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Growth control in cell-cycle mutants of animal cells*

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Summary

In neoplastic cells, regulation of the cell-division cycle is defective, resulting in impaired control of entry of cells into a state of proliferative quiescence and/or of exit from the quiescent state into the division cycle. Conditional cell-cycle mutants represent attractive model systems for studies of mechanisms of regulation underlying the ordered progression in the cell cycle as well as entry into and exit from a state of proliferative quiescence. In this communication, the selection of a series of heat-sensitive (arrested at 39.5° C, exponentially multiplying at 33° C) and cold-sensitive (arrested at 33° C, exponentially multiplying at 39.5° C) cell-cycle mutants and some of their characteristics are described. At the respective nonpermissive temperature, the heat-sensitive as well as the cold-sensitive mutants enter into a state of reversible proliferative quiescence with a cellular DNA content identical to that of cells in G_1 phase. When mutant cells are fused to «wild-type» cells, the phenotype of arrested heat-sensitive cells is expressed in a recessive manner, while cold-sensitive cells behave dominantly under these conditions. Furthermore, the cold-sensitive mutant cells differ from the heat-sensitive cells by their capacity to undergo cellular differentiation with the formation of mast-cell granules when incubated at the nonpermissive temperature. Thus, the state of proliferative quiescence induced in heat-sensitive cells is qualitatively different from that of cold-sensitive cells, and the latter may be used as models in analyzing intracellular processes underlying entry of cells into a state of proliferative quiescence coupled with morphological cell differentiation.

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Zusammenfassung

Neoplasien weisen eine Störung in der Regulation des Zellteilungszyklus auf, indem die Kontrolle des Eintritts von Zellen in einen Zustand der Proliferationsruhe bzw. des Austritts aus der Proliferationsruhe in den Teilungszyklus bei Krebszellen defekt ist. Als vielversprechendes Modellsystem in der Analyse von Regulationsmechanismen, die dem Durchlaufen des Zellteilungszyklus sowie dem Erreichen und Verlassen der Proliferationsruhe zugrunde liegen, haben sich konditionelle Zellzyklus-Mutanten erwiesen. Die Selektion und die Eigenschaften einer Serie hitzeempfindlicher (Arretierung bei 39,5° C, exponentielle Zellvermehrung bei 33° C) und kälteempfindlicher (Arretierung bei 33° C, exponentielle Zellvermehrung bei 39,5° C) Zellzyklus-Mutanten eines Mäuse-Mastozytoms werden beschrieben. Bei der nicht-permissiven Temperatur treten sowohl die hitze- als auch die kälteempfindlichen Mutanten in einen Zustand reversibler Proliferationsruhe mit einem DNS-Gehalt entsprechend der G_1-Phase ein. Bei Fusion mit «Wild-Typ»-Zellen verhielten sich die arretierten hitzeempfindlichen Zellen phänotypisch rezessiv, arretierte kälteempfindliche Zellen dagegen dominant. Ein weiterer Unterschied zwischen den hitze- und kälteempfindlichen Zellzyklus-Mutanten besteht darin, dass sich bei der betreffenden nicht-permissiven Temperatur an den kälteempfindlichen Zellen eine Zelldifferenzierung unter Bildung von Mastzell-Granula nachweisen liess, während an den hitzeempfindlichen Zellen kein derartiger Differenzierungsprozess zu erkennen war. Die in den hitzeempfindlichen Zellzyklus-Mutanten induzierte Proliferationsruhe ist somit qualitativ verschieden von derjenigen in den kälteempfindlichen Mutanten, und letztere bieten sich als Modell für die Analyse der

