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THE CELL CYCLE DURING AMPHIBIAN LIMB REGENERATION

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SUMMARY

The duration of the cell cycle in the blastema of regenerating limbs of axolotls has been measured by means of [^{3}H]thymidine pulse labelling and autoradiography. A chase was required to define the pulse period. An average cell cycle at 20 °C takes 53 h, *S*-phase takes 38 h; including parts of mitosis, G_1 is 10 h and G_2 is 5 h long. The protracted cycle and *S*-phase are consonant with the large genome in axolotls and other urodeles. The rapidly growing blastema probably contains a steady population of about 5000 proliferating cells, as there is a regular withdrawal of differentiating cells from the population. The kinds of determination which exist in this population of cells, or are exerted on it, are briefly considered.

INTRODUCTION

Following the discovery that DNA synthesis for chromosomal replication occurs during interphase and that its synthetic period or *S*-phase can be distinguished from the preceding and following interphase periods or gaps G_1 and G_2, Quastler & Sherman (1959) devised a method of estimating the duration of these phases for unsynchronized proliferating cells. There is an intrinsic interest in the durations of these phases and of the total cell cycle between successive divisions. The durations in the meristems of angiosperms appear to be characteristic for each species, and the *S*-phase and cell cycle lengths are related to the nuclear DNA content with evident evolutionary implications (Van't Hof, 1965; Evans & Rees, 1971). The cell cycle is variable within meristems, however, for subpopulations of cells exist which exhibit prolonged cycles or which are arrested either in G_1 or G_2 (Evans & Van't Hof, 1975). Some support for these concepts can be obtained from studies of vertebrate cells. The exceptionally protracted cell cycles of newts, in comparison to those of other tetrapods, probably reflect their relatively large genomes (Grillo & Urso, 1968; Callan, 1972). Modification of the cell cycle is even more obvious here than in plant meristems, for successive divisions can occur at 15–60-min intervals in amphibian embryos but take as many hours in differentiated tissues.

The relationship between cell division and cell differentiation, often considered to be antagonistic processes, prompted us to investigate the cell cycle in blastemata of regenerating limbs. The blastema is a zone of rapid growth which closely resembles a developing limb-bud and shows some analogies to a root-tip meristem, but differs

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from both in that its cells are derived from previously differentiated tissues. Cell division is obviously required here for regenerative growth and may be essential for cells to transform from one tissue to another. The duration of the cell cycle may impose a constraint on the speed of regeneration and even influence the pattern of limb morphogenesis. We have estimated the duration of the blastemal cell cycle mainly as a contribution to the analysis of limb regeneration, but we believe our results may be of more general interest.

MATERIAL AND METHODS

The labelling of blastemal nuclei after incorporation of tritiated thymidine was recorded in 2 series of experiments on young axolotls, *Ambystoma mexicanum*, maintained at 20 °C. The first series included 8 specimens whose limbs were sampled during a 16-h period following a single injection of the tracer, and 20 specimens subjected to a further injection of unlabelled thymidine (100 × chase) 2 h later and sampled for a 60-h period. The second series included 11 specimens simply injected with tracer and 24 specimens allowed a 2-h labelling time terminated by a 1000 × chase, with sampling up to 72 h. The injections of tracer were staggered in both series, at 10.00 and 22.00 hours, to allow convenient sampling times over the next 3 days.

In preparation for this procedure, white axolotls were reared for about 6 months to reach 6–8 cm and 4–5 g, depending on the series. Their limbs were amputated just above the wrist or ankle and allowed to regenerate for 8–9 days at 20 °C, until a conical blastema appeared. Each specimen was then injected intraperitoneally with 5 μCi [6-^{3}H]thymidine (20·6 μCi/μg) in 25 μl saline. The same volume of chase solution was used, containing 25 or 250 μg thymidine in the different series. Assuming equilibration in a 5-ml body volume, the final concentration of thymidine was about 0·2 mM which is less than has been reported to affect the cell cycle; no adverse effects were noticed. All operations were performed under anaesthetic, using 0·1 % MS 222 (Sandoz).

