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Sister chromatid differential staining and NOR activity of BrdU-resistant sublines

Yan Yongshan, Qian Jin and Xi Xiahui
Institute of Genetics, Academia Sinica, Beijing, China

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Summary. Two 30 μg/ml BrdU-resistant sublines and two 60 μg/ml BrdU-resistant sublines are induced from a Chinese hamster cell line Wg3h (HGPRT$^-$) by one-step and two-step selections, respectively. By inoculating the cells into BrdU-free medium or by adding more BrdU into the culture medium for 26–27 h, it was found that the two BrdU-resistant sublines analysed have very clear sister chromatid differential (SCD) staining patterns. This indicates that some of the nuclear DNA of the BrdU-resistant cells incorporate with BrdU to reach a kinetic balance. Frequencies of sister chromatid exchange (SCE) of the resistant cells are twice to four times as high as those of the Wg3h cells, depending on which BrdU-resistant subline is analysed. The SCE frequencies of the resistant cells also increase with the BrdU concentration in the medium. Analysis of silver-stained nucleolar organizer regions (NORs) indicates that the NOR activity of three out of the four BrdU-resistant sublines is significantly suppressed, i.e., averages of the Ag-NOR number and number of the chromosomes bearing Ag-NORs per cell decrease significantly. The degree of suppression for different BrdU-resistant sublines may be quite different. The suppressed NOR activity of the resistant cells can gradually be restored when the cells are inoculated into BrdU-free medium, but the recovery speed is far lower than that of the Wg3h cells. The suppression of the NOR activity of the BrdU-resistant sublines should be due to BrdU toxicity.

Key words: BrdU-resistant sublines – Sister chromatid differential staining – NOR activity – Somatic cell genetics

Introduction

Thymidine analogue 5′-bromodeoxyuridine (BrdU) can be incorporated into DNA in place of thymidine. If incorporated into DNA for two cell cycles, BrdU can be detected by the fluorescence of such DNA binding dyes as Hoechst 33258 to get differentially labeled sister chromatids on which SCE is based. SCE represents the interchange of DNA replication products at apparently homologous loci within a replicating chromosome. The exchange process presumably involves DNA breakage and reunion. Analysis of SCE formation has already provided information on chromosome structure and been used to detect effects of mutagens. SCE analysis has been carried out on a variety of normal mammalian cells but there is to date no information on SCE in BrdU-resistant cells. Since BrdU-resistant cells can grow in the medium containing high concentrations of BrdU for very long periods, much longer than two cell cycles, the first question to be resolved by SCE analysis is how to get SCD staining patterns in the BrdU-resistant cells. Other interesting questions are whether the SCE frequency of the BrdU-resistant cells is different from that of the Wg3h cells, and if the SCE frequency of the resistant cells can change when the resistants have been subcultured in BrdU-free medium for a very long period.

BrdU has been observed to have many disruptive effects on cells. It can suppress cell differentiated functions (Rogers et al. 1975; Wrathall et al. 1973), interfere with DNA repair (Rommelaere et al. 1974), inhibit activity of ribonucleotide reductase (Meuth et al. 1972), affect the binding of regulatory proteins to DNA (Lin et al. 1972), induce mutations (Litman et al. 1956) and significantly suppress NOR activity of Chinese hamster cells, including diploid cells and cell line Wg3h (Yan Yongshan et al. 1985). Can the BrdU-resistant cells resist the suppression of NOR activity caused by BrdU? Is the suppressed NOR activity of the BrdU-resistants irreversible?

In this article, we report on the induction of the four BrdU-resistant sublines from a Chinese hamster cell line Wg3h, on SCE frequency of the resistant cells which had been subcultured in either BrdU-free medium or the medium containing BrdU for a long period, on NOR activity and on the reversibility of the suppressed NOR activity of the resistant cells.

Materials and methods

1 Induction and isolation of the BrdU-resistant sublines

Wg3h cells were subcultured in Eagle essential medium (MEM) containing both 15% calf serum and 30 μg/ml BrdU (Sigma) first for 16 days at 37 °C in the dark, then followed by subculture under light conditions. Three days later the cells were exposed to sunlight for half an hour. After the 40th subculture, three BrdU-resistant clones appeared which were subsequently exposed to fluorescent light (250 W, 220 V) for one and half hours (distance between the bulb and the cell surface was 20 cm). Two of the clones were named B^r1-30 and B^r2-30, respectively. Another clone was subcultured in MEM containing 60 μg/ml BrdU under viable light for another 16 days, and then exposed to fluorescent light as described above. This clone was named B^r60. Some of B^r2-30 cells were inoculated into MEM containing 60 μg/ml BrdU and then exposed to fluorescent light, the resistant subline was named B^r2-60.

