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TRITIATED URACIL, TRITIATED THYMIDINE, AND BROMODEOXYURIDINE INDUCED MUTATIONS IN EUCARYOTIC CELLS

H. John Burki, E. Moustacchi, and J. E. Cleaver

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H. John Burki,[1] E. Moustacchi,[2] and J.E. Cleaver[3]

1. Group in Structural Biophysics
Lawrence Berkeley Laboratory
University of California, Berkeley
Berkeley, California 94720

2. Institute Du Radium, Biologie
Building 110
Orsay 91405, France

3. Laboratory of Radiobiology
University of California, San Francisco
San Francisco, California 94143

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ABSTRACT

The induction of gene conversion at the ARG-4 locus in strain BZ34 of *Saccharomyces cerevisiae* was examined after the cells incorporated 6-^{3}H uracil under optimum growth conditions for 16 hours, and then received damage at 4°C from tritium decays at very low dose rates of 1.4-27.6 tritium decays per hour. The results were compared to the results of gene conversion induced by ^{60}Co. Each decay of tritium under these conditions was equivalent to a dose of 3.67 rads. This value is very similar to the estimated value of 2.6 rads expected when uniform distribution of dose in the cells is taken into consideration. These results are contrasted with results from Acute $^{3}H_2O$ experiments that suggest a relative biological effectiveness (RBE) of 2.8.

The induction of resistance to 6TG in Chinese hamster ovary (CHO) cells has been studied after incorporation of ^{3}H-methyl thymidine, 6-^{3}H-thymidine, and bromodeoxyuridine under several experimental conditions. The induction of mutations by incorporated 6-^{3}H-thymidine is about three times as effective as the induction of mutations by tritiated-methyl thymidine. Since these results are obtained for cells frozen in the G_1 stage of the cell cycle, it may be influenced by the loss of indirect effects of tritium radiation and by life cycle effects since G_1 is a sensitive time for mutations induced by ionizing radiation. The induction of mutations by BUdR depends on the portion of the DNA that is replicated during exposure to BUdR: early replicating DNA damage is associated with induction of 6TG resistance.

These results suggest that the determination of the RBE for tritium decays in model eucaryotic systems like yeast and cultured Chinese hamster cells will be influenced by the precise experimental conditions employed. In particular, experiments with mammalian cells will be affected by "hot times" for mutagenesis in the cell cycle and "hot positions" within the DNA in the nucleus, and also by the position of tritium decay within the DNA-incorporated molecule.

1. INTRODUCTION

The inventory of tritium produced naturally in the environment is about 1.1×10^8 Ci [1,2]. As a result of atomic weapons testing and the estimated 15-45 kCi/(reactor·GW yr) from fission reactors and other sources, the current world inventory of tritium is 25 to 30 times the natural amount [3,4]. Proposed fusion reactors might release 10^5 times as much tritium to the environment as current fission reactors [5]. Therefore, it is important to ascertain the relative biological effectiveness (RBE) of tritium decays in eucaryotic cells for induced genetic damage.

At this time some disagreement exists on what the quality factor for tritium decay should be, in terms of genetic effects. The data of Dobson and Kwan [6,7] suggest an RBE of almost 2.8 while other studies with similar *in vivo* systems suggest an RBE closer to 1 [8]. The recent studies of Russell [9] on tritium-exposed spermatogonia suggest an RBE of 2.2 for the seven loci test in mice. We undertook studies with yeast and cultured mammalian cells, hoping that these comparatively simple systems for assaying genetic damage would give greater insight to more complicated systems, and yield independent estimates of the tritium RBE. The data summarized here point out some of the difficulties of tritium experiments done with yeast and mammalian cells.

2. MATERIALS AND METHODS

2.1 Diploid Yeast Studies

Strain BZ34, which is heteroallelic at the ARG-4 locus, was used in these studies. Genotype and normal growth conditions are as reported previously [10]. Yeast cells were grown in liquid YEPD medium [10] containing 1% yeast extract, 1% peptone, and 2% dextrose, at 30°C with constant shaking. From 1-50 μCi/ml of 6-^{3}H uracil at specific activity of 20 μCi/mmol (C.E.A., Saclay, France) were added to an exponential phase culture (about 10^6 cells/ml). After 16 hours of growth (6-8 generations, 20-30% budding cells), control and labeled cultures were washed three times with normal saline, resuspended in saline at 5×10^7 cells/ml, and stored at 4°C. The initial number of cells and the frequency of convertants was determined [11]. The plating procedure was repeated at various times after the start of the experiment for periods of up to 3 months. No loss of yeast viability is observed in control cultures not exposed to tritiated uracil during this time period at 4°C. Colonies were scored after 6 days of growth. Aliquots were used to determine the radioactivity per cell, using an NE260

scintillation mixture (Nuclear Enterprise, Scotland). The counting efficiency was 40% as determined, using the channels/ratio method for quenching corrections. The tritium dose rate was 1.4, 5.1, 6.6, and 27.6 d/h in the diploid experiments. Nonlabeled cells prepared in the same manner were exposed to ^{60}Co (dose rate was 3 krad/min,as determined by ferrous sulfate dosimetry).