An arm and a leg were removed at each sampling time, usually from duplicate specimens, and fixed in 3:1 ethanol-acetic acid. Subsequent treatments were adapted from the standard procedures for plant root-tips (see Langridge, O'Malley & Wallace, 1970). Limbs were washed and stained with Feulgen and stripped of epidermis before the blastema was excised and tapped out on a microscope slide in a drop of 45 % acetic acid. The dispersed cells were squashed under a coverslip, which was then removed after freezing in liquid nitrogen, dehydrated in ethanol and air-dried. These preparations were dipped in 50 % Ilford K2 emulsion and stored with silica gel in light-proof boxes at 4 °C for 6 weeks. The autoradiographs were developed in Kodak D19 for 20 min to obtain large grains, fixed, washed, air-dried and mounted in DPX.

The usual terms for phases of the cell cycle are employed: G_1, S, G_2 and M (mitosis). Similarly, their durations and that of the total cycle and the labelling period (when cells are exposed to the tracer) are abbreviated as $Tg1$, Ts, $Tg2$, Tm, Tc and T_l.

RESULTS

The preparation of autoradiographs of well separated cells with numerous large silver grains facilitated scoring nuclei at low magnification. Staining was rather faint after the normal Feulgen technique, but became intense after hydrolysis for 1 h in 5 N HCl at 20 °C. This extracts about half of the thymidine, according to scintillation counting, and thus demands an extended exposure for autoradiography. Most preparations provided 50–100 division figures between mid-prophase and late anaphase. The proportion of these which were labelled at successive sampling times are shown in Fig. 1. According to the analysis of Quastler & Sherman (1959), most phases of the cell cycle can be timed from such graphs under certain conditions.

The critical conditions are that the cells divide repeatedly and are not synchronized, as in a diurnal rhythm, and that the tracer is only incorporated during a defined period. We know that the first condition is met by a population of cells in the blastema and can find no evidence of synchronization. The same proportion of cells incorporated tracer during 2 h in the morning as in the evening, and the number of division figures encountered in each preparation remained fairly constant over a 14-h sampling period during each day, while the specimens were maintained in a dark incubator.

