INTERMITTENT DNA SYNTHESIS AND PERIODIC EXPRESSION OF ENZYME ACTIVITY IN THE CELL CYCLE OF WI-38

ROBERT R. KLEVECZ and LEON N. KAPP

From the Department of Cell Biology, Division of Biology, City of Hope National Medical Center, Duarte, California 91010

ABSTRACT

Synchronous cultures of WI-38 were obtained using an automated system for detachment and partitioning of mitotic cells which operates without the use of inhibitors, altered medium, or lowered temperatures. The generation time in synchronous WI-38 is 19.5 h and the duration of S phase when determined from the percentage of labeled metaphase cells or nuclei is 12 h. DNA replication in WI-38 occurs in three temporally distinct and rapid bursts separated by intervals of greatly reduced synthesis within what is nominally described as the DNA synthetic (S) period. Lactate dehydrogenase (LDH) displayed maxima in G_1 between 2 and 4 h and again at 10 and 16 h. Peaks in LDH activity were coordinated with DNA replication in a fashion similar to that reported for diploid Chinese hamster cells. Oscillations in LDH activity are more pronounced in normal diploid fibroblasts than in established and neoplastic lines.

The finite life-span of diploid cells in culture has been well documented (1–4). It has also been demonstrated that explanted fibroblasts from young individuals emigrate more rapidly from the explant and have a longer in vitro life-span than cells from older donors (2, 5). WI-38, human fibroblasts, have been extensively studied in recent years and attempts have been made to demonstrate age-associated changes in DNA melting temperature (T_m) (6), in RNA synthesis (7), in the level of glycolytic enzymes (8, 9), in lysosomal enzymes (7, 8), and in the metabolism of lipids (10).

Differences in the cell cycle patterns of enzyme activity and DNA replication between heteroploid and diploid Chinese hamster fibroblasts have been reported elsewhere (11–15). In heteroploid cells the pattern of enzyme and protein synthesis can be nearly linear or exponential through the cycle (13). This may be a population effect, since some heteroploid cells (16) and cloned aneuploid derivatives of the parent heteroploid lines display periodicities in enzyme activity (15, 17). However, there is a decrease in the complexity of the temporal pattern of both DNA and enzyme synthesis in cells with long culture histories. This is best exemplified by comparing diploid Don fibroblasts which display three discrete intervals of DNA synthesis within S phase (18) with established aneuploid V79 cells where only a single maximum is detectable in S (17).

It appears that hamster fibroblasts, like rodent cells in general, are able to become established and apparently immortal cell lines without undergoing a phase III (19) crisis, presumably because the loss of proliferative capacity in diploid members of the culture is obscured by the emerging heteroploid population. Greater than 85% of Don cells in this laboratory are diploid but maintenance of the diploid state requires careful attention to

subculturing procedure and rigorous avoidance of confluency (20). There was reason to question whether or not Don should be considered an established cell line (21) and it seemed that the significance of the observed differences between these cells and the established aneuploid V79 (17) and heteroploid G3 (13) hamster lines might be enhanced by examination of the WI-38 human diploid line.

Synchronous cultures of WI-38 were obtained using an automated system for detachment and partitioning of mitotic cells (17) which operates without the use of inhibitors, altered medium, or lowered temperatures, all of which can act to reduce the number of cells traversing the cell cycle (22). The results indicate that enzyme oscillations and discontinuous DNA synthesis are characteristic of normal diploid fibroblasts and are more pronounced in such cells than in established and neoplastic lines. Peaks in lactate dehydrogenase (LDH) activity are coordinated with DNA replication rate in a fashion similar to that reported for Don Chinese hamster cells (11).

MATERIALS AND METHODS

Cell Line and Culture Technique

Culture techniques have been described previously (12). Tests for mycoplasma contamination are performed monthly. WI-38 cells were obtained from Dr. Leonard Hayflick (Stanford University) at the 12th passage, were subcultured 1:2 every 3 or 4 days, and were frozen in liquid nitrogen between the 16th and 18th passage. Except where noted all experiments described in this study were done on cells which were inoculated into roller bottles between the 20th and 24th passage.

Cell Synchrony

WI-38 cells were brought into a synchronous state by repeated selection and automated detachment of mitotic cells from roller bottles. Cells were subcultured at a density of 5×10^7 cells per bottle into roller bottles (840 cm^2 growth area) which had been previously incubated with medium for 30 min. The roller apparatus was programmed to increase its speed from 0.5 to 100 rpm at hourly intervals, selectively detaching rounded mitotic cells. After a 5 min detachment interval at 100 rpm, the roller resumed its regular feeding speed and the detached cells in medium were pumped from the individual roller bottles into growth flasks. 25 ml of fresh medium were then pumped into each roller bottle and the roller bottles were then permitted to turn at the slow speed for another hour. This process was repeated at intervals of 1 h. Repetition of this operation for 24 h generated a series of growth flasks, and each flask had a selected synchronous culture separated in the cell cycle from the other flasks by 1 h.

