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Temporal Sequence of Mutation for 6-thioguanine Resistance in Synchronised Chinese Hamster Cells

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Summary. The temporal sequence of mutation to 6-thioguanine resistance was studied in Chinese hamster ovary cells synchronised in S phase. The number of resistant mutants induced following exposure to pulses of bromodeoxyuridine and subsequent selection was significantly greater if BUdR pulses were administered during the first, rather than the second half of S phase. The results are interpreted as indicating that the gene responsible for 6-thioguanine resistance is replicated during the first half of S phase.

Introduction

Several attempts have been made to determine the temporal sequence of gene replication in mammalian cells. To date, mainly biochemical methods have been used to establish the sequence (Balàzs and Schildkraut 1971; Tobia, Schildkraut and Maio 1971; Balázs, Brown and Schildkraut 1973) of replication. Here we report a genetic method for studying the time of replication of a single gene. Orkin and Littlefield (1971) examined the frequency of 5-bromodeoxyuridine and 6-thioguanine resistant mutants after nitrosoguanidine pulses applied in different periods of the S phase of synchronised hamster (BHK) cells. In these experiments mutagenesis was independent of the cell cycle.

Numerous papers have demonstrated that BUdR results in: physicochemical alterations of DNA (Chargaff 1960): suppression of differentiated functions (Stockdale, Okazaki, Maneroff and Holtzer 1964; Coleman, Coleman and Hartline 1969; O'Neill and Stockdale 1974): altered binding of E. coli Lac repressor to its operator DNA (Lin and Riggs 1972): an alteration in base composition of RNA synthesized by chromatin and/or DNA of mouse fibroblasts (Hill, Tsuboi and Baserga 1974). BUdR incorporated into DNA may result in mispairing and act as a mutagen (Trautner, Swartz and Kornberg 1962). Its mutagenic effect was first reported for phage (Freese 1959; Litman 1956) and more recently for mammalian cells (Chu, Sun and Chang 1972; Huberman and Heidelberger 1972; Stark and Littlefield 1974).

Our goal was to apply BUdR as a mutagen to study the temporal order of mutation for 6-thioguanine resistance in synchronised Chinese hamster cells. The 6-thioguanine resistance was chosen since the basis for the resistance is known, namely the loss or reduced activity of the enzyme hypoxanthine-guanine-phosphoribosyl-transferase (HGPRT) (EC 2.4.2.8), which converts guanine and hypoxanthine or their analogues into nucleotides (see review: Thompson and Baker 1960). Accepting the suggestion that "one might expect 6-thioguanine to be preferable for selecting HGPRT-Chinese hamster cells", then application of a BUdR pulse to cells synchronised in S phase may result in an increase in the frequency of 6-thioguanine resistance if the gene responsible for the expression of such resistance replicates during the time of the BUdR pulse.

Materials and Methods

Cell and cultivation: $CHOK_1$ cells (Kao and Puck 1967) were cultivated in 100 mm ∅ glass Petri dishes in F12 medium (Ham 1965) (GIBCO) supplemented with 5% foetal calf serum (Flow Lab.). The generation time was 12.5 hours (M:0.83, G_1:4.7, S:4.0, G_2:2.9 hours).

Plating experiments: A known number of cells was inoculated into 60 mm ∅ glass Petri dishes. After 8 days of incubation, the developed colonies were fixed with formaldehyde and stained with crystal violet.

Cell synchronisation: Cells were synchronised at late G_1 by a slight modification of the method of Meyn et al. (1973). Samples of 5×10^5 cells in logarithmic growth were plated in 180 mm ∅ glass Petri dishes in F12 + 5FC medium. 10^{-2}M thymidine (TdR) (Fluka) was added to the cultures for 10 hours, followed by incubation in F12 + 5FC medium for 10.5-11 hours. Finally hydroxyurea (10^{-3}M) (Sigma) was administered for 10 hours.