2.2 Mammalian Cell Studies

2.2.1 Asynchronous populations

Tritium labeling and freezing conditions: Chinese hamster ovary (CHO) cells were grown as described previously [12]. The cultures were then labeled with 5 methyl-^{3}H-thymidine or 6-^{3}H-thymidine in a way that would produce a uniform distribution of ^{3}H in both strands of DNA throughout the hamster genome. The cultures were labeled for about five generations (60 h) with a large volume (150 ml) of medium containing ^{3}H-methyl- or 6-^{3}H-thymidine at a high concentration and low specificity (0.3 μCi/ml, 30 μmol, 0.01 Ci/mmol), and 1 μmol deoxycytidine. Cells were then rinsed with a balanced salt solution (Saline A,GIBCO), trypsinized and resuspended in growth medium containing 10% dimethyl sulfoxide. About 5×10^{6} cells per milliliter in 10-ml ampules were frozen at 1°C/min and stored in a Revco freezer at -79°C. Unlabeled cells were frozen at the same time. At the time of freezing, cultures were confluent, and both labeled and unlabeled frozen cultures consisted of more than 90% G_1 cells, as determined by flow microfluorimetry.

Specific activity determinations: ^{3}H activity was determined by fixing known numbers of cells with 4% perchloric acid at 4°C, rinsing them in 4% perchloric acid at 4°C, and then digesting them in 10% perchloric acid at 90°C for 30 min. The ^{3}H activity soluble in hot perchloric acid was measured with a water-miscible scintillation mixture (Aquasol), and counting efficiencies were determined with ^{3}H-toluene internal quench standards. The activity was calculated in decays per cell per day. The total number of decays per cell was varied by thawing cells at 2 to 3 week intervals for up to 6 months from the time of freezing.

Irradiation conditions: X-ray doses of 300-1000 rads were delivered to some frozen ampules of unlabeled cells at dry ice temperatures from a G.E. Maxitron X-ray machine (300 kVp) without added filtration. The dose rate of 250 rads/min was checked with lithium fluoride thermoluminescent dosimeters in the same exposure geometry at room temperature.

Mutation frequency determinations: To determine the frequency of mutations among surviving cells induced by ^{3}H decays or X-rays, ampules were thawed rapidly to 37°C and cells were resuspended in 100-150 ml of growth medium containing 10 μmol thymidine and 4 μmol deoxycytidine and allowed to grow for 7 days for mutation expression. At the end of 7 days, cultures were trypsinized and cells were resuspended and inoculated at a range of densities in normal medium and in medium containing 0.06 mmol 6-thioguanine. Colonies were fixed 7 days later and the plating efficiencies were calculated. Mutation frequencies were calculated from the ratio of the plating efficiency in 6-thioguanine to that in normal medium.

2.2.2 Synchronous populations

Cell culture and synchronization methods: Details of the growth of CHO-kk cells are described in detail elsewhere [12].

For synchronous experiments the cells were grown for several days in a logarithmic growth phase, in roller bottles turned at 0.5 rev/min. They were subcultured into one or more bottles at 2 to 3 × 10^{7} cells per bottle. After 48 h, the cells were synchronized in a 37°C room using a "Cell Cycle Analyzer" (Talandic Research Corporation, Pasadena, California). After synchrony and attachment to 75 cm^{2} tissue-culture flasks, the cells were labeled with 1 mmol BUdR + 1 μCi/ml ^{3}H-BUdR for 1 h. After labeling, the cells were washed and grown in 10^{-5}M TdR + 10^{-6}M CdR to "chase" the BUdR during the first part of the "expression" period. The rest of the cells were grown for mutagenesis.

2.3 Cell Life Cycle Progress Analysis After Mitotic Detachment

(a) Cell volume spectroscopy: The model size and volume distribution of the trypsinized synchronous cells at different positions in the cell cycle were determined, using a Coulter Counter Model ZBI matched to a Coulter Channelyzer.

