

## Isolation of Temperature-Sensitive Mutants of L-Cells\*

L. H. Thompson,† R. Mankovitz,‡ R. M. Baker,§ J. E. Till,  
L. Siminovitch, and G. F. Whitmore

DEPARTMENTS OF MEDICAL BIOPHYSICS AND MEDICAL CELL BIOLOGY, UNIVERSITY OF TORONTO;  
AND THE ONTARIO CANCER INSTITUTE, TORONTO, ONTARIO

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**Abstract.** Procedures are described for the isolation of conditional lethal mutants of mouse L-60T cells. The mutant lines were temperature sensitive by the following criteria: (a) colony-forming ability, (b) growth in suspension culture, and (c) rate of uptake of tritiated-thymidine.

The development and use of conditional lethal mutations has considerably facilitated the analysis of function in viruses and bacteria; as a result, the main features of regulation in these materials have been outlined. However, it is not yet clear how applicable these models of regulation are to eucaryotic cells and, in particular, whether or not they provide a basis for understanding the processes involved in the differentiation of mammalian cells. Although techniques have been developed for genetic studies with somatic cell hybrids,<sup>1, 2</sup> progress in this area has been severely inhibited by the limited numbers and kinds of mutants available. Drug resistant lines<sup>3, 4</sup> and a few auxotrophs<sup>5-7</sup> have been reported, and the only other major source of mutants is through the establishment of cell lines derived from the somatic tissues of patients with known genetically determined defects.<sup>8</sup> If techniques were developed for the isolation of conditional lethal mutants of somatic mammalian cells, it should be possible to obtain a much broader spectrum of mutations. In this paper we describe the isolation of temperature-sensitive mutants of mouse L-cells.

**Materials and Methods.** Mouse L-60T cells<sup>9</sup> were grown in suspension or as monolayer cultures using medium CMRL 1066<sup>10</sup> lacking nucleosides and coenzymes, and supplemented with 10% fetal bovine serum. For most of the experiments detailed here, the permissive temperature was 34°C and the nonpermissive temperature 38.5°C, although temperatures of 33° and 39°C were used in certain early experiments. Temperature regulation in water baths containing suspension cultures was within  $\pm 0.1^\circ$  and in incubators  $\pm 0.2^\circ$ . At 34° and 38.5°C the wild-type population had doubling times of about 18 and 16 hr, respectively.

Incorporation of radioactive precursors into acid-insoluble material was measured for a 30-min pulse in growth medium and the final concentration of these precursors during the pulse was always 1.6  $\mu$ Ci/ml. All radioactive material was obtained from Amersham-Searle, and the specific activities of the stock solutions of radioactive precursors were: tritiated thymidine (<sup>3</sup>H-TdR), 21.9 Ci/mM; tritiated uridine (<sup>3</sup>H-Ur), 15.3 Ci/mM; and tritiated lysine, 11.6 Ci/mM. Label incorporated into acid-precipitable material was measured using methods previously described.<sup>11</sup> The drug 1- $\beta$ -D-arabinofuranosylcytosine (ara-C) was obtained from Sigma Chemical Co., and the mutagen *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine (MNNG) was supplied by Mann Research Laboratories.

Cell volume measurements were made on samples removed from suspension cultures at various times, diluted extensively in cold phosphate-buffered saline<sup>12</sup> and passed as single cells through a Coulter-type aperture attached to a pulse height analysis system.<sup>13</sup>

**Results.** (1) **Isolation of temperature-sensitive mutants:** The selection procedure that yielded the initial set of temperature-sensitive clones of L-60T cells is shown in Figure 1a. An exponentially growing spinner culture at 33°C was treated with the mutagen MNNG (0.5 µg/ml) for 3 hr, which reduced the surviving fraction of cells to approximately 0.2. After 3 days, the temperature of the culture was raised to 39°C; 24 hr later <sup>3</sup>H-TdR (21.9 Ci/mM) was added at a concentration of 2 µCi/ml and left in the culture for 2 days. Cells cycling during this interval would be expected to incorporate the <sup>3</sup>H-TdR and be lethally irradiated<sup>14</sup> whereas temperature-sensitive cells would be arrested and insensitive to the killing agent. Upon removal of the <sup>3</sup>H-TdR, the cells were placed in bottles at 33°C to allow multiplication of the survivors. After about 2 weeks enough cells had accumulated to start a suspension culture. The <sup>3</sup>H-TdR selec-