In initial experiments the detached mitotic cells were immediately dispensed manually into scintillation vials at concentrations of 5×10^4 cells per vial so that multiple enzyme and DNA determinations could be made hourly as described previously (14). At this same time cell counts and mitotic index determinations were made.

Calibration of Synchrony

At the end of the program period, individual cultures were harvested from glass or plastic growth flasks using saline-EDTA (0.14 M NaCl, 0.01 M EDTA, pH 8.0). The last sample was collected immediately after detachment from the roller bottles. These cells were centrifuged at 100 *g* and resuspended in a small volume of Tris-saline (0.14 M NaCl, 0.01 M Tris, pH 8). Individual portions were removed for: (*a*) determination of mitotic index; (*b*) cell counts; (*c*) enzyme and protein determinations; and (*d*) estimates of DNA synthetic rate and percent labeled nuclei.

DNA Replication

[*Methyl*-^{3}H]thymidine (sp act, 6.7 or 50.3 Ci/mmol) was purchased from New England Nuclear (Boston, Mass.). 1 h after the final mitotic selection, medium from growth flasks was removed, 1 or 5 μCi/ml [^{3}H]thymidine was added, and the medium was replaced and allowed to incubate for an additional 30 min or 1 h. After harvesting, an aliquot of the sonicated cell suspension was precipitated with 10% TCA and collected on Whatman GF/C glass fiber filters. The filters were quickly washed twice more with 5 ml of cold 10% TCA, extracted once in 5 ml 1:1 ethanol:ether at room temperature, and finally extracted in 5 ml ether and counted in Aquasol (New England Nuclear). In two experiments [^{3}H]TdR labeling and acid extraction were performed on monolayers growing in scintillation vials by aspiration of medium followed by two washes with Hanks' balanced salt solution. Monolayers were then extracted with 4°C TCA twice, rinsed once in 4°C 10% potassium acetate in 80% ethanol, once in room temperature 95% ethanol, once in 1:1 ethanol:ether, and once in ether. Upon addition of Aquasol or 2,5-diphenyloxazole-dioxane the dried monolayer became transparent and could be counted at 25–30% efficiency in a Beckman LS-233 ambient temperature counter (Beckman Instruments, Inc., Fullerton, Calif.).

Enzyme Assays

Cell suspensions were sonicated for 30 s in an external sonifier (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.). in 1.0 ml of 0.01 M Tris-HCl, pH 8.0 containing 0.14 M NaCl. All assays were performed at 37°C in a Gilford 2400 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) and activities expressed as nanomoles of substrate utilized per minute per 10^6 cells. Assay mixtures for individual enzymes have been described elsewhere (12).

DNA Fluorescence

DNA content per cell was determined by reaction of 3,5-diaminobenzoic acid with the 2-deoxy sugar of DNA as described previously (18). Fluorescence was measured using an Aminco-Bowman spectrofluorometer (American Instrument Co., Inc., Silver Spring, Md.), excitation 420 nm, emission 520 nm.

RESULTS

Synchronization of WI-38 proved to be a difficult problem. Standard methods such as Colcemid-enhanced mitotic selection and S-phase arrest failed. Simple mitotic selection yielded a vanishingly small population of cells. An automated apparatus was constructed which synchronized by mitotic selection. Since the cells are grown in roller bottles and since the selection conditions (speed of revolution) can be optimized, reasonable numbers of mitotic cells can be selected. Selection and harvesting of mitotic cells from roller bottles has been described by Lindahl and Sorenby (23). However, their system differs from this one in that it involved centrifugation and cold storage and made no provision for automated dispension of the cells. Other methods have been applied to study the WI-38 cell cycle. A synchronous wave of DNA replication and cell division involving a fraction of the population can be obtained either by "wounding" a confluent monolayer (24) or by treating it with fresh medium and fetal calf serum (25). Under these circumstances processes unique to replicating cells can be studied by radiochemical means. However, in order to examine enzyme activity and other cell functions in normal diploid cells, it was necessary to obtain a large, pure population of dividing cells. Otherwise, the background level of enzyme activity in the confluent, nondividing population might obscure any periodicity.