All the BrdU-resistant sublines are still growing on MEM containing BrdU very well (six months to date).

2 Growth curve of Wg3h cells

In order to determine if Wg3h cells could grow under normal light conditions in the medium containing a high concentration of BrdU, the cells were subcultured under viable light for 72 h in MEM containing 30 μg/ml BrdU. After 23 h, the cells were exposed to fluorescent light for one and half hours, and counted periodically. Trypan blue-exclusion was used for determining the viability of the cells.

3 Cell treatment for SCD analysis

B^r2-30 cells were first grown in MEM containing 30 μg/ml BrdU and then were inoculated into the following culture media: BrdU-free MEM; MEM containing 39 μg/ml BrdU. Some B^r2-30 cells had already been subcultured in BrdU-free MEM for 15 days before being transferred into MEM containing 30 μg/ml BrdU.

B^r1-30 cells grown in MEM containing 30 μg/ml BrdU were then subcultured in MEM containing 39 μg/ml BrdU.

When all the cells had been in the culture media mentioned above for 25 h, colchicine (Fluka) was added to a final concentration of 0.02 μg/ml for another 2 h before the cell harvest. The cells were trypsinized and treated with 0.075 M KCl for 15 min, and then fixed with a mixture of methanol and glacial acetic acid (3 : 1) three times. Chromosome preparation was performed according to the air-dry method.

To determine if the BrdU-resistants' nuclei had been incorporated with BrdU, we put B^r2-30 cells and freshly prepared mouse bone marrow cells together in order to get a mixed chromosome preparation of the two kinds of the cells for the SCD analysis.

4 Cell treatment for Ag-NOR analysis

B^r1-30 and B^r2-30 cells were grown in MEM containing 30 μg/ml BrdU; B^r2-60 and B^r60 cells, in medium containing 60 μg/ml BrdU. In order to test whether or not the suppressed NOR activity of the BrdU-resistant cells could be restored, the cells were subcultured in BrdU-free MEM: 11 and 26 days for the B^r60 cells and 27 days for the B^r2-60 cells. At the logarithmic stage, colchicine was added and the chromosome preparation was performed as described above.

Wg3h cells were inoculated into MEM containing 30 μg/ml BrdU and subcultured in the dark for 5 days. Some were immediately harvested for the chromosome preparation, the rest were transferred into BrdU-free MEM for another 3 day-culture and then harvested for the chromosome preparation.

Before the cell fixation, the procedure was performed under the safelight.

5 Silver-staining

Six to ten day-old slides were treated with 50% $AgNO_3$, covered with clean coverslips and incubated in a moisture chamber either at 37 °C for 26 h or at 58 °C for 6 h. When the cells appeared brown, the slide were washed with distilled water. Some slides were stained with 1 : 30 Giemsa for 1–3 min. The number of chromosome bearing 1 Ag-NORs and 2 Ag-NORs was recorded, respectively, for each cell examined.

6 Tkase assay

Assay of the Tkase activity of both BrdU-resistant sublines and Wg3h cells was carried out according to the Harris method (Harris 1975).

Results

1 Establishment of the BrdU-resistant sublines

After a 12 h inoculation, Wg3h cells begin to enter the logarithmic stage, they reach the top of the growth

curve 62 h later (Fig. 1). After exposure to fluorescent light, however, the number of Wg3h cells growing in the MEM containing 30 μg/ml BrdU decreases rapidly. By the third day after exposure, not only the cell number falls into the curve bottom, but cell viability is also very low (only 35.6%). It is thus clear that Wg3h cells are difficult to subculture under these conditions (i.e., 30 μg/ml BrdU plus visible light). In contrast, the cells of the four resistant sublines grow very well under the same conditions. Thus, they are believed to be BrdU-resistant sublines.

