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Cell Biology

Quantized generation time in mammalian cells as an expression of the cellular clock

(cell cycle/transition probability/enzyme oscillations)

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ABSTRACT **The distribution of possible generation times in mammalian cells does not appear to be continuous within the limits of range for each cell type; rather, generation time is quantized in multiples of 3–4 hr. Synchronous cultures of Chinese hamster V79 cells were prepared using manual and automated methods to select and stage mitotic cells. Using synchronous cultures and time-lapse video tape microscopy, it was possible to show that generation times within a population of mitotically selected cells normally disperse in a quantized fashion, with intervals of 3–4 hr occurring between bursts in division. In addition, at temperatures above 37°, V79 cells have a 7.5–8.5 hr modal cell cycle, while at temperatures from 36.5° to 33.5° the modal cell cycle is 11–12 hr long. A survey of the synchrony literature reveals that the tendency to preferred generation times holds between cell lines. The distribution of modal generation times from a variety of different cell types forms a series with a similar interval but with a greater range of values than that observed here for V79 cells. To satisfy the published data and the work presented here, I propose a subcycle, G_q, which has a traverse time equal to the period of the clock. The period appears to be fixed at close to the same value in all mammalian somatic cells. The timekeeping mechanism appears to be temperature compensated, since the time required to traverse G_q is constant at temperatures between 34° and 39°. It is suggested that cell cycle time increases at lower temperatures, lower serum concentration, and high cell densities because the number of rounds of traverse through G_q increases.**

Many processes and activities in eukaryotic organisms display overt daily rhythms which have sufficient inertia to persist in the absence of external stimuli. These circadian phenomena have been treated analytically (1) and evidence has been marshalled for the existence of a "driving oscillator." While it is generally agreed that underlying such overt rhythms there exists an endogenous, cellularly based clock whose time constants are generated by means of coupled metabolic oscillations (2, 3), identifying the driving oscillator with any particular element of organismic chemistry has proven difficult (4, 5). The difficulties stem from the fact that most known self-sustaining biochemical oscillations vary in period from a few seconds to 15 min (6), so that individually the periodicities are too short to generate or entrain to the circadian period (3). Too, the clock maintains nearly perfect temperature compensation while most biochemical reactions are strongly temperature dependent (5).

In attempting to describe the mammalian cell cycle in terms of a clock one must account for existing data (7, 8) which would suggest that timekeeping in this system is imprecise (9), as well as the apparent lack of temperature compensation. Prior attempts to characterize cell cycle traverse have left a portion of the cycle indeterminant (10) or have resorted to probabilistic descriptions, arguing that variances in generation time as large as 20% of the mean can only be explained in this manner (11–13). Most recently Smith and Martin (11) have approached the problem by considering that certain portions of the cycle ($S+G_2$) were deterministic while others (the A state of G_1) were probabilistic. This model rests most heavily on the observation that the fraction of undivided cells with time follows a simple negative exponential curve. It suffers from a lack of connection with the biochemistry of the cell cycle.

In this work I propose that generation times are distributed polymodally and that cell division, the initiation of DNA synthesis, and the expression of certain enzymes are timed by a system which has some characteristics in common with the circadian clock. The cell cycle is envisioned as being built up of multiples of a fundamental 4 hr period. When it is allowed that individual cells in a population may have any one of a series of generation times, and that it is this subcycle which is timed and which initiates other events in the cycle, then the timekeeping capabilities of the cell appear more precise.

MATERIALS AND METHODS

Cell Growth and Synchronization. Stock cultures of V79 cells were maintained in McCoy's 5a medium as described previously (14). Synchronization was accomplished in most cases by detachment of mitotic cells from the surface of roller bottles using a cell cycle analyzer (Talandic Research Corp., Pasadena, Calif.) as described elsewhere (15). In some instances mitotic cells were detached by manual agitation of culture flasks.

