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# High-Frequency Reactivation of X-Linked Genes in Chinese Hamster × Human Hybrid Cells

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**Abstract**—*Three genes on the human inactive X chromosome retained in the Chinese hamster × human hybrid cell line X8/6T2 have been reactivated using the demethylating agent, 5-azacytidine (5-aza-CR). Pulse-labeling and histochemical methods permitted detection and measurement of reactivation rates of the hypoxanthine phosphoribosyltransferase* (Hpt) *and glucose-6-phosphate dehydrogenase* (G6pd) *genes within 48 h of treatment. About 50% of the cells became active for these genes, which represents a reactivation rate some 30-fold greater than previously reported in similar systems. The phosphoglycerate kinase* (Pgk) *gene was not reactivated as frequently as the* Hpt *or* G6pd *genes. Segregation analysis of progeny of treated cells showed that enzyme-positive and enzyme-negative cells were produced in proportions supporting the notion that 5-aza-CR causes demethylation by replicative loss and that demethylation leads to reactivation.*

## INTRODUCTION

X inactivation is the dosage compensation system in mammalian females which establishes equal expression of X-linked genes between males and females (1, 2). Early in embryogenesis one of the X chromosomes in females becomes heterochromatic, late replicating, and genetically inert over most of its length (3). In normal somatic cells, X-chromosome inactivation is extremely stable, because X-linked markers on the inactive X are reexpressed very rarely (4–7). Riggs (8) and Holliday and Pugh (9) proposed that inactivation could be carried out and stably maintained by a system which differentially methylates the X chromosomes. Support for the methylation hypothesis came from three experimental approaches. First, it was found that inactive X-chromosome DNA functions in transformation less efficiently than active X-chromosome DNA (10–13). Second, the inactive X chromosome in certain interspecific hybrid cell lines can be reactivated at relatively high frequencies when hybrid cells are subjected to the demethylating agent, 5-azacytidine (5-aza-CR) (13–16). Third, methylation differences between the active and inactive X chromosome have been documented for several X-linked genes using DNA probes (17–20).

There are, however, some inconsistencies which suggest that X inactivation is not maintained solely by methylation. First, the only regions in which consistent methylation differences between the active and inactive X have been found is in the putative control regions of genes where the active X is less



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methylated than the inactive X. Methylation patterns outside of these regions vary (21–24). In particular, there are a number of examples in which the active X is more methylated than the inactive X (18, 22, 23). Second, the inactive X in mouse extraembryonic membranes apparently is not regulated by modification (25). Third, although it is difficult to assign an exact frequency, 5-aza-CR does not reactivate the inactive X chromosome in normal human diploid cells at a frequency greater than $10^{-6}$ (24), whereas the frequency of 5-aza-CR induced reactivation in hybrid cells is $10^{-2}$–$10^{-4}$ (13–16). A similar observation has been made in mouse embryonal carcinoma cells in which 5-aza-CR increases the frequency of reactivation in some cell lines but not in others (26). Unless the biochemical activity of 5-aza-CR is different in these cells, the findings suggest that an additional restraint besides methylation is imposed on the inactive X.

In an earlier report (27), we described our findings using selection experiments to detect reactivation of the inactive X chromosome in the Chinese hamster × human hybrid cell line, X8/6T2. One criticism of selection experiments is that reactivation is not scored until cells have formed a clone one to two weeks after selection has been applied. Moreover, since X8/6T2 cells divide before, during, and after exposure to 5-aza-CR, there is already a sizable clone before selection. For these reasons, we have used methods to detect derepression of inactive genes in unselected populations and in small clones of cells shortly after drug treatment, as well as standard cloning techniques. Our results show that derepression of the hypoxanthine phosphoribosyltransferase (*Hpt*) and glucose-6-phosphate dehydrogenase (*G6pd*) genes is easily detected shortly after treatment with 5-aza-CR. Derepression of these two genes occurs at a high frequency. The results of segregation analysis appear to support the conventional notion that 5-aza-CR causes demethylation by replicative loss and that demethylation leads to reactivation.

## MATERIALS AND METHODS

*Cell Culture.* The human × hamster hybrid cell line X8/6T2 has been described previously (27, 28). The rodent parent was the Chinese hamster ovary cell line CHOYH21 (29), which is deficient in both hypoxanthine phosphoribosyltransferase (HPRT) and glucose-6-phosphate dehydrogenase (G6PD). The human parent was a 5X fibroblast cell line (30). Cells were grown in standard medium containing RPMI 1640 + 15% v/v fetal calf serum and 0.04 mg/ml garamycin. 5-Azacytidine (5-aza-CR) reactivation experiments were done essentially according to the method first described by Mohandas et al. (14). In clone counting experiments $1 \times 10^3$–$5 \times 10^4$ cells were plated in 79-cm$^2$ tissue culture dishes in standard medium. The next day varying amounts of 5-aza-CR (frozen stock 1 mg/ml) were added directly to the medium, and the cells were incubated for 24 h. Afterwards, the cells were allowed to recover in standard medium for two days before they were incubated in HAT medium ($2 \times 10^{-4}$ M, hypoxanthine, $4 \times 10^{-7}$ M amethopterine, and $1.6 \times 10^{-5}$ M thymidine in standard medium). Colonies formed in one to two weeks, and they were stained in 20% ethanol and 0.5% crystal violet (Allied Chemical). Cells were also treated by the same methods with varying concentrations of cytosine arabinoside (frozen stock 1 mM). To test viability, $1 \times 10^3$–$5 \times 10^4$ cells were plated and treated as above but were not subjected to HAT medium. In clone isolation experiments 40 cells were plated in 79-cm$^2$ tissue culture dishes in standard medium. Cells were treated with 4 $\mu$M 5-aza-CR, given two days to recover, and selected in HAT or in 5 $\mu$g/ml 6-thioguanine (6TG). Clones were picked and expanded for analysis and storage. One to three clones were picked from each plate.