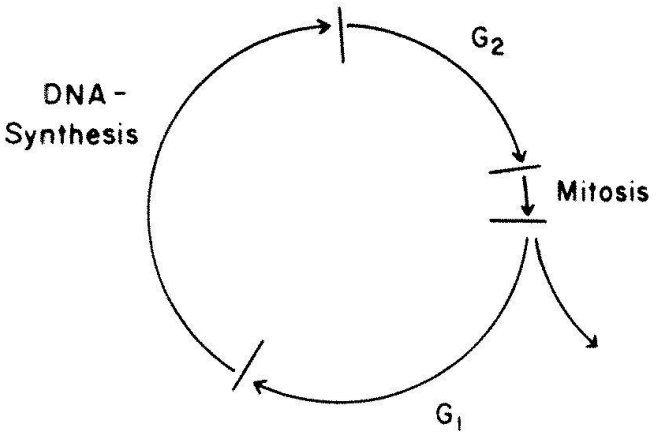

Fig. 1. The cell division cycle of animal cells.

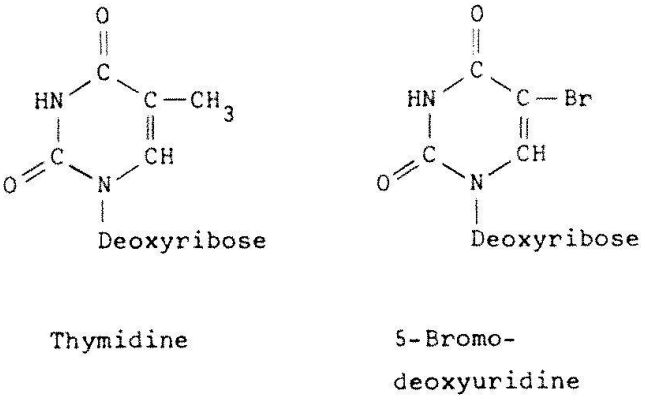

Fig. 2. Chemical structure of the DNA precursor, thymidine, and of its analog, 5-bromo-deoxyuridine.

intrazellulären Prozesse beim Eintritt normaler Zellen in die Proliferationsruhe unter gleichzeitiger Zelldifferenzierung an.

In the cell division cycle of mammalian and other animal cells, i.e. between two successive cell divisions, four different phases can be distinguished (fig. 1): G_1 phase; S phase in which the cellular genome in the nucleus is replicated; G_2 phase; and mitosis. Cells in mitosis are easily identified by their morphology, e.g. by condensation of nuclear chromatin into a specific number of chromosomes, while cells in S phase may be recognized autoradiographically by their capacity to incorporate ^{3}H-labeled thymidine into DNA. Cells in G_1 phase and those in G_2 phase may be distinguished by cytofluorometric determination of their DNA content (Tobey et al., 1975).

When animal cells are cultured in vitro under appropriate culture conditions, they may multiply exponentially over prolonged periods of time, resulting in an essentially constant duration of successive cell division cycles of usually below 30 hours. On the other hand, in the intact organism and under growth-limiting culture conditions, many cells have stopped their progression in the cell cycle between mitosis and S phase and are in a state of proliferative quiescence which may last many months, or even indefinitely. In this context, neoplasia may be defined as a defect in the control of entry into and/or exit from such a state of proliferative quiescence.

In the investigation of individual cellular functions responsible for ordered progression in the cell cycle and for entry into and exit from proliferative quiescence, conditional cell-cycle mutants of animal cells are considered to be promising model systems (Prescott, 1976; Baserga, 1978; Basilico, 1978; Simchen, 1978; Siminovitch et al., 1978). Cell-cycle mutants are operationally defined as mutants that are arrested, under the nonpermissive condition, in a specific phase of the cell cycle. The mutants described so far are heat-sensitive or cold-sensitive, and most of them are arrested in G_1 phase (i.e. between mitosis and S phase) at the nonpermissive temperature.