(b) Flow cytometry (flow microfluorimetry): Cells were collected by gentle centrifugation at different times after mitotic detachment and fixed at 0°C, using a fixative containing three parts 200 mmol $MgCl_2$ + one part absolute alcohol, and then kept at 4°C. They were then stained with chromomycin A3 (100 μgm/ml,Cal Biochemical), for at least 1 hour. Analysis of the fluorescence was made, using an instrument as described previously [12].

(c) Uptake of tritiated BUdR: Usually 10^{6} cells were washed three times with cold 10% TCA, and then kept at 90°C for 1 hour to hydrolyze the cell DNA for a determination of the

amount of precursor incorporated into DNA at different times. The radioactive mixture was added to PCS solubilizer (Amersham and Searle Co.) for liquid scintillation counting. The absolute counting efficiency for the samples was determined by using tritium quench standards and the channels ratio method.

2.4 X Radiation In Cycle

After synchronization, the cells were washed two times with Pucks Saline A (PSA) and then irradiated with 50 kVp X-rays. The beam was filtered with 2.5 mg/cm^2 of aluminum and the beam half-value thickness was 8 mm of H_2O. The dose rate was 300 ± 12 rads/min. After irradiation the cells were trypsinized and plating efficiency was determined. Most of the cells were grown for 8 days before they were "challenged" with 6-thioguanine media.

2.5 Selection For Drug Resistance

Clones resistant to 5 μgm/ml of 6TG were selected after an expression time of 8 days had passed. After 8 days in 6TG media the number of clones was determined by counting the number of colonies stained with 1% methylene blue. Experiments were designed to insure that at least 100 clones above background were obtained after treatment at peak times for BUdR mutagenesis, to insure 10% statistical reliability at the peak time of mutagenesis.

It is important to note that in the usual experiment the cells were kept in a log growth stage for the entire "expression period". The usual procedure was initially to use an aliquot of about 2×10^6 cells, permit growth in a roller bottle for 2 days and subculture to 2×10^6 cells, and then subculture again after 3 more days. Growth curves of cells after these treatments showed that 7-16 population doubling times occurred under the experimental conditions.

3. RESULTS AND DISCUSSION

3.1 Yeast Cell Systems

The survival curves after exposure to incorporated 6-^{3}H uracil obtained after four experiments at four different dose rates showed no dose rate effect for damage accumulated at 4°C [10]. The shapes of the diploid survival curves as well as a haploid derived from them are similar to that found for X or gamma radiation. The effect of 1 rad of external radiation is equivalent to that obtained with 0.4-1 decay per cell of tritium. A calculation of the physical dose per tritium decay, ignoring edge effects and vacuoles, gives 2.6 rad/decay for a diploid

cell with an average radius of 2 micron. (In other unpublished reports, Burki and Moustacchi found that the killing efficiency for yeast depended on the relative amount of the tritium that decayed in the DNA of the cells.)

The induction of gene conversion at the ARG-4 locus by gamma rays has the following characteristics: a rapid linear increase in the low dose range (where no killing is observed) and a less rapid increase with higher doses (Fig. 1A, 1B). Twelve convertants/(10^5 viable cells·krad) were found for the initial portion of the curve (Fig. 1A). The portion of the convertants, due to radiation-induced mutation at the same locus, results in less than 0.2 revertants/(10^5 viable cells·krad). For tritium-induced gene conversion, one finds the same biphasic response with an initial slope giving 44 convertants/(10^5 viable cells·10^3 disintegrations)(Fig. 1B). The "rad equivalence" for tritium decay is then 3.67 rad/decay. Thus the RBE for gene conversion, using these assumptions, is 3.67/2.6 = 1.4.

Figure 1A also shows the results of Ito and Kobayashi [13] for gene conversion in another strain of yeast at another locus, the TRP-5 locus. This locus is apparently about one half as sensitive as the ARG-4 locus above, with 64 convertants/(10^5 viable cells·krad). The reason for the reduced sensitivity is related to the different loci examined and to the fact that the cells of Ito and Kobayashi were not in log stage but in stationary phase during irradiation.

Figure 1C shows data for induced gene conversion after exposure to tritiated water at two specific activities. A dose rate effect is apparent even though a dose rate effect is not seen after gamma radiation [13,14].

Using several assumptions in their experiments, Ito and Kobayashi [13] calculate the dose to the cells from the tritiated water. They assume that the water content of yeast is 70% throughout; that the intracellular water is quickly equilibrated with added ^3H_2O; and that the dimension of the yeast cell is large compared to the average range of the tritium beta particle. For the same dose rate of ^{60}Co (0.3 krads/h), one can calculate from the data an RBE of 2.8.