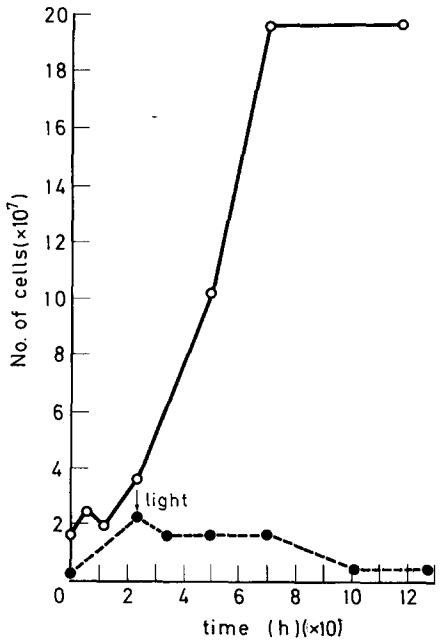

Fig. 1. Growth curve of Wg3h cells. ●------● 30 μg/ml BrdU + light exposure; ○———○ untreated Wg3h cells

2 SCD observation

It was found that B^r2-30 and B^r1-30 cells have very clear SCD patterns in both the BrdU-free group and the BrdU group (Fig. 2 a). None of the SCD patterns can be found in slides of the resistant cells growing in MEM containing a consistant concentration of 30 μg/ml BrdU. It is also found that the chromosomes of B^r2-30 cells are more lightly stained than those of the normal mouse cells (Fig. 2 b).

As shown in Table 1, the SCE frequency of B^r2-30 cells is about twice as high as that of the Wg3h cells. The frequency of the SCE of the B^r2-30 cells in group 3 is significantly higher than that of both group 2 and group 4 (i.e., $0.01 > P > 0.001$, and $P < 0.001$, respectively). There is no difference in SCE frequency between group 2 and group 4. In comparison with the B^r2-30 cells, the SCE frequency of B^r1-30 is far higher than that of the B^r2-30 cells, and about four times as high as that of the Wg3h cells.

3 Ag-NOR distribution of both BrdU-resistant sublines and Wg3h cells

It is shown in Table 2 that for B^r2-30 cells the number of the Ag-NORs and the chromosomes bearing Ag-NORs per cell does not significantly differ from that of Wg3h cells ($P > 0.05$) but for the other three sublines, the number of the Ag-NORs and the chromosomes bearing Ag-NORs per cell is far smaller than in the Wg3h cells. For example, the Ag-NOR number per B^r1-30 and B^r60 cell is 4.4 and 6.2, respectively, less than that in the Wg3h cells. The number of the chromosomes bearing Ag-NORs per B^r1-30 and B^r60 cell decreases from 10.42 to 8.63 and 7.76, respectively. In

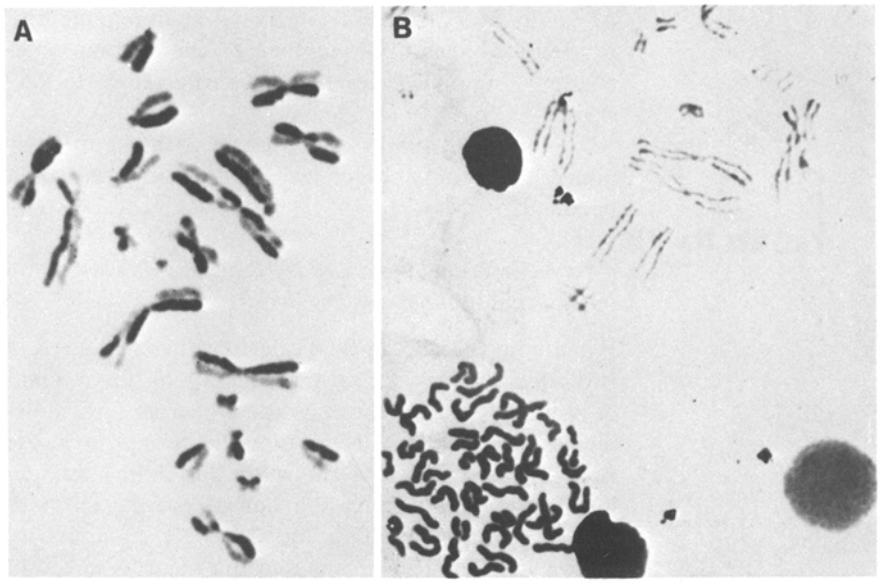

Fig. 2. A SCE pattern of the BrdU-resistant cells; **B** SCD staining of resistant cells which show a lighter staining (*upper on right*) than normal mouse cells (*bottom on left*)