In Fig. 1 *a* the yield of mitotic cells from the roller bottles is plotted as a function of time after initiation of the automated selection regime. The ability of the instrument to select a constant fraction of the population is apparent. Approximately twice as many cells are removed by the first (purge) selection as are removed in subsequent ones. The wisdom of discarding this first sample is attested to by the low mitotic index (67%). Subsequent selections yielded very nearly equal populations of 5×10^5 cells with a higher percentage of mitotic cells (>85%). As mentioned previously, the instrument selects cells in all stages from prometaphase to early G_1 with occasional contaminating interphase cells. In order to obtain reasonable estimates of initial synchrony it is necessary to score all mitotic stages in the determination of initial synchrony. Additional estimates of the degree of synchrony are given by the increase in cell number relative to the initial cell inoculum (Fig. 1 *c*) and by measuring the entry of cells into S phase. Diploid cells plate with a lower efficiency than do established tissue culture lines. In order to make accurate determinations of the time of doubling in cell number it was first necessary to determine the plating efficiency of the detached mitotic cells. The kinetics of attachment and the approach to maximum plating efficiency are shown in Fig. 1 *b*. Mitotic cells detached from exponential, preconfluent cultures show a plating efficiency of approximately 0.5 which is midway between previously reported values (26, 27). There was a tendency for the plating efficiency to fall to 0.2 or 0.3 if mitotic cells detached from nearly confluent monolayers were used. The duration of S phase in 20–24th passage cells is between 11.5 and 12.5 h, again in agreement with values already in the literature (28). The duration of S was also determined using percent labeled nuclei (PLN) from synchronous cultures and here also it was found to be about 12 h long. This comparison was considered important in view of the additional temporal structure resolved using the PLN data.

LDH has been used in this laboratory as a marker enzyme in cell cycle studies and its activity has been assayed in a large variety of cells. The LDH activity pattern of WI-38 cells is shown in Fig. 2. Four independent experiments performed over a 4 mo period are shown. In these and other experiments three peaks in LDH activity can be observed. It is apparent that the time of occurrence of individual peaks is not rigorously determined and although the three peaked pattern is strikingly reproducible, the exact time of occurrence can be shifted, presumably in response to some uncontrolled changes in the physiological conditions of

