Theor Appl Genet (1987) 74:147–153



# A cytogenetic characterization comparing a rat 6TG-resistant strain and 6TG-sensitive clones

**Yan Yongshan, Fen Shang and Liu Lianrui**

Institute of Genetics, Academia, Beijing, China

Received December 10, 1986; Accepted January 10, 1987
Communicated by Hu Han

**Summary.** A 8.3 μg/ml 6-thioguanine (6TG)-resistant strain was isolated from a rat tetraploid cell line by step-by-step selection in 6TG-medium. In the 6TG-resistant cell population 51% of the cells were tetraploid and 35% of the cells were hypertetraploid, i.e., one chromosome more than a tetraploid. The 6TG-resistant strain grew very well in RPMI 1640 medium with intervals of three days between subcultures. The 6TG-resistant cells all have a homogeneously staining region (HSRs) in one of the X chromosomes which do not stain after chromosome C-banding. They also possess a higher NORs activity and much lower frequency of sister chromatid exchanges (SCE). When the 6TG-resistant RCT cells were subcultured in 6TG-free medium for three days, their SCE frequency did not change. 5′-bromodeoxyuridine (BrdU) significantly suppressed the NORs activity for both 6TG-resistant cells and 6TG-sensitive cells ($P < 0.001$).

**Key words:** Rat tetraploid strain – 6TG-resistance – SCE – NORs activity – HSRs-X chromosome

## Introduction

6TG has been wildly used to select against those cells expressing the enzyme hypoxanthine posphoribosyl transferase (HPRT). HAT medium was used to select hybrids having 6TG-resistant cells as one of the parents (Littlefield 1964). Induction and isolation of the 6TG-resistant cells are thus important in investigations on somatic cell hybridization and drug-resistance.

It is a known fact that silver-stained nucleolar organizer regions (Ag-NORs) reflect the activity of rRNA genes rather than merely their presence (Miller et al. 1976). We previously reported that the NORs activity in BrdU-resistant cells was much lower than that found in BrdU-sensitive cells (Yan Yongshan et al. 1985). Is the NORs activity in the 6TG-resistant cells also suppressed, and what about their cytogenetical characteristics?

SCE reflects an interchange between DNA molecules at homologous loci within a replicating chromosome. It has been used both for monitoring the exposure of cells to mutagenic carcinogens and for detecting and differentiating between certain human diseases although the mechanism for the SCE process remains unclear (Latt 1981). There is very little information available on SCE frequency in drug-resistant cells. We have reported that SCE frequencies in BrdU-resistant cells were twice to four times higher than those found in BrdU-sensitive parent cells (Yan Yongshan et al. 1985) but data on SCE frequency in 6TG-resistant cells is lacking.

In the present paper, we report on the induction and isolation of a 6TG-resistant strain, a HSRs-X chromosome, the comparison in NORs activity and SCE frequency between the TG-resistant cells and TG-sensitive cells, and observations made on a scanning electron microscope.

## Materials and methods

*Induction of 6TG-resistant strain*

RC cells, a tetraploid cell line from a rat sarcoma (Yan Yongshan et al. 1987), was subcultured in RPMI 1640 containing 6TG (1.8 μg/ml, Koch-Light, England). The 6TG-medium was renewed every three days. After 23 days of culture, several 6TG-resistant clones having diameters ranging from 0.3 to 0.5 cm appeared. The 6TG-resistant cells were subcultured in medium containing a higher concentration of 6TG, eg. the cells grew in a medium containing 3.3 μg TG/ml

*Abbreviations:* 6TG = 6-thioguanine; HSRs = homogeneously staining region; NORs = nucleolar organizer region; SCE = sister chromatid exchange; BrdU = 5′-bromodeoxyuridine; HPRT = Hypoxanthine phosphoribosyl transferase

for 15 days, then in one containing 5.0 μg 6TG/ml for another 3 days until finally the TG concentration in the medium was increased to 8.3 μg 6TG/ml. In all cases the cells grew very well with intervals of three days between subcultures. The 6TG-resistant strain was named RCT. The RCT strain was subcultured for 55 passages.

Some RCT cells were subcultured in selective HAT medium (Littlefield 1964) to test their ability to survive in HAT. The HAT medium was renewed every three days.