Table 1. SCE frequencies of BrdU-resistant sublines and Wg3h cells

Cell type	No. of group	BrdU concentration in MEM (μg/ml)		No. of cells exmined	SCE/cell ($\bar{X}\pm 1.96$ SE)	SCE/chromosome ($\bar{X}\pm 1.96$ SE)
		Original[a]	27 h before cell harvest			
Wg3h	1	0	30	91	3.93±0.43	0.19±0.02
B^r2-30	2	30	0	59	7.69±0.71	0.368±0.034
	3	30	39	70	9.66±0.88	0.452±0.062
	4	0	30	69	7.35±0.77	0.334±0.033
	5	30	30	340	0	0
B^r1-30	6	30	39	40	15.80±2.17	0.668±0.79

[a] The cells grew either in BrdU-free MEM or in MEM containing 30 μg/ml BrdU more than 15 days

Table 2. Ag-NORs distribution of BrdU-resistant sublines and Wg3h cells

Cell type	Concentration of BrdU (μg/ml)[a]	No. of cells examined	No. of chromosomes bearing Ag-NORs per cell ($\bar{X}\pm 1.96$ SE)			No. of Ag-NORs/cell ($\bar{X}\pm 1.96$ SE)
			Total	With 1 Ag-	With 2 Ag-	
Wg3h	0	52	10.42±0.57	2.12±0.19	8.18±0.40	18.53±0.94
B^r2-30	30	51	11.22±0.59	4.20±0.36	7.00±0.55	18.20±1.02
B^r1-30	30	52	8.63±0.52	3.13±0.48	5.50±0.57	14.13±0.97
B^r2-60	60	55	4.38±0.42	1.78±0.31	2.59±0.41	6.98±0.76
B^r60	60	50	7.76±0.63	3.16±0.48	4.60±0.41	12.38±0.95
Wg3h	30*	51	8.47±0.66	3.24±0.43	5.22±0.60	13.70±1.20

[a] Wg3h cells grew in MEM containing 30 μg/ml BrdU for 5 days before cell harvest

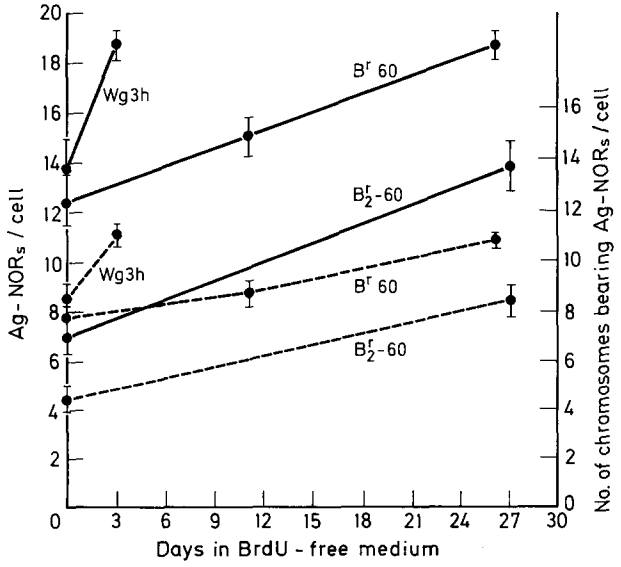

Fig. 3. Ag-NORs distribution of BrdU-resistant sublines and Wg3h cells in BrdU-free MEM. ●———● No. of Ag-NORs per cell; ●--------● No. of chromosomes bearing Ag-NORs per cell

comparison with Wg3h cells, the number of the chromosomes bearing 2 Ag-NORs per B^r1-30 and B^r60 cell decreases from 8.18–5.50 and 4.60, respectively. The number of the Ag-NORs and the chromosomes bearing Ag-NORs in B^r2-60 cells is the smallest among the four resistant sublines. The number of the chromosomes bearing 2 Ag-NORs per B^r2-60 cell decreases to 2.59 (Fig. 5).

Among the 50 cells examined in each group mentioned above, only one or two NOR associations could be found.

4 Recovery of the suppressed NOR activity of both the resistant sublines and Wg3h cells

When both B^r2-60 and B^r60 cells have been cultured in BrdU-free medium for several generations, the number of Ag-NORs and chromosomes bearing Ag-NORs increases gradually with the time the cells grow in the BrdU-free medium. As shown in Fig. 3, the straight lines representing either the number of the chromosomes bearing Ag-NORs or Ag-NOR number are almost parallel. This means that the recovery speed of

Table 3. TKase activity of both BrdU-resistant sublines and Wg3h cells

Cell type	Concentration of BrdU resisted (μg/ml)	TKase activity (cpm/10^7 cells)
Wg3h	0	17,361
B^r2-30	30	22,355
B^r2-60	60	1,334
B^r60	60	3,734

the NOR activity for the two resistant sublines is almost same. It can also be seen in Fig. 3 that both the numbers of Ag-NORs and chromosomes bearing Ag-NORs in B^r60 cells which have been cultured in the BrdU-free medium for 26 h are restored to the level of the normal Wg3h cells. For B^r2-60 cells, However, the numbers of Ag-NORs and the chromosomes bearing Ag-NORs only recover to the level of the Wg3h cells which have been subcultured in MEM containing 30 μg/ml BrdU for 5 days.