The method of selecting conditional cell-cycle mutants that has been applied most frequently is based on the use of 5-bromodeoxyuridine (BrdUrd) which is an analog of the normal DNA precursor substance, thymidine (dThd) (fig. 2). If 5-bromodeoxyuridine is present in the culture medium, it is efficiently incorporated by cells in S phase into their DNA. Cells containing 5-bromodeoxyuridine in place of thymidine in their DNA differ from cells containing normal DNA by their high sensitivity to light in the wave-length region around 400 nm. Cells with 5-bromodeoxyuridine-containing DNA may thus be killed selectively by irradiation with 400 nm light (Puck et al., 1967). The principle of selection based on the use of 5-bromodeoxyuridine and applied for isolation of heat-sensitive cell-cycle mutants is depicted in figure 3.

In our studies, cultured cells of a murine mast-cell tumor termed P-815 mastocytoma (Dunn et al., 1957; Schindler et al., 1959; Green et al., 1960) were used as the parent population for selection of both heat-sensi-

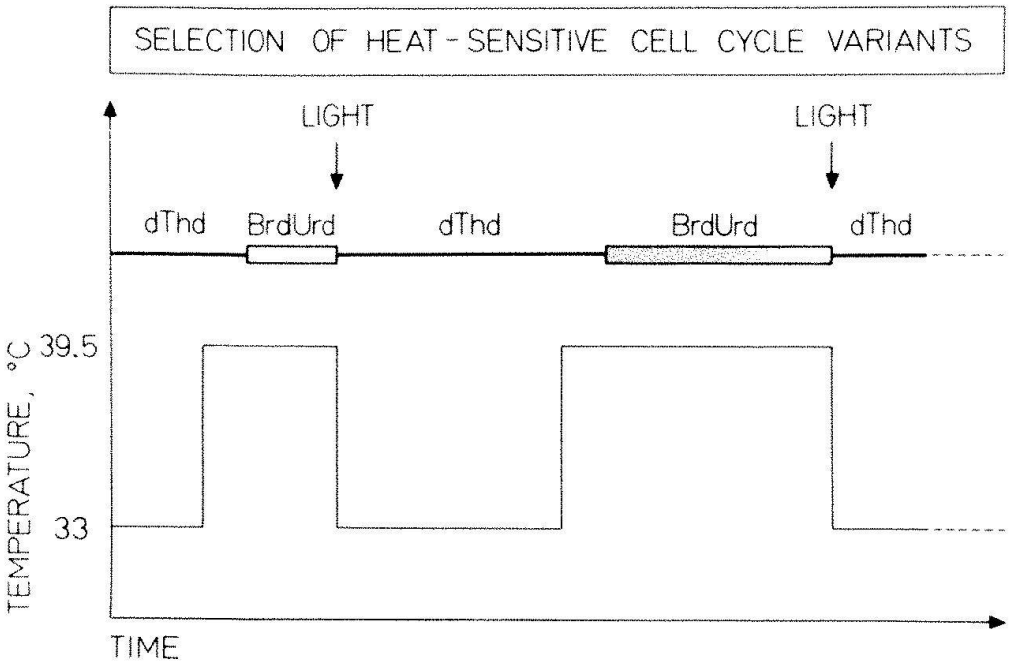

Fig. 3. General procedure used for selection of heat-sensitive cell-cycle mutants based on incubation with 5-bromodeoxyuridine (BrdUrd) at the nonpermissive temperature, followed by irradiation with light (wave length: 400 nm) and incubation of surviving cells at the permissive temperature with thymidine (dThd).