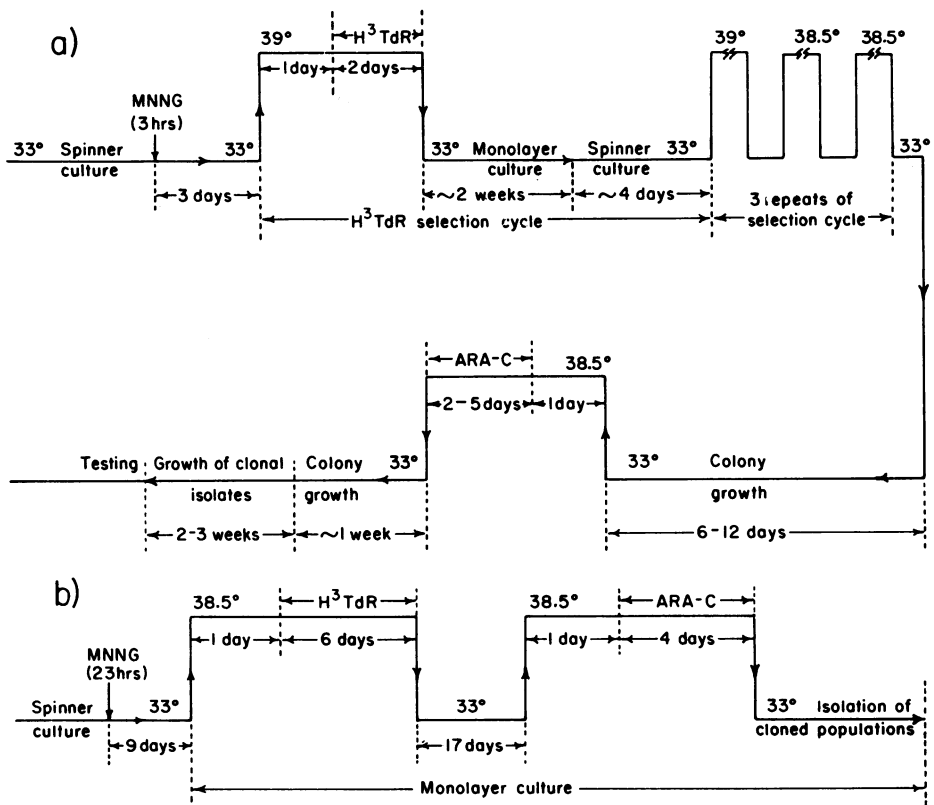


FIG. 1.—Schematic diagrams of the selection procedures used to obtain temperature-sensitive mutants. (a) A selection which consisted of four selection cycles with <sup>3</sup>H-TdR followed by one selection with ara-C. After the second cycle with <sup>3</sup>H-TdR the nonpermissive temperature was lowered from 39° to 38.5°C, which was used as the nonpermissive temperature in all later experiments. (b) A selection which involved a single treatment with <sup>3</sup>H-TdR followed by a treatment with ara-C.

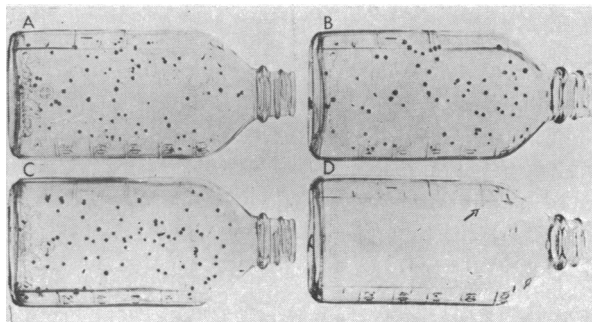
tion cycle was then repeated to enrich further for temperature-sensitive cells. Following a total of four selections with  $^3\text{H-TdR}$ , single cells were plated and allowed to form small colonies. A final selection was made by treating these small colonies at  $38.5^\circ$  with ara-C at a concentration of  $40\ \mu\text{M}$ , to eliminate any clones<sup>15</sup> which were not temperature sensitive but simply resistant to  $^3\text{H-TdR}$ . After surviving colonies had grown for about 10 days, several were isolated and grown into mass cultures. The testing of each clonal isolation for conditional temperature sensitivity was then performed.

