Synergism of Xist RNA, DNA Methylation, and Histone Hypoacetylation in Maintaining X Chromosome Inactivation

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Abstract. Xist RNA expression, methylation of CpG islands, and hypoacetylation of histone H4 are distinguishing features of inactive X chromatin. Here, we show that these silencing mechanisms act synergistically to maintain the inactive state. Xist RNA has been shown to be essential for initiation of X inactivation, but not required for maintenance. We have developed a system in which the reactivation frequency of individual X-linked genes can be assessed quantitatively. Using a conditional mutant *Xist* allele, we provide direct evidence for that loss of Xist RNA destabilizes the inactive state in somatic cells, leading to an increased reactivation fre-

quency of an X-linked *GFP* transgene and of the endogenous hypoxanthine phosphoribosyl transferase (*Hprt*) gene in mouse embryonic fibroblasts. Demethylation of DNA, using 5-azadC or by introducing a mutation in *Dnmt1*, and inhibition of histone hypoacetylation using trichostatin A further increases reactivation in *Xist* mutant fibroblasts, indicating a synergistic interaction of X chromosome silencing mechanisms.

Key words: X chromosome • *Xist* gene • DNA methylation • histone deacetylase • gene silencing

Introduction

In mammals, equal X-linked gene dosage between the sexes is achieved by X chromosome inactivation in females. The inactivated X chromosome resembles constitutive heterochromatin in that it is condensed in interphase (Barr and Carr, 1962), hypoacetylated on histone H4 (Jeppesen and Turner, 1993), and replicates late in S phase (Priest et al., 1967). It is also methylated on CpG islands of housekeeping genes (Norris et al., 1991) and is enriched in histone macroH2A1, a histone H2A variant with a large nonhistone domain (Costanzi and Pehrson, 1998). The inactive X (Xi)[1] expresses Xist (Borsani et al., 1991; Brockdorff et al., 1991; Brown et al., 1991), a nuclear untranslated RNA (Brockdorff et al., 1992) that coats the chromosome in cis (Clemson et al., 1996). Both X chromosomes of an undifferentiated embryonic female cell are active, and X inactivation is initiated at the time of differentiation in vitro or in vivo (Monk and Harper, 1979). Once an X chromosome is

inactivated in a cell, the inactive state of the chromosome is clonally inherited through many rounds of cell division.

The most remarkable feature of Xi chromatin is its stability with respect to reactivation. Overall experimental reactivation of the entire chromosome in somatic cells has not been observed. Reactivation of one or few genes on the Xi has been seen, but the rate of reactivation is low, on the order of 10^{-5} to 10^{-4}, in cultured somatic cells (Mohandas et al., 1981; Graves, 1982). Many lines of evidence indicate that multiple molecular mechanisms are responsible for the high fidelity of maintenance of X inactivation (for review see Migeon, 1994), but the contribution of individual mechanisms to silencing and their complex relationship remains to be elucidated.

Methylation of CpG dinucleotides in the promoter region of repressed genes has long been thought of as a mechanism stabilizing X chromosome inactivation. 5-azadC, an inhibitor of DNA methyltransferase 1 (Dnmt1), the major enzyme responsible for maintaining genomic methylation patterns, has been used to derepress several Xi-linked genes, providing the experimental evidence for the importance of methylation in X inactivation (Mohandas et al., 1981; Graves, 1982). Methylation of the CpG island of hypoxanthine phosphoribosyl transferase (*Hprt*) follows X inactivation by several days, implying that methylation plays a maintenance role (Lock et al., 1987). An in

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[1]*Abbreviations used in this paper:* GFP, green fluorescent protein; HAT, hypoxanthine/aminopterin/thymidine; *Hprt*, hypoxanthine phosphoribosyl transferase; IAP, intracisternal A particle; ICF, immunodeficiency centromeric instability facial anomalies; TSA, trichostatin A; Xa, active X; Xi, inactive X.

vivo demonstration of the importance of CpG methylation in X inactivation maintenance is the instability of silencing on the Xi of ICF (immunodeficiency centromeric instability facial anomalies) patients. The Xi of patients suffering from ICF syndrome is hypomethylated at all CpG islands analyzed (Hansen et al., 2000). The syndrome is caused by a defect in the DNA methyltransferase DNMT3B (Hansen et al., 1999; Okano et al., 1999; Xu et al., 1999), suggesting that the enzyme may be responsible for establishing CpG methylation on Xi. X inactivation in the virtual absence of CpG methylation is less stable, as evidenced by reactivation of some loci in cells from these patients (Hansen et al., 2000).

Underacetylation of NH_2-terminal lysine residues on histones H3 and H4 is another feature of Xi chromatin (Jeppesen and Turner, 1993). It is well established that transcriptional activity of genes is regulated by histone acetylation. Histone deacetylation generally correlates with transcriptional silencing and high levels of acetylation with transcriptional activity (Cheung et al., 2000). However, reactivation of X-inactivated genes by altering histone acetylation levels has not been reported. The appearance of a hypoacetylated X chromosome is a late event during differentiation and X inactivation, suggesting that deacetylation of histones is a maintenance rather than establishment mechanism (Keohane et al., 1996).

