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Genetics

Genetic analysis of the G1 period: Isolation of mutants (or variants) with a G1 period from a Chinese hamster cell line lacking G1

(cell cycle/V79 cells/somatic cell genetics/complementation)

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ABSTRACT **Cells of the Chinese hamster line V79-8 multiply without a G1 period (i.e., they are G1$^-$) and have an average generation time of 9.5 hr. After mutagenesis and selection we have derived five stable mutants (or variants) of this line that have longer generation times. In each case the increase in generation time is due solely to the introduction of a G1 period into the cell cycle, with no measurable effect on S, G2, or M. Fusions among these five G1$^+$ mutant lines and another presumably nonmutant G1$^+$ line (V79-743) produce hybrid cells lacking a G1 period in all but one case. These complementation tests define five complementation groups among these six G1$^+$ cell lines. The six G1$^+$ lines represent five different causes or bases for the presence of a G1 period. The two G1$^+$ mutants belonging to complementation group V are temperature sensitive for expression of the G1$^+$ phenotype (G1 ≅ 0, 4, and 6 hr at 33°, 37°, and 39°, respectively). In all cases the G1$^-$ state is dominant over the G1$^+$ state, suggesting that the presence of G1 represents a "deficient" condition. Mutants of this type may be useful in the analysis of the switch from G1$^-$ to G1$^+$ that occurs normally in cleaving embryos and in elucidation of the genetic mechanism(s) responsible for the presence of a measurable G1 in most cells.**

Rates of division for cells of multicellular organisms are generally regulated by the control over transit through the G1 period of the cell cycle (for a recent review see ref. 1). Both reversible and irreversible closing down of cell reproduction are achieved by arrest of cells in G1. (Cells that have ceased reproduction in G1 are sometimes described as having entered a G_0 state.) In cell types with different reproductive rates the lengths of the G1 periods are correspondingly modulated, possibly by transient arrest at a specific point between mitosis and the start of the S period (1). The most rapidly proliferating cells, exemplified by blastomeres of cleaving embryos (e.g., see refs. 1–3) and certain erythropoietic cells (4), lack a G1 period entirely (i.e., they are G1$^-$), presumably signifying the complete absence of restraint on reproduction. Thus, an understanding of how cell reproduction is regulated will require more information about the event(s) that determine the transit through G1 and of how these events are interrupted to achieve either transient or long-term G1 arrest.

In the simple eukaryote yeast, transit through G1 requires the expression of specific genes (5, 6). Considerable evidence suggests that the same is true in mammalian cells (for reviews see refs. 1 and 7). Indeed, the existence of at least two different genes whose expressions are required for G1 transit in mammalian cells is suggested by studies on temperature-sensitive (ts) mutants (7, 8).

We have begun a genetic analysis of G1 using the G1$^-$ Chinese hamster lung cell line V79-8 (9). The G1$^-$ nature of the V79-8 line has been demonstrated both by autoradiographic techniques (9–11) and by the premature chromosome condensation technique of cell-cycle analysis (ref. 12; P. N. Rao, personal communication). The G1$^-$ phenotype of V79-8 is dominant in *intraspecific* cell fusions, i.e., fusions of V79-8 cells with the cell lines of Chinese hamster that have a G1 period (G1$^+$ cells) produce only G1$^-$ hybrids (11). In addition, Chinese hamster V79-8 cells have been hybridized with two different ts cell-cycle mutants [AF8 of Syrian hamster and B54 of mouse (8)] that each blocks specifically in the G1 period at the restrictive temperature. All such hybrids lack the ts phenotype, indicating that V79-8 can correct the defects of these two ts G1 mutants (11). From these observations we conclude that the G1$^-$ V79-8 cell expresses at least two functions that are associated with G1 (corrects defects of ts G1 mutants in *interspecific* cell fusions), but expresses additional information that "erases" G1 (dominance of the G1$^-$ phenotype in *intraspecific* cell fusions). We suppose that the G1$^-$ phenotype reflects the full expression of a gene(s), and loss of such expression (either partial or complete) of this gene(s) results in delayed transit from mitosis to S, i.e., creates a G1 period. By analogy with conventional genetic analysis, the G1$^-$ state may be considered as the "wild-type" condition (shows expression of the G1-erasing mechanism), and the G1$^+$ state may be considered as a "deficient" condition (lacks expression of this mechanism). In this view, the switch from the G1$^-$ state of rapidly dividing early blastomeres in an embryo to the G1$^+$ state for cells in later development might be brought about by "repression" of the cell cycle gene(s) that allow(s) immediate transit from mitosis to S.