Autoradiography: The slides were dipped in Ilford K5 liquid emulsion and exposed for 6 days. After development and fixation, they were stained with 5% Giemsa for 20 minutes.

Cell progression after synchronisation: The cellular progression throughout the cycle was measured by

^{3}HTdR incorporation, ^{3}H TdR autoradiography and mitotic counting. For the determination of ^{3}H TdR incorporation, 5×10^4 cells were synchronised in 60 mm Ø glass Petri dishes. After synchronisation 0.1 μCi/ml ^{3}H TdR (spec. act.: 19.6 mCi/mM) (UVVVR, Prague) was added. At hourly intervals the cells were harvested with trypsin, collected on Sartorius membrane filters (0.45 μ pore size, 25 mm Ø), rinsed twice with 10 ml of Saline and twice with 10 ml of 5% TCA, transferred to scintillation vials, and counted in a Packard liquid scintillation spectrometer using PPO (Reanal, Hung.), POPOP (Serva) scintillation fluid. The percent labelled cells was determined by autoradiography of synchronised cells, after 15 minutes pulse labelling with 0.5 μCi/ml ^{3}H TdR. For scoring the mitotic index the synchronised cells were arrested by means of colcemide (0.2 μg/ml) (Ciba) for 2 hours.

Bromodeoxyuridine (BUdR) mutagenesis and selection of mutant cells: The S phase (which lasts for 4 hours in our hand) was arbitrarily divided into two parts and 10^{-4}M BUdR (Sigma) pulses were administered, in F12 + 5FC, TdR medium, during the first and second half, respectively, followed by a 10^{-4}M TdR chase. Synchronised cells without a BUdR pulse served as controls. After an expression time of 72 hours the cells were trypsinised and 10^6 cells were seeded into 100 mm Ø Petri dishes containing the selective medium with 5 μg/ml 6-thioguanine (Sigma). (The total number of cells selected is given in Table 1.) After incubation for 8-10 days in selective medium, the colonies that developed were counted and the frequency of induced 6-thioguanine resistant mutations per 10^6 cells was calculated.

Determination of the replication pattern of the X chromosome in synchronised cells: ^{3}H TdR pulses (0.5 μCi/ml for 15 minutes) were given to aliquots of synchronised cells at 0, 1, 2, 3 and 4 hours after release from the hydroxyurea block. Chromosome preparations were made at the next mitosis and processed for autoradiography.

HGPRT assay: The HGPRT activity of the selected colonies was measured in both intact and lysed cells. For the determination of HGPRT in the former, 5×10^4 cells were inoculated into 60 mm Ø plastic dishes (Greiner). After 12 hours aminopterin (4×10^{-7} M) (Schuchardt) was addded for 3 hours and this was followed by incubation with 0.6 μCi/ml ^{14}C hypoxanthine (spec. act.: 6.96 mCi/mM) (Inst. of Isotopes, Hung.) for an additional 4 hours. The cells were harvested with trypsin and the acid insoluble radioactivity counted as described above. The HGPRT activity from the lysed cells was determined according to the method of Harris and Cook (1969). The reaction mixture consisted of 8 mμ moles hypoxanthine-8-^{14}C (spec. act.: 6.96 mCi/mM) 50 mμ moles 5-phosphorylribose 1-pyrophospate (Calbiochem) 500 mμ moles Tris HCl pH = 7.4, and 10 μl of lysed cells. The enzyme activity was expressed in terms of cpm of inosinic acid formed per μg protein per hour.

Results and Discussion

Presuming that BUdR exerts its mutagenic effect by base-analog substitution and subsequent mispairing, then BUdR administered to synchronised cultures incorporating into newly replicated DNA may preferentially mutagenize the genes replicating during the time of addition. Consequently the application of a BUdR pulse to cells synchronised in S phase may increase the frequency of 6-thioguanine resistance if the gene responsible for the expression replicates during the time of the pulse.