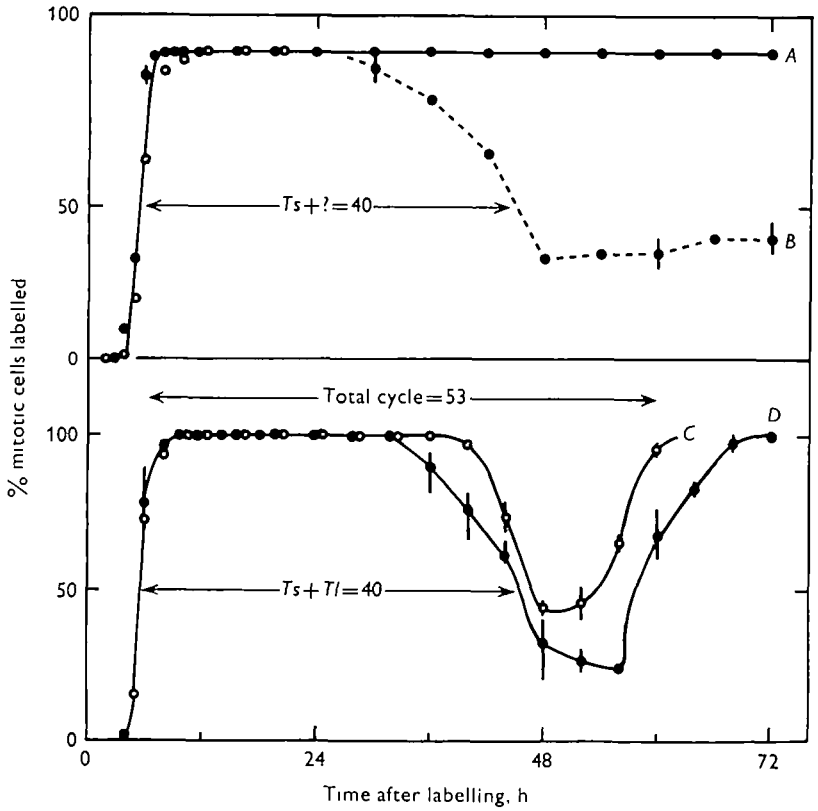
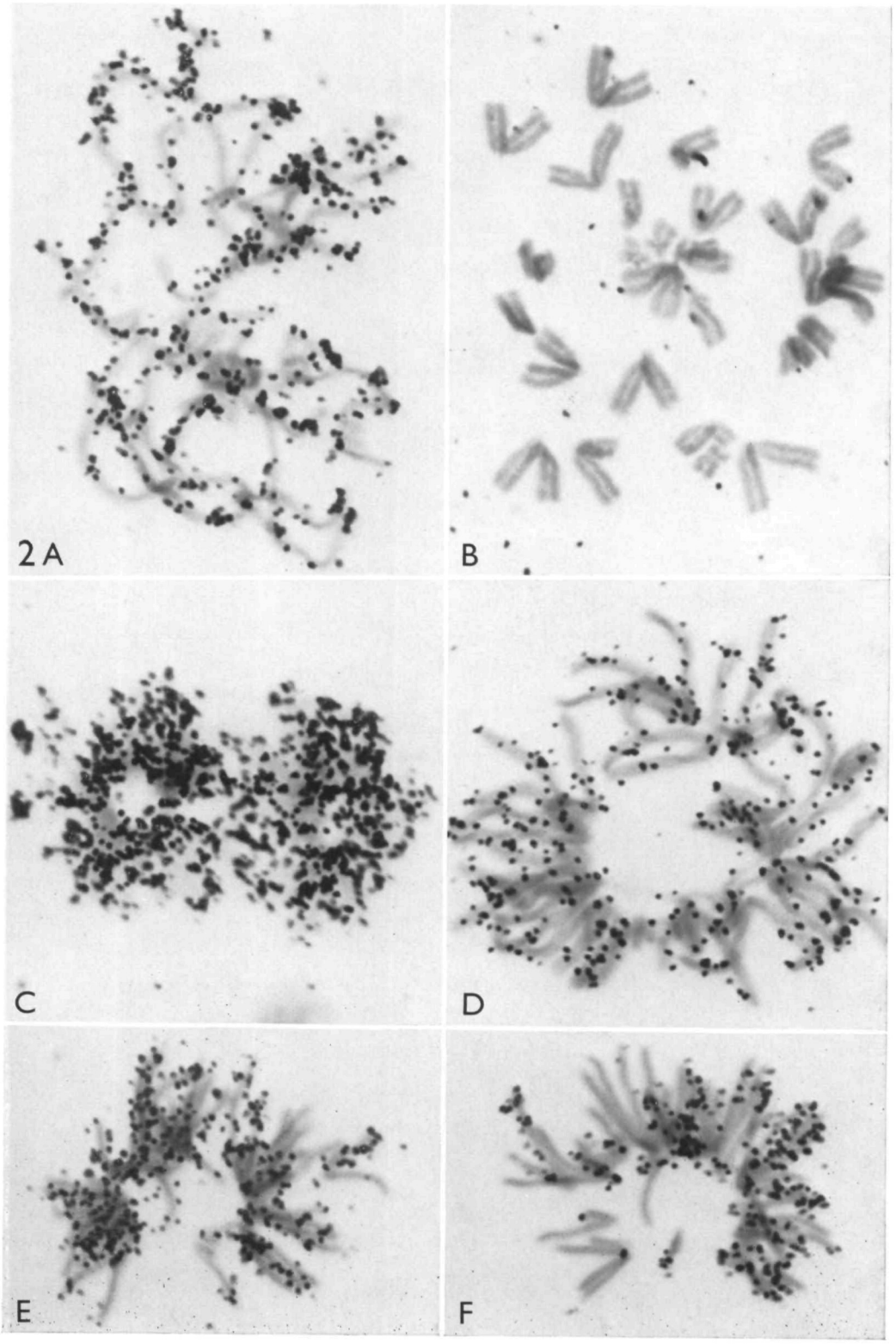


