A METHOD FOR IMPROVING SYNCHRONY IN THE G_2 PHASE OF THE CELL CYCLE

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Presently available techniques for synchronizing mammalian cells allow the preparation and analysis of populations in the mitotic, G_1, and S phases of the cell cycle. However, synchronization by selective detachment of mitotic cells from monolayers (Robbins and Marcus, 1964; Robbins and Scharff, 1966) or by exposure of random cells to 2 mM thymidine (Xeros, 1962; Bootsma

et al., 1964) yields populations which lose their synchrony by the time they reach the G_2 phase, and therefore systematic study of the morphological and biochemical events which immediately precede mitosis has been seriously hampered. In this report we describe a combination of existing methods which provides a population with improved synchrony in G_2.

MATERIALS AND METHODS

Stock suspension cultures of HeLa cells (S_3 strain) were maintained at between 2 and 4 $\times$ 10^5 cells/ml by daily dilution with fresh growth medium (Eagle, 1959); all procedures were carried out at 37.5°C with prewarmed glassware and media. Randomly growing cultures were exposed to 2 mM thymidine for 12 hr. The cells were then gently pelleted (1000 *g* for 1 min), resuspended at a density of 4 $\times$ 10^5 cells/ml in Eagle's minimum essential medium (MEM) (monolayer formulation) containing 3½% each of calf and fetal calf serum, and inoculated into Blake bottles at 2 $\times$ 10^7 cells/bottle. The cultures were gased with 5% CO_2 and tightly stoppered. 8.5 hr later the bottles were vigorously shaken to dislodge degenerating cells and debris, rinsed twice with 40 ml of Eagle's medium for suspension culture (without serum) as previously described (Robbins and Scharff, 1966), and inoculated with 50 ml of suspension culture medium with 3½% each of calf and fetal calf serum. 30–90 min later, mitotic cells were preferentially detached by gentle agitation of the medium (Robbins and Marcus, 1964; Robbins and Scharff, 1966); populations with 95–99% mitotic cells were routinely obtained. The cells were then placed in suspension culture, and thymidine was added to a final concentration of 2 mM. After 8 hr, the cells were pelleted, resuspended in fresh medium, and allowed to proceed through S, G_2, and into mitosis. The degree of synchrony was assessed by simultaneously monitoring the incorporation of thymidine-^{14}C into TCA[1]-precipitable material (Robbins and Scharff, 1966) and the mitotic index. For comparative purposes cells synchronized by the selective detachment method alone or by routine double thymidine blockade were also studied. For the latter, cells growing at a concentration of 3 $\times$ 10^5 cells/ml were exposed to 2 mM thymidine for 12 hr and resuspended in fresh growth medium for 10 hr. They were then treated a second time with thymidine for 12 hr and again resuspended in fresh medium. In some experiments mitotic index determinations were facilitated by adding colchicine (0.2 μg/ml) at 6 hr into the S phase and monitoring the subsequent accumulation of metaphase-arrested cells.

[1] Abbreviation used: TCA, trichloroacetic acid.

RESULTS AND DISCUSSION

Fig. 1, A–C, depicts in composite form the time course of DNA synthesis and accumulation of metaphase-arrested cells in populations synchronized by: Fig. 1 A, selective detachment of mitotic cells alone; Fig. 1 B, double thymidine blockade of random cells, and Fig. 1 C, the combined technique of selective detachment followed by thymidine blockade. In Fig. 1 A, 0 hr refers to 6 hr after harvesting the mitotic cells, while in 1 B and 1 C it coincides with the removal of the thymidine blockades. In all three instances a char-