To measure cell doubling times, 0.8–2.0 × $10^5$ cells were plated in 25-$cm^2$ tissue culture flasks and the next day either counted or treated with 4 μM 5-aza-CR for 24 h. The treated cells were either counted or given standard medium and then counted at times following exposure. Cell doubling times were similarly determined for untreated cells and for cells treated with cytosine arabinoside.

X8/6T2HAT cells are a HAT-resistant derivative of X8/6T2 obtained by treating 1 × $10^6$ cells with 4 μM 5-aza-CR, allowing two days for recovery, and selecting in HAT medium. X8/6T25AC cultures are 5-aza-CR-treated X8/6T2 cells grown in standard medium without HAT selection. Both 405 and 520 are female human fibroblasts derived from skin biopsies, and CHO are Chinese hamster ovary cells. UT-2 cells were obtained from Dr. Joe Goldstein (31).

*Histochemical Labeling Experiments.* Cells (2.5 × $10^4$–5 × $10^5$) were plated in 10-$cm^2$ tissue culture dishes in standard medium. The following day, the cells were subjected to 4 μM 5-aza-CR for 24 h. Cells were labeled with 5–7 μCi of [$^3$H]hypoxanthine (NEN) at 0.145 μg/ml for 4 h at various times after treatment. After label was removed, the cells were washed once in PBS before fixing with several changes of 100% methanol for a total of 20 min. The methanol was rinsed off in water, and the dishes were air dried. The lips of the dishes were cut off, and emulsion (Kodak NTB-2) was layered over the cells for three days. After developing the emulsion (Kodak D-19), the cells were stained in one part Tetrachrome (Chroma-Gesellschaft, Schmid & Co.) to two parts Gurr's buffer for 1 min. Control HPRT$^+$ and HPRT$^-$ cultures were assayed simultaneously in each experiment.

*Histochemical Staining for G6PD.* Cells (2.5 × $10^4$–5 × $10^5$) were plated in 10-$cm^2$ tissue culture dishes in standard medium. The next day, they were treated with 4 μM 5-aza-CR. At various times after treatment, the cells were stained for G6PD activity according to Rosenstraus and Chasin's (29) modification of Wajntal and DeMars' method (32). Staining developed in the tissue culture incubator for 1 h. Cells were fixed in 1% formaldehyde. Control G6PD$^+$ and G6PD$^-$ cultures were assayed simultaneously in each experiment.

*Mixing Experiments.* To determine the degree of cross-feeding in the above histochemical labeling and staining experiments, X8/6T2 cells (HPRT$^-$, G6PD$^-$) were mixed with either X8/6T2HAT (HPRT$^+$) or CHO (G6PD$^+$) cells in ratios of 0:100, 10:90, 30:70, 50:50, 70:30, 90:10, and 100:0 in 10-$cm^2$ tissue culture dishes containing a total of 4 × $10^5$ cells. The next day, the mixtures were histochemically labeled for HPRT or stained for G6PD.

*Segregation Analysis.* Cells (5 × $10^5$) were plated into three 25-$cm^2$ tissue culture flasks in standard medium. The next day they were treated with 4 μM 5-aza-CR for 24 h. 5-aza-CR was removed. From one flask the cells were trypsinized, counted, and plated in eight to ten 10-$cm^2$ tissue culture dishes at 100–150 cells per dish in standard medium. The remaining flasks received standard medium. After 12 h and 24 h, the latter two flasks were handled as the first flask was. The dishes were monitored until clones of 25–100 cells had formed, usually in four to six days. The clones were either histochemically labeled for HPRT or stained for G6PD.

*Enzyme Electrophoresis.* Human and hamster forms of G6PD and phosphoglycerate kinase (PGK) were distinguished by cellulose polyacetate strip electrophoresis. Confluent 75-$cm^2$ tissue culture flasks of cells were harvested by trypsinization, washed once in PBS, pelleted, and resuspended in 5 μl of PBS. Three-day cultures are cells treated with 4 μM 5-aza-CR for 24 h and harvested two days later. Lysates were made by freeze-thawing three times. The lysates were centrifuged at 12,000*g* for 5 min. Lysates were used

fresh or stored at 4°C, and they were diluted in PBS before electrophoresis. PGK electrophoresis was performed according to the conditions described by Meera Khan (33) and G6PD electrophoresis was done in ice-cold hemoglobin buffer (Gelman) at 350 V for 30 min. PGK was stained according to the method described by Reddy et al. (34).

*Chromosome Preparations.* Cells ($1 \times 10^5$–$1 \times 10^6$) were plated in 75-cm$^2$ tissue culture flasks in standard medium. When necessary, the following day the cells were treated with 4 $\mu$M 5-aza-CR for 24 h. Replication banded metaphases were prepared according to the method of Goto et al. (35) and Willard (36).