Both of these sets of yeast experiments involve some uncertainty in their tritium dose assumptions. In the case of the experiment of Burki and Moustacchi, tritiated uracil may not mimic the overall labelling pattern of tritiated water. However, Ito and Kobayashi's experiment may also not mimic the chronic disposition of water in the cell. These experiments suggest

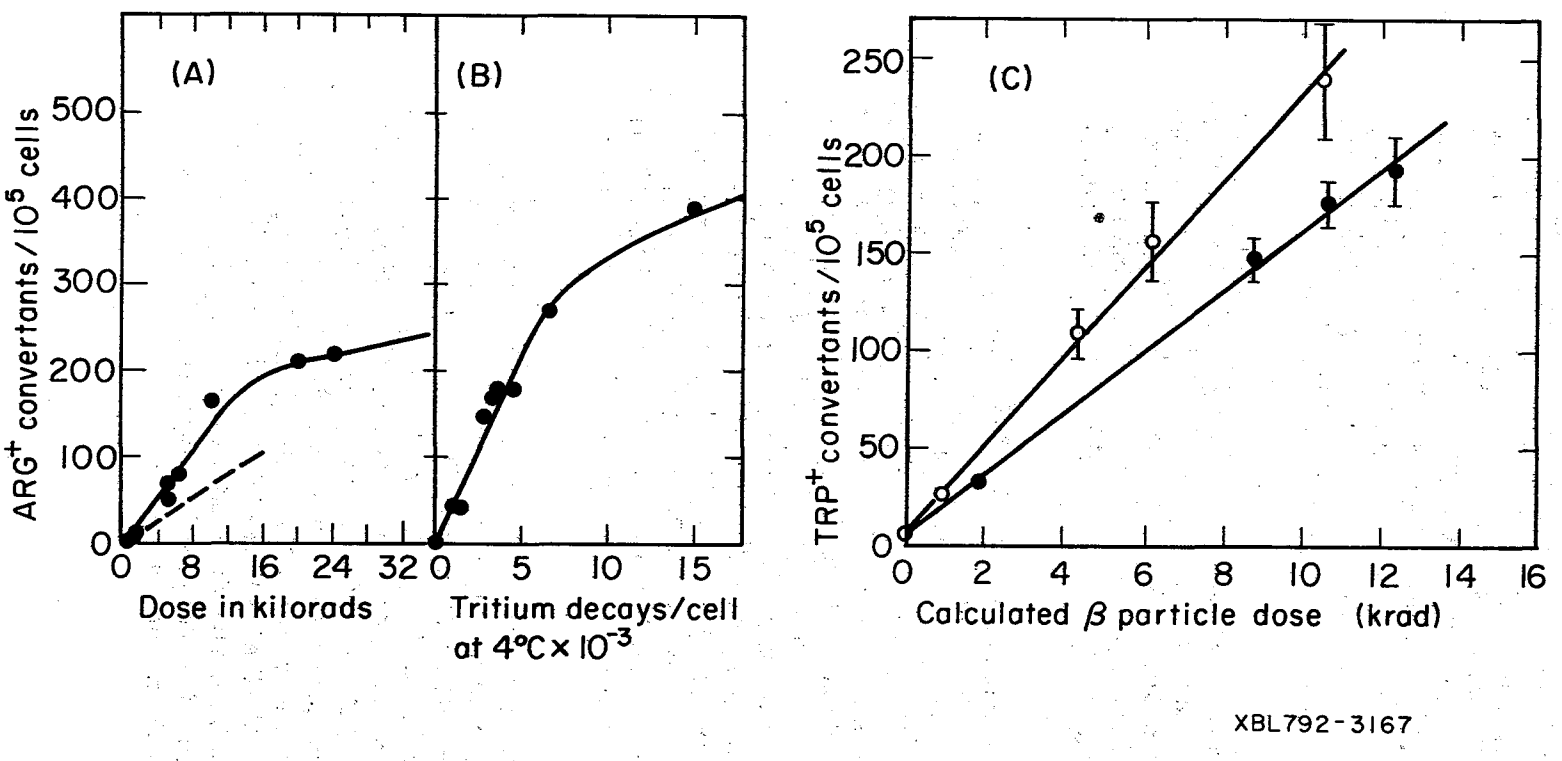

Fig. 1 A) Gene conversion induced by gamma rays at the ARG-4 locus. The solid circles (●) are data points from Burki and Moustacchi [10]. The dotted line is gene conversion induced by gamma rays at the TRP-5 locus from the data of Ito and Kobayashi [13].