Xist RNA is essential for the initiation of X chromosome inactivation in cis (Penny et al., 1996; Marahrens et al., 1997). However, several lines of evidence indicate that after X inactivation has been established, Xist is no longer required for maintenance. In studies using mouse–human somatic cell hybrids, a human Xi chromosome fragment that lacked the XIST gene remained transcriptionally silent and its sensitivity to reactivation by 5-azadC did not increase (Brown and Willard, 1994). Similarly, in human leukemia cells, an Xi-derived isodicentric chromosome maintained its inactive state despite missing the XIST gene (Rack et al., 1994). To directly address the role of continued Xist RNA expression in karyotypically normal somatic cells, we have previously generated a conditional Xist allele (Csankovszki et al., 1999) using the Cre-loxP system (Sauer and Henderson, 1988). After Cre-mediated deletion of Xist in primary mouse embryonic fibroblasts, the Xi remained silent (Csankovszki et al., 1999), again arguing that, in the absence of Xist RNA, other silencing mechanisms are sufficient to keep Xi silent. Another study used embryonic stem cells expressing an inducible Xist cDNA transgene, in which the timing of expression can be experimentally manipulated (Wutz and Jaenisch, 2000). This approach defined an initial differentiation time window in which X inactivation is reversible and Xist dependent, followed by irreversible and Xist-independent X inactivation in fully differentiated cells (Wutz and Jaenisch, 2000).

Nevertheless, continued transcription of Xist RNA and its close association with Xi throughout the lifetime of the female mammal suggests a role for Xist in somatic cells (Clemson et al., 1996). Indeed, in rodent–human somatic cell hybrids where the human XIST RNA does not localize correctly to Xi (Clemson et al., 1998; Hansen et al., 1998), the stability of silencing is greatly reduced. It has been speculated that reduced efficiency of silencing in hybrid cells is due to the absence of correctly functioning XIST RNA (Clemson et al., 1998; Hansen et al., 1998). Furthermore, deletion of Xist in mouse embryonic fibroblasts disrupts preferential localization of histone macroH2A1 to Xi, and this alteration of chromatin may also lead to decreased stability of silencing (Csankovszki et al., 1999).

To assess the possible role of Xist RNA in X inactivation maintenance and to study the relative contribution of Xist, DNA methylation, and histone hypoacetylation, we developed a system in which even low levels of reactivation can be quantitatively measured. Reactivation of two markers on Xi were studied, a green fluorescent protein (GFP) transgene, GFP (Hadjantonakis et al., 1998), and the endogenous Hprt gene. Using a conditional deletion of Xist (Csankovszki et al., 1999), we provide direct evidence for the first time that Xist RNA contributes to silencing in somatic cells. Additionally, using the drugs 5-azadC and trichostatin A (TSA) and by introducing a mutation in the Dnmt1 gene (Jackson-Grusby et al., 2001), we show that Xist RNA, DNA methylation, and histone hypoacetylation act synergistically to achieve a highly stable inactive state.

Materials and Methods

Mice and Preparation of Mouse Embryonic Fibroblasts

The $Xist^{2lox}$ (Csankovszki et al., 1999), the $Xist^\Delta$ (Marahrens et al., 1997), the GFP mice (Hadjantonakis et al., 1998), and the $Hprt^\Delta$ mice (Hooper et al., 1987) have been described elsewhere. To obtain mice carrying the GFP transgene and the $Xist^{2lox}$ allele in cis, germline recombinants were generated from double heterozygous females. Recombination frequency was 40% ($n = 115$). Similarly, we obtained mice with the $Hprt^\Delta$ and $Xist^\Delta$ alleles in cis from mice heterozygous for both mutations in trans (27% recombination frequency, $n = 81$). Finally, $Xist^\Delta;Hprt^\Delta/Xist^+;Hprt^\Delta$ females were mated to $Xist^{2lox};Hprt^+;GFP/Y$ males to generate Xist conditional mutant fibroblasts and $Xist^+;Hprt^+;GFP/Y$ males to generate controls. The $Dnmt^{2lox}$ (Jackson-Grusby et al., 2001) and $Dnmt^S$ (Lei et al., 1996) mutations were bred into the colony to create Xist, Dnmt1 double conditional knockout fibroblasts.

Primary mouse embryonic fibroblasts were derived from dissociation and trypsinization of embryonic day 14 embryos, cultured, and when necessary immortalized with SV-40 T-antigen (Jat et al., 1986). For Cre-mediated recombination in fibroblasts, cells were infected with an adenovirus vector carrying the gene for Cre recombinase (Anton and Graham, 1995). Infection was carried out in monolayer culture in DME with 2% fetal calf serum for 2 h. The lowest possible multiplicity of infection that yielded 100% recombination without cytotoxic effects was experimentally determined. Uninfected cells were treated identically, without the addition of virus. After infection, cells were grown in DME with 15% fetal calf serum with antibiotics. When appropriate, 5-azadC (Sigma-Aldrich) was added to the cultures to a final concentration of 0.3 μM, and TSA (Sigma-Aldrich) to final concentration of 500 nM.

Southern Blotting

To analyze the efficiency of Cre-mediated Xist deletion, genomic DNA of infected cells was digested with XbaI, blotted, and hybridized with probe 7, a 1.1-kb EcoRI-XbaI fragment at the beginning of exon 7. To analyze recombination at the Dnmt1 locus, genomic DNA was digested with SpeI and hybridized with the HV probe (Jackson-Grusby et al., 2001). To analyze demethylation of genomic DNA, an HpaII digest was performed, and the blot was hybridized with a probe covering the gag coding region of intracisternal A particle (IAP) element (nucleotides 1570–1899, sequence data available from GenBank/EMBL/DDBJ under accession no. M17551) (Walsh et al., 1998).

FACS® Analysis

Fibroblast cultures were trypsinized to obtain single cell suspension and resuspended in complete medium. Propidium iodide was added to 1 μg/

ml. Viable cells were gated using scatter properties and exclusion of propidium iodide. 100,000–500,000 cells were analyzed for each bulk sample and 10,000 cells for poorly growing hypoxanthine/aminopterin/thymidine (HAT)–resistant clones. To isolate GFP-positive clones, single GFP-positive cells were sorted into wells of a 96-well plate containing DME with 15% fetal calf serum. After 2 wk, 20–30% of the wells contained fibroblast clones, of which ~30% were GFP-positive or contained a high percentage of GFP-positive cells.