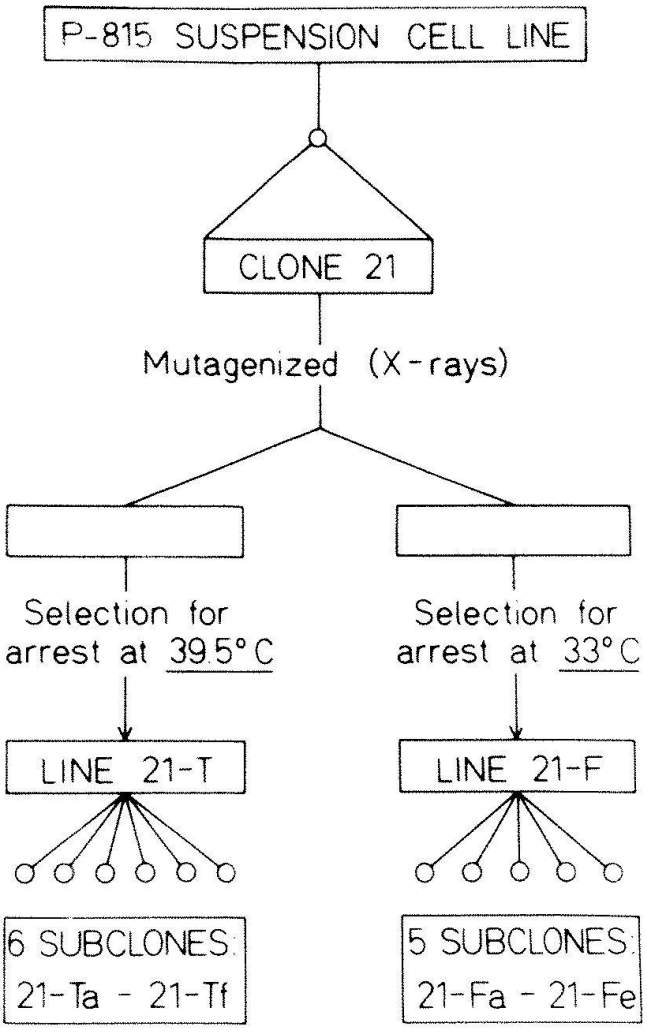

Fig. 4. Selection and nomenclature of heat- and cold-sensitive cell-cycle mutants of the P-815 mastocytoma line.

tive (hs, multiplying at 33° C, arrested at 39.5° C) and cold-sensitive (cs, multiplying at 39.5° C, arrested at 33° C) cell-cycle mutants as follows (fig. 4). A clonal subline (termed clone 21 or K 21 and representing the "wild-type" control) of the P-815 mastocytoma was subjected to mutagenization by treating 2.5×10^7 cells with 250 rads of X-rays. The culture was then split and subjected to the selection procedure for hs and cs mutants described above. Of the two variant cell lines (21-T and 21-F) thus obtained, clonal sublines were derived by isolation of single cells. The six hs subclones to be used in further studies were designated as 21-Ta – 21-Tf, while the five cs subclones were designated as 21-Fa – 21-Fe.

The results on cell multiplication of the hs subclones 21-Ta – 21-Tf and of the wild-type K 21 line at 33° C and 39.5° C are illustrated in figure 5. Whereas K 21 cells exhibited exponential proliferation at both incubation temperatures, multiplication of all six 21-T subclones nearly ceased after the first 24 hours at 39.5° C. On the other hand, multiplication of 21-T cells at 33° C was similar to that of K 21 cells. After 6–12 days at 39.5° C, cell multiplication of 21-T cells was resumed. This was shown to reflect the outgrowth of revertants with properties similar to those of K 21 cells. The five cs 21-F subclones exhibited exponential cell multiplication at 39.5°