B) Gene conversion induced by ^{3}H-uracil decay in cells at 4°C at the ARG-4 locus.

C) Tritiated water-induced gene conversion at the TRP-5 locus. Open circles (o) = 31 mCi/ml, and solid circles (●) = 62 mCi/ml. (Calculated beta particle dose rate is (o) = 0.3 krad/h, and (●) = 0.5 krad/h.) Modified from Ito and Kobayashi [13].

that even in simple yeast systems much remains to be done to define the RBE for tritium-induced genetic damage.

3.2 Mammalian Cell Experiments

Although the results for 6-^{3}H-thymidine suggest a small threshold (Fig. 2), the production of clones resistant to 6TG in cells frozen in the G_1 stage of the cell cycle appears to be a linear function of the dose of X-rays or the dose of accumulated tritium decays. These results show a position effect for tritium decay as has been seen in procaryotic cells [15-19]. It is interesting to point out that a "local effect" for tritium-induced strand breaks of the DNA does not occur in the same experiments [20].

The induction of mutations by X-ray exposure in the frozen stage is about one third as effective as results found at normal temperature with asynchronous cells; however, the latter curve appears to be curvilinear (Fig. 2B). These results suggest different mechanisms for direct and indirect effects in inducting 6TG resistance.

Ignoring the small threshold on the effect of 6-^{3}H-thymidine, the slopes of the curves enable us to calculate an RBE for the two types of tritium label with respect to X-rays. Assuming that theoretical calculations, made on the distribution of ^{3}H decays within spheres of the size of mammalian nuclei, can estimate correctly the beta dose from tritium decays to be 0.2-0.4 rad/decay [21,22], then the values determined for the 5 methyl-thymidine for mutagenesis of 0.11-0.28 [20] suggest an RBE of about 0.67. The average value of the ratio of 6-^{3}H-thymidine/5-^{3}H-methyl-thymidine is 2.95 ± 1.05, suggesting an RBE value of 2 ± 0.7. However, even though position effects are important, one must remember that this "position effect" may occur for only a few sites in the DNA as in the case of bacteria [16,18].

Besides the position effects of tritium there are two other difficulties in the efforts to determine the RBE for tritium damage in mammalian cell systems: the position of the cells in the cell cycle, and the position of the tritium within the DNA. The first problem is seen when the induction of 6TG resistance in CHO cells is measured as a function of cell cycle time after cell exposure to 450 rads of X-rays (Fig. 3) [23]. Late G_1 cells are three times more sensitive than cells at other times in the cell cycle. Thus, this factor must now be taken into account in the determination of RBE, since asynchronous populations will reflect a composite of the "age response" for mutagenesis in the cycle.