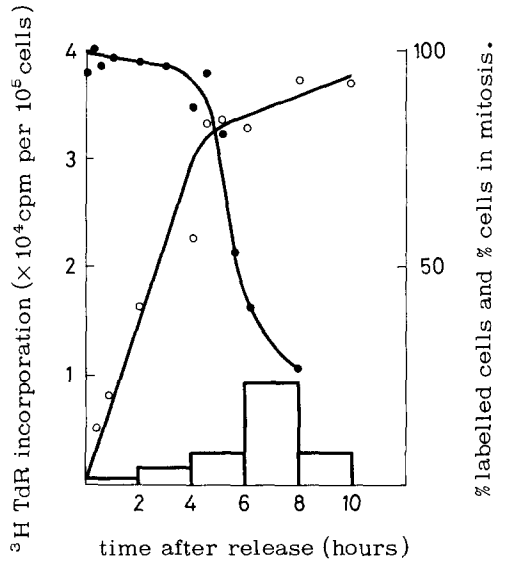

Fig. 1. Cell progression after synchronisation: The cellular progression was studied by determining the ^{3}H TdR incorporation after addition of 0.1 μCi/ml ^{3}H TdR (spec. act.: 19.6 Ci/mM) (o——o).

The % labelled cells was measured after 15 minutes pulse-labelling with ^{3}H TdR (0.5 μCi/ml; spec. act.: 19.6 Ci/mM) and autoradiography (●——●). Percent cells in mitosis was scored. Each column represents % cells in mitosis after 2 hours mitotic arrest with 0.2 μg/ml colcemide

The action of BUdR pulses on the appearance of 6-thioguanine resistant mutants in synchronised cells

Cell synchronisation and BUdR mutagenesis were carried out according to the techniques presented in Materials and Methods. The quality of synchronisation was determined by ^{3}H TdR incorporation, subsequent mitotic counting and ^{3}H TdR autoradiography (Fig. 1).

Administering BUdR pulses during the first and the second half of the S phase and selecting the 6-thioguanine resistant mutants that developed, the frequency of induced mutants was calculated. The mutation frequency was significantly higher if BUdR was administered during the first half of the S phase (Table 1), indicating that the gene in question replicates at that time. The slight increase over background

Table 1. 6-thioguanine resistant mutants in synchronised $CHOK_1$ cells[1]

Experiment	BUdR pulse	Days for expression	Total number of cells selected	Total number of mutant clones	Mutant frequency/10^6 cells	Induced mutant frequency/10^6 cells
1	0	3	3×10^6	2	0.83	-
	0 - 2		4×10^6	18	5.6	4.77
	2 - 4		3×10^6	3	1.25	0.42
2	0	3	4×10^6	3	0.94	-
	0 - 2		4×10^6	14	4.37	3.43
	2 - 4		4×10^6	7	2.18	1.24
3	0	3	7×10^6	0	0	-
	0 - 2		10^7	31	4.0	4.0
	2 - 4		7×10^6	3	0.57	0.57
4	0	3	4×10^6	0	0	-
	0 - 2		7×10^6	20	3.57	3.57
	2 - 4		7×10^6	4	0.71	0.71
5	0	3	3×10^6	0	0	-
	0 - 2		8×10^6	25	3.9	3.9
	2 - 4		6×10^6	4	0.83	0.83

[1] The treatment of the cells and selection are described in the text. Days of expression: the time elapsed from mutagen treatment to the beginning of selection.