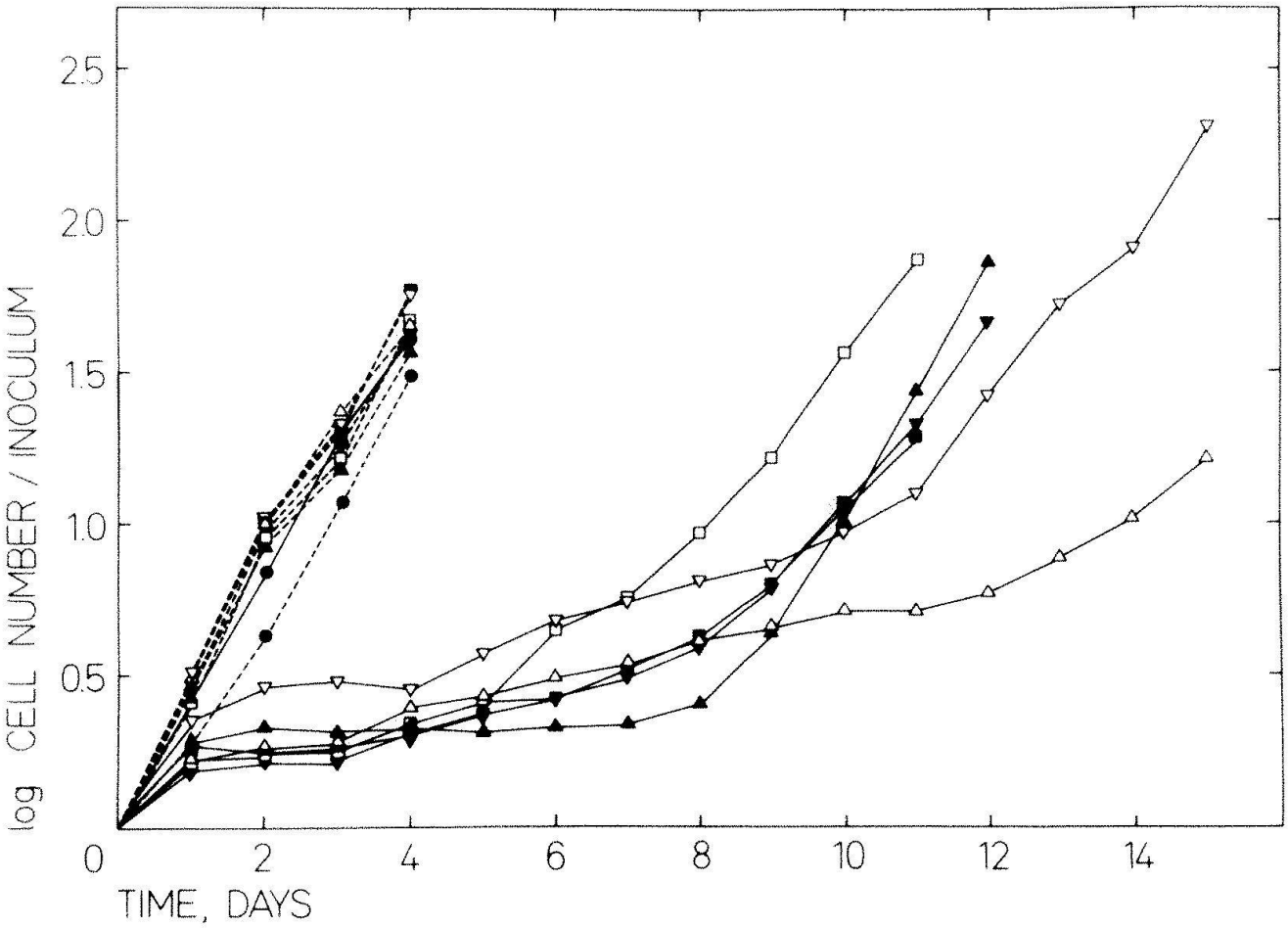

Fig. 5. Multiplication of "wild-type" K 21 cells and of 6 subclones of the hs 21-T line at 33 °C (———) and at 39.5 °C (– – –).

● K 21
△ 21-Ta
▲ 21-Tb
▽ 21-Tc
▼ 21-Td
□ 21-Te
■ 21-Tf

C, whereas the cell multiplication rate decreased to minimal levels after the first 3 days of incubation at 33° C (data not shown).