A simplified version of the above selection scheme also produced one temperature-sensitive clone. As shown in Figure 1b, a two-step selection was performed with monolayer cultures using a single  $^3\text{H-TdR}$  treatment followed by an ara-C treatment. Nine days after a spinner culture had been treated with  $0.5\ \mu\text{g/ml}$  of MNNG for 23 hr (surviving fraction approximately 0.1), two 32-oz bottles were each inoculated with  $2 \times 10^6$  cells and incubated at  $38.5^\circ\text{C}$ . After 24 hr,  $^3\text{H-TdR}$  ( $1\ \mu\text{Ci/ml}$ ) was added for 6 days. The cultures were then given fresh medium lacking  $^3\text{H-TdR}$  and incubated at  $33^\circ\text{C}$  until several macroscopic colonies had appeared. These colonies were shifted to  $38.5^\circ\text{C}$  and a day later were treated for 4 days with ara-C as in the original selection procedure. Those colonies which survived were allowed to grow before being picked and tested.

A total of eight clones were isolated by using the first selection method, seven of which *tsA1-tsA7*, showed obvious temperature-sensitive properties in the tests described below. The remaining clone exhibited questionable temperature sensitivity in preliminary tests and has not been examined further. From the second selection procedure three clones were isolated, at least one of which, *tsB1*, showed pronounced temperature-sensitive properties. Most of the data to be described has been obtained with a single clone, *tsA1*, isolated by the first procedure, but similar data have been obtained for the other clones and will be referred to briefly.

(2) **Temperature sensitivity of clonal isolate *tsA1* with respect to colony-forming ability:** Clones from the above selection experiments were grown into mass cultures and tested for temperature sensitivity by plating and by growth in suspension culture. Figure 2 illustrates the results of a plating experiment

FIG. 2.—Temperature sensitivity of clonal isolate *tsA1* as shown by plating single cells. (A) Wild-type cells incubated at  $34^\circ\text{C}$  for 16 days; 100 cells inoculated; plating efficiency (P.E.) = 80%. (B) Wild type incubated at  $38.5^\circ\text{C}$  for 14 days; 100 cells inoculated; P.E. = 67%. (C) *tsA1* cells incubated at  $34^\circ\text{C}$  for 16 days; 100 cells inoculated; P.E. = 72%. (D) *tsA1* incubated at  $38.5^\circ\text{C}$  for 14 days;  $10^5$  cells inoculated; P.E.  $\approx 10^{-8}$ . The arrow designates the only macroscopic colony.



in which clone *tsA1* was compared with the parental wild-type L-cells. At the permissive temperature (34°C), both cell types grew well. The wild-type cells (Fig. 2a) had a plating efficiency of 80%, while for *tsA1* cells (Fig. 2c) the value was 72%. There was no appreciable difference in colony size. In contrast, at the nonpermissive temperature (38.5°C) the wild-type cells grew while the *tsA1* cells did not. The wild-type cells (Fig. 2b) had a plating efficiency of 67%, whereas there were no survivors when as many as  $10^4$  *tsA1* cells were inoculated. Bottles inoculated with  $10^6$  *tsA1* cells (Fig. 2d) showed a background of giant-like cells with an occasional small colony. These results demonstrate that *tsA1* had the properties of a temperature-sensitive mutant.

(3) **Temperature sensitivity of clone *tsA1* as measured by growth in suspension culture.** Tests for temperature-dependent growth of *tsA1* cells in suspension cultures yielded results consistent with the plating experiments, as shown in Figure 3. The control culture of wild-type cells used in this experiment had