## RESULTS

We showed previously that the human inactive X chromosome harbored in the Chinese hamster × human hybrid X8/6T2 readily reactivates when treated with the demethylating agent, 5-aza-CR (27). The inactive X chromosome was reactivated by treatment with 5-aza-CR, and reactivated clones were selected in HAT medium. Originally, we obtained HAT-resistant (HAT$^R$) clones at a maximum frequency of $1.4 \times 10^{-2}$ using four days recovery. However, under the conditions used in this experiment (two days recovery), the induced reactivation frequency in the previous experiments was $3.8 \times 10^{-4}$. The spontaneous incidence of HAT-resistant clones was $1 \times 10^{-5}$ HAT$^R$. When we repeated these experiments after the X8/6T2 cells had been cultured continuously for over a year, the frequency of 5-aza-CR-induced HAT$^R$ clones had significantly increased. In Table 1 cells treated with 4 $\mu$M 5-aza-CR and given two days for recovery gave rise to roughly half as many clones in HAT as in standard medium. This represents a 20,000-fold increase over the spontaneous incidence of HAT$^R$ clones, which was measured at a frequency of $2.3 \times 10^{-5}$. Treatment with other concentrations of 5-aza-CR is also shown. With 4 $\mu$M 5-aza-CR, there has been approximately a 1000-fold increase in the frequency of reactivant clones over values obtained a year ago.

Figure 1 shows the cell doubling times of X8/6T2 cells with and without 4 $\mu$M 5-aza-CR treatment. The cell doubling times of treated and untreated cells is about 21 h and 11 h, respectively. Treated cells grew slowly the following day and then returned to the untreated rate. It is possible that HAT resistance induced by 5-aza-CR is caused not by demethylation but by retardation of the cell cycle (37, 38). In order to distinguish these possibilities, we treated cells with 0.5 $\mu$M cytosine arabinoside (araC), a level which retards the growth rate of X8/6T2 cells about 50%. No HAT$^R$ clones were obtained in three plates seeded with a thousand cells each. Nor were HAT$^R$ clones induced at 1 $\mu$M or 3 $\mu$M where the growth rate was severely retarded. We conclude that the induction of copious HAT$^R$ clones by 5-aza-CR is not caused by the growth-retarding effects of the drug.

**Table 1.** Frequency of Reactivation by Selection in HAT

| Treatment ($\mu$M 5-aza-CR) | Number of HAT$^R$ colonies | Total number of cells | CE (%)[a] | Reactivation frequency |
|---|---|---|---|---|
| None | 3 | $2 \times 10^5$ | 64.5 | $2.3 \times 10^{-5}$ |
| 0.5 | 0 | $3 \times 10^4$ | 60.2 | $<5.5 \times 10^{-5}$ |
| 1.0 | 44[b] | $5 \times 10^4$ | 52.4 | $1.7 \times 10^{-3}$ |
| 4.0 | 663 | $3 \times 10^3$ | 48.3 | $4.6 \times 10^{-1}$ |
| 40 | 87 | $3 \times 10^3$ | 29.8 | $9.7 \times 10^{-2}$ |

[a]CE = cloning efficiency.
[b]Cells were given three days to recover in this experiment.

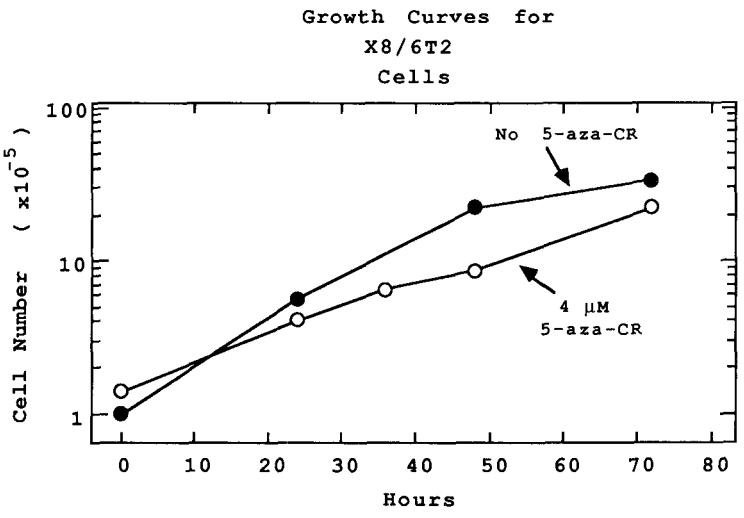


**Fig. 1.** Cell doubling times for 4 μM 5-aza-CR-treated (○) and untreated (●) X8/6T2. Cells were treated in the first 24 h. Each point is the average of two values.

*Early Detection of HPRT Activity.* X8/6T2 cells were treated with 4 μM 5-aza-CR for 24 h and then histochemically labeled with [$^3$H]hypoxanthine at different intervals following treatment. Untreated X8/6T2 and X8/6T2HAT cells were used as negative and positive controls, respectively. The number of grains on control negative cells averaged 6 grains/cell and rarely exceeded 20 grains/cell. Positive controls were heavily labeled with more than 100 grains/cell and label rarely fell short of 40 grains/cell. In the 5-aza-CR time series, a cell was scored positive for HPRT if it was labeled with 40 or more grains. On day 1, when the drug is removed, there is no detectable increase in label over negative controls (Table 2A). HPRT$^+$ cells are first detected on the second day (21%) and are maximal by the third day (84%; average of four experiments). In two experiments, the maximal level of labeling was close to 100%, and in two other experiments the percent of HPRT$^+$ cells was scored between 60% and 80%. In order to determine the amount of spreading of substrate, and hence of label, between positive and negative cells at high cell density, we mixed different ratios of X8/6T2 (HPRT$^-$) to X8/6T2HAT (HPRT$^+$) and scored the percent positive for each mixture (Table 3). Accordance between the mixing ratio and the percent of cells scored positive was seen only in the smaller ratios. The 50:50 mixtures were hardly distinguishable from 0:100 mixtures. Using the mixing experiments as a measure, we conclude that HPRT depression occurs in 30% or more cells after 5-aza-CR treatment.