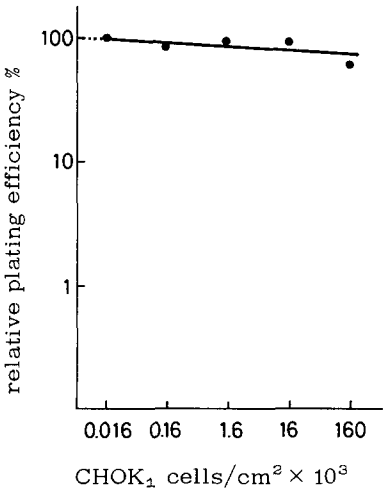

Fig. 2. Reconstruction experiment to assess efficiency of recovery of $HGPRT^-$ mutant cells as a function of wild type ($CHOK_1$) cell density

in the induced mutant frequency in the second half of S phase might mean that the replication time of the gene responsible for the resistance is close to the middle of S phase.

In synchronised cells the BUdR caused a uniform 30% decrease in the plating efficiency, whether it was added during the first or the second half of the S phase. Therefore, the increase in the induced mutant frequency during the first half of the S phase was not the result of selective killing by the drug. BUdR did not disturb the life cycle of the synchronised cells, since the mitotic peak was observed almost at the same time as in the case of the untreated synchronised control. This is in agreement with the data of Meyn et al. (1973).

Optimal conditions for the selection of 6-thioguanine resistant cells

In preliminary experiments 5 μg/ml 6-thioguanine was found to inhibit clonal or mass growth of wild-type cells, while permitting almost 100% colony formation of known $HGPRT^-$ cells. This concentration is 10 times higher than that permitting the growth of wild-type cells.

Variation in the frequency of induced 6-thioguanine resistance could be due to various culture conditions, such as inoculum size and the time required for the expression of the mutant phenotype. It has been shown that frequencies of mutation for Chinese hamster cells are reduced above a certain inoculum size. This reduction is due to metabolic cooperation between mutant and wild type cells (Chu 1971).

To eliminate this source of error, reconstruction experiments were carried out to test for metabolic cooperation between resistant and non-resistant cells. Three hundred $HGPRT^-$ 6-thioguanine resistant cells were seeded together with different numbers of wild type cells and selected in F12 + 5FC medium containing 5 μg/ml 6-thioguanine. The colonies that developed were counted (Fig. 2). Very little metabolic

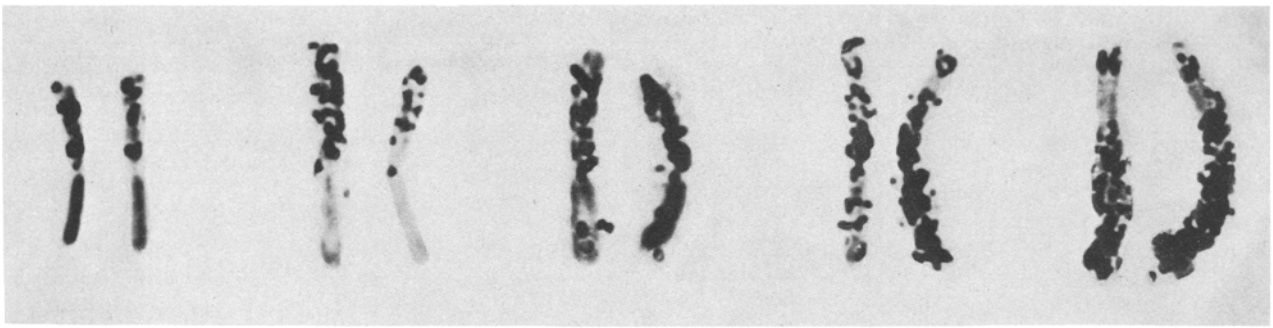

Fig.3. The replication pattern of the X chromosome in synchronised $CHOK_1$ cells.
The numbers represent the time in hours elapsed after synchronisation, when the ^3H TdR pulse was administered

cooperation could be detected. In further selection experiments an inoculum size of 1.6×10^4 cells/cm^2 was used. This resulted in a 20% decrease in the plating efficiency of the known 6-thioguanine resistant cells. This value was taken into consideration in calculating the mutation frequencies.