HAT Selection and Calculation of Reactivation Frequencies

For selection of clones carrying a reactivated *Hprt* gene, the appropriate number of cells were plated and selected in 1× ESQ HAT (Stratagene) containing media for 14 d. After selection, HAT-resistant clones were picked into regular media. For counting the number of HAT-resistant clones, plates were fixed in methanol/acetic acid (3:1) and stained with Giemsa. For Luria-Delbrück fluctuation analysis (Luria and Delbrück, 1943), small independent fibroblast cultures were expanded to the appropriate size, plated at a density equivalent of a 1:9 to 1:6 split in HAT containing medium, and selected, fixed, and stained as above. Reactivation rates were calculated according to the P0 method of Luria and Delbrück (1943) (rate = −ln[proportion of negative cultures]/average culture size).

Analysis of Replication Timing

Cells were grown in the presence of 30 μM BrdU for the last 5 h before fixation. Colcemid (Sigma-Aldrich) was added for the last hour. Cells were fixed in methanol/acetic acid (3:1) and dropped onto slides. Slides were denatured in 70% formamide/2× SSC at 70–74°C for 2 min. BrdU signal was detected using a monoclonal anti-BrdU antibody (Becton Dickinson) followed by fluorescein-conjugated anti–mouse antibody (Vector Laboratories) in blocking buffer (1× PBS, 5% goat serum, 0.2% Tween, 0.2% fish skin gelatin). Slides were washed in 1× PBS/0.2% Tween, dehydrated, and used for DNA FISH without further denaturation of the chromosomes. The Xi was identified with a directly labeled *GFP/Pgk-Puro* probe. The inserts of EGFP-N1 plasmid (CLONTECH Laboratories, Inc.), and pPGKPuro were isolated, pooled, and labeled with Cy3-dCTP (Amersham Pharmacia Biotech) using random priming. Using only the *GFP* insert as a probe yielded identical results, but the signals were weaker. Only results obtained with the *GFP/Pgk-Puro* probe are shown. Hybridization of the probe and washing were performed as described (Panning and Jaenisch, 1996).

Results

Generation of Xist Mutant Mouse Embryonic Fibroblasts

To study the stability of silencing on Xi, we generated fibroblasts with two Xi-linked markers, where reactivation was designed to be detectable even at low frequencies. One marker was an X-linked *GFP* transgene that is subject to X inactivation (Hadjantonakis et al., 1998). When the *GFP* transgene was located on Xi, cells were GFP negative and reactivation could be monitored using FACS® analysis. The insertion site of the transgene was determined by DNA FISH, and *GFP* was mapped to a position near the centromere (Fig. 1 A, and see Fig. 5 A). The second marker was the endogenous X-linked *Hprt* gene, the activity of which is required in order to survive in HAT containing medium. When cells carrying a wild-type *Hprt* allele on Xi and a mutant allele, *Hprt*^Δ (Hooper et al., 1987), on the active X (Xa) are subjected to HAT selection, even a few reactivants in a large population can be isolated. To generate a homogeneous population of cells with defined Xa and Xi chromosomes, a null mutation in the *Xist* gene, *Xist*^Δ (Marahrens et al., 1997), was introduced onto the chromosome carrying *Hprt*^Δ. Since the *Xist*^Δ allele cannot be chosen for X inactivation (Marah-
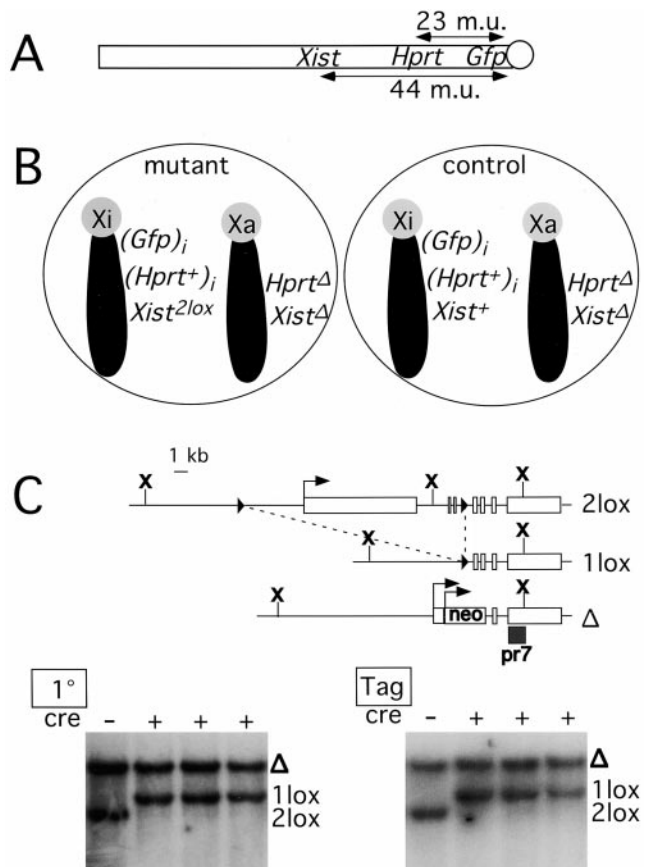

Figure 1. Generation of *Xist* conditional mutant fibroblasts with X-inactivated *GFP* and *Hprt* genes. (A) Map of the X chromosome with approximate genetic distances between genes. (B) Genotypes of *Xist* conditional mutant and control fibroblasts. Both cell types are phenotypically GFP negative and HAT sensitive, as the *GFP* transgene and the only functional *Hprt* allele are on the Xi and are inactivated (i). Conditional mutant cells carry the *Xist*^2lox allele on Xi, whereas controls carry a wild-type *Xist* allele. (C) Cre-mediated deletion of *Xist* after adenovirus-Cre infection. Southern blotting of XbaI-digested DNA hybridized with probe 7 (pr7) indicates 100% recombination in primary (1°) and SV-40 T-antigen–immortalized (Tag) cells.

rens et al., 1998), cells with genotype *Hprt*^+;*GFP/Xist*^Δ; *Hprt*^Δ carry the *GFP* transgene and the *Hprt*^+ allele on Xi.