*Isolation of clones CRC2, CRC3, CRC5 and CRC7*

The RC cells were trypsinized and diluted with the medium to obtain a cell density of 500 cells/ml. A volume of 0.1 ml of the cell suspension were added into each dish (diameter-8 cm) containing five ml of RPMI 1640 medium. The cells were incubated in 5% $CO_2$ at 37 °C. Nineteen days later, several clones were isolated out using a ring technique. Four of them were analyzed cytogenetically and named CRC2, CRC3, CRC5 and CRC7. Rat macrophages were used as feeder cells during the isolation of the clones. The clones were subcultured for 52 passages with intervals of three days between subcultures.

To test the ablility to survive in the 6TG-medium and the HAT medium, all the clones were subcultured in both RPMI 1640 containing 5 μg TG/ml and HAT. The 6TG-medium and HAT were renewed every three days.

*Chromosome analysis*

The cells were pretreated with 0.1 μg colchicine per ml for 90 min and then exposed to 0.075 M potassium chloride (hypotonic treatment) for 20 min. Five drops of a methanol/glacial acetic acid (10:1) fixative were added to the cell suspension. After centrifugation, the cells were fixed in the fixative for an additional 40 min, and then in a methanol/glacial acetic acid (3:1) fixative for 40 min. The chromosome preparation was carried out according to the reutin air-dried method.

Some slides were stained for chromosome counting. At least 130 metaphases were examined for each strain. A diagram of the chromosome distribution was drawn.

Using the trypsin treatment the slides were aged for 1 to 3 days before Giemsa banding. In addition to observing the slides under the microscope, 8 to 10 nicely G-banded metaphases were photographed for each strain. The banded chromosomes were identified, using the system recommended by Levan (1974).

For chromosome C-banding, the slides were treated with 0.1 N HCl for 30 min at room temperature, rinsed in distilled water, and then incubated in saturated $Ba(OH)_2$ for 1 min at 65 °C. After rinsing in distilled water, the slides were incubated in 3XSSC (a solution of 0.045 M sodium citrate and 0.45 M NaCl) at 65 C° for 1–2 h, drying through ethanol. The slides were stained with Giemsa.

*Cell treatment for SCE*

The cells were subcultured in RPMI 1640 medium at 37 °C for 24 h. BrdU (Sigma) was added to a final concentration of 10 μg/ml and/or 30 μg/ml for another 48 h under dark conditions. To test TG influence on the SCE frequency, the RCT cells were subcultured in medium containing TG of 5 μg/ml and 8.3 μg/ml, or in TG-free medium for 3 days. The chromosome preparation and SCE analysis were performed as described in our previous paper (Yan Yongshan et al. 1985). Numbers of both SCE and chromosomes were recorded for each cell examined.

*Ag-NORs analysis*

Some cells were subcultured in the medium containing BrdU (10 μg/ml or 30 μg/ml) for 48 h before the cell harvest for the chromosome preparation in order to test the BrdU effects on the NORs activity. Before cell fixation, the procedure was performed under a safelight.