Time-Lapse Video Tape Analysis. Video tapes were made using a Hitachi HV-16 CCTV camera and a Hitachi SV-512 time-lapse video tape recorder at 48-fold time compression. Cells were examined using a Nikon MS inverted scope with a heat filter and Wrattan 25 and 15 visible light filters. Illumination was continuous at 3 V. Cultures were fixed and stained at the termination of the recording to determine whether growth inhibition from the continuous microscope illumination had occurred. At high light intensities (6 V) and in the absence of either the heat filter or the Wrattan filters a zone of partial growth inhibition coherent with the illuminated portion of the flask could be observed. Temperature was maintained by housing the cells and the microscope in a small incubator heated by a 250 W heater and controller by an RFL model 70 controller. Temperature was controlled to ±0.1°. The Nikon lamp was external to the incubator and illumination was through a clear plastic panel in the incubator. Containing the lamp within the incubator resulted in fluctuations in temperature with changes in illumination, and excessive lamp fatigue. Tapes were analyzed for the occurrence of anaphase figures, which were the most characteristic and easily identified mitotic stage (7). The duration of anaphase was brief and variation in the duration of anaphase was also less than that of metaphase. In low temperature (<33°) experiments mitotic stages were prolonged so that analysis became difficult. In many instances arrest in mitosis appeared to be permanent. At elevated temperatures (39°–40°) the third and fourth divisions were often multipolar, usually tripolar. At extreme high and low temperatures only

Table 1. Survey of modal generation times (T_g) for cultured mammalian cells

Fig. 4 legend number	Cell	T_g (hr)	Ref.	Fig. 4 legend number	Cell	T_g (hr)	Ref.	Fig. 4 legend number	Cell	T_g (hr)	Ref.
1	Squamous cell carcinoma *in vivo*[a]	7.7	30	15	CHO	12.5	15	28	HeLa-S3	20	47
				16	ELD, Ehrlich ascites	15	38	29	KL[e]	22.5	
2	V79-M6-39[b]	7.8	—[c]	17	HeLa	15.5	39		Ham[e]	24.5	
3	V79	8.5	15	18	HeLa[e]	15.5			Ham[e]	21.0	48
4	L5178Y	8.5	26			19.5	16	30	Human kidney	21.3	49
5	L5178Y	8.75	22	19	L-929	15.5	—[f]	31	HeLa-S3	21.8	50
6	V79-753B[d]	9	31	20	L-60T	16	40	32	L-929	22.5	51
7	V79-28513	11	32	21	V79-325	16	41	33	HeLa-S3[g]	23.8	52
8	V79-M6-34[b]	11.25	—[c]	22	HeLa	19	23	34	HeLa	24	53
9	Don	11.5	33	23	HeLa-S3	19	42	35	HeLa	24.5	54
10	BHK	11.5	34	24	Ham[e]	19.4	43	36	HeLa	25	55
11	B14-FAF	11.5	35	25	WI-38	19.5	44	37	HeLa[e]	25	56
12	Rat sarcoma[e]	11.9		26	L-929[e]	19.5	45	38	L-5	26	
		12.8	8	27	L-132 human lung	19.5	46			29	57
13	CHO	12	36					39	CMP	28.5	58
14	Don C	12	37					40	D983	30	59

[a] Labeling index *in vivo*.
[b] Video tape microscopy of synchronous cultures.
[c] R. R. Klevecz, this report.
[d] Slope of curve from random exponential cell cultures.
[e] Cinematography of random culture.
[f] R. R. Klevecz, unpublished.
[g] Multiple brief high thymidine blocks.

a fraction of the cells divided and of those most divided only once.

RESULTS

In examining the literature it is apparent that certain cell cycle times occur with much greater frequency than others. In particular, many cells have cycle times of 11–12 hr, 15–16 hr, 19–20 hr, and 24 hr. A systematic compilation of cell cycle times from published synchrony experiments has been collected in Table 1 and arranged graphically later in this article in Fig. 4. Since these cells were grown under a variety of conditions in a number of laboratories one might reasonably wonder whether the differences represent clonal and therefore genetic or stable epigenetic changes, as opposed to transient shifts in generation time in response to environmental changes. This aspect of the problem has been investigated using synchronous cultures of Chinese hamster V79 cells with a minimum generation time of 7.5–8.5 hr.

Dispersion in cell cycle times

In the experiments which follow, mitotic cells were selected, subcultured, and then examined continuously by time-lapse video tape microscopy. Cell division was scored by noting the time of appearance of anaphase figures. These have been tabulated and arranged as frequency distributions of generation times.