We describe here efforts to identify the gene function(s) responsible for the G1$^-$ and G1$^+$ conditions. These experiments consist of the selection and characterization of mutants (or variants) of the G1$^-$ V79-8 cell line that express a G1.

MATERIALS AND METHODS

Cell Lines and Culture Techniques. Growth characteristics of the G1$^-$ V79-8 and the G1$^+$ V79-743 Chinese hamster lung cell lines have been described (10, 11). The nature of the original V79 cell line is unclear. We do not know for certain whether the G1$^-$ cell arose from a culture of G1$^+$ cells or vice versa, although there is some indication that the V79-743 line (G1$^+$) is characteristic of the original cell population (10). It would appear that V79-743 has always been G1$^+$ and was not selected, at least knowingly, from an otherwise G1$^-$ V79 culture. We will therefore refer to V79-743 as a "nonmutant" G1$^+$ cell. These cells (11) and mutants derived from them all have a modal chromosome number between 20 and 22 chromosomes. Cells were cultured in Dulbecco's modified Eagle's medium plus

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Abbreviations: HAT, hypoxanthine/aminopterin/thymidine; GAMA, guanine/adenine/mycophenolic acid/azaserine; Ou^R, ouabain-resistant; Ou^S, ouabain-sensitive; HPRT$^-$, hypoxanthine phosphoribosyltransferase-deficient; APRT$^-$, adenine phosphoribosyltransferase-deficient; TK$^-$, thymidine kinase-deficient; ts, temperature-sensitive.

Table 1. Cell-cycle parameters (at 37°) of the two V79 lines and five G1+ mutants derived from the G1− V79-8 line

Cell line	G1	S	G2 + M	Generation time
V79-8	0	9.0	0.5	9.5
V79-743	2.0	9.5	0.5	12.0
G1+-1	4.25	9.5	0.75	14.5
G1+-2	2.5	9.0	0.5	12.0
G1+-3	2.0	9.5	0.5	12.0
G1+-4	3.5	9.0	0.5	13.0
G1+-5	4.0	9.5	0.5	13.5

Values are in hr.

~12% fetal calf serum in an atmosphere of 3% CO_2/97% air.

Cell-Cycle Analysis. The "standard" method of analysis was used for all mutant cells and some hybrid cells as follows. Generation times (GT) were calculated from population doubling times, applying a correction for noncycling cells, i.e., cells that did not incorporate [^{3}H]dThd during continuous incubation with the radioisotope for an interval equal to one generation time. The length of the S period was calculated from the percentage of cells labeled autoradiographically in an asynchronous population labeled with [^{3}H]dThd (5 μCi/ml; 50 Ci/mmol) for 10 min (length of S = fraction labeled × GT). The length of G2 was calculated by the labeled mitosis method (13). The length of M, determined by direct microscopic observation, was ~0.5 hr for V79-8 (10). The length of G1 was calculated by subtraction (G1 = GT − S − G2 − M).

The "short" method of cycle analysis was used for initial screening of mutant cells and for most hybrid cells because the large number of hybrid cells characterized made the standard method impractical. Generation time was measured by the standard method. S was measured from the labeling index after a 10-min pulse with [^{3}H]dThd. G2 + M was assumed to be ~1.0 hr (this value was actually determined for some cell hybrids by applying the labeled mitosis method for measuring G2). As an example, the generation time was ~10.0 hr for many hybrids; the labeling index was typically 90%—hence, S = 0.9 × 10 hr = 9 hr. G2 + M = 1.0 hr (see above); hence, G1 = 10 − 9 − 1 = 0 hr.