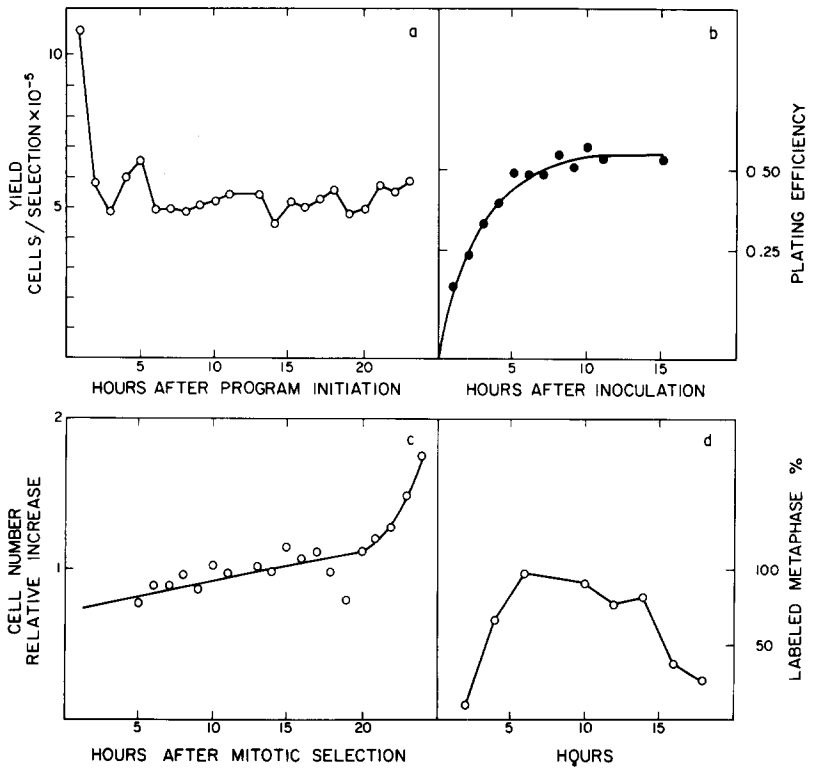

FIGURE 1 Selection and growth of mitotic WI-38 cells. (*a*) Yield of mitotic cells from the automated synchrony apparatus as a function of time after program initiation. Immediately after each fast roll interval, selected cells were dispensed manually into scintillation vials. One sample from each set was counted at this time and the mitotic index determined. (*b*) Attachment and plating efficiency of selected mitotic cells. Kinetics of mitotic attachment were scored by counting cells in suspension before inoculation into scintillation vials. Cells were inoculated at a concentration of 5×10^4 cells per vial and vials were trypsinized at hourly intervals thereafter and the number of attached cells relative to initial inoculation scored. Half-maximum plating efficiency was achieved within 2 h with a maximum efficiency of 0.54. (*c*) Relative increase in cell number after selection and plating. Using a plating efficiency value of 0.54 to adjust the initial inoculum, the relative increase in cell number was scored. Depression at 19 h in this curve is coincident with maximum mitotic index in the synchronous cultures and may represent loss of less tightly attached mitotic cells. Approximate doubling in cell number occurs with a sharp inflection between 20 and 23 h. (*d*) Duration of S and G_2 phases in nonsynchronous WI-38. Cultures of 2×10^6 WI-38 cells were cultured into plastic flasks (Falcon, 75 cm^2) and 24 h later, high specific activity thymidine (50.3 Ci/mmol; 1 μCi/ml) was added to each culture. After 1 h labeling, medium was aspirated and the monolayers washed twice with 37°C medium. At 2 h intervals after the wash, samples were harvested, trypsinized, and fixed for autoradiography. After a 1 wk exposure, slides were developed and scored for the percent of labeled metaphases. By application of this method S phase appears to be between 11.5–12.5 h in length and $G_2 + M/2$, 3–3.5 h. Values are scored as hours after removal of label.

the cells or their environment. As it is currently constituted, the physical setup of the automated synchrony apparatus does not lend itself to taking multiple samples at any given time point. Although the reproducibility of the enzyme assay, once the sample has been harvested and sonicated, is very good, sampling errors and variance in estimated cell number tend to introduce some variation into the curves. On occasion, as is shown in experiment AS43, this can act to obscure one or another of the maxima. Pooling and averaging the results from three independent experiments (Fig. 3) serves to reduce some of the "noise" but it may also diminish the amplitude of the activity change because of slight temporal shifts in the occurrence of the maxima. Presented individually, an experi-

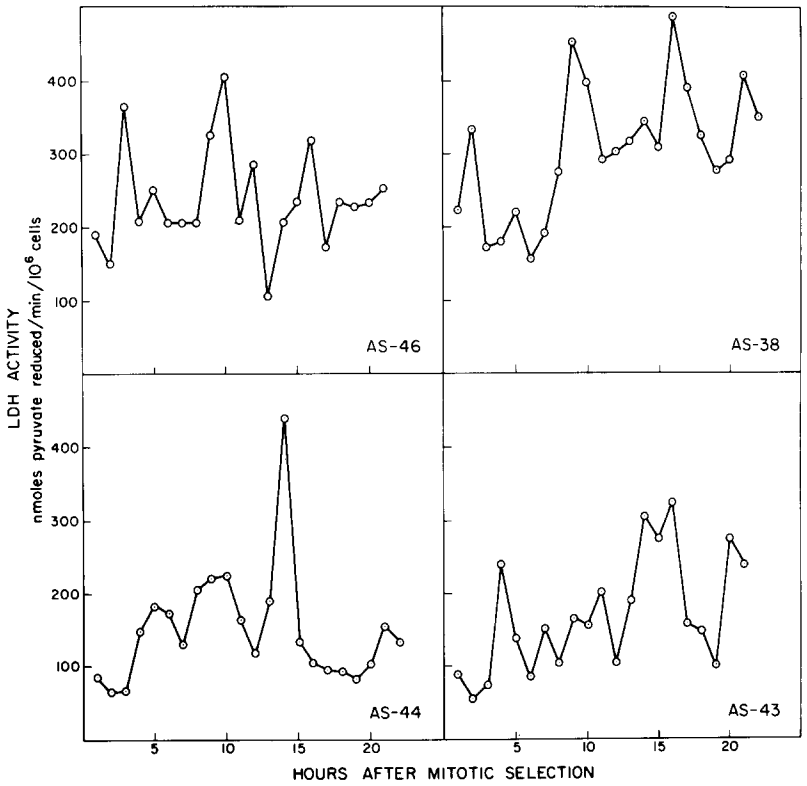

FIGURE 2 LDH activity changes in the cell cycle of WI-38. In experiments AS38 and AS43 cells were harvested from culture flasks in saline EDTA, centrifuged, resuspended in 1 ml of Tris-saline, pH 8.0, and sonicated as described in Materials and Methods. In experiments AS44 and AS46 cells were grown as monolayers in scintillation vials, were washed, and sonicated in 0.01 M Tris, pH 8.0, as described. Results are from four independent experiments performed over a 4 mo period. Activity is expressed as nanomoles of pyruvate reduced per minute per 10^6 cells.

ment such as AS44 (Fig. 2) might be misleading since a statistically significant maximum which occurs at 14 h in this experiment may occur at 15 or 16 h in another. This explanation is offered so that other workers will come to understand that the pooling and averaging of data from several experiments must be applied judiciously (29, 30) if they are not to obscure important temporal structure in the cell cycle.