To study the effect of deletion of *Xist* from Xi, a conditional allele of the gene *Xist*^2lox (Csankovszki et al., 1999) was introduced onto the chromosome. Cells with genotype *Xist*^2lox;*Hprt*^+;*GFP/Xist*^Δ;*Hprt*^Δ will be referred to as conditional mutants. Control cells with genotype *Xist*^+;*Hprt*^+; *GFP/Xist*^Δ;*Hprt*^Δ lack the *Xist*^2lox allele, and therefore cannot delete the *Xist* gene (Fig. 1 B). To induce Cre-mediated deletion of *Xist*^2lox, we infected cells with an adenovirus carrying the gene encoding Cre recombinase (Anton and Graham, 1995). An *Xist*^2lox to *Xist*^1lox recombination was observed in 100% of the cells, as assayed by Southern blotting (Fig. 1 C). Conditional mutants and controls were treated identically, and infections and all further analyses were done in triplicate. Cre-mediated deletion of *Xist* took place over a period of 2–3 d, and Xist RNA levels were undetectable by day 4 (Csankovszki et al., 1999; data not shown). In a previous study, we showed that deletion of

Xist in mouse embryonic fibroblasts does not interfere with late replication timing and underacetylation of histone H4 residues, but it disrupts preferential localization of histone macroH2A1 to Xi (Csankovszki et al., 1999).

Deletion of Xist Leads to Increased Reactivation of GFP and Hprt

GFP expression was analyzed using FACS® in *Xist* mutant and control cells that were infected with adenovirus-Cre or left untreated. GFP fluorescence was compared with autofluorescence, and cells showing greater GFP fluorescence than autofluorescence were counted as positive. We found that the number of cells expressing GFP was dependent on cell density and both the number of cells expressing GFP and the intensity of fluorescence decreased upon long term culture (data not shown). Therefore, care was taken to ensure that an equal number of cells were plated for each sample and cells from the same early passage were used for each experiment. Results of a typical experiment are shown in Fig. 2. Fibroblasts not containing the *GFP* transgene (genotype +/Y) were negative, whereas over 99.9% of cells with *GFP* on Xa (*GFP/Y*) were positive. The majority of cells with *GFP* on Xi were also GFP negative with a small number of positives, ~10–20 in 100,000, representing the spontaneous reactivation frequency (Fig. 2 A). In primary *Xist* conditional mutant fibroblasts after Cre-mediated deletion of *Xist*, the number of GFP-positive cells increased about twofold (Fig. 2 B). This increase cannot be attributed to the effect of adenovirus infection, as the number of GFP-positive cells did not increase in control cells after viral infection. Although the effect is small, we consider it significant as we consistently detected a twofold difference in GFP expressing cells between mutant and control cells in five independent repetitions of the assay.

We considered the possibility that allowing the cells to go through many rounds of cell division in the absence of *Xist* will lead to an increased number of cells reactivating *GFP*. To test this hypothesis, we derived permanent cell lines by SV-40 T-antigen transformation and cultured the cells for over 2 mo. GFP expression was analyzed at various time points (Fig. 2 C). 7 d after adenoviral infection, the transformed cells behaved similarly to primary cells: the conditional mutants exhibited a two- to threefold increase in the number of GFP-positive cells after Cre-mediated deletion of *Xist*, whereas there was no difference between infected and uninfected control cells. However, by day 14 after infection, the difference between the infected and uninfected mutant cells disappeared, and the proportion of GFP-positive cells remained unchanged for >2 mo. Reactivated *GFP* alleles are likely subject to resilencing, consistent with our observations that GFP expression declines over time and with earlier studies that showed that this transgene is subject to nonspecific silencing, even when carried on Xa (Eggan et al., 2000). In addition, it is possible that reactivation of X-inactivated genes confers selective disadvantage on the cells leading to a decrease in the number of GFP-positive cells over time (see below).