In Fig. 1 the generation times of individual mitotically selected cells in a population of synchronous cells were examined under moderate density culture conditions. The occurrence of anaphase in the first synchronous cycle was scored for each of 304 cells. Three independent mitotic selection experiments were compiled to generate the data in Fig. 1. In this cell the generation time appears to be distributed polymodally with maxima centering around 8.5, 12.25, and 15.5 hr. Using either the χ^2 or Kolmogrov–Smirnov tests for goodness of fit, the probability that generation time or its reciprocal, generation rate, is normally distributed is <0.01. Similarly, a fit to a distribution following a negative exponent such as the Poisson gives a χ^2 value of 96 ($P < 0.001$) and therefore the decay in synchrony does not appear to be a random process. Under high density culture conditions or in a suboptimal nutritional state the major mode has been observed to shift from 8 to 11.5 hr (unpublished observations).

Temperature compensation

The eclosion rhythm expressed by pupating *Drosophila pseudoobscura* is one of the better known circadian rhythms (16). That process is considered to be temperature compensated

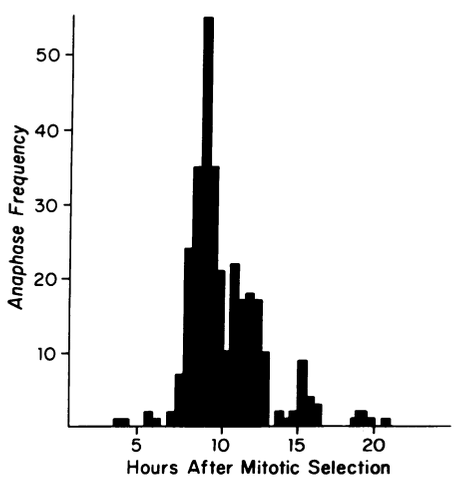

FIG. 1. Polymodal distribution of generation times in synchronous cultures of mitotically selected V79 cells. Mitotic V79 cells were prepared by selection from roller bottles at 37° and a selection speed of 200 rpm. Plastic flasks were inoculated at a density of 1×10^5 cells per cm^2. The occurrence of anaphase figures was recorded from time-lapse video tapes and scored as a function of time after mitotic selection as described under *Materials and Methods*.

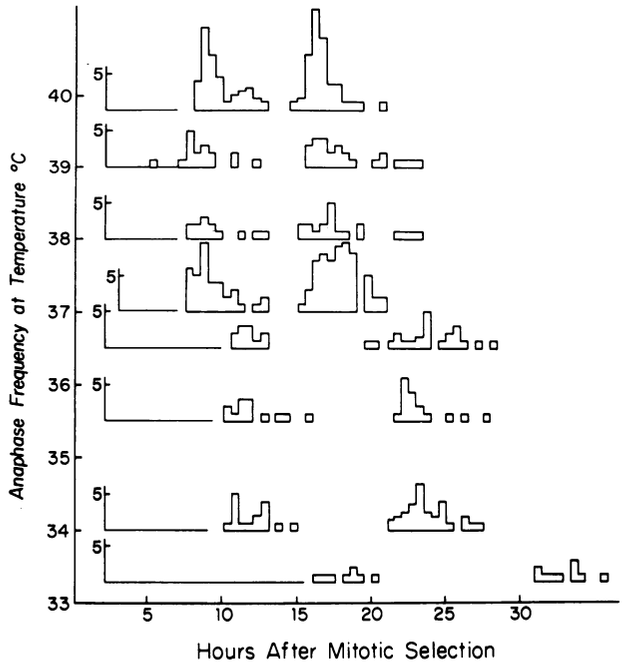

FIG. 2. Generation time of synchronous V79 cultures at temperatures between 34 and 40°. Synchronous cultures of V79 cells were prepared by mitotic selection from roller bottles at 200 rpm using the cell cycle analyzer. Cells were inoculated into flasks at a concentration of 4×10^4 cells per cm^2. Frequency of anaphase figures is scored as a function of the time after mitotic selection at temperatures between 33.3 and 40°. Generation times at various temperatures were determined by measuring the interval between the occurrence of maximum division frequency in the first and second synchronous waves following selection. Values were grouped in half-hour intervals.