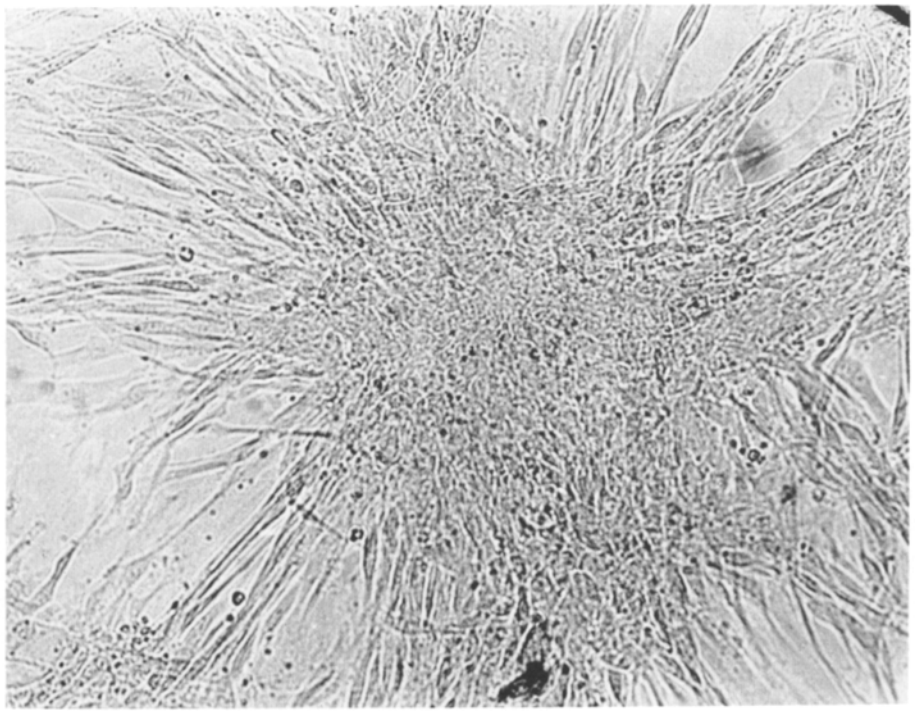


**Fig. 1.** A photograph of living RCT cells

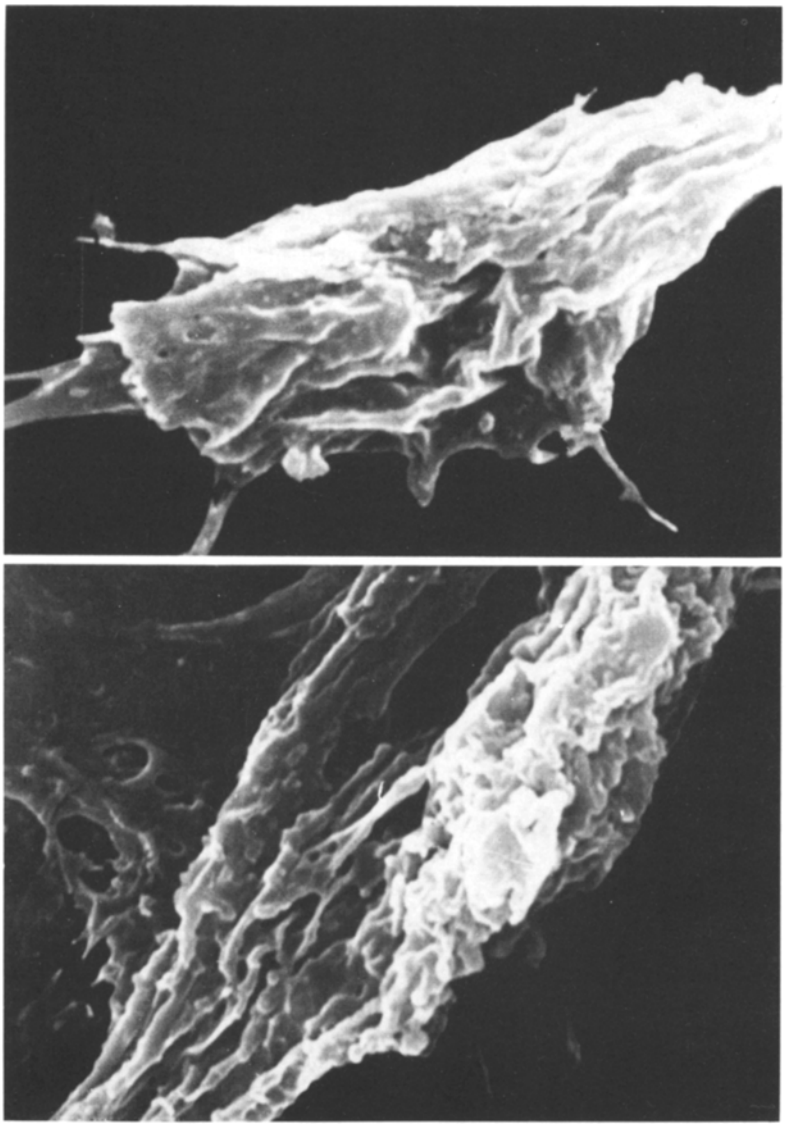


**Fig. 2.** Photographs from the scanning electron microscope of a RCT cell (above) and a rat muscle cell (below) (5,900 X)

One to six day-old slides were treated with 50% $AgNO_3$ containing 1% formalin, covered with clean coverslips and incubated in a moisture chamber at 37 °C for 24 h. When the cells appeared a brown colour, the slides were rinsed in distilled water and stained with 1:20 Giemsa for 1 min.

All data were treated statistically according to the t-test formula.