Next, we analyzed reactivation of an endogenous X-inactivated gene, *Hprt*, by subjecting cells to selection in HAT medium. Since HAT-resistant colonies from rare reacti-
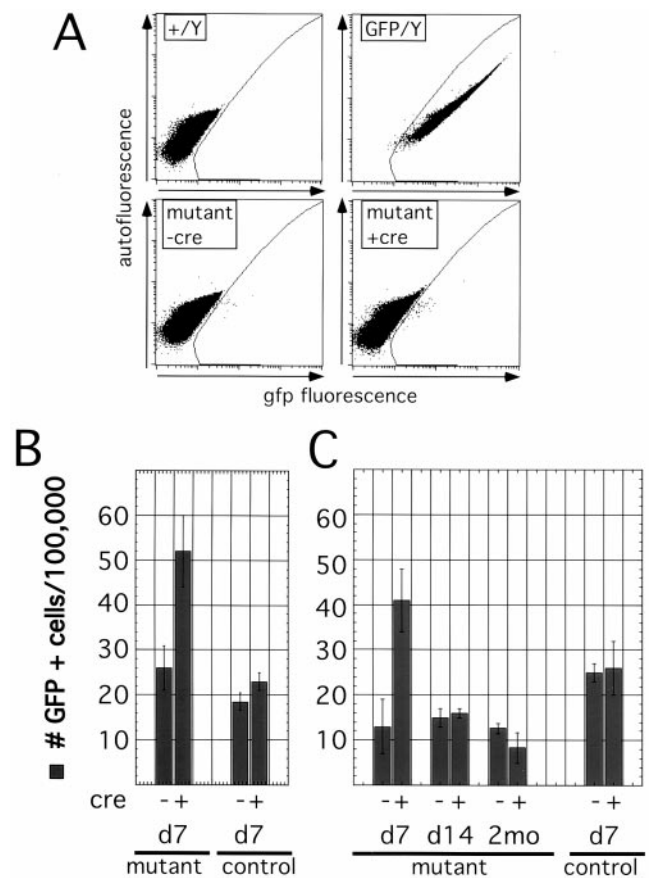

Figure 2. Reactivation of *GFP* in *Xist* mutant fibroblasts. FACS® analysis of cells. (A) Live cells were gated, and their GFP fluorescence was plotted against autofluorescence. Dots to the right of the diagonal represent cells in which GFP fluorescence is greater than autofluorescence and therefore are considered GFP positive. GFP-negative (+/Y) and GFP-positive (*GFP/Y*) populations are shown for control. *Xist* conditional mutant cell populations, before Cre-mediated deletion of *Xist* (−cre), contained a small number of *GFP*-positive cells. After Cre-mediated deletion of *Xist* (+cre), the number of *GFP*-positive cells increased. (B) Number of *GFP*-positive cells in primary conditional mutants and in controls with or without adenovirus-Cre infection on day 7 after infection. In *Xist* conditional mutants, Cre-mediated deletion of *Xist* led to a twofold increase in GFP-positive cells, whereas controls remained unchanged. (C) Long-term culture of SV-40 T-antigen–transformed conditional mutant cells. FACS® analysis was performed at various time points after adenovirus-Cre infection. Although initially we observed an increase in the number of GFP-positive cells after Cre-mediated deletion of *Xist*, the number decreased after another week in culture to reach the level of spontaneous reactivation and remained at that level for the duration of the experiment.

vants can only be obtained in permanent cell lines, these experiments were performed in SV-40 T-antigen–immortalized cells. Two million cells were plated and selected in HAT medium for 2 wk, after which plates were fixed and stained to count the number of colonies (Fig. 3 A). Very few (<1/plate) colonies were seen on control plates and on plates containing conditional mutant fibroblasts before Cre-mediated *Xist*, indicating that the spontaneous reactivation frequency of *Hprt* is very low. However, after dele-
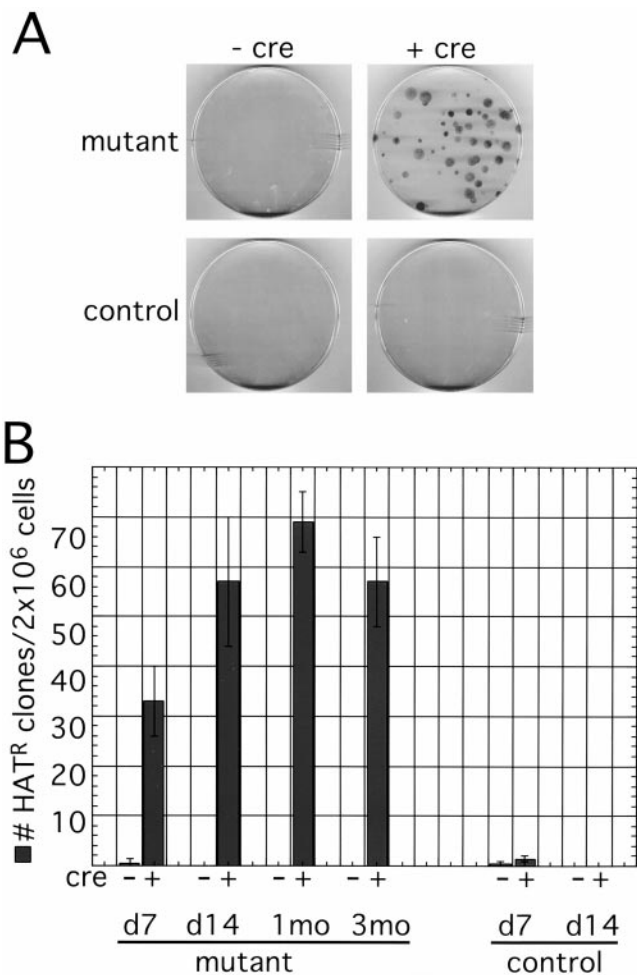
Figure 3. Reactivation of *Hprt* in *Xist* mutant fibroblasts. (A) HAT selection of *Xist* conditional mutant and control SV-40 T-antigen–immortalized fibroblasts with (+cre) or without (−cre) adenovirus-Cre infection. 2×10^6 cells were plated, selected in HAT media, and then fixed and stained. Cells that reactivated the Xi-linked *Hprt* gene were able to proliferate and form colonies. No or very few colonies were observed on control plates and plates containing conditional mutants before adenovirus-Cre infection. However, after Cre-mediated deletion of *Xist* in conditional mutants, HAT-resistant colonies were routinely detected. (B) The proportion of HAT-resistant cells in the culture does not change significantly after culturing cells for 7 and 14 d, and 1 and 3 mo after Cre-mediated deletion of *Xist*.

tion of *Xist*, a small but significant number of cells reactivated *Hprt*, and *Xist* mutant cultures yielded ∼30–70 HAT-resistant colonies per plate. The proportion of HAT-resistant cell in the population increased for the first 4 wk in culture indicating ongoing reactivation of Hprt. Furthermore, HAT-resistant cells were detected even after 3 mo in culture (Fig. 3 B). Northern analysis of total RNA indicated that different clones transcribed different amounts of Hprt RNA (data not shown). We conclude that deletion of *Xist* leads to an increased, albeit low, frequency of reactivation of an endogenous X-inactivated gene, *Hprt*, indicating that Xist RNA contributes to stabilizing the inactive state in the maintenance phase of X inactivation.