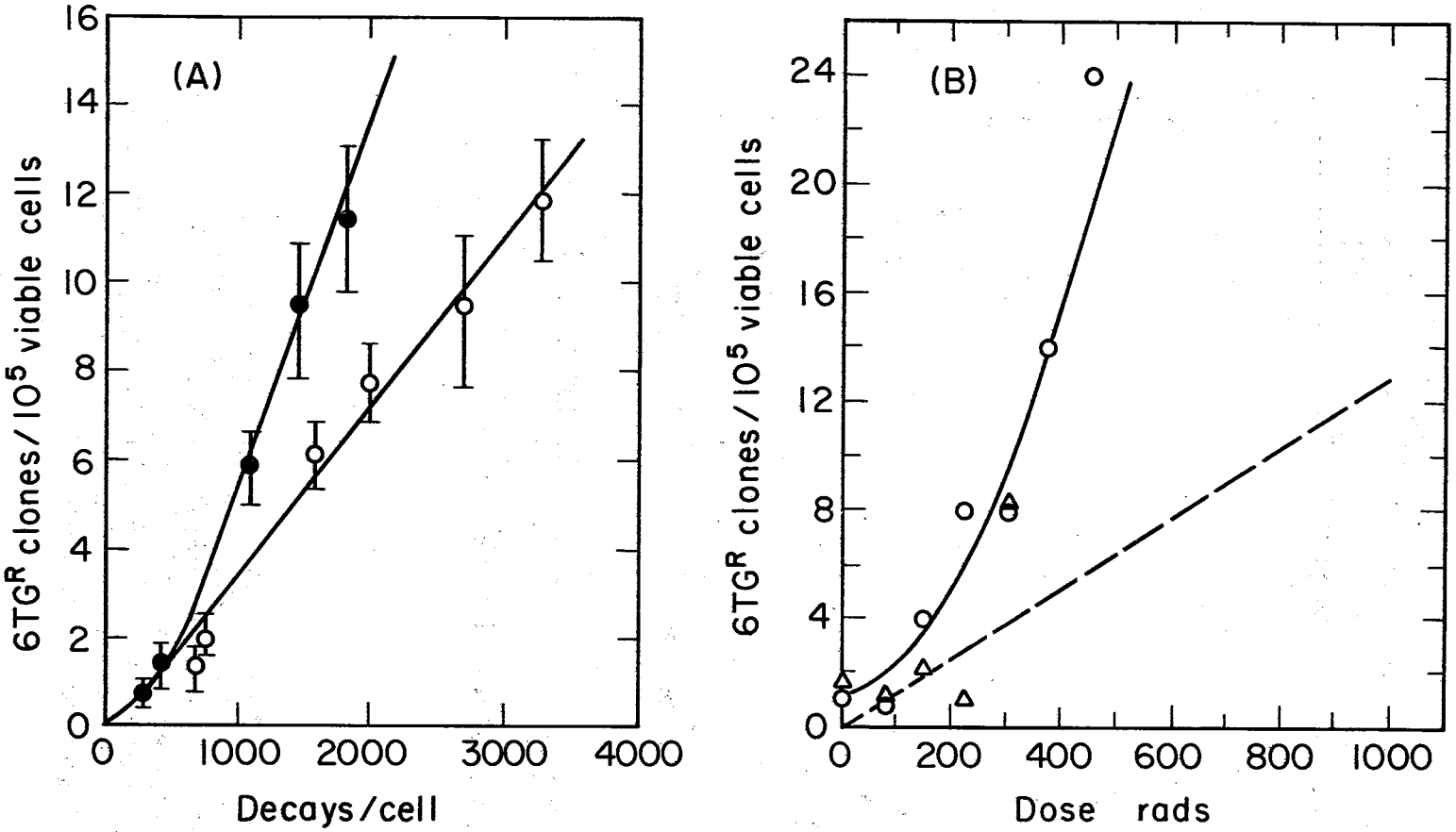

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Fig. 2 A) The frequency of 6-thioguanine resistant clones per 10^5 viable cells is given as a function of the number of ^3H decays in frozen cells at -79°C in the G_1 stage of the cell cycle. Solid circles (●) = 6-^{3}H-TdR, open circles (o) = 5-methyl ^{3}H-TdR (x) controls. (Modified from Cleaver [20].)

B) Circles and triangles (o, Δ) indicate the frequency of 6-thioguanine resistant clones per 10^5 viable cells as a function of the dose of 50 kVp X-rays at room temperature in asynchronous log growth cells [23], and dashed line (---) indicates the stationary phase G_1 cells in the frozen state at -79°C, irradiated with 300 kVp X-rays. (Modified from Cleaver [20].)