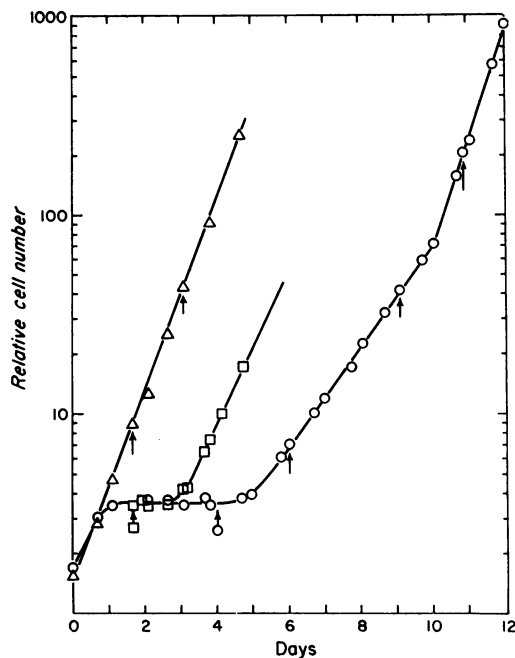


FIG. 3.—Growth curves of wild-type and *tsA1* cells in spinner-flask suspension culture. At time zero, when both the wild type ( $\Delta$ ) and *tsA1* ( $\circ$ ) were growing exponentially at 34°C, the temperature was raised to 38.5°C. At 40 hr the *tsA1* culture was diluted and split into two cultures. One of the new cultures was immediately placed at 34°C; the other was kept at 38.5°C until day 4, when it was returned to 34°C. The arrows indicate when dilutions were made.

been kept at the permissive temperature since the time the selection experiments were begun, a period of about 4 months. Raising the temperature from 34° to 38.5°C produced a striking difference in growth between the wild-type and *tsA1* cultures. The cells in the control culture continued to increase in number with a 16-hr doubling time until the culture was discarded. In contrast, the number of *tsA1* cells increased approximately twofold during the first 24 hr after the temperature shift to 38.5°C, and then showed no further increase while held at 38.5°C. After 40 hr the culture of *tsA1* was split into two cultures, one of which was immediately cooled to 34°C where it resumed growth after 1 day (Fig. 3), rapidly achieving a doubling time comparable to that of the wild-type cells at

34°C. The other culture of *tsA1* cells, which remained at 38.5°C for a total of 96 hr, showed a longer-lasting effect of maintenance at the nonpermissive temperature. Although cell division began again within 24 hr, the cells grew more slowly, with a doubling time of about 28 hr, for the next 5 days. Thereafter, a return to a normal doubling time was observed. These results show that *tsA1* cells are temperature sensitive with respect to growth in suspension culture. They also indicate that the kinetics of growth of *tsA1* cells returned to the permissive temperature are dependent on the time interval spent at the nonpermissive temperature.

**(4) Growth of wild-type and mutant *tsA1* cells as a function of temperature :**

A further series of growth curves was constructed to test for the effect of different temperatures on the growth of wild-type and *tsA1* cells. All cultures were derived from cells maintained initially at 34°C. After the growth rate at a new temperature had stabilized, a doubling time for the population was obtained from the exponential region of each growth curve if such an exponential portion existed. These values of doubling time are plotted in Figure 4. The doubling time of *tsA1* cells increased abruptly at temperatures above 37°C while a sizable increase in the doubling time of wild-type cells did not become apparent until 40°C.

Stable growth rates were not obtained in the temperature regions indicated by the dashed lines in Figure 4. However, pronounced differences were observed between the shapes of the curves of cell number versus time for the wild type as compared to the mutant populations. As previously shown in Figure 3, *tsA1* cells raised to 38.5°C approximately doubled in number and then showed no division for several days. At 38.0°C *tsA1* cells appeared to terminate division but over a period of about 12 days and after a 14-fold increase in cell number. In contrast to the gradual termination of growth seen for *tsA1* at temperatures from 37.5° to 38.5°C, wild-type cells exhibited exponential growth curves for temperatures up to 40.0°C while at 40.5°C no growth whatever was detectable. From these results it seems most unlikely that the cessation of growth of *tsA1* cells at temperatures just above 37°C is simply the result of an increase in the temperature sensitivity of the same mechanism which prevents the growth of wild-type cells above 40°C.

**(5) Incorporation of radioactive precursors :** The fact that the temperature sensitivity of the *tsA1* cells was apparently not due to a nonspecific loss of metabolic function prompted a preliminary investigation of the rate of uptake of various labeled precursors into acid-insoluble material, during a 30-min pulse,

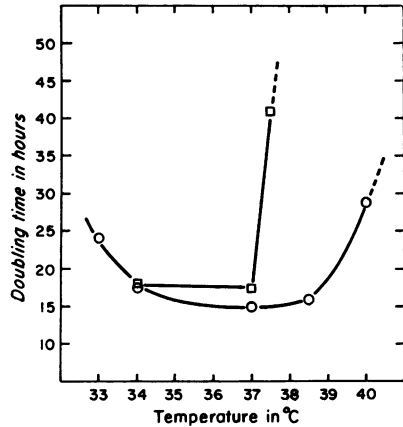


FIG. 4.—Relationship between population doubling time and temperature for wild type (O) and the mutant *tsA1* (□) grown in spinner flasks. Each doubling time value was obtained from a growth curve based on cell counts over periods as long as 14 days.