For the five mutant cell lines listed in Table 1 (G1+ -1 through G1+ -5) the cell cycle was analyzed by both methods, and the calculated G1 lengths were the same in each case. Autoradiography was performed by coating slides with Kodak NTB2 liquid emulsion, exposing them for 21–40 days, developing them, and examining them as described (10).

Induction and Detection of G1+ Cells. A culture of V79-8 G1− cells was treated with 1.0 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml for 4 hr. This treatment kills ~65% of the cells. Allowing 3 days for phenotypic expression of induced mutations, the cells were next incubated with 5 μCi of [^{3}H]dThd per ml for 2 hr to kill cells in S. Ten hours after removal of exogenous [^{3}H]dThd (~S period), cells were again treated for 2 hr with [^{3}H]dThd followed by 10 hr without [^{3}H]dThd. Five to six treatments with [^{3}H]dThd were used in this manner to select for more slowly growing cells (see Fig. 1 for selection scheme). After the last [^{3}H]dThd application, surviving cells were allowed to form colonies (7–10 days). Individual colonies were harvested, cultured as clonal lines, and screened for the presence of a G1 period. Candidates for a G1+ phenotype were then subjected to the standard method of cell-cycle analysis.

Cell Fusion and Identification of Hybrids. Cell fusion was induced with polyethylene glycol (14). Hybrids were selected by one of three systems: (*i*) the hypoxanthine/aminopterin/thymidine (HAT) system for isolating hybrids between hypoxanthine phosphoribosyltransferase-deficient (HPRT−) cells and thymidine kinase-deficient (TK−) cells (15); (*ii*) the guanine/adenine/mycophenolic acid/azaserine (GAMA) system, for HPRT− × adenine phosphoribosyltransferase deficient (APRT−) cells (16) (this technique was used for most of the complementation tests); and (*iii*) the ouabain (Ou)–HAT system for crosses between cells doubly marked by HPRT− and Ou resistance (Ou^R) and HPRT+ and Ou-sensitive (Ou^S) cells (17).

HPRT− cells were obtained by selection in medium containing 10 μg of 6-thioguanine per ml. APRT− cells were selected on the basis of their resistance to 100 μg of 6-diaminopurine per ml. All HPRT− and APRT− cell lines were selected after one-step mutagenesis with methylnitronitrosoguanidine. HPRT− Ou^R cells were obtained by selecting for spontaneous Ou^R subclones of HPRT− cells based on their ability to proliferate in 1 mM ouabain. Ou^R clones appeared at frequencies ranging from 3×10^{-6} to 5×10^{-7}, depending on the subline.

Reversion frequencies of all HPRT−, APRT−, and HPRT− Ou^R lines were $<10^{-6}$ and in most cases were undetectable. Hybrids were selected by their ability to grow continuously in the appropriate selective medium. All hybrids were near tetraploid (~40–44 chromosomes). In these and earlier studies of *intraspecific* hybrids with V79 cells (11) minimal chromosome loss has been seen during subsequent cultivation. The frequency of hybrid formation varied from 2×10^{-3} to 8×10^{-5}, depending on the particular cross and selective system.