For determination of cell-cycle distribution, cells were prepared for cytofluorometric determination of DNA content as described by Grieder et al. (1975). Typical results obtained for the hs subclone 21-Tb are presented in figure 6. It is seen that at the time when the cells were transferred from the permissive temperature of 33° C to 39.5° C, their distribution in the cell cycle was typical of that of an exponentially multiplying population, with two peaks corresponding to cells in G_1 phase and in G_2 phase and mitosis, respectively, which are connected by a broad distribution of cells in S phase with intermediate DNA values. After 8 hours of incubation at 39.5° C, the percentage of cells in G_1 phase already had increased, and after 24 hours at the nonpermissive temperature, nearly all cells had a DNA content typical of G_1 phase. It may, therefore, be concluded that at 39.5° C these cells were arrested between mitosis and S phase and thus may be considered to be true cell-cycle mutants. Similar results were obtained by cytofluorometric analysis of the other 21-T subclones and after transfer of the cs 21-F subclones to the nonpermissive temperature of 33° C. The arrest of cell multiplication of 21-T and 21-F cells at the respective nonpermissive temperature was reversible, i.e. cell multiplication was resumed within 24 hours if cells were brought back to the permissive temperature. Thus, these results indicate that at the nonpermissive temperature, both the hs

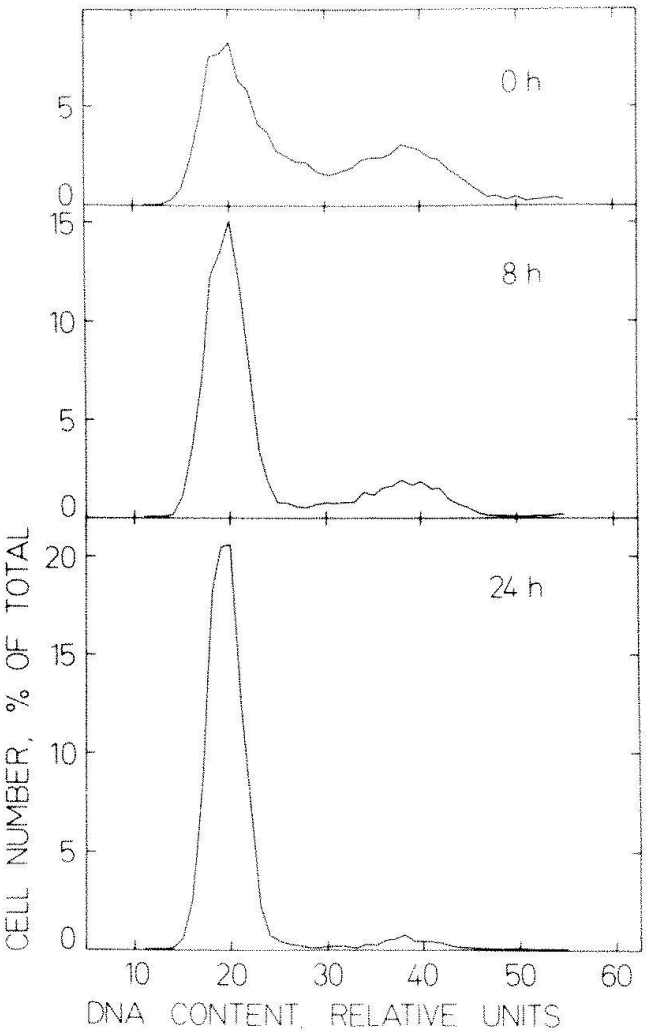

Fig. 6. Cell cycle position of 21-Tb cells, as determined by cytofluorometric measurements of cellular DNA content, at different times after transfer of cells from 33°C to 39.5°C.

and the cs cells accumulated in a state of reversible proliferative quiescence which is located between mitosis and S phase.