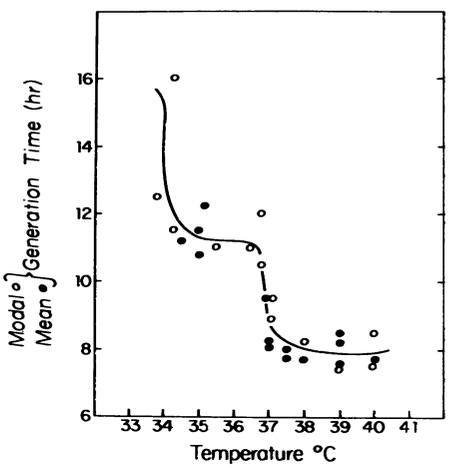

FIG. 3. Variation in generation time with temperature. Modal generation times from synchronous V79 cultures (○) from Fig. 2 and population doubling time (mean) (●) values from experiments using asynchronous cultures are plotted as a function of temperature.

because flies will emerge at the same subjective time of day independent of the temperature at which they are raised. Emergence is thus gated, and the clock that times emergence acts independently of the temperature even though development time increases with decreasing temperature.

To see if an analogous situation might exist in cultured mammalian cells we have investigated the effect of temperatures between 32° and 41° on the cell cycle of V79 cells. Fig. 2 shows a compilation of experiments in which the frequency of occurrence of anaphase figures was noted in the second and third synchronous division following mitotic selection. At temperatures below 33° and above 40° the majority of cells were unable to complete more than one cell cycle. Comparisons of variation in generation times with temperature were made using the second complete cell cycle, and because of the tendency for generation times to disperse with increasing density, only the first two cycles were measured. Between temperatures of 37° and 40° the second synchronous cell cycle requires 7.5 hr to complete. At temperatures between 36.5 and 33.5° the second synchronous cycle required 11.5 hr to complete. The temperature coefficient for cell cycle times at 34 versus 36.5° is close to 1.0. Similarly the temperature coefficient between 37.5 and 40° approaches unity.

The results of the temperature compensation studies suggested another series of experiments using random cell cultures growing at 35.5, 37.5, and 39.5°. Cells were grown at the high and low temperatures for 24 hr prior to beginning the experiment. Cell counts were performed at intervals over the ensuing 60 hr. From the results of Fig. 2 one might expect that the population doubling time of random cultures would be the same at 39.5 and 37.5° but different at 35.5 and 37.5 even though the temperature increment (2°) was the same in both cases. However, the differences in population doubling time at the higher and lower temperatures might not be so notable as with the synchronous cultures since the doubling time will be an average of the individual cell generation times and therefore dependent upon the distribution among the possible generation times. The data from the synchrony experiments of Fig. 2 and the population doubling times from random growth curves are plotted together in Fig. 3. One practical conclusion from this graph is the indication that certain temperatures, i.e., those between 36.5° and 37°, may be poor for maintaining synchrony in a population.

DISCUSSION

Frequency distributions of generation times compiled from cinematographic studies of cells in culture show a skewing toward longer generation times and considerable variance, often greater than 20% of the mean. In the past, intermitotic times were scored on random exponential populations of cells with relatively long generation times (7, 8, 14). In one of the earliest studies by Hsu (7), HeLa cells with a 26 hr modal generation time were examined. It seems likely that even if cells in this population were expressing a quantized division interval it would have been difficult to detect since the increment, 4 hr/26 hr, represents only a 15% change in generation time and would in all likelihood have been obscured by the variance between individual cells. However, in one instance reported by Marin and Bender (17) L929 cells did show a clear bimodal distribution in generation time. A second possibility which has not been explored here is that the variance is generated in random exponential populations by virtue of interactions between cells at different stages in their division cycle. It was in recognition of these points that synchronous V79 cells with an 8 hr generation time were used, with the expectation that a 4 hr increment, representing a 50% change in generation time, would be more easily detectable.