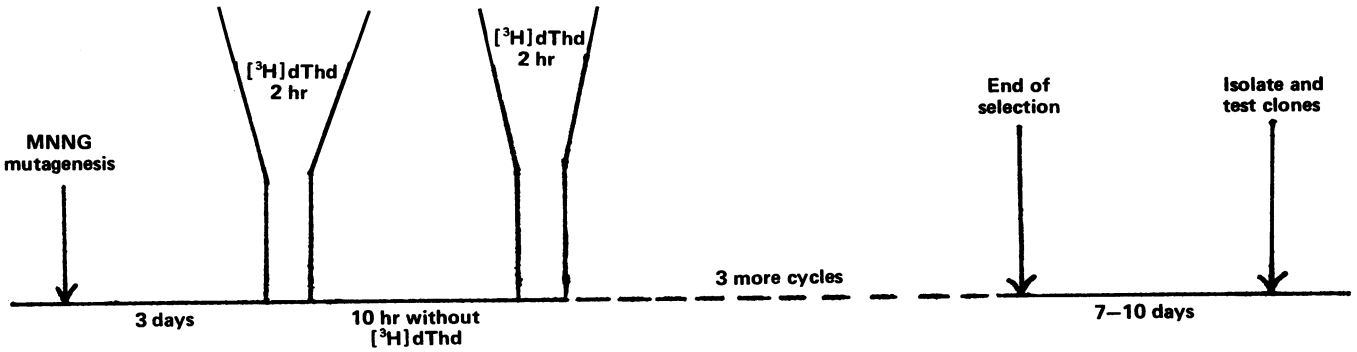

FIG. 1. Diagram of [^{3}H]dThd suicide selection used for the isolation of G1+ mutants from the G1− V79-8 cell line. Five to six cycles, each consisting of a 2-hr treatment with 5 μCi of [^{3}H]dThd (specific activity ~50 Ci/mmol) were used. The rationale of this procedure is that short pulses (i.e., 2 hr) of [^{3}H]dThd should preferentially kill G1− cells and allow survival of cells with an increased period of non-S during their cell cycles (e.g., G1+ cells). The 10-hr period between administration of [^{3}H]dThd is intended to allow any cells that were in G1 during one [^{3}H]dThd period to traverse S before the next round of selection. MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

After isolation, hybrid clones were subcultured for several generations in the appropriate selective media to ensure their hybrid nature, followed by transient cultivation in a medium such as Dulbecco's modified Eagle's medium plus hypoxanthine/thymidine (in the case of HAT) to prevent poisoning of hybrids by residual drugs, and finally placed into Dulbecco's modified Eagle's medium for cell-cycle analysis.

RESULTS

Isolation of G1$^+$ Mutants. After treatment of the G1$^-$ cells with methylnitronitrosoguanidine and phenotypic expression, cultures were subjected to the selection scheme outlined in Fig. 1. Because ~95% of the cell cycle of the G1$^-$ V79-8 line is occupied by S, a pulse (~2 hr) of high levels of [^{3}H]dThd kills a large fraction of the G1$^-$ cells and allows preferential survival of cells whose cycles have an increased period occupied by non-S (e.g., addition of a G1 and/or a G2 period).

Two selections involving five to six rounds of [^{3}H]dThd treatment were performed on a total of ~10^6 survivors of mutagenesis. Fifty colonies were isolated and their cell cycles analyzed with the short method (see *Materials and Methods*). The cells in most of these colonies (43 of 50) had either a short generation time and high labeling indices (e.g., representing surviving G1$^-$ cells) or a long generation time and high labeling indices (e.g., cells that presumably have an increased length of S). Seven clones had an increased generation time and a lower labeling index compared to the original G1$^-$ line and therefore were subjected to analysis by the standard method of cell cycle analysis (see *Materials and Methods*) to determine whether they were indeed G1$^+$. During these analyses two putative Gl$^+$ lines appeared to "revert" back to G1$^-$, as suggested by their progressively decreasing generation times; these were discarded. The remaining five putative G1$^+$ mutants have retained their characteristic increased generation times for over 6 months of culture and are the subject of the remainder of this report.

The results of cell-cycle analysis of the five G1$^+$ mutants and of the presumably *nonmutant* (see *Materials and Methods*) G1$^+$ line V79-743 are given in Table 1. Each mutant has an increased GT that is due exclusively to the appearance of a G1 period. The generation times of the five mutants were not detectably shortened when the cells were grown in Dulbecco's medium supplemented with all of the nonessential amino acids (data not shown). This suggests that the appearance of G1 in the mutants is not due to creation of some degree of auxotrophy for one or another nonessential amino acid.