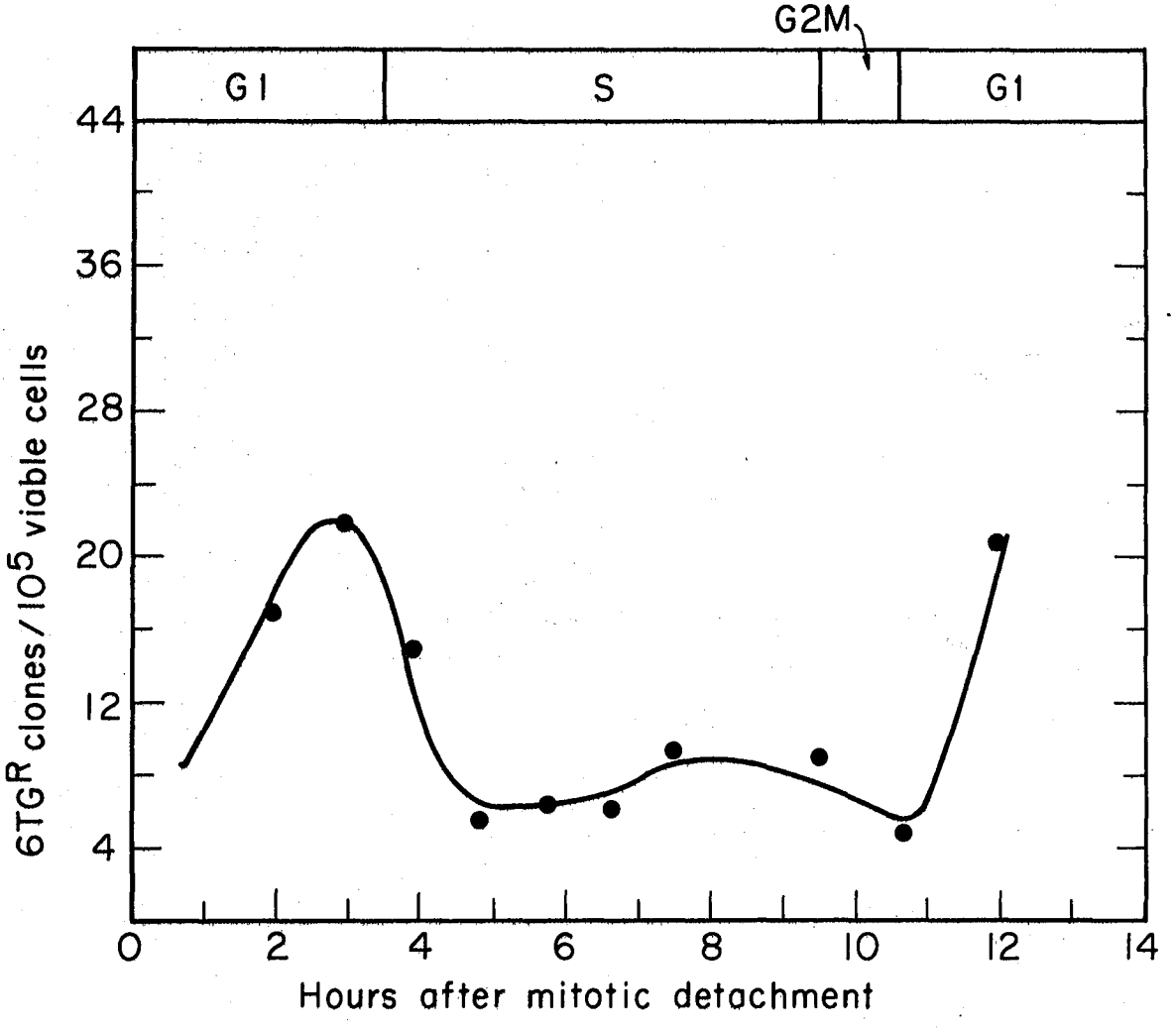

Fig. 3 Cells are synchronized as described in the text section titled "Materials and Methods." They are then exposed to 450 rads of filtered 50 kVp X-rays at 300 rads/min at different hours of the cell cycle in Pucks Saline A at room temperature. The surviving cells are grown for 8 days before growth in 5 µgm/ml 6-thioguanine to measure the number of drug resistant clones.

The second difficulty is related to the position of the gene associated with the genetic locus being tested. The position of the gene(s) related to the induction of 6TG resistance is in the early replicating DNA, as has been suggested by Burki and Aebersold [12] from BUdR pulse mutagenesis studies. It has been known for several years that there are "hot spots" for genetic damage in DNA. For example, the induction of 6TG resistance after a one-hour pulse of BUdR (1 mmol) and a chase with TdR and CdR is given in Fig. 4. Some of the "hot spots" that have been reported are summarized in Table I. Although these "hot spots" are very interesting and are the subject of much of our recent work, they complicate the design of tritium experiments and suggest that the distribution of the tritium within the nucleus may be important.

4. CONCLUSIONS

The induction of gene conversion in diploid yeast was determined after exposure to ^{60}Co radiation and after exposure to tritiated uracil for 16 hours. The results permit calculation of an RBE of less than one for tritium gene conversion. In contrast to the results of gene conversion induced by tritiated uracil are the studies of tritiated water, which suggest an RBE of 2.8. A number of factors will influence the results, including dose rate, localization of tritium within the cells, genetic locus used, and the state of the cell population. Further studies are required to determine the RBE of tritium decay for genetic recombination in yeast.

In cultured mammalian cells there are several complicating factors that will influence the determination of the RBE for tritium. They include the position of the tritium on the molecule used to label DNA, i.e., "position effect," since 6-^{3}H-thymidine is more effective than ^{3}H-methyl thymidine; the stage of the cell cycle when the damage occurs, since there are "hot times" for mutagenesis in the cell cycle; and the region of the DNA which is damaged, because there are "hot positions" within the nuclear DNA for different genetic endpoints. Care must be taken in future experimental design to insure that the standard radiation exposure precisely duplicates the conditions of tritium exposure.