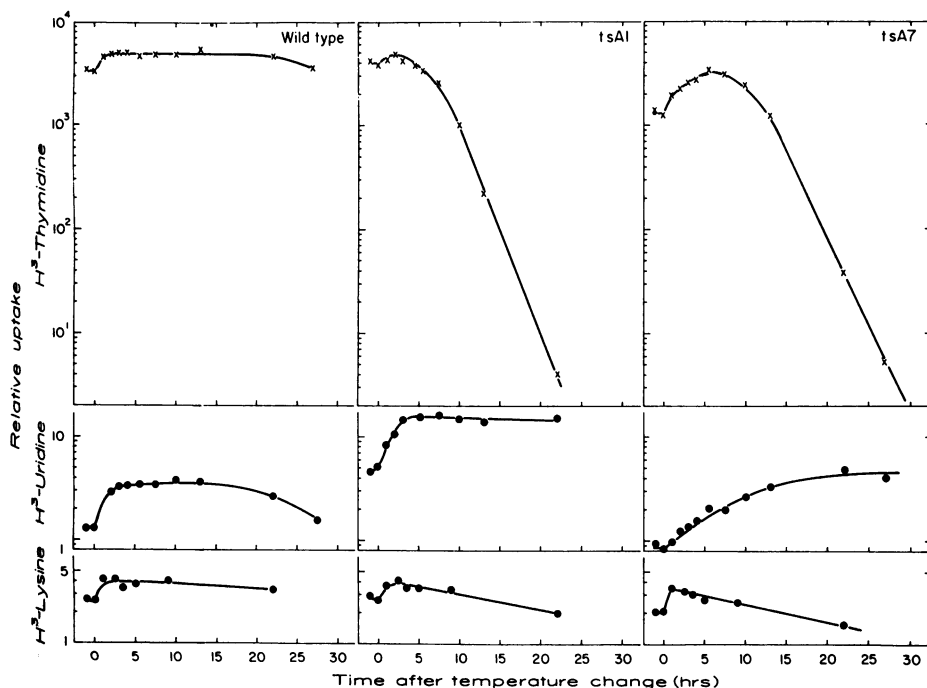


FIG. 5.—The rates of incorporation of  $^3\text{H-TdR}$ ,  $^3\text{H-Ur}$ , and  $^3\text{H-lysine}$  into acid insoluble material in wild-type, *tsA1*, and *tsA7* cells. Incorporation per cell was measured over a 30-min period in suspension cultures both before and after a temperature shift from  $34^\circ$  to  $38.5^\circ\text{C}$ .

as a function of time after raising the temperature to  $38.5^\circ\text{C}$ . Figure 5 shows that for the wild-type population the temperature increase produced immediate increases in the rates of thymidine, uridine, and lysine incorporation and that new equilibrium values appeared to be rapidly established. For *tsA1* cells also the temperature increase resulted in an increased uptake of uridine and lysine, but in an immediate and marked decline in the incorporation of thymidine.

In addition to the experiments with *tsA1*, uptake measurements were made also on all of the other temperature-sensitive clones. The third column of Figure 5 shows data obtained for clone *tsA7*. The main differences between the results obtained with clone *tsA7*, as contrasted to *tsA1*, were the initial increase in the uptake of thymidine which preceded the rapid decline, and the lower rate of increase in the uptake of uridine. These data suggest that the temperature sensitivity of *tsA7*, as well as the other isolates, may result from changes in nucleotide metabolism or nucleic acid synthesis.

(6) **Cell volume changes:** One very noticeable feature of the growth of all of the temperature-sensitive isolates at  $38.5^\circ\text{C}$  was a rapid increase in cell size. Measurements of mean cell volume of *tsA1* over a period of 5 days at  $38.5^\circ\text{C}$  showed a linear increase in average cell size to a value 3.3 times that obtained at  $34^\circ\text{C}$ . Under similar conditions wild-type cells showed no change in average cell volume.