Fig. 1. Estimation of the cell cycle from labelled mitosis curves. The 2 experiments are distinguished by open and closed circles. Vertical lines show the range of observations in 2–4 samples. Unchased injections of [^{3}H]thymidine label all dividing nuclei in samples taken 8–72 h after injection (*A*), but heavily labelled mitoses (*B*) from the same samples show a spurious resemblance to a Quastler–Sherman plot. A 2-h pulse of [^{3}H]thymidine followed by a 100× chase (*C*) or 1000× chase (*D*) allows most phases of the cycle to be measured as 50% intercepts.

Continuous labelling series

The condition of a finite pulse is not met after simply injecting the radioactive tracer. While unlabelled division figures were seen up to 8 h after the injection, giving a maximum estimate of *Tg*2, all divisions were labelled thereafter (Fig. 1.*A*) As only cells in *S*-phase should incorporate tracer, this means that an appreciable


2A
B
C
D
E
F

amount of tracer must persist for a period equivalent to the rest of the cycle (*Tc–Ts*). If only heavily labelled divisions are scored, however, the graph declines between 24 and 48 h and then remains steady at about 40% (Fig. 1*B*, broken line). This graph appears more like the expected Quastler–Sherman plot, but it cannot provide reliable estimates of *Ts* or *Tc* either in practice or theory. In practice, the interval between ascending and descending 50% intercepts (at 5 and 45 h in Fig. 1*B*) represents the sum of *Ts* and an undefined T_t; calculation of *Tc* fails for lack of a second peak of labelled divisions. The theoretical aspect of this failure is that the category of lightly labelled dividing cells (which would be scored as unlabelled after smaller doses of tracer or shorter autoradiographic exposure) compounds 2 categories: the second division of cells which were in *S*-phase at the time of injection and the first division of cells which only entered *S*-phase later when the tracer was somewhat depleted. The distinction between heavily- and lightly-labelled nuclei was obvious, with background counts below 20 grains (Fig. 2).

Discounting a minor fraction of obviously differentiated cells with elongated nuclei, the proportion of blastemal interphase nuclei which were labelled, increased with sampling time from about 45% initially to a maximum of 90% after 72 h. Since the dividing cells were invariably labelled after 8 h yet only 50–60% of all blastemal cells were labelled in samples at 10–20 h, it seems that almost half the cells had already reverted to a non-dividing state, presumably as a prelude to overt differentiation before the tracer was injected. Samples taken after 20 h also contained some partly differentiated labelled cells, which suggests that the final division cycle prior to differentiation is not especially protracted. The population of blastemal cells examined here has thus probably achieved a steady state equilibrium, with proliferation balancing the withdrawal of differentiating cells. Division figures amounted to about 1% of all blastemal cells, or 2% of the labelled cells in samples between 2 and 20 h. As all proliferating cells must be labelled by the end of this period, 2% is taken as the true mitotic index in order to calculate *Tm*. This must give an arbitrary and low value of *Tm*, however, because only clear mitotic figures were scored comprising mid-prophase to late anaphase, examples of which are shown in Fig. 2.

Fig. 2. Autoradiographs of division figures after a 2-h pulse with [^{3}H]thymidine; all × 1800.

A, B, labelled prophase and unlabelled metaphase from the same preparation, fixed after 6 h.

C, D, heavily labelled anaphase and more lightly labelled metaphase, both from the same specimen but in samples taken after 18 and 64 h, respectively.

E, F, two halves of a labelled anaphase fixed after 72 h, showing an unequal distribution of silver grains.

Both light labelling (D) and unequal partition of label (E, F) usually indicate cells which are dividing for the second time since incorporating the tracer.