When Wg3h cells were subcultured in MEM containing 30 μg/ml BrdU for 5 days, followed by inoculation into the BrdU-free medium for another 3 days, the numbers of Ag-NORs and the chromosomes bearing Ag-NORs can increase to normal levels.

Similarly, one or two NOR associations could be found among the 50 cells analysed in each group.

5 Tkase activity

In comparison with the Wg3h cells, B^r2-30 cells have higher TKase activity. However, the TKase activity of B^r60 and B^r2-60 cells is reduced by 78.5% and 91.3%, respectively (Table 3).

Discussion

Four BrdU-resistant sublines were induced from a Chinese hamster cell line Wg3h by means of one-step and two-step selections. The analysis of TKase activity indicates that the B^r2-60 subline has an extremely low TKase activity, only as high as 7.7% of that in the Wg3h cells, and is considered as a BrdU-resistant subline with TK^-. The B^r2-30 subline has a higher TKase activity than the Wg3h cells.

Similar results have been found in previous reports (Toliver et al. 1976; Bradley et al. 1982; Cullen et al. 1978). B^r60 is a resistant subline with a very low TKase activity. A variety of cell lines resistant to BrdU have been associated with either the loss of TKase activity (Kit et al. 1963) or a deficiency in thymidine uptake system (Breslow et al. 1969). There are only a few cases reported in which BrdU resistance is not associated with the exclusion of BrdU from DNA (Toliver et al. 1967; Davidson et al. 1973; Kaufman et al. 1977).

As reported here, the SCD staining patterns of the BrdU-resistant cells couls be obtained by means of adding more BrdU into the culture medium or by inoculating the cells into BrdU-free medium. This indicates that some of the nuclear DNA of the resistant cells are incorporated with BrdU. During the cell proliferation, the incorporation of BrdU has kept in a kinetic balance as shown in Fig. 4.

SCE analysis demonstrates that SCE frequencies of the BrdU-resistant sublines (B^r2-30 and B^r1-30) analysed are much higher than those found in the Wg3h cells. Higher concentrations of BrdU in the medium can significantly increase the SCE frequency of the resistant cells. This is similar to that found in normal cells.

The SCE frequency of B^r2-30 cells growing in the BrdU-free medium for a long period does not decrease and is also significantly higher than that in the Wg3h cells. For the B^r2-30 cells, the frequency of 7.35 to 7.69 SCE per cell might be their hereditary SCE frequency. Our results also indicate that SCE frequency for different BrdU-resistant sublines could be quite different.

Results of the NOR activity analysis indicate that even though the resistant sublines can resist the high concentration of BrdU, three out of the four resistant sublines can not resist the suppression of NOR activity caused by BrdU. The suppression of the NOR activity expresses a significant decrease in both the number of Ag-NORs and the chromosomes bearing Ag-NORs per cell. The decrease in the number of the chromosomes bearing 2 Ag-NORs per cell compares with the increase in the number of the chromosomes bearing 1 Ag-NORs per cell. It implies that under BrdU effects, (a) one of the sister chromatids of the BrdU-resistant cells might lack an adequate number of the gene copies, leading to minute amounts of stainable materials which might be difficult to be detected in the silver staining technique or, (b) the genes though present, failed to express. The

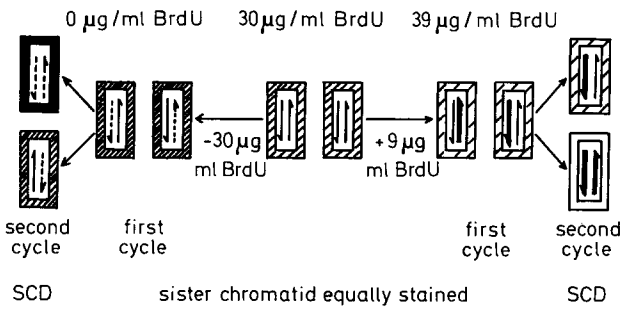

Fig. 4. Sister chromatid differentiation of B^r2-30 cells by BrdU-dye technique. → Nuclear DNA has incorporated with BrdU; → More BrdU incorporated into the nuclear DNA; → A trait of BrdU in the nuclear DNA

