM of three determinations.

Since 25 μM hydroxyurea reduced but did not completely abolish G_1, we investigated the effect of 50 μM hydroxyurea. This level of drug (Fig. 3) caused (*i*) a 4-hr increase in generation time, (*ii*) a 6-hr increase in S, and (*iii*) a decrease in G_1 of ≈2 hr (from 2.9 to 0.9). The higher concentration of hydroxyurea did not alter the length of G_1 + M/2.

These results indicated that, even at a hydroxyurea concentration that significantly increases the generation time, a small but reproducibly measurable G_1 period of ≈1 hr occurs. One possible explanation for this is that hydroxyurea has an unrecognized effect on cell growth that is distinct from its effect on DNA synthesis and, by this effect, causes a short G_1 period to be retained. To test this possibility, we investigated the effect of 50 μM hydroxyurea on the cell cycle of V79-8 cells. This cell line does not have a G_1 period (10–12) and is the parent line from which the G_1^+-1 cells were derived (14). In these studies, 50 μM hydroxyurea increased the generation time of V79-8 cells from a control value of 8.4 hr to 12.7 hr. At this drug level, however, no G_1 period could be detected. It seems unlikely, therefore, that some unknown effect of hydroxyurea causes the 1-hr G_1 observed in G_1^+-1 cells.

We examined the effects of hydroxyurea on a second cell line, CHO, whose cell cycle normally has a G_1 period. This is in contrast to the G_1^+-1 cell line in which appearance of G_1 was presumably induced by mutation of the G_1^- line V79-8 (14). The results of these studies are shown in Table 2.

In the absence of any drug, CHO cells have a generation time of 13.6 hr and a G_1 period of 2.4 hr. At 40 μM hydroxyurea, which does not alter the generation time, S is increased by 1.4 hr with a corresponding decrease of G_1 to 1 hr (i.e., to 40% of the control value). As seen in Table 2, higher levels of hydroxyurea increase the generation time and the length of S, but G_1 is not further decreased below the value of ≈1 hr. As previously noted for the G_1^+-1 cells, none of the hydroxyurea concentrations used altered the value of G_2 + M/2 measured for CHO cells.

Table 1. Effect of hydroxyurea on cell cycle of G_1^+-1 cells

	Time, hr		
	Generation	S period	G_1 period
Experiment 1			
Control	12.8	7.9	3.3
Hydroxyurea (5 μM)	12.8	8.9	2.3
Experiment 2			
Control	13.2	9.1	2.5
Hydroxyurea (50 μM)	13.4	10.4	1.4

Results represent single determinations.

DISCUSSION

Our results show that low concentrations of hydroxyurea (≈10 μM) increase the length of S period without changing the generation time. Lengthening of S is accommodated by a shortening of G_1. The G_1 period is shortened by 70% in G_1^+-1 cells and by 60% in CHO cells. We conclude that at least most of G_1 is expendable and therefore does not contain specific events required for initiation of DNA synthesis. Instead, the data lead to the conclusion that most of G_1 in G_1^+-1 cells and CHO cells represents the completion of the growth required to initiate S.

The results reported here are similar to the recent results of Singer and Johnston with yeast (17). Genetic studies in yeast have defined a point in the cell cycle termed "start" that occurs at or close to the G_1/S border (18). Low concentrations of hydroxyurea that do *not* increase the generation time in yeast increase the length of S and cause a corresponding decrease in G_1 (17). In this system, however, "G_1" (i.e., the time between M and start) can actually be decreased to zero. Hydroxyurea thus produces qualitatively similar effects in yeast and the two mammalian cell lines we have studied.

In addition to the studies reported here, numerous other observations support the hypothesis that G_1 is primarily a period of generalized cell growth rather than a set of specific cellular processes that regulate entry into S. For example, (*i*) some cells, including the V79-8 line used here, do not exhibit a G_1 (10–12)—i.e., they are phenotypically G_1^-; (*ii*) G_1^- cells can be induced to exhibit a G_1 period by conditions that slow cell growth without affecting the length of the chromosome cycle (13); (*iii*) G_1^+ "mutants" may be derived from G_1^- parental cell lines. The majority of these G_1^+ mutants have slower growth rates as evidenced by decreased rates of protein synthesis relative to those of the parental G_1^- cells, but the length of the chromosome cycle remains unaltered (13). The significance of these observations in relationship to the nature of G_1 has been discussed previously (19). As pointed out by Cooper (20), the view that G_1 is simply a period of growth suggests a unified picture of cell reproduction in both prokaryotes and eukaryotes.