*Early Detection of G6PD Activity.* Using histochemical staining, we examined the reactivation of the *G6pd* gene by 5-aza-CR. The Chinese hamster ovary cell parent is a *G6pd* mutant which goes unstained by this technique. Reactivation can be simply assayed by noting the appearance of a purple precipitate on positively staining cells. Although positive control cells show some variability from cell to cell in the extent of staining, mixing experiments showed that it was possible to measure quite accurately the percent of cells positive for G6PD activity (Table 3). In the 5-aza-CR time series, very little G6PD was detected on the first or the second days (Table 2B). However, by the third day the percent of cells staining positively reached a maximum of about 50% (average of third and fourth days). For both HPRT and G6PD, there is some

**Table 2.** Early Detection of Reactivation Induced by 5-aza-CR in X8/6T2 Cells[a]

| Time after initiation of treatment (days) | Experiment number I | II | III | IV |
|---|---|---|---|---|
| A. Percent of cells scored positive for HPRT activity | | | | |
| Control | 0 | 0 | 0 | 0 |
| 1 | 0 | 0 | — | — |
| 2 | 21.0 | — | — | — |
| 3 | 98.5 | 62.4 | 95.0 | 80.1 |
| 4 | 93.5 | 65.5 | — | — |
| B. Percent of cells scored positive for G6PD activity | | | | |
| Control | 0.5 | 0 | 0 | |
| 1 | 0 | 0 | 0 | |
| 2 | — | 3.5 | 2.0 | |
| 3 | 24.7 | 59 | 48.2 | |
| 4 | — | 61 | 57.7 | |

[a]Over 200 cells were scored for each point.

variation in maximum frequencies between experiments, the cause of which is unknown. We conclude that G6PD derepression occurs in 30–60% of the cells.

*Segregation of Activated from Inactive Genes.* When X8/6T2 cells were plated at low density, treated with 4 μM 5-aza-CR for 24 h, then stained after small clones of 25–100 cells had formed, nearly all of the clones stained positively for G6PD ($^{265}/_{281}$ = 94.3%); however, almost all of the clones were mosaic. The implication of this experiment is that most cells after treatment with 5-aza-CR will give rise to two lineages of cells—one G6PD$^+$ and the other G6PD$^-$. In order to determine the time at which active and inactive genes segregate from one another, X8/6T2 cells were treated with 4 μM 5-aza-CR for 24 h, trypsinized either immediately or after 12 h or after 24 h, and then plated at low density to allow clones to form. We scored fully negative, fully positive, and mosaic clones in material established 24, 36, and 48 h after the beginning of 5-aza-CR treatment. Examples of each type of clone are shown in Fig. 2. The G6PD results are the average of three experiments, and the range is shown in parentheses (Table 4). For G6PD, mosaic clones were predominant in 24-h material (67.2%); in 36-h material mosaics decline (41.9%), positives increase (4.1% to 17.1%), and negatives increase (28.8% to 41.0%); in 48-h material mosaics show a further decline (15.7%), positives increase further (38.8%), and negatives increase slightly (45.5%). For HPRT, from 24 to 48 h mosaics drop from 38.9% to 9.0%, positives increase from 2.0% to 28.0%, and negatives increase slightly from 59.1% to 63.0%.

It is unlikely that phenotype instability plays a large part in this experiment, because if it did the percent of mosaics would remain high relative to the percent of positives in all material. Indeed, the induced HPRT$^+$ and G6PD$^+$ phenotypes appear to be quite stable in X8/6T2 cells treated with 5-aza-CR and passaged nonselectively for many generations. After 90 generations, 43% of X8/6T2 cells grew in HAT and 45% stained positively for G6PD. In another experiment, a similarly derepressed and nonselectively maintained X8/6T25AC culture was monitored every four generations from the 10th to the 22nd generation for percent G6PD$^+$, and no change in frequency (25%) was observed. These

**Table 3.** Mixing Experiments[a]

| | Exp. I (% positive) | | |
|---|---|---|---|
| A. HAT$^+$:HAT$^-$ | | | |
| 0:100 | 0 | | |
| 10:90 | 9.5 | | |
| 30:70 | 55 | | |
| 50:50 | 82 | | |
| 70:30 | 92 | | |
| 90:10 | 98 | | |
| 100:0 | 100 | | |
| | Exp. (% Positive) | | |
| | I | II | III |
| B. G6PD$^+$:G6PD$^-$ | | | |
| 0:100 | 0 | 0 | 0 |
| 10:90 | 11 | 10 | 16.5 |
| 30:70 | 20 | 36 | 32.5 |
| 50:50 | 50 | 54 | 45 |
| 70:30 | 63 | 80 | 72 |
| 90:10 | 92 | 91 | 88 |
| 100:0 | 100 | 100 | 96.5 |

[a]Over 200 cells scored for each point.