To determine if the hs and cs phenotypes of the 21-T and 21-F cell-cycle mutants are expressed in a dominant or recessive manner, the mutant cells were arrested at the nonpermissive temperature and fused to "wild-type" (WT) P-815 cells that had been arrested by incubation in culture medium containing a low concentration (0.01%) of serum. As a means of identifying homokaryons and heterokaryons, the cells were induced to take up latex particles of different sizes. For cell-to-cell fusion, a method based on the effects of polyethylene glycol and dimethyl sulfoxide, as described in detail elsewhere (Zimmermann et al., 1981), was used. After fusion, the cells were reincubated at the respective nonpermissive temperature in medium containing 10% serum and [^{3}H]thymidine. At different times, DNA-synthetic activity of mono- and binuclear cells was determined by autoradiography. As illustrated schematically in figure 7, the 21-T x WT heterokaryons entered the S phase under these conditions, indicating that the hs 21-T phenotype was recessive, whereas the cs 21-F cells behaved in a dominant manner, i.e. the heterokaryons did not enter the S phase for at least 48 hours, as reported in detail elsewhere (Zimmermann et al., 1981). These results are compatible with the assumption that the hs 21-T cells contain a heat-labile gene product which is essential for traverse through G_1 phase, and which is inactivated upon shift to 39.5° C, resulting in proliferative quiescence of cells with a DNA content typical of G_1 phase. On the other hand, the dominant expression of the cs phenotype of 21-F cells supports the assumption that these cells contain a heat-labile gene product which in its "active" form (i.e. the form present at 33° C) induces a state of proliferative quiescence which is also located between mitosis and S phase.

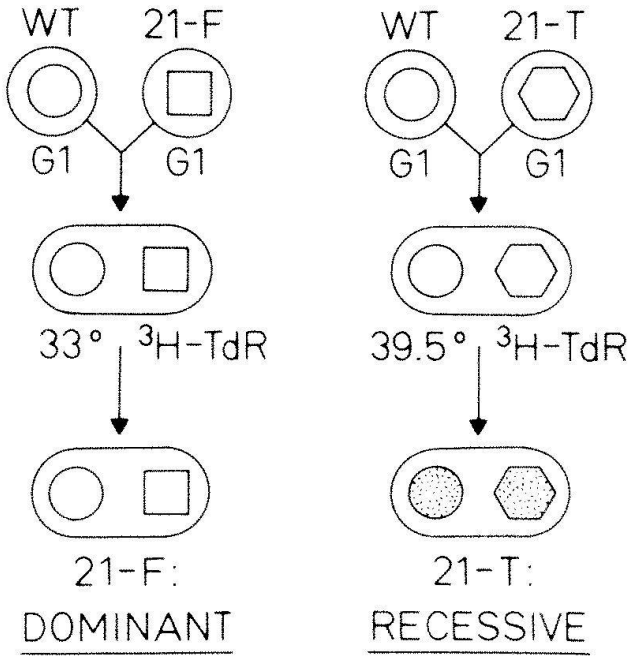

Fig. 7. Autoradiographic analysis of the capacity to enter S phase after fusion of "wild-type" (WT) cells arrested by serum deprivation with cs 21-F cells arrested at 33°C or hs 21-T cells arrested at 39.5°C. After cell-to-cell fusion, the cultures were incubated at the temperature indicated in medium containing [^{3}H]-thymidine (^{3}H-TdR) and 10% serum. DNA-synthesizing nuclei (i.e. covered by silver grains formed by decay of ^{3}H during autoradiography) are depicted by the black dots.