*Scanning electron microscopy*

Primary cultures of rat muscle cells and the RCT cells were subcultured separately in medium on coverslips for 20 h, rinsed 3 times in PBS, dehydrated in a gradiated alcohol series and then treated with glutaraldehyde. The fixed cells were sputtercoated with a layer of gold and examined in a AMRAY electron microscope operating at 30 KV.

## Results

*Establishment of 6TG-resistant strain*

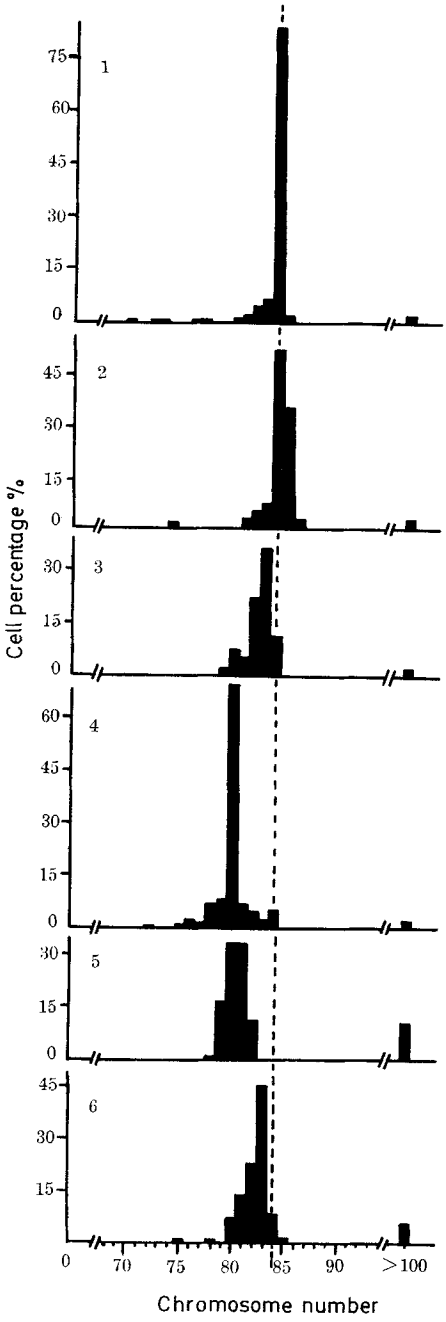
When the RC cells were subcultured in 6TG-medium for two weeks, most of the cells died and only a few clones appeared. These cells, renamed RCT, were resistant to higher concentrations of 6TG (8.3 μg/ml). The RCT cells could be a spontaneus mutation of the RC cells under the selective force of the step-by-step selection in the 6TG-medium.

The RCT cells are fibroblast-like cells (Fig. 1). Scanning electron microscopy showed that on the surface of the RCT cells there are no microvilli as found on normal rat muscle cells (Fig. 2).

The RCT cells had no metaphase after a three day inoculation in HAT medium and died within two weeks.

*Karyotype characteristics*

As shown in Fig. 3, the RCT strain has a mean chromosome number of 84 and 85, the latter number appearing in 35% of the cells. For CRC3, CRC5, CRC7




and CRC2 clones, the mean number of the cromosomes is 83, 80, 80 and 81, and 83, respectively.

Chromosome C-banding analysis indicates that for each pair of chromosomes of the RCT cells there are four copies of rat chromosomes with the same C-banding patterns, in other words, the C-banding patterns of the RCT cells do not differ from those of rat diploid cells, including C-banding size and location on the chromosomes (Fig. 4).

Chromosome G-banding patterns of RCT cells are shown in Fig. 5. Each pair of RCT chromosomes has doubled in chromosome number for rat diploid cells having the same G-banding patterns, except for the X chromosomes in which one of the X chromosomes has a larger HSRs.

*NORs activity*

When the cells grew in the BrdU-free medium, the number of Ag-NORs per cell for RC, CRC3, CRC7 and CRC2 is 14.33, 14.78, 13.64 and 13.35, respectively, but for the RCT cells it is 18.08. It is obvious that the NORs activity of the RCT cells is significantly higher than others ($P < 0.001$) (Fig. 6).

Growing for 48 h in medium containing 30 μg/ml BrdU, the NORs activity of the RCT, RC and CRC3 decreased significantly to 61%, 53.6% and 72%, respectively, of their original level ($P < 0.001$) (Table 1).