Patchy Reactivation of X-inactivated Genes in Xist Mutant Fibroblasts

As the nature of Xist RNA–mediated silencing has not been studied in detail in somatic cells, we next examined whether Xist RNA coordinately silences genes on Xi or whether genes are regulated independently of one another. First, we isolated HAT-resistant clones with a reactivated *Hprt* allele and asked whether these cells are also GFP-positive (Fig. 4). We analyzed two types of clones, fast-growing ones that grew almost as well as the unselected population ($n = 6$), and slow-growing ones that divided barely enough to yield sufficient cells for FACS® analysis ($n = 7$). In *Xist* mutant and *Xist* wild-type bulk populations before HAT selection, the number of GFP-positive cells was ∼1 in 10,000. Among fast-growing clones, the variation was large, but on average the clones contained 10–20 times more GFP-positive cells than the unselected populations. In slow-growing clones, the number of GFP-positive cells was on average another 10-fold higher (Fig. 4). Yet, even in the clones with the highest proportion of GFP-positive cells, over 98% of cells remained GFP negative. Therefore, most cells that reactivated *Hprt* generally did not also reactivate *GFP*. However, a chromosome that reactivated *Hprt* was more prone to reactivate *GFP* than a chromosome with an inactive *Hprt* gene.

It is interesting to note that HAT-resistant clones with a higher proportion of GFP-positive cells grew slower than those with fewer GFP-positive cells. This result is consistent with the conclusion that reactivation of X-inactivated genes confers selective disadvantage on the cells. In clones with a higher proportion of GFP-positive cells, the more extensive reactivation is more detrimental to cell growth.

Finally, we isolated GFP-positive clones by sorting and expanding individual GFP-positive cells, and tested whether these clones also reactivated *Hprt*. Similarly to the HAT-resistant clones, GFP-positive clones did not grow well, and most died under HAT selection without yielding a single colony. However, occasionally (2 of the 42 clones analyzed), the clone grew well in HAT-containing medium, indicating that most, if not all, cells of the clone were also Hprt positive. We conclude that most clones that reactivated one X-linked gene do not also reactivate another. Yet, there is a certain level of cooperativity in reactivation, as a chromosome that reactivated one gene on Xi, is more likely to reactivate another one than a chromosome that has not reactivated any gene.

X Chromosomes with Reactivated Genes Remain Late Replicating

An Xi chromosome after deletion of *Xist* remains late replicating when analyzed in bulk population with presumably very few of the cells containing reactivated genes (Csankovszki et al., 1999). We wished to see whether in the reactivated clones, Xi, or a cytologically visible portion of it, became early replicating. We analyzed two slow-growing HAT-resistant clones with a high proportion of GFP-positive cells and one GFP-positive clone that was also HAT resistant. As the rest of the clones grew too poorly to obtain sufficient numbers of cells for the assay, we pooled small HAT-resistant colonies and GFP-positive
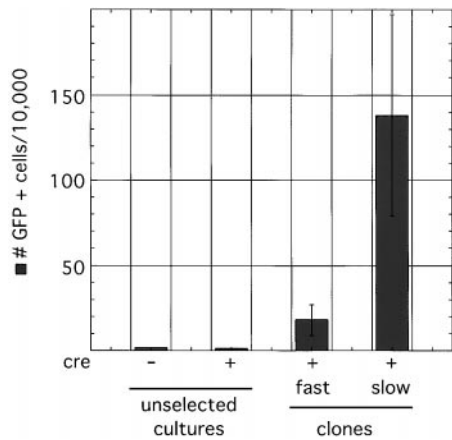
Figure 4. FACS® analysis of *Hprt*-positive clones. The number of GFP-positive cells is higher in HAT-resistant clones than in bulk unselected cultures. Slow-growing clones reactivated GFP in a higher proportion of cells than fast-growing ones.

Figure 5. Late-replicating Xi chromosomes in clones with reactivated *GFP* and *Hprt*. (A) Mapping of the *GFP* transgene insertion site by DNA FISH. A Cy3-labeled *GFP/Pgk-Puro* probe (red) was hybridized to denatured chromosomes (DAPI, blue). Enlargement of a single X chromosome is shown with the centromere staining brighter with DAPI than the rest of the chromosome. The arrow shows the site of transgene integration near the centromere. The doublet signal corresponds to sister chromatids. (B) Analysis of replication timing of the inactive chromosome. BrdU incorporation into late-replicating regions of the genome was detected using a monoclonal anti-BrdU antibody and fluorescein-anti–mouse antibody (green) on DAPI-stained metaphase chromosome spreads (blue). Xi (arrow) was identified using the *GFP/Pgk-Puro* probe (red). In all clones analyzed, the Xi with reactivated genes was late replicating. (C) An enlargement of a single late-replicating Xi.

clones, and the assay was also performed on the pools. BrdU incorporation into late-replicating regions was detected using an anti-BrdU antibody and Xi was identified by DNA FISH using a probe that detects the *GFP* transgene. In all clones and pools analyzed, the Xi marked by the *GFP* transgene was late replicating (Fig. 5, B and C). These results indicate that even after reactivating one or more genes on Xi, the chromosome as a whole remained inactive. We conclude that reactivation of Xi-linked genes after loss of Xist RNA occurs at one or a few loci and does not result in a detectable change in late replication of the chromosome.