The fluctuations in enzyme activity are at least as great as those shown by diploid and pseudodiploid Chinese hamster fibroblasts, Don and Don C (11) and considerably greater than those in V79 (18). Summarizing from the averaged values of Fig. 3, activity maxima occur at 4, 10, and 16 h into the WI-38 cycle. These times correspond respectively to the G_1/S boundary, mid-S phase, and late S or G_2.

The kinetics of DNA synthesis through the S

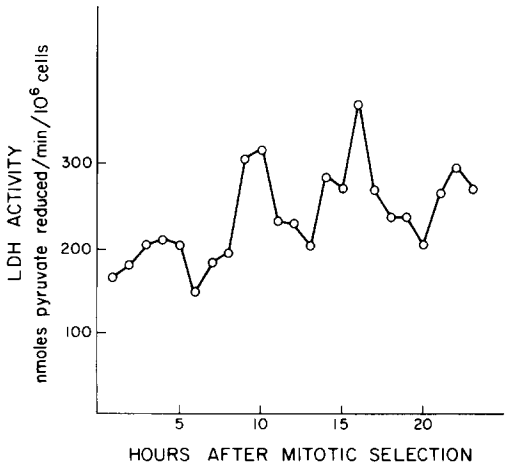

FIGURE 3 LDH activity in the WI-38 cell cycle. Average LDH activity from pooled data of three independent experiments, AS38, AS43, AS46.