Recessiveness of G1$^+$ Phenotype of Mutant G1$^+$-1. To test for the dominance compared to the recessiveness of the G1$^+$ phenotype of G1$^+$-1, we crossed an HPRT$^-$ derivative of G1$^+$-1, G1$^+$-1(HPRT$^-$), with an APRT$^-$ derivative of G1$^-$ V79-8, V79-8(APRT$^-$), and analyzed the cell cycles of the hybrids (isolated in GAMA medium) by the standard method. The results are shown in Table 2. The five hybrid lines (defined by their ability to grow in GAMA and by their near tetraploidy) had generation times ranging from 9.5 to 11 hr and lacked a G1 period (i.e., were G1$^-$). As a control, near-tetraploid clones of the G1$^-$ V79-8 line were isolated after self-fusion [i.e., V79-8(HPRT$^-$) × V79-8(APRT$^-$)]. The generation times of the self-hybrids ranged from 9.0 to 10.5 hr, and all hybrids were G1$^-$. The results of the fusions between G1$^+$-1 and V79-8 (Table 2) show that the G1$^+$ phenotype of the G1$^+$-1 mutant is *recessive* to the G1$^-$ phenotype of V79-8. This confirms the dominance of the G1$^-$ phenotype reported earlier (11) in *intraspecific* cell hybrids.

Complementation Tests between G1$^+$ Cells. Assuming that these G1$^+$ mutants represent recessive defects in different functions, hybrids between two different mutants would be expected to be G1$^-$ due to complementation. As an initial test for such complementation, hybrid lines were formed by fusion of G1$^+$-1(HPRT$^-$) with G1$^+$ V79-743 (TK$^-$), isolated using HAT, and their cycles were analyzed by the standard method. All six hybrid lines tested were G1$^-$ (Table 2). Therefore, G1$^+$-1 and V79-743 complement each other in somatic cell hybrids. Such complementation suggests that G1$^+$-1 and V79-743 express a G1 for different reasons.

Table 2. Cell-cycle parameters of hybrids from two different crosses: mutant G1$^+$-1 × V79-8 (G1$^-$) and mutant G1$^+$-1 × V79-743 (G1$^+$)

Cell	G1	S	G2 + M	Generation time
G1$^+$-1	4.25	9.5	0.75	14.5
V79-8(G1$^-$)	0	9.0	0.5	9.5
Hybrids				
1	0	10.0	1.0	11.0
2	0	8.5	1.0	9.5
3	0	9.0	1.25	10.0
4	0	9.0	1.0	10.0
5	0	10.25	0.75	11.0
G1$^+$-1	4.25	9.5	0.75	14.5
V79-743(G1$^+$)	2.0	9.5	0.5	12.0
Hybrids				
1	0	9.0	0.75	9.5
2	0	9.0	1.0	10.0
3	0	9.25	0.75	10.0
4	0	9.5	1.0	10.5
5	0	8.5	0.75	9.0
6	0	9.0	1.0	10.0

All cell hybrids in both crosses are G1$^-$. Cell-cycle analysis was done by the standard technique. Values are in hr.

In a similar manner the five G1$^+$ mutants and the G1$^+$ line V79-743 were crossed with one another to determine the number of complementation groups for the G1$^+$ phenotype. For each cross (e.g., G1$^+$-1 × G1$^+$-2) five to seven individual hybrid clones were isolated, and their cell cycles were analyzed by the short method described in *Materials and Methods*. As a control, G1$^+$-1 was self-crossed using HPRT$^-$ and APRT$^-$ markers to ask whether fusion and formation of a near-tetraploid cell had any effect on the presence or absence of G1. The G1$^+$-1/G1$^+$-1 hybrids derived from this fusion were all G1$^+$, indicating the expected absence of complementation (Table 3). The results of all the complementation tests are given in Table 3. Complementation (formation of G1$^-$ hybrids from two different G1$^+$ parents) was found in all fusions except for G1$^+$-4 × G1$^+$-5. The complementation groups defined by the crosses of the six G1$^+$ lines are summarized in Table 4. The five mutant lines define four complementation groups and the nonmutant line V79-743 represents a fifth complementation group. Thus, these cell lines tentatively define five different bases or causes for the presence of a G1 period. Furthermore, in each case the G1$^+$ mutant phenotype behaves recessively.