Quantized Variation in Generation Times. The generation times of synchronous cells were determined from published data and are displayed schematically in Fig. 4. With a few exceptions only mitotic selection synchronies showing the subsequent mitotic wave were included. The distribution of generation times forms a series with a repeating interval best estimated as between 3.5 and 4 hr. Many cell lines are capable of expressing more than one modal generation time. V79 cells

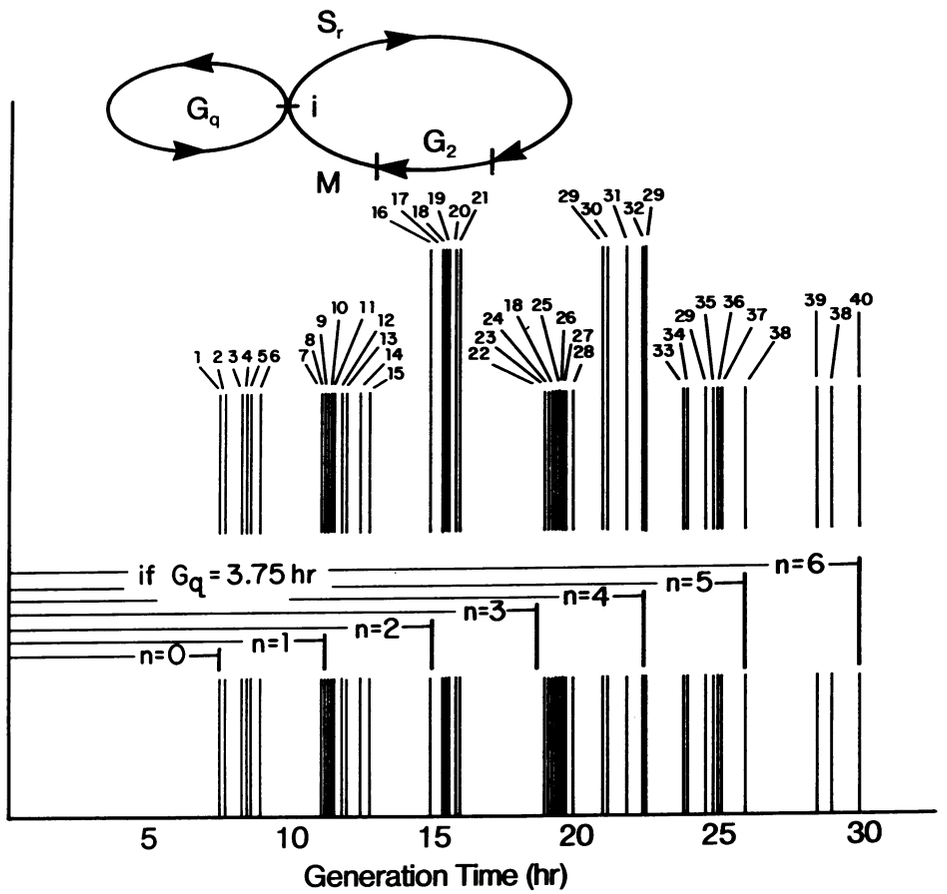

FIG. 4. Quantized variation in generation times of mammalian cell lines. Generation times were determined from the published data on cells synchronized by mitotic selection or from time-lapse cinematography of random cultures. The list is not exhaustive but represents a sampling of papers published between 1961 and the present in which the stated generation time could be directly confirmed in the data. Wherever possible modal generation times were obtained; that is, the time of maximum mitotic index rather than the center of mass of the mitotic wave. Numbers above each line in the figure refer to the reference number in Table 1. The calculation of possible generation times uses the simple expression $T_g = nG_q + 2G_q$. To obtain a fit to the data of Fig. 1, values for the probability of emergence from G_q and the density function describing dispersion will have to be determined. T_g = generation time in hours. G_q (quantized G) = incremental increase in generation time in hours.

grown in different laboratories divide with cell cycle times of 8.5, 11.5, and 16 hr; HeLa cells divide with cell cycle times centering around 15.5, 19.5, 22.5, and 24–25 hr; and L cells divide with cell cycles of 15.5–16, 19, 22.5, and 26 hr. Even though a variety of cell lines growing under variable conditions was considered, the intervals between preferred generation times appeared to be similar. In some experiments with V79 cells we have observed a double peak in anaphase frequency; the first at 11 hr and the second at 12.5 hr. This double wave also appears to be expressed in other cell lines (Fig. 4) as a wide band at 11–12.5 hr and again at twice that generation time; 22 and 25 hr. The significance of this observation is unclear at present.