*SCE analysis*

If the cells grew in medium containing 10 μg/ml BrdU for 48 h, the SCE frequency per chromosome for the CRC3, CRC7 and CRC2 is 0.302, 0.319 and 0.264, respectively, but for the RCT cells, it is 0.192, signi-

**Fig. 3.** A diagram of chromosome distribution: *1* RC line; *2* RCT strain; *3* Clone CRC3; *4* Clone CRC5; *5* Clone CRC7; *6* Clone CRC2

**Table 1.** Ag-NORs distribution in the 6TG-resistant cells and 6TG-sensitive cells

| Cell type | 6TG concentration (μg/ml) | BrdU treatment | | No. of cells examined | Ag-NORs/cell (X±S.E.) |
|---|---|---|---|---|---|
| | | Concentration (μg/ml) | Time (h) | | |
| RCT | 5 | 0 | 0 | 60 | 18.08±0.44 |
| | 5 | 10 | 48 | 65 | 11.85±0.45 |
| | 8.3 | 30 | 48 | 44 | 11.02±0.41 |
| RC | 0 | 0 | 0 | 64 | 14.33±0.45 |
| | 0 | 30 | 48 | 40 | 7.68±0.52 |
| CRC3 | 0 | 0 | 0 | 94 | 14.78±0.30 |
| | 0 | 30 | 48 | 56 | 10.64±0.49 |
| CRC7 | 0 | 0 | 0 | 50 | 13.64±0.33 |
| CRC2 | 0 | 0 | 0 | 46 | 13.35±0.58 |