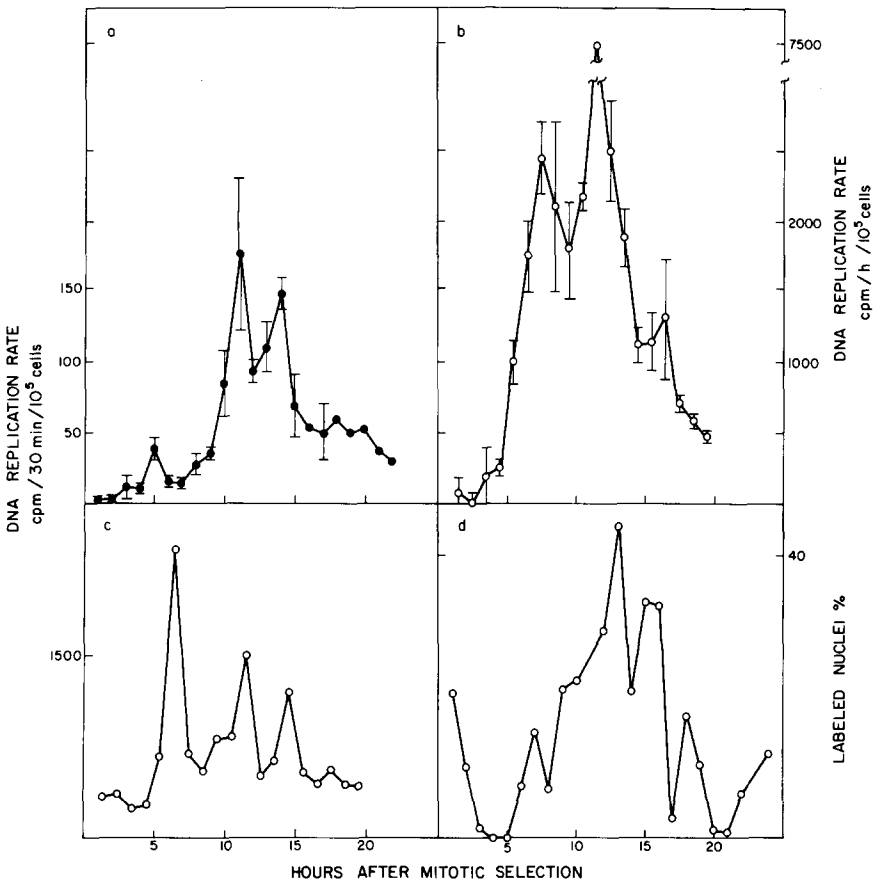

FIGURE 4 Kinetics of tritiated thymidine incorporation into DNA in the cell cycle of WI-38. (*a*) Cells were dispensed automatically into growth flasks and 24 h after program initiation all cultures were exposed to 5 μCi/ml [^{3}H]TdR (6.7 Ci/mmol) for 30 min. Cultures were harvested as described in Fig. 2 and in Materials and Methods. Three 100 microliter aliquots were precipitated in TCA and collected on GF/C glass fiber filters. Bars indicate standard errors. (*b*) Cells were dispensed manually into three scintillation vials at hourly intervals and 24 h after the first mitotic selection all cultures were labeled with 5 μCi/ml [^{3}H]TdR (50.3 Ci/mmol) for 1 h. Scintillation vials were then treated as described in Materials and Methods. Bars indicate standard errors. (*c*) Cells were dispensed manually into scintillation vials and treated as described in Fig. 4 *b*. Cultures were labeled with 5 μCi/ml [^{3}H]TdR (50.3 Ci/mmol) for 30 min. (*d*) Cells were labeled and harvested as described in Fig. 4 *a*. 0.5 ml of each sample was centrifuged, fixed in 50% acetic acid, and squash preparations were made. Autoradiographs were prepared and after 3 wk exposure, slides were developed.

phase of WI-38 were determined using autoradiographic and radiochemical measures of tritiated thymidine incorporation. Pulse labeling studies revealed considerable variations in apparent synthetic rate within this substage. Maxima in DNA replication rate occurred at 5–7 h and again between 11–12 and 14–16 h. Experiments showing the degree of reproducibility between multiple determinations of incorporation from individual samples (Fig. 4 *a*), and from multiple samples from each time point (Fig. 4 *b*), as well as extremes in the observed pattern of incorporation (Fig. 4 *c*), are presented in order to describe this phenomenon as completely and accurately as possible. In every experiment measuring the replication rate, a very sharp peak in incorporation was detected between 5 and 7 h after mitosis. The apparent synthetic rate in the first (early S) phase peak as determined by the tritiated thymidine incorporation rate is considerably more variable from experiment to

experiment than are the second two peaks, and may reflect very abrupt and synchronous initiation of a large number of replicating units.

The duration of S phase as determined from PLN after a brief exposure to high specific activity thymidine (Fig. 4 *d*) or from percent labeled metaphases (Fig. 1 *d*) is between 11.5 and 12.5 h. Most interesting in this regard is the fact that values for PLN do not exceed 50% in our experiments, regardless of the length of exposure of the autoradiograph. In an autoradiographic series exposed for 1, 3, and 9 wk the values for PLN and the overall appearance of the curve were unchanged. This finding can be explained by suggesting that periods of active DNA replication in WI-38 are brief and that slight temporal shifts in the onset of each burst of DNA synthesis result in less than the maximal number of cells being at the synthetic point when label is introduced. The unlabeled cells would be those which have either passed or have not yet reached this synthetic point. In part it may also be a measure of the fact that at this passage number only 70% of the cells incorporate label after prolonged (24–30 h) exposure to thymidine (31). The results cannot be easily accounted for by suggesting the presence of noncycling cells since a greater fraction are labeled in the second and third bursts of DNA synthesis than in the first peak.

The existence of three discrete cell populations with variable lengths of G_1 and G_2 and a single short S phase cnnot be invoked to account for the three discrete bursts in DNA synthesis. Autoradiographs prepared from cells labeled continuously for intervals of 1–17 h, from the time of mitotic selection until harvest, showed a single increase in PLN to a maximal value of 80% at 11 h with a midpoint between 5–6 h, in agreement with total incorporation data (Table I). In the experiment shown, the cultures were at the 29th passage. The observed labeling index is in reasonable agreement with the recent findings of Cristofalo and Sharf (31) showing a gradual decline in the PLN with increased numbers of population doublings. In summary, continuous labeling studies give no indication of population heterogeneity.

The most convincing evidence for synchronous initiation of replication comes from fluorometric determinations of DNA content per cell using Hinegardner's (32) modification of the Kissane and Robbins's (33) technique. In Table II, fluorescence increases abruptly between 4 and 6 h at the same time as total incorporation from pulse labeling shows a peak. Thereafter there appears to be a plateau period with no further increase in fluorescence until 10 h into the cell cycle. Somewhat surprisingly, DNA fluorescence seems to be a better measure of discontinuous DNA synthesis than does thymidine incorporation. This may indicate, as we have suggested before (12, 18), that changes in pool size and transport act to obscure rather than enhance periodic synthesis in the cell cycle.

Most interesting is the fact that the increase in fluorescence which occurs at 5 h is almost completely lost between hours 6 and 9. This has been repeatedly observed in experiments with WI-38 as well as with other cell types. The precise meaning of this observation will doubtless become clear in future experiments but it seems likely that early replicating DNA may undergo amplification and subsequent catabolism.