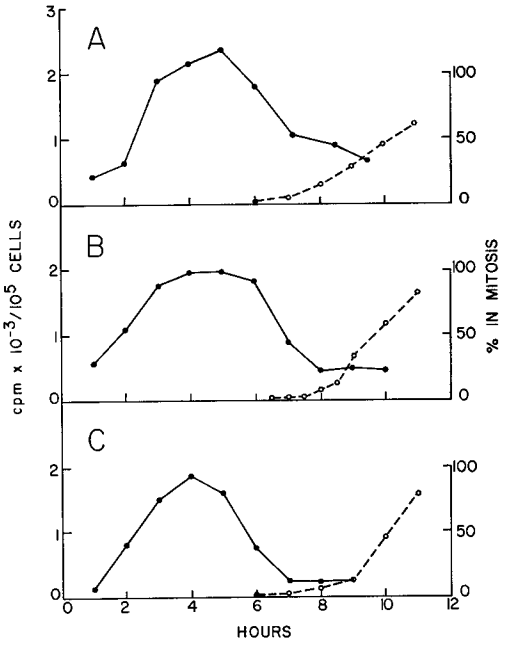

FIGURE 1 DNA synthesis and mitosis in synchronized HeLa cell populations. A, selective detachment of mitotic cells. Randomly growing cells in suspension culture were exposed to 2 mM thymidine for 12 hr, resuspended in fresh medium, and placed into monolayer cultures from which pure metaphase cells were harvested 9–10 hr later. 0 hr in Fig. 1 A refers to 6 hr after metaphase. B, double thymidine blockade. Randomly growing cells were exposed to 2 mM thymidine for two successive 12-hr intervals separated by 10 hr of growth without inhibitor; 0 hr refers to the time at which the second thymidine blockade was removed. C, combined techniques. Metaphase cells obtained as in Fig. 1 A were placed in suspension culture with 2 mM thymidine for 10 hr and then resuspended in fresh medium (0 hr in figure). DNA synthesis (●——●) and mitosis (○----○) were monitored as described in Materials and Methods.

acteristic rise and fall in DNA synthesis rate is followed by a progressive accumulation of cells in mitosis; however, there are significant differences in the degree of overlap between these two events. In Fig. 1 A metaphase cells begin to appear 7 hr into the S phase, at which time the thymidine-^{14}C incorporation is 40% of the peak value; by 9 hr about 25% of the cells are in metaphase arrest but thymidine-^{14}C incorporation is still 30% of the mid-S value. The overlap of these two parameters obviously precludes isolation of a meaningful G_2 population. The relatively poor synchrony of the preparation in Fig. 1 A is also apparent from the fact that the population as a whole enters mitosis gradually over a 4 hr interval. Double thymidine blockade (Fig. 1 B) leads to some improvement in synchrony; the rate of DNA synthesis now declines to about 25% of the peak value before significant numbers of metaphase cells begin to accumulate (8 hr), but there is still no interval during which a usable G_2 preparation may be obtained. In contrast, the combined techniques generate a reasonably distinct G_2 phase (Fig. 1 C); the rate of DNA synthesis declines to a low constant level at 7 hr, which is about 10% of the peak value, while the metaphase index does not begin to rise until 9 hr. This 2 hr interval which we may designate as G_2 is in good agreement with that derived by radioautographic timing methods (Quastler and Sherman, 1959) in randomly growing HeLa cells (E. Robbins, unpublished results).

Cell populations are inherently heterogeneous with respect to the length of their life cycle as well as its component phases. Consequently, the longer the time that elapses between synchronization and assay, the less synchronous the population will be. When mitotic cells are collected by selective detachment they maintain a usable degree of synchrony throughout G_1 and S, but by the time they reach G_2 synchrony has significantly deteriorated. If, however, G_1 cells are arrested with thymidine as they arrive at the G_1-S transition, and are then released, synchrony is re-established and its decay is transposed several hours further into the cycle, leading to the results we have described above (Fig. 1 C). With double thymidine blockades significant numbers of cells are still synthesizing DNA 10 hr after the first thymidine blockade is removed; they are arrested in S upon application of the second blockade, because thymidine detains cells in their traverse of S as well as preventing the entrance of G_1 cells into the DNA synthetic phase (Galavazi and Bootsma, 1966). These S-arrested cells resume DNA synthesis upon release of the second block and reach metaphase earlier than with the combined techniques (Figs. 1 B and 1 C).

Despite the requirement for an initially synchronized mitotic population, the present procedure can yield quantities of cells sufficient for many types of biochemical assays (e.g. Pederson and Robbins, 1970). Under ordinary circumstances about 400 mg of 95–99% pure mitotic cells may be collected from 50 Blake bottles inoculated with 2×10^7 cells/bottle, and by the time these cells reach the subsequent G_2 phase their mass has doubled. The improved G_2 synchrony herein described may facilitate exploration of ultrastructural and biochemical transitions occurring at this important phase of the cell cycle.

SUMMARY

Metaphase HeLa cells were obtained by selective detachment from monolayers and were allowed to pass through the subsequent G_1 phase in the presence of 2 mM thymidine. Following resuspension in fresh medium, the cells moved through the S and G_2 phases with a degree of synchrony unattainable with other techniques.

Dr. Robbins holds a United States Public Health Service Research Career Development Award.

This study was supported by grants from the National Institutes of Health (GM 14582, GM 876, and AI 04153), the National Science Foundation, and the American Cancer Society Inc.

Received for publication 11 November 1970.

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