When no complementation was observed in the initial cross, G1$^+$-1 (APRT$^-$) × G1$^+$-3(HPRT$^-$), a second cross was done using different subclones of each G1$^+$ mutant, each with different selection markers (Table 3). The initial cross yielded only G1$^+$ hybrids with G1 periods of about the same length as the G1$^+$-1 parent, i.e., 3–4 hr. However, when G1$^+$-1(HPRT$^-$ Ou^R) was fused with G1$^+$-3(HPRT$^+$ Ou^S), all hybrids tested had shorter generation times than either parent and were G1$^-$; the hybrids had generation times of 9.5–10.5 hr compared to 14 hr

Table 3. Summary of complementation tests

	G1⁺-1	G1⁺-2	G1⁺-3	G1⁺-4	G1⁺-5
V79-743 (G1⁺)	c	c	c	c	c
G1⁺-1	nc	c	c*	c	c
G1⁺-2			c	c	c
XG1⁺-3				c	c
G1⁺-4					nc*

Results of complementation tests between the nonmutant V79-743 (G1⁺) and five G1⁺ mutant clones isolated from V79-8 (G1⁻). c, Complementation occurred, as indicated by the formation of G1⁻ cell hybrids. nc, *No* complementation, as indicated by the formation of G1⁺ cell hybrids. Each test for complementation was done by examination for the G1 phenotype of at least five individual hybrid clones. For any given cross all hybrids consistently showed the same G1 phenotype, all were G1⁺ or all were G1⁻. The G1 phenotypes of the hybrids were determined with the short technique of cell-cycle analysis (except for the cross V79-743 × G1⁺-1 where the standard technique was used).

* Cases in which more than one complementation test was performed (see *Results*).

for G1⁺-1 and 12 hr for G1⁺-3. In this second cross the cells probably have at least in part different "secondary" genetic backgrounds, i.e., in the first cross G1⁺-3 was remutagenized to obtain the HPRT⁻ marker and in the second cross it was not. We assume that unidentified secondary mutations possibly induced during selection for these markers are somehow responsible for the observed noncomplementation in the initial cross and complementation in subsequent crosses.

A third cross between G1⁺-1 and G1⁺-3 was done using the reverse arrangement of the selection markers present in the second cross, i.e., using G1⁺-1(HPRT⁺ Ou^S) × G1⁺-3(HPRT⁻ Ou^R). Again, the five hybrids analyzed had generation times of 9.0–10.0 hr and were all G1⁻, indicating complementation between G1⁺-1 and G1⁺-3. Although we cannot explain the absence of complementation in the G1⁺-1(APRT⁻) × G1⁺-3(HPRT⁻) cross, we have assigned G1⁺-1 and G1⁺-3 to different complementation groups on the basis of the complementation found in two out of three crosses. Assignment to two complementation groups is also consistent with the observation that G1⁺-1 and G1⁺-3 have G1 periods of different average lengths, i.e., 4 hr and 2.0 hr, respectively.

Fusion between G1⁺-4 and G1⁺-5 consistently yielded G1⁺ hybrids in crosses with two sets of selection markers: G1⁺-4(HPRT⁻) × G1⁺-5(APRT⁻) and G1⁺-4(HPRT⁺ Ou^S) × G1⁺-5(HPRT⁻ Ou^R). The G1 periods for all 14 hybrids analyzed were 3–5 hr. Therefore, G1⁺-4 and G1⁺-5 have been assigned to the same G1⁺ complementation group.

The results with crosses between G1⁺-1 and G1⁺-3 serve as a warning that failure to observe complementation could be due to secondary genetic changes created during mutagenesis to obtain selection markers rather than to mutation of the same gene in both partners of a cell fusion.

Tests for ts of G1⁺ Phenotype. To determine whether any of the five G1⁺ mutants were ts for the expression of G1, the G1 lengths of the G1⁻ parent line and the G1⁺ mutants were determined at 33°, 37°, and 39°. The results are given in Fig. 2. The G1⁻ parent is G1⁻ at all three temperatures. Mutants G1⁺-1, G1⁺-2, and G1⁺-3 are G1⁺ at all temperatures tested (Fig. 2). G1⁺-4 and G1⁺-5, which are in the same complementation group (see Tables 3 and 4), have longer GTs at 37° and 39° than the G1⁻ parent line, but have essentially the same generation time as the G1⁻ parent line at 33° (data not shown). Standard cell-cycle analysis of G1⁺-4 and G1⁺-5 showed that both mutants are essentially G1⁻ at 33° and, in contrast, G1⁺ at 37° and 39°. G1⁺-4 and G1⁺-5 most likely represent descendants of one original G1⁺ cell because they fail to complement, are both ts for the expression of G1 were isolated in the same mutant hunt, and have about equal G1 lengths.