Although our studies indicate that the *majority* of time a cell spends in G_1 is not a prerequisite for entry into S, we have not been able to reduce G_1 to zero. It is conceivable, therefore, that the minimum 1-hour G_1 period we have observed represents

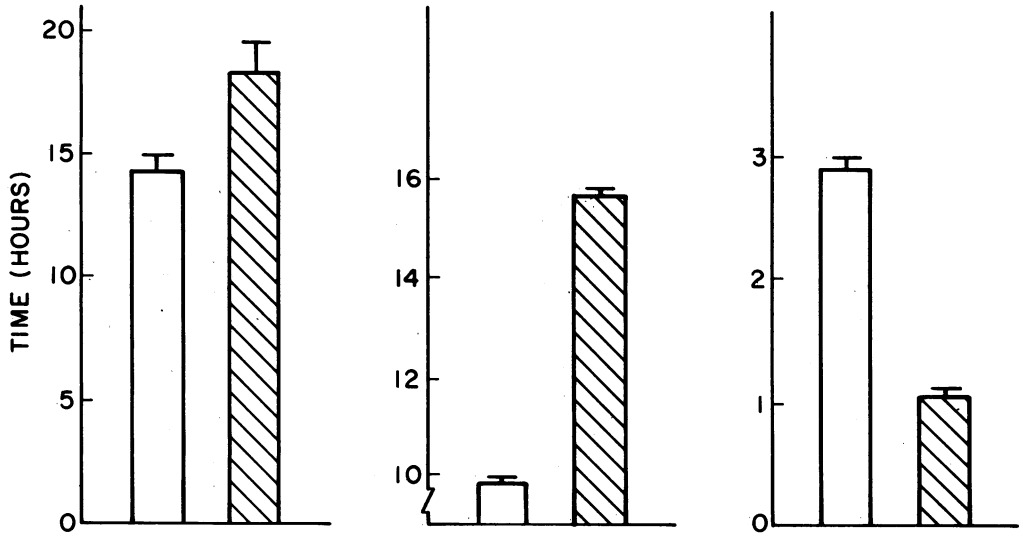

FIG. 3. Effect of 50 μM hydroxyurea on the cell cycle of G_1^+-1 cells. (*Left*) Generation time. (*Middle*) S period. (*Right*) G_1 period. □, Control; ▨, presence of hydroxyurea.

a period during which some specific event or events must occur in order to enter S. This, however, does not alter the conclusion that the bulk of G_1 is not part of the chromosome cycle. The use of hydroxyurea and other agents that decrease the rate of DNA synthesis might provide an experimental approach to study the genetic and molecular nature of such a putative "G_1-specific event," which would presumably occur in the minimum G_1 period of ≈1 hour. We are still confronted with the enigma of an irreducible G_1 in some cells and a complete absence of G_1 in others.

Finally, it is clear that regulation of the rate of proliferation of animal cells occurs between M and S. In our studies, the cells were in logarithmic phase without any known limitations on proliferation. This represents a less complicated situation compared with other commonly used systems such as cells arrested in G_1 or G_0 *in vitro* or *in vivo*. In these systems, the time between release from arrest [by growth factors, nutrients, or other means (1, 2, 21)] and the entry into S may contain any number of additional cellular events that do not occur between M and S in continuously proliferating cells. These "non-cell-cycle events" would likely be necessary to bring an arrested cell to a state in which it would enter and continually traverse the cell cycle without any impediments. In summary, our results indicate that, when a cell continuously traverses the cell cycle, the majority of G_1 is simply a period of time used for completion of the growth cycle.

Table 2. Effect of hydroxyurea on cell cycle of CHO cells

Hydroxyurea, μM	Time, hr		
	Generation	S period	G_1 period
0	13.6 ± 0.4	10.1 ± 0.3	2.40 ± 0.10
40	13.6 ± 0.2	11.5 ± 0.2	1.02 ± 0.03
60	14.6 ± 0.4	12.4 ± 0.4	1.13 ± 0.05
80	15.9 ± 0.5	13.7 ± 0.4	1.15 ± 0.06

Results represent mean ± SEM of three determinations.

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