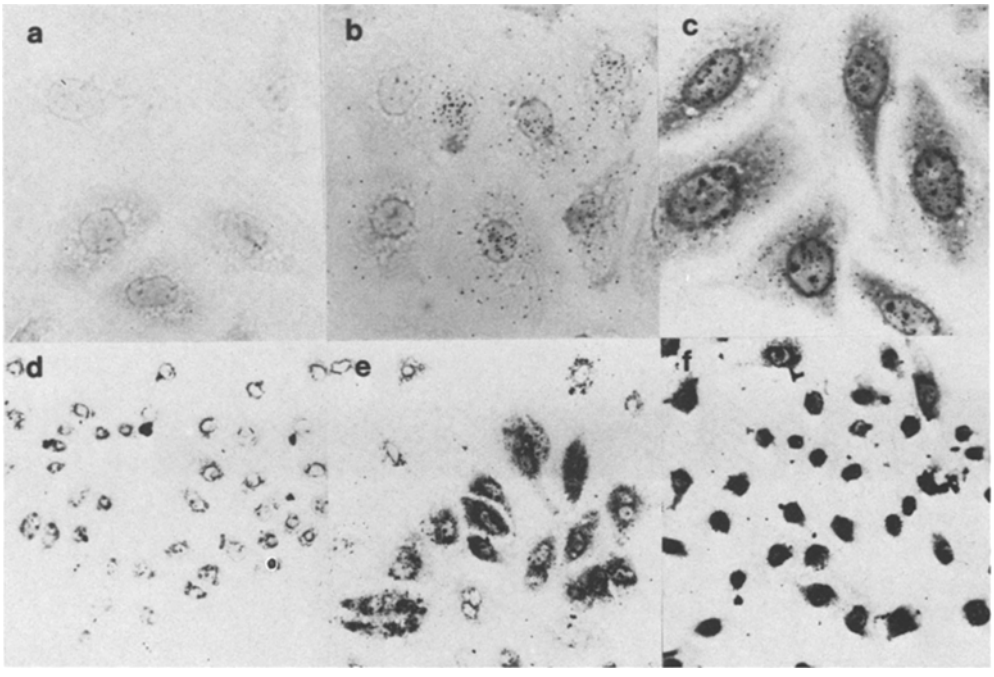


**Fig. 2.** HPRT histochemical labeling (A–C) and G6PD histochemical staining (D–F) in small clones of X8/6T2 cells established 24, 36, and 48 h after beginning treatment with 4 μM 5-aza-CR: (A) 24-h HPRT negative; (B) 24-h HPRT mosaic; (C) 24-h HPRT positive; (D) 48-h G6PD negative; (E) 24-h G6PD mosaic; (F) 48-h G6PD positive.

experiments also imply that selection in culture is not distorting the frequency of reactivated cells.

*Electrophoresis of Human X-linked Genes.* The G6PD mutation in YH21 cells has not been reported to revert. In order to confirm that the G6PD activity in three-day cultures was originating from the human X chromosome, we performed cellulose polyacetate strip electrophoresis. Human G6PD activity was found in a 5-aza-CR-treated culture and in the human fibroblast cell line 405SV, while no G6PD activity was detected in untreated X8/6T2 cultures (Fig. 3). Hamster and human G6PD activities are separable in this system. Therefore, it is the *G6pd* gene from the human inactive X chromosome which is derepressed by 5-aza-CR.

In order to assay for the possible reactivation of the human *Pgk* gene, we performed cellulose polyacetate strip electrophoresis on three-day cultures of X8/6T2 cells, on X8/6T25AC cells, and on X8/6T2HAT cells. A three-day culture did not have an additional band running at the human position unless we overloaded protein onto the strip (Fig. 4). In a mixture of 10% human to 90% hamster protein, the human band was easily separated from the hamster. Active PGK is a monomer, and the induced enzyme should migrate at the human position. In X8/6T25AC cells no induced band was detected, whereas in X8/6T2HAT cells a very faint band could be seen representing less than 5% activity. Therefore, PGK was derepressed at less than 5% activity in 5-aza-CR-treated X8/6T2 cells.

*Coreactivation of X-linked Genes.* When X8/6T2 cells were plated at low density, treated with 4 μM 5-aza-CR for 24 h, and after three days grown under HAT selection until clones had formed, 98% ($^{70}/_{71}$) of the clones stained positively for G6PD, but only 9.9% of the clones were fully positive. Coreactivation was further examined in HAT-

**Table 4.** Percent of Negative, Positive, and Mosaic HPRT and G6PD Clones Established at Different Times Following 5-Azacytidine Treatment

| Times after beginning treatment clones were established (h) | HPRT clones | | | | G6PD clones | | | |
|---|---|---|---|---|---|---|---|---|
| | Neg. (%) | Pos. (%) | Mosaic (%) | Total No. | Neg. (%) | Pos. (%) | Mosaic (%) | Total No. |
| 24 | 59.1 | 2.0 | 38.9 | 149 | 28.8 (22.3–33.2) | 4.1 (2.8–4.8) | 67.2 (62.2–72.9) | 846 |
| 36 | 44.2 | 21.4 | 34.4 | 154 | 41.0 (36.0–47.4) | 17.1 (11.6–26.0) | 41.9 (38.0–46.6) | 1065 |
| 48 | 63.0 | 28.0 | 9.0 | 87 | 45.5 (35.8–51.1) | 38.8 (28.2–55.6) | 15.7 (3.6–22.1) | 607 |