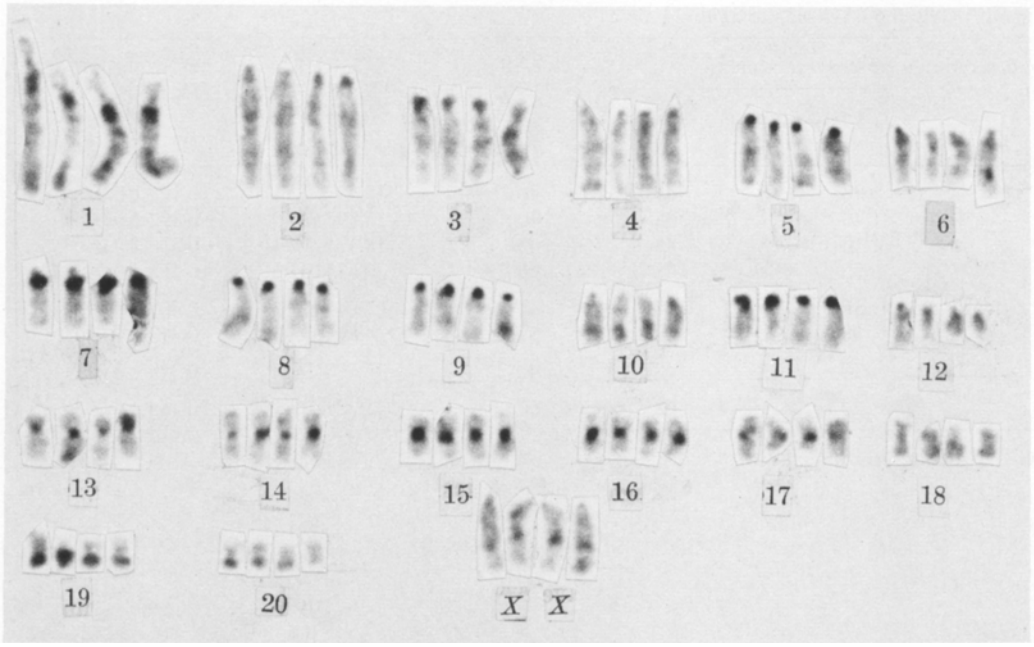



**Fig. 4.** Chromosome C-banding pattern of RCT cells

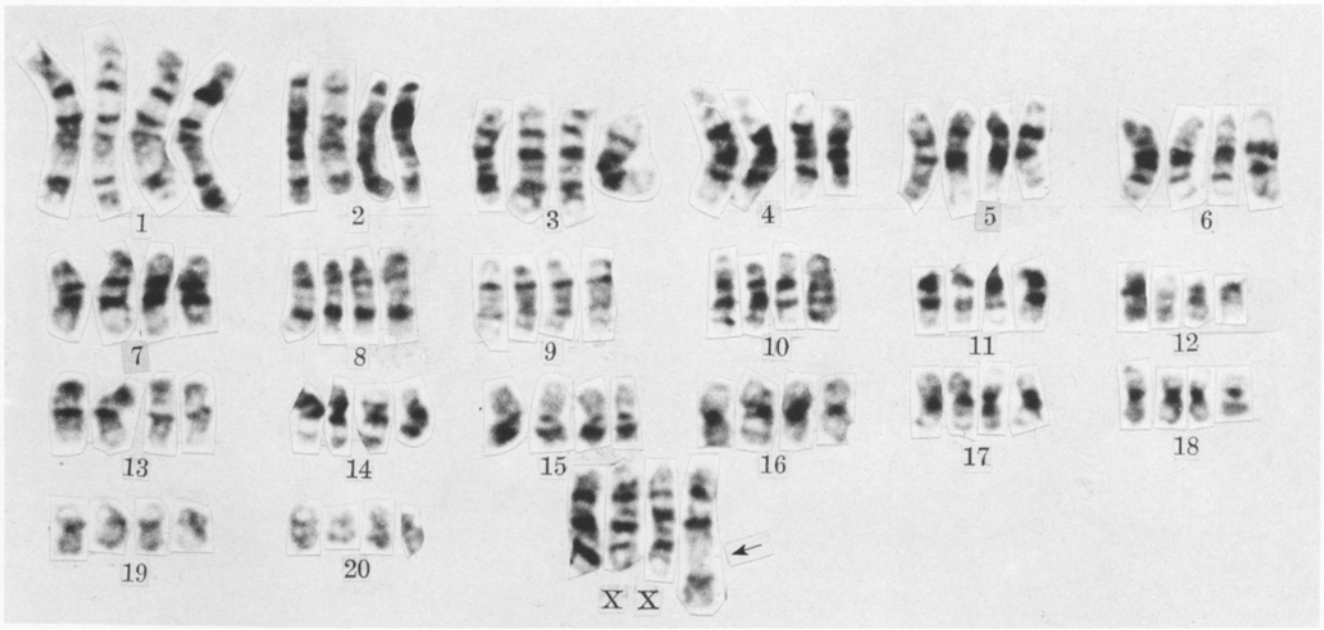



**Fig. 5.** Chromosome G-banding pattern of the cells. A arrow shows a HSRs in one of the X chromosomes

ficantly lower than the others ($P < 0.001$) (Fig. 7). When the BrdU concentration increases from 10 μg/ml to 30 μg/ml, the SCE frequency per chromosome does not increase ($P > 0.05$) (Table 2).

If the TG concentration increases from 5 μg/ml to 8.3 μg/ml, the SCE frequency does not change ($P > 0.05$). When the RCT cells grows in TG-free medium for 3 days, the SCE frequency doesn't change ($P > 0.05$).

## Discussion

In comparison with parent RC cells, 35% of the RCT cells have a mean chromosome number of 85, ie., one chromosome more than the RC cells. The other clones lose one to four chromosomes. It seems likely that TG-resistance in RCT cells is accompanied by an increase in the chromosome number.

**Table 2.** SCE frequencies in RCT cells and 6TG-sensitive cells

| Cell type | 6TG concentration (μg/ml) | BrdU treatment | | Cell no. | SCE/cell (X±S.E.) | SCE/chromosome (X±S.E.) |
|---|---|---|---|---|---|---|
| | | Concentration (μg/ml) | Time (h) | | | |
| RCT | 0[a] | 10 | 48 | 40 | 15.53±0.84 | 0.191±0.010 |
| | 5 | 10 | 48 | 70 | 15.39±0.46 | 0.192±0.006 |
| | 8.3 | 10 | 48 | 42 | 18.12±0.74 | 0.229±0.085 |
| | 8.3 | 30 | 48 | 40 | 20.53±0.91 | 0.258±0.011 |
| CRC3 | 0 | 10 | 48 | 46 | 23.24±1.17 | 0.302±0.015 |
| | 0 | 30 | 48 | 52 | 24.87±1.03 | 0.317±0.013 |
| CRC7 | 0 | 10 | 48 | 42 | 24.69±0.87 | 0.319±0.011 |
| CRC2 | 0 | 10 | 48 | 50 | 21.32±0.66 | 0.264±0.008 |