TABLE I

Labeled Nuclei in Synchronous WI-38 Continuously Exposed to Tritiated Thymidine

Hours after mitotic selection*	Percent labeled nuclei
1	<0.4
2	4.5
3	12.0
4	25.3
5	—
6	50.4
7	73.0
8	66.9
9	64.2
10	72.5
11	80.5
12	—
13	68.6
14	64.5
15	—
16	—
17	64.4

* Synchronous cultures were labeled continuously from time of mitotic selection until harvest with 1 μCi/ml [^{3}H]TdR (sp act 6 Ci/mmol). All cultures were harvested 17 h after program initiation. In this experiment cells were inoculated into roller bottles at passage 29.

DISCUSSION

Our findings establish that enzyme oscillations are a characteristic of normal diploid fibroblasts and

TABLE II

DNA Fluorescence in Synchronous WI-38

Hours after mitotic selection	Fluorescence*
1	76.6 ± 4.2
2	70.9 ± 1.1
3	82.5 ± 3.1
4	85.5 ± 4.4
5	120.9 ± 12.7
6	115.7 ± 7.3
7	77.9 ± 5.5
8	90.8 ± 7.7
9	85.2 ± 3.5
10	97.5 ± 2.04
11	140.6 ± 13.4
12	131.2 ± 14.3
13	129.4 ± 9.2
14	125.6 ± 10.3
15	142.5 ± 7.8
16	161.2 ± 15.0
17	157.5 ± 13.7
18	114.4 ± 8.2
19	82.5 ± 3.6
20	103.1 ± 11.4
21	97.5 ± 5.6

* Values are in arbitrary fluorescence units per 10^3 cells, but 0.077 fluorescence units corresponds to 6.98 pg DNA. Standard error of the mean is indicated in each case. Mitotic cells were selected hourly from four roller bottles and then dispensed through a Gilson fraction collector (Gilson Medical Electronics, Inc., Middleton, Wis.) into individual scintillation vials at a concentration of approximately 4×10^5 cells per vial. 19 h after the first selection all cultures were harvested and treated as described in Materials and Methods.

in fact are more pronounced in such cells than in established (31, 15, 17) and neoplastic lines (16, 29, 34–37). The duration of S phase in synchronous WI-38 cells, when determined from the percentage of labeled metaphase cells or nuclei, is 12 h. Direct fluorometric measurement of DNA content per cell and radiochemical determinations of DNA replication rate indicated several abrupt changes in the rate of DNA synthesis through S phase, with the possibility that there may be intervals when no DNA is being synthesized within what is nominally described as the DNA synthetic period. The greater total length of S in WI-38 permitted a more detailed examination of the sequence of synthetic events in early S phase and enabled us to resolve three discrete bursts in DNA synthesis.

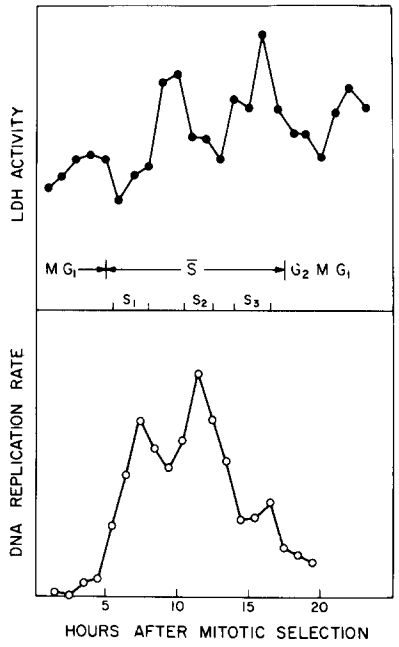

FIGURE 5 Temporal coordination between LDH activity and rate of tritiated thymidine incorporation. Average LDH activity from Fig. 3 and the rate of [^{3}H]TdR incorporation into DNA from Fig. 4 *b* have been plotted together to demonstrate the reciprocity between maxima in the two processes. Best estimates of the length of cell cycle substages were made from autoradiographic and pulse labeling studies of Figs. 1 and 4. Proposed subdivision of the S phase ($\bar{S}$) into three separate substages (S_1, S_2, and S_3) is indicated.

LDH displayed maxima in G_1 between 2 and 4 h and again at 10 and 16 h. Changes in enzyme activity through the cell cycle were extremely sharp in keeping with our expectation for diploid cells of limited passage number.