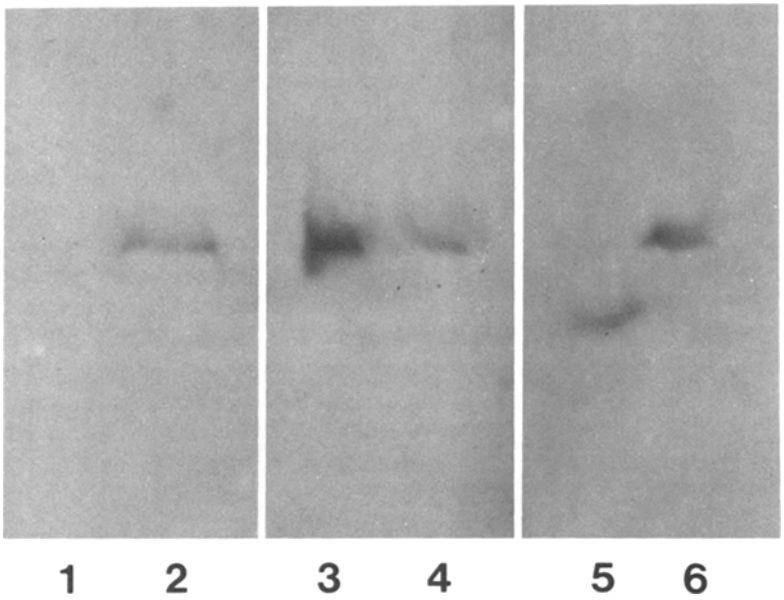


**Fig. 3.** Cellulose polyacetate strip electrophoresis of G6PD. Lane 1, untreated X8/6T2 hybrid; lanes 2, 4, and 6, a three-day culture of X8/6T2 treated with 4 $\mu$M 5-aza-CR; lane 3, human female fibroblast, 520; lane 5, Chinese hamster UT-2.

selected and 6TG-selected clones induced by 5-aza-CR treatment (Table 5). G6PD was assayed by histochemical staining, and PGK was assayed by cellulose polyacetate strip electrophoresis. Nine of ten 6TG-selected clones ($HPRT^-$) were $G6PD^-$. One clone (TG-1) was mosaic with 26.5% positive cells. Additionally, all of the 6TG-selected clones were $PGK^-$. On the other hand, 15 of 15 HAT-selected clones were $G6PD^+$. Six of these (40%) were mosaic, with an average of 52.8% positive cells. Hence, 40% of the clones were mosaic when picked and expanded compared to 88.1% when selected in HAT but not picked. Perhaps $HPRT^-$ cells can survive by cross-feeding in unpicked clones. Seven of 15 (47%) HAT-selected clones were also $PGK^+$. In all of these clones there was clearly less human PGK activity compared to hamster PGK activity, suggesting that these clones were mosaic for PGK or the *Pgk* gene was not expressed at full activity.

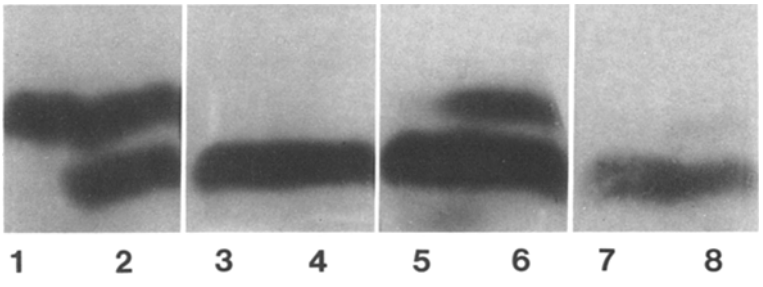


**Fig. 4.** Cellulose polyacetate strip electrophoresis of PGK. Lane 1, SV40-transformed human fibroblast 405; lane 2, 1:1 mixture of SV40-transformed human fibroblast 405 and untreated X8/6T2 hybrid; lane 3, untreated X8/6T2 hybrid; lane 4, 5-aza-CR-treated three-day culture of X8/6T2 hybrid; lane 5, two times more of 5-aza-CR-treated three-day culture of X8/6T2 hybrid; lane 6, 1:10 mixture of SV40-transformed human fibroblast 405 and untreated X8/6T2 hybrid; lane 7, X8/6T25AC hybrid; lane 8, X8/6T2HAT hybrid.

**Table 5.** Coreactivation Patterns of $HAT^R$ and $6TG^R$ Clones of X8/6T2

| Hybrid lines[a] | Selective agent | Cells $G6PD^+$ (%) | PGK |
|---|---|---|---|
| TG-1 | 6TG | 26.5 | – |
| TG-2 | 6TG | 0 | – |
| TG-3 | 6TG | 0 | – |
| TG-4 | 6TG | 0 | – |
| TG-5 | 6TG | 0 | – |
| TG-6 | 6TG | 0 | – |
| TG-7 | 6TG | 0 | – |
| TG-8 | 6TG | 0 | – |
| TG-9 | 6TG | 0 | – |
| TG-10 | 6TG | 0 | – |
| H-1A | HAT | 41.1 | + |
| H-1B | HAT | 67.8 | + |
| H-3A | HAT | 98.5 | + |
| H-3B | HAT | 100 | – |
| H-4A | HAT | 100 | – |
| H-4B | HAT | 97.5 | + |
| H-4C | HAT | 49.4 | + |
| H-5A | HAT | 33.3 | – |
| H-5B | HAT | 86.0 | – |
| H-6A | HAT | 100 | – |
| H-6B | HAT | 38.9 | – |
| H-7 | HAT | 95.7 | – |
| H-8 | HAT | 100 | + |
| H-9 | HAT | 99.5 | + |
| H-10 | HAT | 97.5 | – |

[a]Each number represents a separate plate.

We have also examined coreactivation of G6PD in spontaneous and 1 $\mu$M 5-aza-CR-induced $HPRT^+$ clones. None of the spontaneous (0/3) and 1 $\mu$M 5-aza-CR-induced (0/6) $HPRT^+$ clones were $G6PD^+$.