(7) **Other temperature-sensitive clonal isolates:** In addition to *tsA1*, the seven other mutant clones also have been tested for their plating efficiency,

suspension growth at 38.5°C, and rate of return to normal growth following temperature arrest. The plating efficiencies at 38.5°C ranged from a low of about  $10^{-5}$  for *tsA1* to a high of 0.014 for *tsB1*. All of the mutant clones showed approximately a 1.5- to 2-fold total increase in cell number as suspension cultures, and this occurred during the first 24 hr after the temperature shift up. Cultures incubated at 38.5°C for 72 hr and then shifted to 34°C showed very marked differences in their resumption of normal growth rates. These data would appear to suggest that at least several independent mutations have been isolated. However, an alternative explanation is that a single mutant has undergone a series of alterations during the time required for growth to mass populations and for testing. This poses the question as to what extent an individual clone maintains its phenotype upon recloning. The results of a preliminary investigation of the phenotypic stability of *tsA1* are presented in the following section.

(8) **Subclones of mutant *tsA1*:** After initial testing of the *tsA1* cells, 10 subclones of this mutant were isolated and tested for temperature sensitivity by plating, growth in suspension culture, and uptake of radioactive precursors into acid-insoluble material. In all the tests, 9 of the 10 subclones were very similar to the parental *tsA1* population while the remaining clone, *tsA1S2*, exhibited altered properties. With inocula of  $10^4$  cells the parental population and nine of the subclones produced no colonies at 38.5°C. With inocula of  $10^5$  cells all of the subclones produced a variable background of giant cells containing the occasional cell cluster or "pseudocolony" composed of large cells. At this concentration, however, *tsA1S2* produced noticeably more growth than the other subclones, implying that many of the cells had undergone several divisions although no colonies were actually detected. In suspension culture at 38.5°C, only *tsA1S2* failed to yield arrest and recovery curves similar to those depicted in Figure 3. It showed instead an asymptotic type of growth curve with a 50-fold increase in cell number before multiplication ceased after approximately 14 days. In uptake experiments, only subclone *tsA1S2* was different from the parental *tsA1* line in that the decline of thymidine uptake was much less pronounced.

**Discussion.** The isolation of eight temperature-sensitive clones in two separate selection experiments shows that it is feasible to isolate such mutants from a heteroploid line of mammalian cells. There was a possibility that the procedures simply selected for mutants pre-existing in the populations. However, very recently we have obtained conditional mutants from populations cloned at 38.5°C to select against such pre-existing mutants.

The most extensive analysis of phenotype has been made with clone *tsA1*, but further experiments will be needed to delineate its defective function. In this mutant, as well as the others, cell division at 38.5°C appears to continue for about one generation before arrest. This suggests that a gene product must decay before the phenotype is expressed. It is also of interest that data not presented here suggest that populations maintained at the nonpermissive temperature for as long as 11 days still contain an appreciable fraction of viable cells.

Analysis of the phenotypes of the various mutants indicated noticeable differences among them. At first sight this would suggest that we have isolated

lines containing independent mutations. However, such a conclusion may be premature in view of the result that one of the 10 subclones of *tsA1* appeared to differ significantly from the parental line.

The exact chromosomal nature of the conditional lethal mutations which we have isolated cannot as yet be specified. There is, of course, no method of genetic analysis available which would allow one to state whether or not these are point mutations. The problem in this particular case is compounded by the fact that L-cell lines are highly heteroploid and are known to undergo extensive karyotypic fluctuations. Changes in phenotype could occur due to chromosomal rearrangements or to loss of specific chromosomes. It seems unlikely that the observed temperature sensitivity is due to chromosomal deletion, since preliminary results indicate that partial revertants can be isolated from temperature sensitive populations.

The availability of these conditional lethal mutants of L-cells and those reported recently by Naha<sup>16</sup> for a heteroploid line of African green monkey kidney cells should accelerate developments in somatic cell genetics. However, it will also be important to show whether or not the methods can be applied to cells with diploid karyotypes and to cells capable of differentiation. In this regard, it is of interest that we have recently been able to isolate a temperature-sensitive mutant of the Chinese hamster line V79-S171,<sup>17</sup> which appears to have a chromosome complement which is near diploid.

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† Requests for reprints may be sent to Dr. Thompson, The Ontario Cancer Institute, 500 Sherbourne Street, Toronto 284, Ontario, Canada.

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