Synergism of Xist RNA, DNA Methylation, and Histone Hypoacetylation in X Chromosome Silencing

Next, we examined the relationship between Xist RNA-mediated silencing and inactivation by other mechanisms. DNA methylation and hypoacetylation of core histones are believed to contribute to inactivation of X-linked genes (Cedar, 1988; Keohane et al., 1998). A low frequency of reactivation of X-linked genes has been observed after demethylating cells using 5-azadC (Mohandas et al., 1981; Graves, 1982). Transcriptional activation of silenced genes has also been seen after treating cells with TSA, a potent inhibitor of histone deacetylases (Yoshida et al., 1995). We wanted to see whether the *Xist* mutant cells with their already compromised ability to silence Xi are more sensitive to demethylation and/or inhibition of histone deacetylation than wild-type cells, leading to a further increase in the number of cells reactivating *GFP* and *Hprt*.

Adenovirus-Cre–infected and –uninfected *Xist* conditional mutant cells were treated with 5-azadC and/or TSA, and the number of cells that reactivated *GFP* was analyzed using FACS® (Fig. 6 A). Due to the toxicity of the drugs, the experiments were performed in SV-40 T-antigen–transformed cells. The cells were infected with adenovirus-Cre, allowed to recover, and then treated twice with 5-azadC on days 7 and 9 after infection. After allowing the cells to go through several rounds of cell division to a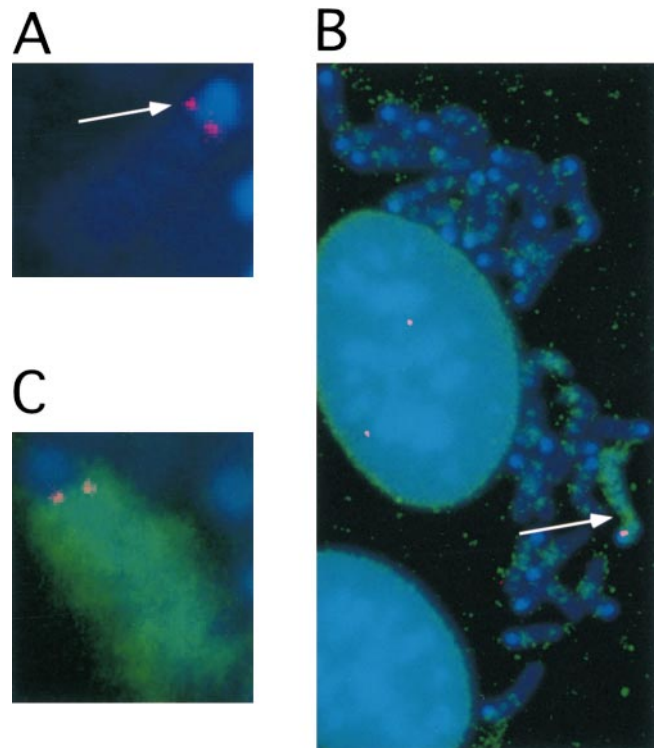chieve demethylation of DNA, half of the cultures were treated with TSA on day 12 after infection. FACS® analysis was performed on day 13. By day 13 after infection, the *Xist* deletion–induced increase in the number of *GFP* reactivants disappeared, and we could no longer observe a difference between *Xist* wild-type and *Xist*-deficient cells without drug treatment (Fig. 2 C). Inhibition of histone deacetylases by itself had no effect on the number of GFP-positive cells, whether or not *Xist* was deleted. 5-azadC–induced demethylation increased the number of *GFP*-positive cells by ∼20-fold in cells that did not delete *Xist*. However, the combined effect of *Xist* deletion and 5-azadC treatment was a 30–40-fold increase in GFP reactivants. 5-azadC treatment followed by TSA further increased the number of *GFP*-positive cells by another two-fold in both *Xist* mutant and control cells. These results are summarized in Table I. The ≤60-fold increase in the number of cells reactivating GFP indicates that the effects of Xist RNA deletion, 5-azadC, and TSA treatments are synergistic, rather than simply additive.
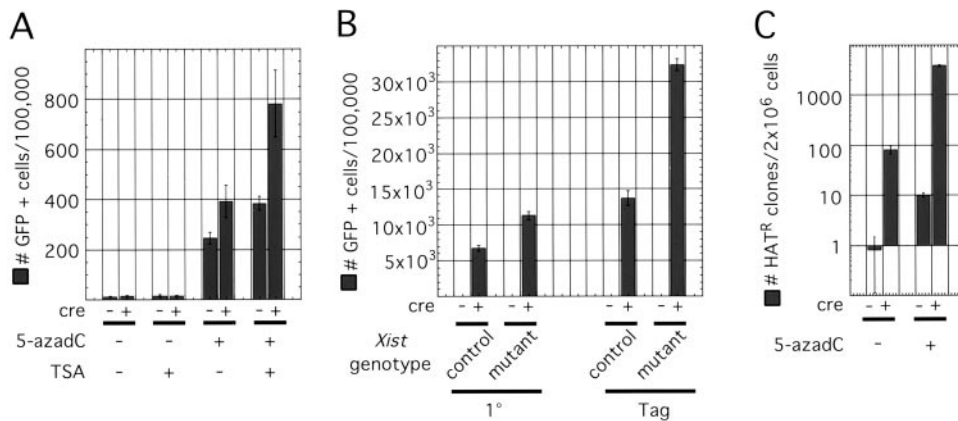
Figure 6. Synergism of X chromosome silencing mechanism. (A) FACS® analysis of *GFP* reactivation in Xist mutant fibroblasts with or without Cre infection treated with 5-azadC and/or TSA. TSA by itself had no effect. However, 5-azadC by itself, or followed by TSA treatment, led to significant *GFP* reactivation. *Xist* deleted cells were more sensitive to the treatment than those that did not delete *Xist*. (B) *GFP* reactivation in *Xist/Dnmt1* double conditional mutant fibroblasts. Control cells ($Dnmt1^{2lox/S}$ and $Xist^{+/\Delta}$) delete *Dnmt1* upon adenovirus-Cre infection, whereas *Xist* mutant cells ($Dnmt1^{2lox/S}$ and $Xist^{2lox/\Delta}$) delete both *Dnmt1* and *Xist*. Both primary (1°) and SV-40 T-antigen–immortalized (Tag) cells were analyzed. Deletion of *Dnmt1* alone led to reactivation of *GFP* in ≤7% of primary and ≤15% of T-antigen–transformed cells. Deletion of *Xist* also in addition to *Dnmt1* increased the number of GFP-positive cells about twofold. (C) The number of HAT-resistant colonies in *Xist* conditional mutant treated with 5-azadC. Cre-mediated deletion of *Xist* had a more significant effect on the number of HAT-resistant cells than 5-azadC treatment. However, deletion of *Xist* followed by 5-azadC treatment reactivated *Hprt* in a much higher proportion of cells than either treatment alone. Note the use of log scale.