Pulse-chase series

The condition of pulse labelling was approached by the 100× chase injection (Fig. 1*C*) and perhaps achieved by the 1000× chase (Fig. 1*D*). Neither of these treatments imposed any noticeable synchronization or depression of cell division, nor any significant delay in those phases of the cell cycle which could be determined in the continuous-labelling series. Consider first the results of the 1000× chase. The

Table 1. *Duration of cell cycle phases (mean and range in hours)*

Series	G_1	S	G_2	M	Total cycle
No chase	—	40	4·5	1	—
100× chase	3·2 (0–7)	40 (32–48)	4·8 (4–8)	1	49 (44–56)
1000× chase	9·5 (5–14)	38 (30–46)	4·5 (3–8)	1	53 (48–60)

gradual decline of this graph (Fig. 1*D*) between 32 and 56 h and its failure to reach zero, as well as the gradual ascent to a second peak in comparison to the abrupt initial increase of labelled divisions at 4–8 h, all reflect a considerable variation of cycle times (cf. Langridge *et al.* 1970). The most unusual features of this graph are the prolonged initial plateau from 8 to 32 h and the 100% value attained by the second peak. Both of these features indicate a prolonged *S*-phase which occupies a major part of the cell cycle. The 50% intercepts of the graph yield the following estimates of average phase durations:

$$\text{Origin to 1st intercept, } Tg2 + 0{\cdot}5Tm = 5 \text{ h.}$$
$$\text{1st to 2nd intercept, } Ts + T_l = 40 \text{ h.}$$
$$\text{1st to 3rd intercept, } Tc = 53 \text{ h.}$$

The sum of $Ts + T_l$ occupies 75% of the cell cycle on this basis, or 77% when calculated from the areas of the graph according to Gerecke (1970). These data can be corrected for the 2-h pulse period and for Tm (mitotic index × Tc, 2% of 53 h = 1 h approx.). $Tg1$ can then be obtained by subtracting all the other phases from Tc, thus providing the best estimates shown in Table 1. The variation of phases has been deduced from the maximum and minimum values (Fig. 1*D*) and is expressed as a range of times in Table 1.

An identical treatment of the data from the 100× chase gives lower estimates for Tc and hence for $Tg1$ (Table 1). We think these estimates are less reliable than the previous ones for the following reason. Since injected tracer persists for 15 h or so, there is probably an endogenous pool of thymidine which dilutes the tracer and reduces the effectiveness of a chase. If equal in amount to the injected tracer, such a pool would halve its specific activity and thereby reduce a 100× injection to a 50× chase. The chase not only reduces the incorporation of tracer but also retards its depletion, in proportion to the lowered specific activity. A cell which entered the prolonged *S*-phase during the early chase period, therefore, might incorporate almost half as much tracer (at 2% of initial specific activity for about 20× T_l) as other cells incorporated during the pulse period. The extra lightly-labelled divisions postulated

by this argument are certainly present in the shoulder of the 100× chase data 36–44 h (Fig. 1*C*), and they could well augment the second divisions of cells labelled during the pulse period to produce a spurious advance in the second peak of the graph at 52–60 h. If this argument is applied to the 1000× chase, then the class of late-labelled cells would have been considered unlabelled in comparison to the background grains in the autoradiographs (Fig. 2).

DISCUSSION

The technique adopted here is less laborious than the conventional method of cutting sections and we believe it permits mitotic figures to be scored more easily and efficiently. We have already emphasized the discrepancy between results obtained after simply injecting tracer and those obtained by a pulse-chase treatment. The results provide consistent evidence that tracer persists for a considerable period,

Table 2. *Duration of cell cycle phases in regenerating tissues (hours)*

Tissue	$G_1+0{\cdot}5\ M$	S	$G_2+0{\cdot}5\ M$	Total	Reference
Newt lens, 22 °C	(32–)43	19(–30)	2·5	65	Zalik & Yamada (1967)
Newt limb blastema and connective tissue,* 20 °C	3	34–41	4·5	45	Grillo (1971)
	15	30–40	5	56	
Axolotl limb blastema, 20 °C	10	38	5	53	present results