Earlier we noted a reciprocal relationship between maximum rate of tritiated thymidine incorporation into DNA and achievement of maximum enzyme levels in Don C (13), and the observation was later extended to other cell lines (16, 18). This reciprocal relationship which has always been phenomenological, holds for WI-38 (Fig. 5) as well. Recently, however, bursts in synthesis of a number of enzymes in V79 have been shown to follow closely increases in DNA content (38) and it now appears that thymidine incorporation does not define changes in DNA content as precisely as direct fluorometric measures.

Synchronous initiation of clusters of replicating units with pauses between synthetic bursts offers

an explanation for the disparity in length of S phase between early cleavage embryos and somatic cells. In preblastula embryos of the amphibian *Xenopus* DNA synthesis is completed in 10 min (39). At gastrulation, the autoradiographic pattern of synthesis changes (40) and in *Rana*, S phase begins to lengthen, reaching 9 h at neurulation (41) and 22 h in the adult kidney (42). Similarly, in *Drosophila* DNA synthesis requires less than 3 min in early embryos and 600 min in cultured somatic cells (43). The rate of movement of the replicative fork is not strikingly different in somatic cells as opposed to early embryos (44, 43) nor are there a sufficiently greater number of initiation sites in embryos as judged from electron microscope analysis (43) to explain the 200-fold increase in the duration of S phase in somatic cells. On the basis of the work presented here, it seems more reasonable to suggest that the temporal sequence of initiation is closely regulated in differentiated somatic cells and that clusters of replicating units related by function are synthesized and processed intermittently. There is, in addition, some evidence to suggest that the increasing length of S which occurs during differentiation is at least partially reversed in cultured cells, as a correlate of increased culture history. In rabbit brain cells S phase takes 25 h to complete (44) and in passage 20 WI-38 DNA synthesis requires 12 h. HeLa cells, on the other hand, require only 6–8 h to complete S (44). In the established aneuploid Chinese hamster line, V79 (15), DNA synthesis is completed in 5.5 h. In contrast to what we have observed for WI-38 cells, DNA replication rate in the cell cycle of Chinese hamster V79 and Don cells is a unimodal function beginning 2 h after mitosis. It reaches a maximum rate between 6 and 7 h after mitosis and then decreases uniformly as the cells enter G_2 (15, 17). However, DNA determinations using the Kissane and Robbins's procedure do reveal some residual temporal structure (38). The unimodal pattern of thymidine incorporation, which has been generally considered to be true for all cells (18), appears now to be characteristic only of some established cell lines and heteroploid tumor cells (45). In the latter case, possible cell cycle heterogeneity prevents one from knowing whether individual cells are replicating DNA unimodally as the V79 line does or in a manner more like WI-38. Population effects can be eliminated in V79 since it is very homogeneous with regard to chromosome number and cell cycle time. Such a profound difference in the organization of macromolecular events between cells might be expected to have other manifestations.

Macieira-Coelho et al. (28) have observed considerable heterogeneity in the DNA and RNA content and generation time of WI-38 cells approaching phase III. RNA and protein synthesis continue but the number of cells synthesizing DNA is greatly reduced. Cell size increases and cells appear to arrest in G_2 as well as in G_1. Willingham et al. (46) have shown that cyclic AMP arrests 3T3 cells in G_1 and G_2 but stimulates DNA synthesis in cells in S phase. Very recently, Ryan and Cristofalo (47) have observed an age-associated decrease in histone acetylation. Histone acetylation has been shown to be temporally associated with DNA replication and consequently it seems that the aged cells may arrest in portions of the cell cycle which are incompatible with acetylation. Gelfant and Smith (48) suggest that senescence results from the arrest of cells in either G_1 or G_2.

The immortality of cultured tumor cells is in contrast with the senescent changes experienced by normal diploid cells. It appears that in transformed cells a temporal derangement occurs in the initiation of DNA synthesis. Consequently, the synthesis of DNA, which in normal diploid cells is a discrete and stepwise process in tumor cells is initiated asynchronously. In the sense that S phase is dominant over other cell cycle substages (49), this may act to obscure potential arrest points in the cell cycle. It seems reasonable to propose that normal cells are able to divide and maintain a relatively homogeneous chromosome number, cell size, and optimal generation time because of the highly ordered nature of their metabolic, biosynthetic, and degradative processes. Evolutionarily, cells must have exploited the fact that feedback systems oscillate and used the rhythmic expression of various processes for predictive and timing purposes. Specifically, we suggest that cellular senescence results from a loss in temporal organization of biosynthetic and degradative processes with the consequent arrest and differentiation of cells in portions of the cell cycle from which they can no longer be stimulated to divide.

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