[a] The cells grew in the 6TG-free medium for three days

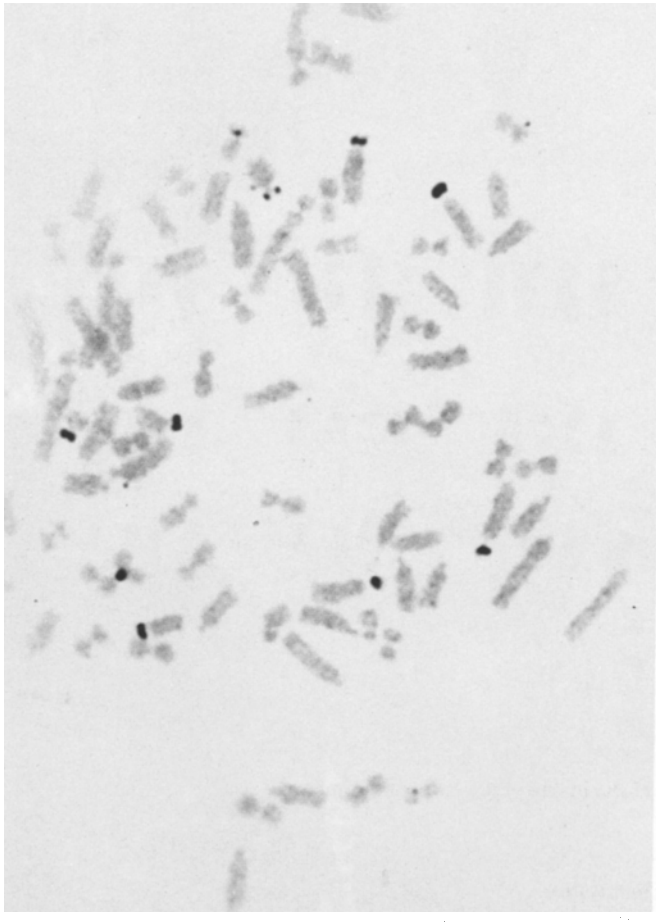


**Fig. 6.** Ag-NORs pattern on the chromosomes of RCT cells

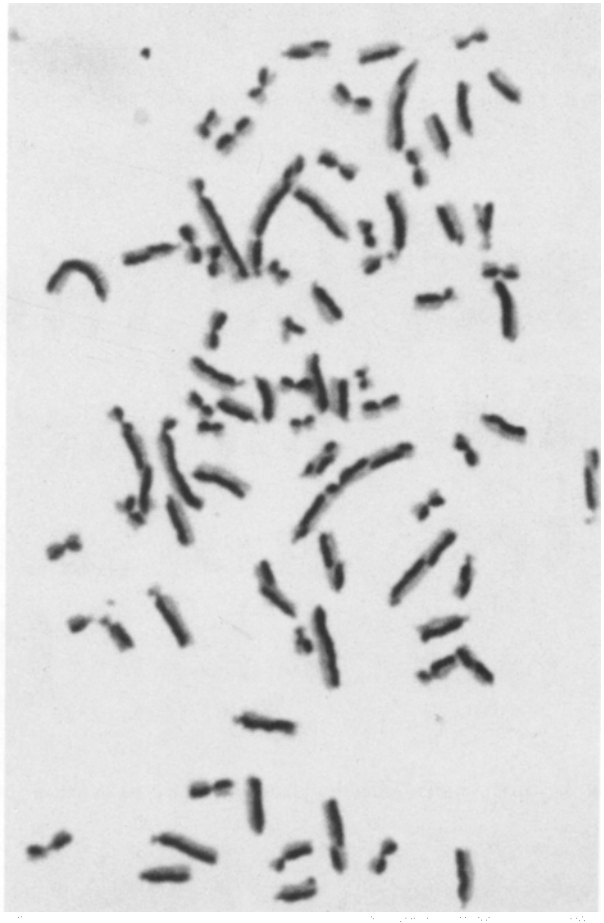


**Fig. 7.** SCE pattern of RCT cells

RCT cells are resistant to 6TG of 8.3 μg/ml and do not survive in HAT medium, implying that the RCT cells should be deficient in HPRT.