Another factor which can lead to variation of mutant frequencies is the time necessary for the expression of the mutant character. In our system the cells were grown in fresh F12 + 5FC medium for 72 hours after the BUdR pulses and before selection. This time proved to be sufficient for the recovery of surviving cells from sublethal effects, and for expression and fixation of the mutant character (Orkin and Littlefield 1971).

The effect of synchronisation on the survival and the life cycle of survived cells

The synchronisation processes themselves often result in poor survival because of unbalanced growth and the toxic action of the synchronizing drugs. The combined TdR-hydroxyurea treatment applied in these experiments reduced the plating efficiency to 30% of the random population. To determine whether the cells could enter mitosis after the synchronisation, the culture was examined after various intervals for mitotic figures. A mitotic peak was observed at a time compatible with the known life cycle data. Moreover, the percentage of mitotic cells was in good correlation with the survival data (Fig.1).

There is good evidence which indicates that the HGPRT enzyme is determined by X-chromosomal genes in Chinese hamster cells (Westerveld, Visser and Freeke 1971). Since the biochemical basis of 6-thioguanine resistance lies in the lack or decreased activity of HGPRT, we analysed the time of replication of the X-chromosome in synchronised cells. ^{3}HTdR pulses were administered at different points in S phase, followed by chromosome preparations in mitosis (Fig.3). We obtained the same replication pattern as was previously published by Hsu (1964) for the Chinese hamster DON line. From the results presented above, it seems clear that the cells which survive the synchronisation regiment have a normal life cycle and pattern of chromosome replication.

Characterisation of mutant colonies obtained after the BUdR pulses

Changes in phenotypes of mammalian cells could be caused by mutations in genes with epistatic functions, karyotype changes, mitotic recombination and by extranuclear changes (De Mars 1974). Genes might also be switched on or off by processes of differentiation. Recently the true mutant nature of hypoxanthine analogue resistance has been questioned by several authors (Harris 1971; van Zeeland, De Ruijter and Simons 1974), who propose that stable shifts in phenotypic expression may arise. In Chinse hamster cells, however, there is good evidence that 6-thioguanine preferentially selects for $HGPRT^-$ mutants, because the enzyme of Chinse hamster origin has greater substrate specificity for 6-thioguanine than for other hypoxanthine analogues (Thompson and Baker 1960).

The real mutant nature of the resistant colonies obtained after the BUdR pulses is indicated by several lines of evidence (Table 2).

Table 2. HGPRT activity and growth of mutant colonies in HAT and in a medium containing 5μg/ml 6-thioguanine

Colony	HGPRT activity: Intact cells cpm/10^4 cells/4h	HGPRT activity: Lysed cells cpm/μg protein/h	Growth in HAT	Growth in 6-thioguanine
11	0		-	+
12	0		-	+
13	0		-	+
14	0	7.95	-	+
15	31		-	+
16	11		-	+
17	0	4.06	-	+
31	0			
32	0	20.10	-	+
33	28.4		-	+
34	8		-	+
$CHOK_1$	255	329	+	-

1. None of the 11 colonies (selected on a random basis) grew in HAT medium which supports the growth of the $HGPRT^-$ cells only (Szybalski, Szybalska and Ragni 1962).

2. None of the 11 colonies incorporated ^{14}C-hypoxanthine.

3. There was reduced HGPRT activity in the 3 clones tested.

4. After growing in nonselective medium for about 15 generations before the above tests were carried out, the cells retained their mutant character.

The data here presented strongly indicate that the gene responsible for the HGPRT enzyme replicates during the first half of the S phase (using shorter BUdR pulses we have preliminary evidence that the HGPRT gene replicates in the second hour of S phase). Whether these mutants have alterations in their regulatory or structural genes still has to be explored.

It is hoped that this approach may be useful for further genetic mapping of the replication order of different genes in mammalian cells. We are currently exploring different and gentler means of synchronization, so as to avoid the introduction of non-specific damage into the genome.

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