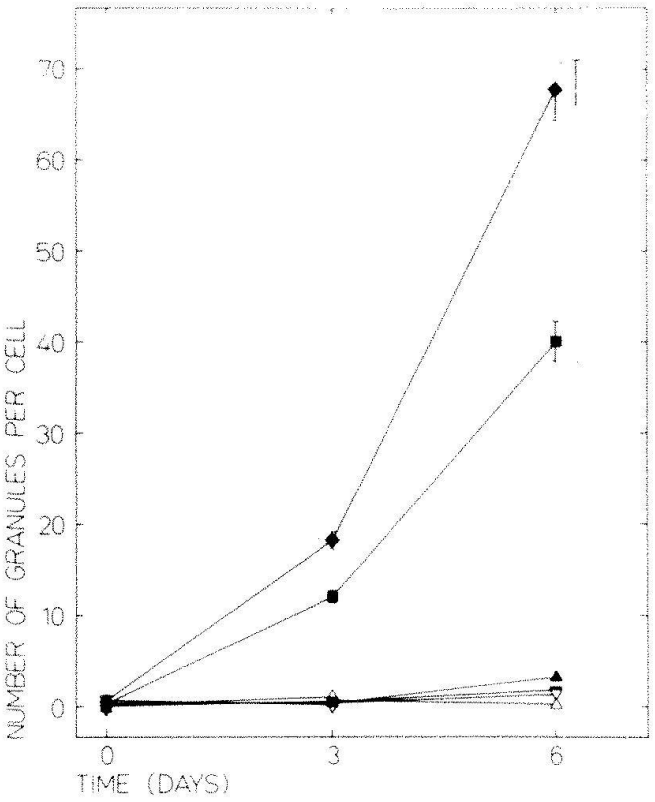

Fig. 8. Formation of metachromatically staining mast-cell granules by cs and hs cell-cycle mutants and "wild-type" (K 21) mastocytoma cells at two different temperatures.
△ K 21 cells at 33 °C
▽ K 21 cells at 39.5 °C
◆ 21-Fb cells at 33 °C
■ 21-Fc cells at 33 °C
▲ 21-Tb cells at 39.5 °C
▼ 21-Tf cells at 39.5 °C

As an additional possibility to characterize the states of proliferative quiescence attained by 21-T and 21-F cells at the respective nonpermissive temperature, the degree of cellular differentiation of these mutants of the mastocytoma line was evaluated after staining of fixed cells with toluidine blue. With this procedure, heparin-containing granules which are a typical constituent of normal mast cells may be identified by their metachromatic staining properties. As seen in figure 8, "wild-type" K 21 cells contained very few, if any, metachromatically staining granules, and similar results were obtained for 21-Tb and 21-Tf cells during culture at 33° C and after shift to 39.5° C. On the other hand, upon incubation of 21-Fb and 21-Fc cells at the nonpermissive temperature of 33° C, the average number of mast-cell granules per cell increased markedly.

In conclusion, the results obtained, as summarized in table 1, are compatible with the assumption that the gene product responsible for the cs phenotype of 21-F cells may be a pleiotropic effector inducing both a state of reversible proliferative quiescence and the formation of mast-cell granules, i.e. specialized cellular organelles, during incubation at 33° C, while at 39.5° C, this gene product would be inactive. On the other hand, lack of activity of a gene product required for traverse through G_1 phase, as exemplified by the hs 21-T cell-cycle mutants at 39.5° C, apparently is insufficient for induction of mast-cell differentiation. It may, therefore, be appropriate to distinguish between qualitatively different states of proliferative quiescence. The state of quiescence induced in 21-F cells at 33° C, being characterized by (a) dominant expression in cell fusion experiments and (b) cellular differentiation, may represent a useful model system because mechanisms similar to those responsible for induction of proliferative quiescence and cellular differentiation in 21-F cells at 33° C may be operative also in normal cells. The mutation underlying the cs phenotype of 21-F cells may thus be a means to identify one of the elements in the regulation of the transition of cells from exponential proliferation into a state of proliferative quiescence.

Table 1. Characteristics of heat- and cold-sensitive cell-cycle mutants of the P-815 murine mastocytoma line.

	hs mutants (21-T)	cs mutants (21-F)
Cell cycle arrest at	39.5 °C	33 °C
Cell proliferation at	33 °C	39.5 °C
Arrested in	G_1	G_1 (or "G_0")
Reversibility of arrest	reversible	reversible
Expression of ts phenotype in heterokaryons	recessive	dominant
Cell differentiation of arrested cells	no	yes
Cell cycle arrest induced by	absence of active gene product	presence of active gene product (pleiotropic effector?)

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