Table 4. G1⁺ complementation groups

Group	Member(s)
I	V79-743
II	G1⁺-1
III	G1⁺-2
IV	G1⁺-3
V	G1⁺-4, -5

Summary of the five G1⁺ complementation groups defined by fusions between the various G1⁺ lines.

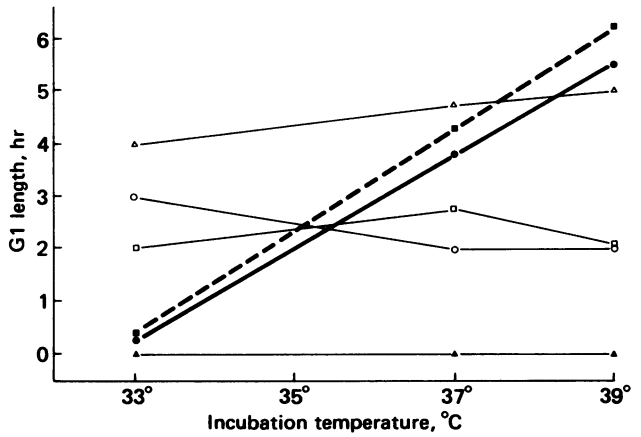

FIG. 2. The length of G1 periods at three temperatures for the five G1⁺ mutants and the parental G1⁻ V79-8 line. ▲, G1⁻ V79-8; △, G1⁺-1; □, G1⁺-2; ○, G1⁺-3; ●, G1⁺-4; ■, G1⁺-5. The length of G1 for mutants G1⁺-4 and G1⁺-5 decreased with temperature and was nearly 0 at 33°. In each case the cells were kept for several generations at the growth temperatures to be tested before measurement of G1 length. G1 length for mutants G1⁺-4 and G1⁺-5 was determined by the standard technique.

DISCUSSION

When V79-8 (G1⁻) cells are fused to G1⁺ cells of Chinese hamster (e.g., V79-743 or CHO), the hybrid cell products are always G1⁻ (11). This dominance of the G1⁻ phenotype in *intraspecific* hybrids suggests that V79-8 expresses a function(s) that is absent in established G1⁺ lines of Chinese hamster cells. Therefore, we supposed that the function(s) responsible for the absence of G1 could be impaired by mutation of the gene(s) coding for such function(s). Two mutant searches using chemical mutagenesis and selection by [^{3}H]dThd suicide have yielded five stable G1⁺ mutants. These G1⁺ mutants have increased generation times compared to the G1⁻ parent (V79-8) due solely to the induction of a G1 period, while S, G2, and M are not detectably affected.

The simplest explanation for the presence of a G1 period in the mutants is that the function of a putative G1-erasing gene has been eliminated or reduced by mutation. However, the situation seems more complex than this because fusions among the five G1⁺ mutants and one presumably nonmutant G1⁺ V79-743 line have demonstrated five G1⁺ complementation groups. These five complementation groups show differences in the average length of the G1 periods (see Table 1). Thus, we conclude that the complementation groups define five different genes all of whose unimpaired functions are required for immediate transit from mitosis to the initiation of DNA replica-

tion. Since the G1$^+$ phenotype is recessive in all five complementation groups, we assume the G1$^+$ mutants and V79-743 have G1 periods because they lack full expression of one of the five putative genes identified by the complementation tests. Further mutant searches should enable us to obtain an estimate of how many complementation groups for the G1$^+$ phenotype can be defined for the V79-8 cell line.

Although we have referred to the G1$^+$ cells derived from G1$^-$ V79-8 as mutants, the evidence that the G1$^+$ phenotypes are the result of mutation is indirect. Conceivably, a stable change from a G1$^-$ to G1$^+$ phenotype could be the result of stable changes in the regulation of gene expression. Concerning their genetic nature, we offer the following considerations:

(*i*) The five G1$^+$ cell clones have retained the G1$^+$ phenotype during more than 6 months of continuous cultivation.