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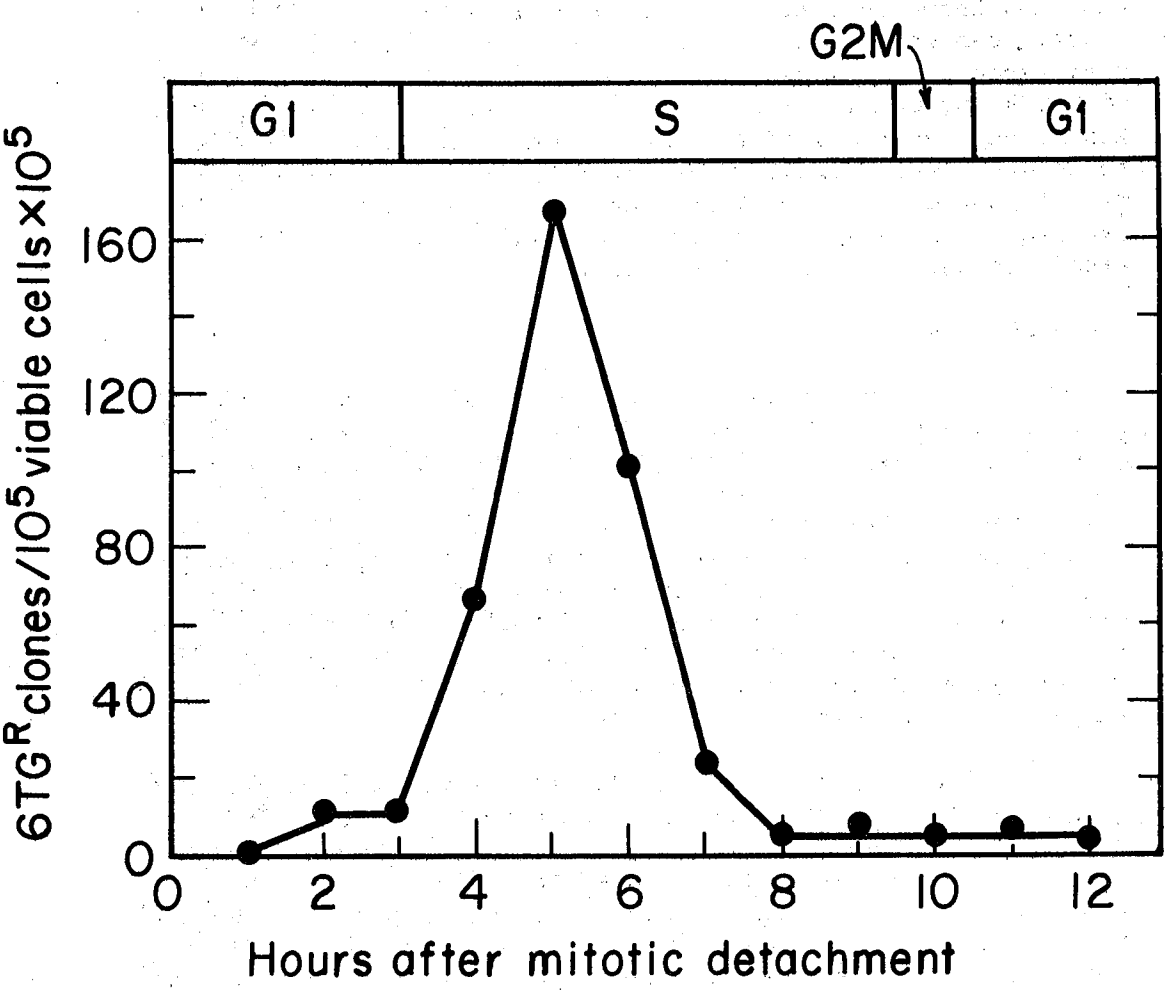

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Fig. 4 The induction of resistance to 6-thioguanine by 1 mM pulses of BUdR in the presence of 2×10^{-6} M FUdR + 1 μCi/ml ^{3}H BUdR for one hour. (Times after mitotic detachment are the middle point of the one-hour labeling period.) After this treatment, the cells are grown in media containing 10^{-5} M TdR and 10^{-6} M CdR for a 6-day expression period.

Table I. "Hot Spots" Within Mammalian DNA

System	Endpoint	Time of Replication in 'S' period	Reference
CHO-BUdR	$6TG^R$	1.5-2.5 h	[12]
V79-BUdR	$6TG^R$	1-2 h	[24]
V79 ^{125}IUdR	Reproductive Death	4-5 h	[25,26]
L5178Y BUdR+ Light	Ala Reversion	1-2 h	[27]
BHK21 BUdR+ 313 Light	T^S reversion	2-4 h	[28]
Syrian Hamster BUdR + near UV	$6TG^R$	Early S	[29]

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TECHNICAL INFORMATION DEPARTMENT
LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
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