To satisfy the existing data and the work presented here, I propose in Fig. 4 a subcycle, G_q, which has a traverse time equal to the period of the clock. The period appears to be fixed at close to the same value in all somatic cells. In the interest of keeping the familiar terminology, G_q is visualized as a second cycle appended to S+G_2+M at the point *i*. A more formally correct model would be one in which the cycle is described simply in multiples of G_q with the other cell cycle substages, such as the initiation of DNA synthesis, being indicated as sequences of dependent or independent events. Differences in the length of G_1 in cells of the same culture can be envisioned as being a consequence of the gated entry of cells into S. The exit from G_q into S is probabilistic in the sense that it depends on the environment with respect to cell density, nutrition and the presence of mitosis stimulating factors.* Should the environment be nonpermissive when the cell reaches *i*, the cell enters or reenters G_q. G_1 arrest (or G_0) can be viewed as a consequence of a more permanently unfavorable environment leading to an indefinite number of G_q cycles in which certain cell functions continue, while those which are dependent upon the initiation of DNA synthesis cease. Whether this situation, in which multiple rounds through G_q occur, and which may lead to a condition not unlike unbalanced growth, can explain the differences in composition between quiescent (G_0) cells and normally cycling cells is problematical (29).

The diagram drawn here bears some superficial resemblance to the cell cycle diagram drawn by Temin (10). G_q differs from IG_1 in having a specified time interval and in its identification with the cellular clock. It enables one to describe the cycle and specify its duration in terms of multiples of the single constant G_q. There may also be a similarity of the point *i* at the disjunction of G_q and S+G_2+M to Pardee's restriction point, R; the arrest point for isoleucine or serum deprivation and cAMP arrest. In their work, both Pardee (18) and Temin (10) found an arrest point and in both cases it appeared to occur some hours before the beginning of S phase.

*We expect that the equation of Smith and Martin (11) will fit the distribution of generation times only as a first approximation.

The model provides a means of explaining the heterogeneity of G_1 as it is expressed by serum-starved or nutritionally depleted cells upon refeeding; this heterogeneity can be thought of as arising from the gated entry of cells into S depending on their physiological state at the time of stimulation.

It is worth pointing out that the interval between preferred generation times is the same as the interval between enzyme activity maxima (19) or between bursts in thymidine incorporation (15). This later observation is taken as indicating that initiation occurs in discrete temporal and spatial clusters within S phase (15, 20, 21).

Several years ago I (14) observed oscillations in the activity of a number of enzymes in synchronized mammalian cells and suggested in a general way that this might be an expression of the cellular clock. Oscillations with periods of 3–4 hr were observed for a number of enzymes that had no obligatory connection with other periodic events such as DNA synthesis. Other workers have observed similar intracyclic fluctuations in a variety of cellular constituents (22–28). Subsequently, it was shown that the oscillations involved protein synthesis and degradation as well as modulation and that the system displayed inertia in the sense that inhibition of DNA and RNA synthesis damped, but did not obliterate the enzyme oscillations (14). It has been known for some time that V79 cells, which normally divide with an 8.5 hr generation time, display two maxima in lactate dehydrogenase activity, whereas Don cells, which normally divide with a 12 hr generation time, display three maxima (19). The results of the temperature compensation experiments suggest that the generation time and the number of enzyme maxima may be manipulated simultaneously.

At present we have no basis for distinguishing a biochemical oscillation that is the clock from one that is driven by the clock. The overt oscillations observed in enzyme activity may be merely an expression of the driving oscillator or they may possibly be evidence for a clock composed of multiple coupled oscillators as Pavlidis (3) has suggested. As an extension of these observations it seems reasonable to hypothesize that rapid degradation as well as synthesis of the proteins involved is necessary to generate adequate amplitudes, and sufficiently stable limit cycles, to serve as a timekeeping mechanism (4).

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