*Chromosome Analysis.* In order to be certain that the human inactive X chromosome was present and unrearranged in X8/6T2 cells, we examined chromosome spreads which were late-replication banded (RBG). X8/6T2 cells contained on average 35–40 chromosomes with one late X chromosome in 17 of 31 metaphases (55%) and two late X chromosomes in two of 31 metaphases (6%). The X chromosome appeared to be unrearranged. The number of X chromosomes per cell may have dropped since it was recorded by Gartler et al. (27).

We have examined the number of late X chromosomes in 5-aza-CR-treated two-day and three-day cultures to investigate whether the reactivated X chromosome had changed its replication timing relative to control untreated X8/6T2 cells. Two day cultures had one late X chromosome in 19 of 40 metaphases (48%) and two late X chromosomes in three of 40 metaphases (8%). Three-day cultures had one late X chromosome in 15 of 39 metaphases (38%) and two late X chromosomes in five of 39 metaphases (13%). Early replicating X chromosomes were not observed, and overall the number of late X chromosomes did not decrease. Therefore, 5-aza-CR does not induce chromosome-wide changes in replication timing at early times after treatment. Additionally, we carefully examined the bands Xq26-Xq28, where the *Hpt* and *G6pd* genes are located (39), for changes in replication timing in three-day cultures relative to the same bands in untreated cultures. A band was scored as late replicating if, and only if, it was quenched in staining. For each band, the number of early and late replicating events was not different between untreated and three-day cultures (Table 6), nor did bands Xq26-Xq28 change in replication timing with respect to the band Xq22. We conclude that at the standard level of microscopic resolution, the replication timing of the human X chromosome does not change due to derepression of genes on it.

## DISCUSSION

In this paper, we have shown that two genes on the human inactive X chromosome in the Chinese hamster × human hybrid cell line X8/6T2 undergo high-frequency reactivation when cells were treated with the demethylating agent, 5-aza-CR. High-frequency reacti-

**Table 6.** Replication Timing of Distal Tip of Inactive X Chromosome

| | Bands scored late (% of times) | | | |
|---|---|---|---|---|
| | Xq26 | Xq27 | Xq28 | Total No. |
| Control | 36.4 | 100 | 51.5 | 33 |
| 3-day culture | 42.4 | 100 | 57.6 | 33 |

vation has been measured both in clones of selected cells and in large populations and small clones of unselected cells. The frequency of reactivation in unselected cells was measured at 30% or greater for *Hpt* and at 50% for *G6pd* (Table 2). In clonal studies, the *Hpt* gene was reactivated at a frequency of about 45% (Table 1). *G6pd* was active in 15 of 15 HAT-selected clones and in one of ten 6TG-selected clones (Table 5). Additionally, *Pgk* was active in seven of 15 HAT-selected clones and in zero of ten 6-TG selected clones (Table 5). *Hpt* and *G6pd* reactivation correlate well at 4 $\mu$M 5-aza-CR but poorly at 1 $\mu$M 5-aza-CR. We determined that segregation of active from inactive genes for both HPRT and G6PD occurred after the first cell division after 5-aza-CR had been removed, because there is a 10-fold increase of fully positive clones in 48-h material (Table 4). These experiments are not sensitive enough to determine the exact moment when *Hpt, G6pd,* and *Pgk* become active, but we suggest that activity begins after the segregation event, because no positive cells were scored immediately following 5-aza-CR treatment and very few cells scored positive one day after treatment.

In our previous report (27), the frequency of HPRT reactivation was quite comparable to rates found in other hybrid systems. At 4 $\mu$M 5-aza-CR the maximum frequency of $HAT^R$ clones we observed was 1.4%. The reason for the change in reactivation frequency of the *Hpt* gene in X8/6T2 cells is not clear. The cells have been grown in large populations and have not been cloned, frozen, or bottlenecked since our original observations. It is conceivable that there existed a subpopulation of cells which were highly reactivatable and that these cells have been fortuitously selected. If 5-aza-CR were metabolized more efficiently in faster growing cells, then, when 5-aza-CR is applied, it might result in larger fluxes in methylation levels. Another possibility is that small, gradual changes have accumulated independently in all cells. For instance, methyl groups may have been lost over time, and now much less demethylation is required in order to achieve expression. In either case, we suggest that highly efficient demethylation is causing massive derepression in the absence of other restraints.

The current model for the mechanism by which 5-aza-CR induces demethylation proposes that 5-aza-CR is incorporated into DNA where it binds and inactivates the maintenance methylase so that no methylation of newly synthesized strands can occur (40–42). The DNA is hemimethylated at this point, and another round of replication is required to segregate fully methylated from fully unmethylated DNA duplexes. According to the model, fully methylated strands are inactive, and fully unmethylated strands are active. Recently, a different mechanism of demethylation has been demonstrated in Friend erythroleukemia cells in which hexamethylenebisacetamide can induce active enzymatic demethylation of both strands without the requirement of two rounds of replication (43).