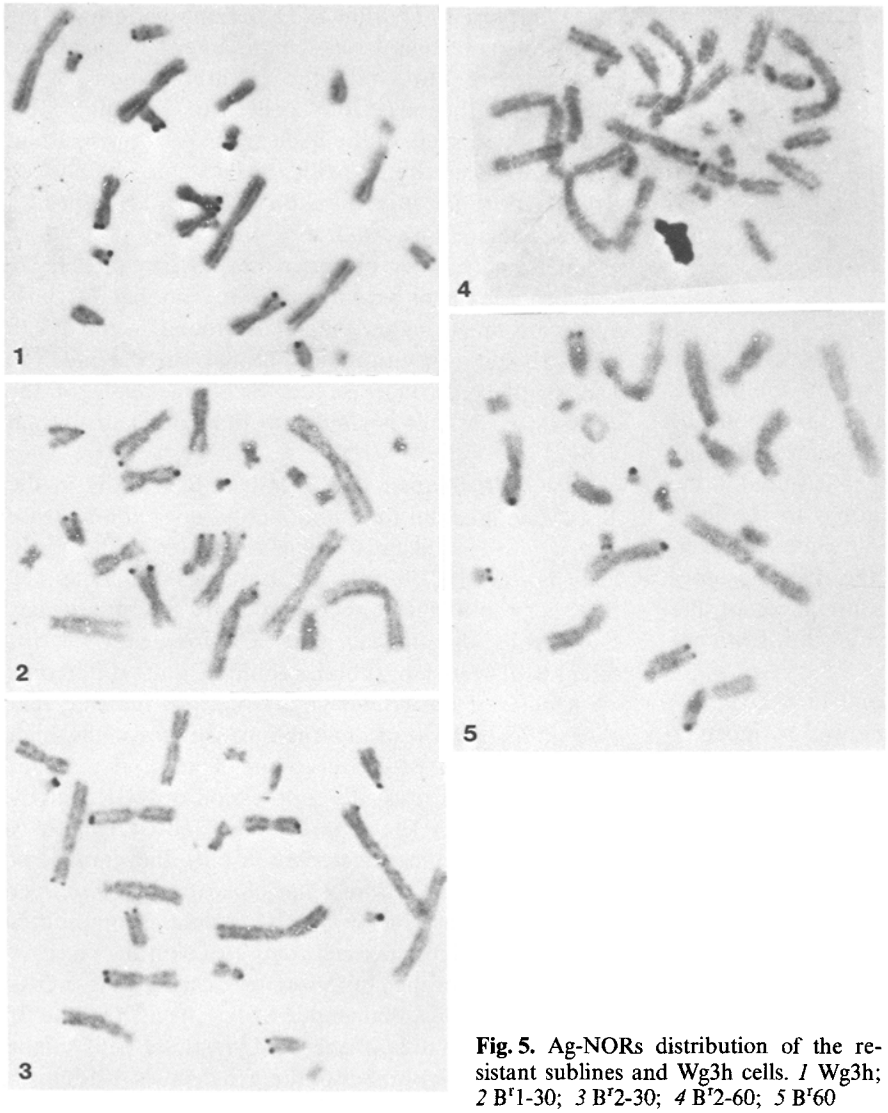

Fig. 5. Ag-NORs distribution of the resistant sublines and Wg3h cells. *1* Wg3h; *2* B^r1-30; *3* B^r2-30; *4* B^r2-60; *5* B^r60

frequencies of the Ag-NORs association in both the resistant cells and Wg3h cells are extremely low, there is no significant difference between the resistants and the Wg3h cells. The degree of the suppression of the NOR activity in the different BrdU-resistant sublines could be quite different. Since all the BrdU-resistant sublines have been growing in MEM containing BrdU the reversion could be avoided.

If the BrdU-resistant cells are subcultured in BrdU-free medium for a long period, the suppressed NOR activity can gradually restore the number of the Ag-NORs and the chromosomes bearing Ag-NORs per cell. The recovery speed is far slower than that of the Wg3h cells. It implies that the suppression of the NOR activity of the BrdU-resistant cells might be due to BrdU toxicity similar to that observed in Wg3h cells.

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