HSRs are cytological manifestations of gene amplification, eg., amplified dihydrofolate reductase genes are localized in HSRs in methotrexate-resistant cells (Nunberg et al. 1978), HSRs chromosomes increased DNA content in human neuroblastoma cells (Balaban-Malenbann et al. 1979), and HSRs have extremely high transcription activity for genes coding for 18s+28s rRNA (Yan Yongshan et al. 1985). It is interesting that amplified drug-resistance markers contained in HSRs are much more stable than those carried on double minutes (Hamlin et al. 1984). HSRs have been found in methotrexate-resistant Chinese hamster cells and human neuroblastoma cells (Biedler et al. 1976), in a methotrexate-resistant mouse melanoma cell line (Bostock et al. 1979), a mouse adrenocortical tumor cell line (George et al. 1980), a rat hepatoma cell line (Miller et al. 1979), and in a 6TG-resistant rat strain, as reported here. Our results also indicate that HSRs are not only located on autochromosomes as described in previous papers, but also on the X chromosome. The HSRs in the RCT cells are not stained after chromosome C-banding which is the same as that found in human cells but differs from that found in Chinese hamster cells (Biedler et al. 1976) and mouse cells (Bostock et al. 1979).

For the 6TG-resistant RCT cells, the number of Ag-NORs per cell increased by 26% in comparision with their parent RC cells. The relationship between 6TG-resistance and the extremely high NORs activity remains to be investigated. It is clear that NORs activity of both 6TG-resistant cells and 6TG-sensitive cells is significantly suppressed by BrdU.

The SCE frequency in the 6TG-resistant RCT cells is much lower than that found in the 6TG-sensitive cells. Since SCE contains information about both chromosomal DNA breakage and repair, our results might imply that the chromosomes of the RCT cells are quite stable. The much lower SCE frequency and much higher NORs activity in the 6TG-resistant RCT cells are contrary to that found in BrdU-resistant cells (Yan Yongshan et al. 1985).

## References


Biedler JL, Spengler BA (1976) Metaphase chromosome anomaly: association with drug resistance and cell-specific products. Science 191:185–187

Bostock CJ, Clark EM, Harding NGL, Mounts PM, Tyler-Smith C, Heyningen V, Walker PMB (1979) The development of resistance to methotrexate in a mouse melanoma cell line. Chromosoma 74:153–177

Balaban-Malenbaun G, Grove G, Gilbert F (1979) Increased DNA content of HSR-marker chromosomes of human neuroblastoma cells. Exp Cell Res 119:419–423

George DL, Francke U (1980) Homogeneously staining chromosome regions and double minutes in a mouse adrenocortical tumor cell line. Cytogenet Cell Genet 28:217–226

Hamlin JL, Milbrandt JD, Heintz NH, Azizkhan JC (1984) DNA sequence amplification in mammalin cells. Int Rev Cytol 90:31–76

Littlefield JW (1964) Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinanta. Science 145:709–710

Levan G (1974) Nomenclature for G-bands in rat chromosomes. Hereditas 77:37–52

Latt SA (1981) Sister chromatid exchange formation. Annu Rev Genet 15:11–55

Miller OJ, Tantravahi R, Miller DA, Yu L-C (1979) Marked increase in ribosomal RNA gene multiplicity in a rat hepatoma cell line. Chromosoma 71:183–195

Nunberg JH, Kaufman RJ, Schimke RT, Urlaub G, Chasin LA (1978) Amplified dihydrofolate reductase genes are localized to a homogeneously staining regions of a single chromosome in a methotrexate-resistant Chinese hamster ovaey cell line. Proc Natl Acad Sci USA 75: 5553–5556

Yan Yongshan, Fen Shang, Liu Lianrui, Zhen Huixin and Fen Zhifan (1987) A rat nontumorigenic tetraploid cill line and it's characteristics. Acta Genet Sin (in press)

Yan Yongshan (1985) NOR activity and giant chromosomes with HSR of hybrid clone derived from a four-karyon. Acta Genet Sin 12:454–461

Yan Yongshan, Qian Jin and Xi Xiahui (1985) Sister chromatid differential staining and NORs activity of BrdU-resistant sublines. Theor Appl Genet 70:529–535

Yan Yongshan (1986) Influence of both coculture and BrdU on NOR activity of mouse, rat and human cells. Theor Appl Genet 72:700–705