* Believed to be redifferentiating cells.

sufficiently to perturb or mask the form of a labelled-mitosis curve. Previous reports of the persistence of injected tracer in adult newts (Grillo, Urso & O'Brian, 1965) and incidental observations at higher doses in newts and axolotls (O'Steen & Walker, 1961; Steen, 1968) have been generally ignored in amphibian cell cycle studies, although such persistence can be detected in them (e.g. Grillo & Urso, 1968). For this reason we doubt the reliability of 2 previous attempts to analyse the cell cycle in regenerating tissues of *Triturus viridescens*, even though they provided results quite similar to our own (Table 2) and a 54-h cycle has apparently been recorded in tissue cultures of *T. viridescens* (Wilson, 1975). These earlier studies did not employ a chase and yielded results which resemble those we have interpreted in terms of continuous labelling (Fig. 1*B*).

The protracted cell cycles and *S*-phases of regenerating urodele tissues might be expected on the basis of their large genomes (see Introduction), although it is not evident from Table 2 that these times are longer in newts than axolotls, as would be predicted on the same basis. These times are so much larger than those recorded for anurans or even plants, at about the same temperature, that we doubt if a more detailed comparison would prove informative. We note, however, that Van't Hof's (1965) formula $Tc = 5{\cdot}26 + 1{\cdot}27Ts$ applies to axolotls.

The range of estimates shown in Tables 1 and 2 includes a genuine variability of

cycle phases among the proliferating cells, besides the inevitable errors of measurement. We have examined sections of blastemata to see whether there is a spatial component to such variability, but find both labelling and cell division to be fairly uniformly distributed throughout the blastema. This observation and the rather rapid differentiation of some labelled cells do not support the contention of Grillo (1971) that redifferentiating connective tissue cells show a longer cell cycle than undifferentiated blastemal cells (see Table 2), and we doubt if the data he obtained warranted such a conclusion. We have to consider the proliferating population of blastemal cells as a single entity until subpopulations with different characteristics can be identified. Meanwhile, we can calculate the size of the population recognized in terms of the average cell cycle. We estimate there were about 10000 undifferentiated cells in our more complete preparations, based on 100 division figures and an overall mitotic index of 1% in sample counts. At least half of these cells become labelled in 16 h after a single injection of tracer, when all proliferating cells should have been able to incorporate it ($Tc - Ts = 15$ h). The proliferating population thus consists of about 5000 cells in the conical blastema, derived by dedifferentiation of stump tissues. The size of this population is probably fairly constant during the major growth period, so that the average division produces one cell which is left behind to redifferentiate and another which will divide again. According to cell counts in newt limbs (Chalkley, 1954) the original population produced by dedifferentiation consists of a few thousand cells. We thus perceive a reasonable analogy to the apical meristem of plant roots, for the blastemal meristem acts as a self-perpetuating steady-state population which is responsible for most of the growth of the regenerate.

This blastemal meristem is apparently identical to the progress zone postulated by Smith, Lewis, Crawley & Wolpert (1974) as a zone where positional information for regional specificity within the limb has not yet been finally determined. The model described by Smith *et al.* (1974) seems a plausible basis for considering the amount of regulation evident in limb development and regeneration, if only in a single dimension. There is certainly a regional predisposition inherent in the blastemal population (Iten & Bryant, 1975; Stocum, 1975) and indeed in the cells which must dedifferentiate as its source; besides strong tendencies to reproduce the part of the body from which the cells originated, such as left- or right-handedness or limbs rather than tails. There may even be a weak predisposition to retain a previous histological determination, for regenerates derived from cartilage grafts which have an adequate muscle supply in young axolotls (Wallace, Maden & Wallace, 1974; Maden & Wallace, 1975) are virtually devoid of muscle in older specimens (Namenwirth, 1974). To what extent these features are determined by the whole animal, old limb-stump, blastemal meristem or its constituent cells remains to be resolved. All affect the pattern of growth, however, and so are likely to be mediated by the blastemal cell cycle.