We believe our results support the former model of 5-aza-CR action in the derepression of inactive genes in X8/6T2 cells. First, activity is not observed until one day after 5-aza-CR is removed, which is equivalent to about one cell doubling. No activity was detected at any time during 5-aza-CR treatment (data not shown), and full activity is not reached until two days after 5-aza-CR is removed. Second, segregation experiments followed the pattern predicted by a passive model. In 24-h material, the cells have doubled once in 5-aza-CR, and the DNA should be hemimethylated. This DNA should segregate both fully methylated and fully unmethylated DNA duplexes. Most clones established at 24 h after treatment were either negative or mosaic (Table 4), indicating the DNA was either hemimethylated or fully methylated at the time the clone was established. In 48-h material, the DNA should be either fully methylated or fully unmethylated. Positive clones increased and mosaics decreased con-

tinuously in the 24 h following 5-aza-CR treatment. Clones established at 48 h were mostly either negative or positive. About 15% of the clones were mosaic, and these could have arisen by (1) two cells of opposite phenotype falling together at plating, (2) cells mixing due to cell movement, (3) cells with hemimethylated DNA not cycling in the one-day period prior to trypsinization, or (4) cells bearing unstable phenotypes at early periods or due to cell manipulation. A small number of positives arose in 24-h material, and they could come from cells traveling rapidly through two cell divisions after the beginning of treatment or from negative sib cells dying early in the clone's formation. We have not distinguished these possibilities. An active demethylation model predicts that activation of a culture should be rapid and that segregation of activated from inactive genes should be complete in 24-hour material. Since neither of these properties of active demethylation was found, our data support the conventional model.

With highly efficient demethylation, the model predicts that genes should reactivate together. Each cell makes two hemimethylated duplexes when they replicate in the presence of 5-aza-CR so that the active genes segregate with the newly synthesized unmethylated strands and the inactive genes segregate with the old methylated strands. According to this interpretation, each gene is controlled independently by methylation, say at its 5′ end, and highly efficient demethylation by replicative loss causes derepression of many syntenic genes at a high frequency and together. Coreactivation is not 100% in X8/6T2 cells because there is G6PD mosaicism in HAT-selected clones (Table 5). This implies that 5-aza-CR can be incorporated around both the *Hpt* and *G6pd* genes in one duplex and around only the *Hpt* gene in the other duplex. The alternative interpretation of our data is that the *Hpt* and *G6pd* genes are under the control of a single regulator gene which activates them when it is demethylated. This hypothesis predicts that *Hpt* and *G6pd* should almost always be reactivated together. While Hors-Cayla et al. (16) observed that X-linked genes were not independently reactivated in their hamster × human hybrid, Lester et al. (13) found X-linked genes reactivated independently in their mouse × human hybrid. It seems unlikely that the single regulator would work in one hybrid and not the other. Our results with spontaneous and 1 μM 5-aza-CR-induced $HPRT^+$ clones support the former hypothesis because none of these clones were $G6PD^+$ (%). Moreover, the mosaicism seen in Table 5 also supports this hypothesis.

Nearly all X8/6T2 cells carry at least one inactive X chromosome, because 94.3% of the clones that formed after 5-aza-CR treatment stained positively for G6PD. Karyotype analysis, on the other hand, showed that 61% of the cells carried one or more late X chromosomes, which is probably an underestimate of the real value due to the strict criteria on the identification of the X. Two late chromosomes were seen in 13% of the metaphases. According to the demethylation model, these cells would have a higher probability of reactivation. This observation could explain how reactivation exceeds 50% in some experiments.

We have been unable to detect any change in the replication timing of the X chromosome either in two-day or three-day cultures of 5-aza-CR-treated X8/6T2 cells. Moreover, at standard resolution there was no difference in the replication timing of Xq26-Xq28 between untreated X8/6T2 and a three-day culture of 5-aza-CR-treated cells. Previous reports do not offer a consistent view of 5-aza-CR's effect on replication timing. Graves and Young (15) found late Xs in an HPRT reactivant from a mouse × teratocarcinoma hybrid cell line. Hors-Cayla et al. (16) found two reactivants for PGK, GLA, HPRT, and G6PD with late replicating X chromosomes and one reactivant for GLA and HPRT with an early replicating X chromosome in their hamster × human hybrid cell line. It was

possible that replication timing changes are restricted to small regions of chromosomes; therefore, Schmid et al. (44) examined replication timing of bands of the inactive X in a mouse × human hybrid line. They reported a change in timing in the band Xq26 where the *Hpt* is located relative to Xq22 band.

We carried out a similar experiment, except we have looked shortly after treatment and at unselected cells. If we were to detect a change, it would be seen in roughly half of the metaphases, because only half the cells are expressing HPRT and G6PD. Since no change was detected, the experiment might indicate that changes in replication timing in the hybrid system occur secondarily and later than changes in expression. In support of this idea, Paterno et al. (26) saw no 5-aza-CR-induced changes in replication timing of the whole X chromosome in reactivated mouse embryonal carcinoma cells at times following treatment, but found early replicating Xs in seven of nine clones isolated in HAT. On the other hand, Jablonka et al. (45) and Shafer and Priest (46) have described replication timing changes soon after 5-aza-CR treatment in gerbil and human cells, respectively. We suggest that changes in replication timing may be species specific or cell-type specific; however, since the data as a whole are not consistent, it is not possible to evaluate their importance with respect to expression at this time. Perhaps the only sure way to determine if 5-aza-CR is having an effect on the replication of a reactivated gene is to fractionate early- and late-replicating DNA and test for the presence of the gene in each fraction (47).

In conclusion, we have shown that the *Hpt* and *G6pd* genes from the inactive human X chromosome in X8/6T2 cells are reactivated together and at high frequency, while the *Pgk* gene was reactivated at a lower frequency. The kinetics of expression and segregation experiments support a passive model of demethylation leading to the expression of two frequently reactivated genes. No change in the timing of replication of the X chromosome or of the bands Xq26-Xq28 was seen as a result of expression. We believe that X8/6T2 cells will be useful in testing hypotheses concerning the relationship of methylation, chromatin structure, replication timing, and other molecular properties of the inactive X chromosome to X inactivation. By comparing the molecular properties of genes on the inactive X chromosome which reactivate at different rates, we hope to dissect the molecular mechanism underlying the maintenance of X inactivation.

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