(*ii*) At least one subclone of each G1$^+$ line (e.g., G1$^+$-1 HPRT$^-$ derived from G1$^+$-1) has been isolated, and all the subclones retain their characteristic G1$^+$ phenotype.

(*iii*) One of the G1$^+$ complementation groups (G1$^+$-4 and G1$^+$-5 of group V) is ts for the G1$^+$ phenotype (G1$^-$ at 33° and G1$^+$ at 37° and 39°), thus representing strong evidence for true mutation. Nevertheless, we must apply the designation "mutation" with caution because there is at least one case in which the change from G1$^-$ and G1$^+$ *is* nonmutational. This occurs in the switch from G1$^-$ cycles for blastomeres of early embryos to G1$^+$ cell cycles for cells of later embryos (3). This switch could involve "repression" of one or more genes whose full expression is required for immediate entry into S.

We have no direct evidence that the G1$^+$ mutants result from defects in gene functions specifically associated with G1. It is conceivable that defects that slow cell growth slightly by affecting general cell functions, e.g., a "leaky" mutation in a tRNA synthetase gene that slows the rate of protein synthesis, could cause the appearance of a G1 period in a G1$^-$ cell. We can say that (*i*) most likely none of the mutants displays a G1 due to partial auxotrophy for a nonessential amino acid because the G1 period is not erased for mutant cells growing in medium supplemented with nonessential amino acids, and (*ii*) none of the mutations noticeably affects transit through S, G2, or M.

The results indicate that the transit from telophase to the S phase depends upon fulfillment of at least several requirements. The time taken for such transit, i.e., the length of G1, presumably depends on the efficiency with which these requirements are met. Thus, our results and interpretation of them can be summarized by Scheme 1:

Requirements: *i*
ii
iii
iv
v

Telophase ——————→ S phase

Cell type	Requirements for S fulfilled
G1$^-$	All
G1$^+$-1	All except *i*
G1$^+$-2	All except *ii*
G1$^+$-3	All except *iii*
etc.	
G1$^+$-1/G1$^+$-2 hybrid	All
G1$^+$-1/G1$^+$-3 hybrid	All
etc.	

SCHEME 1.

Here we have identified five such putative requirements by genetic analysis. We suggest that these five complementation groups represent five genes whose fully expressed functions are necessary for the immediate transit from telophase to the S phase. Impairment of any one of these functions results in the appearance of a G1 period. Complete loss of any of these functions would presumably completely inhibit transit from mitosis to S. Finally, the scheme does not necessarily imply any particular sequence in which the requirements must be fulfilled, although a sequence may exist.

We may ask whether any of the G1$^+$ mutants isolated thus far represent defects in functions normally involved in the expression of G1 *in vivo*. This question may be approached using cell fusion to determine whether the experimentally derived G1$^+$ mutants are in the same complementation group(s) as cells with *nonmutant* or "natural" G1$^+$ phenotype(s). We have done several crosses of this type by fusing cells of each of the five G1$^+$ mutants with cells of the G1$^+$ line V79-743 and found complementation in each case (Table 3). Assuming that the G1 period in V79-743 is natural in the sense that it is based on the mechanism responsible for the G1 period in normal cells *in vivo*, we conclude that none of the G1$^+$ mutants affects that gene function presumed to cause G1 normally. Perhaps this reasoning contains an oversimplified view of the G1 period, i.e., the G1 periods in different cells may be due to modulation of different genes. Indeed, fusion between cells of two nonmutant G1$^+$ lines, V79-743 and CHO, produces G1$^-$ hybrids (unpublished observations). This complementation suggests that these two lines have G1 periods for different reasons. The derivation from V79-8 of more G1$^+$ mutants representing additional complementation groups may yield mutants that are not complementary to one or another "natural" G1$^+$ cell line(s) or to normal G1$^+$ cells in primary cultures. Analyses of such "noncomplementing" mutants should aid in identifying genes that normally underlie the presence of a G1 period and modulation of its length.

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