5-azadC treatment leads to limited genomic demethylation (Fig. 7 A). More extensive demethylation can be achieved by deleting *Dnmt1*. To study how X inactivation is maintained in the absence of Xist RNA and DNA methylation, we bred a *Dnmt1* conditional allele (Jackson-Grusby et al., 2001) into the *Xist* mutant colony and generated double conditional mutant fibroblasts. Fibroblast genotypes were $Xist^{2lox};GFP/Xist^{\Delta},Dnmt1^{2lox/S}$, for *Xist* conditional mutants, and $Xist^{+};GFP/Xist^{\Delta},Dnmt1^{2lox/S}$, for control. The $Dnmt1^{S}$ allele is a constitutive null mutation of the gene (Lei et al., 1996). Upon adenovirus-Cre infection of these cells, both *Xist* and *Dnmt1* were deleted in *Xist* mutant cells, whereas only *Dnmt1* was deleted in the controls (Fig. 7 B). Deletion of *Dnmt1* led to a more pronounced demethylation of bulk genomic DNA than 5-azadC treatment (Fig. 7). Primary *Dnmt1* mutant fibroblasts arrested within 1 wk of adenovirus-Cre infection, whereas SV-40 T-antigen–transformed cells continued dividing, although at a much slower rate than wild-type cells (Jackson-Grusby et al., 2001). We analyzed both primary and transformed cells 7 d after infection. A much higher proportion of cells reactivated *GFP* in the *Dnmt1* mutants than in 5-azadC–treated cells (Fig. 6 B), most likely as a

result of the *Dnmt1* mutants being much more demethylated. Transformed cells consistently yielded more *GFP*, reactivants possibly because of their increased ability to proliferate and further demethylate or as a result of decreased stability of silencing in transformed cells. *Xist/Dnmt1* double mutants reactivated *GFP* in about twice as many cells as *Dnmt1* single mutants, indicating again that *Xist* and DNA methylation cooperate to silence GFP on Xi. GFP was reactivated in ≥30% of *Dnmt1*/*Xist* double mutant cells, representing an almost 3,000-fold increase over controls (Table I). Interestingly, the *Dnmt1* mutation, or even limited demethylation using 5-azadC, has a much more significant effect on the number of GFP reactivants than the *Xist* mutation, arguing that at least for the X-linked GFP transgene, DNA methylation is a more important contributor to silencing than *Xist*.

To study the combined effect of demethylation and *Xist* deletion on an endogenous X-inactivated gene, we analyzed *Hprt* reactivation in adenovirus-Cre–infected or –uninfected *Xist* conditional mutant cells, with or without 5-azadC treatment (Fig. 6 C). In adenovirus-Cre–infected (*Xist* deleted) cultures without drug treatment, we observed an ~100-fold enrichment for HAT-resistant cells

Table I. Synergistic Effect of X Chromosome Silencing Mechanisms on the Repression of GFP and Hprt

Xist RNA	Histone macroH2A enrichment*	DNA methylation	Histone hypoacetylation	Increase GFP	Increase Hprt
+	+	+	+	1×	1×
Δ	−	+	+	2–3×	100×
+	+	5-azadC	+	19×	12×
+	+	+	TSA	1×	ND
Δ	−	5-azadC	+	30×	4,800×
+	+	5-azadC	TSA	29×	ND
Δ	−	5-azadC	TSA	60×	ND
+	+	Δ	+	1,500×	ND
Δ	−	Δ	+	2,500×	ND

Fold increase in the number of *GFP* and *Hprt* expressing cells following deletion of *Xist* and/or *Dnmt1*, inhibition of DNA methylation using 5-azadC, and/or inhibition of histone deacetylation using TSA. Basal level of reactivation in untreated wild-type cells is designated as 1×. +, intact; Δ, a null mutation in the gene *Xist* or *Dnmt1*; 5-azadC, cells were demethylated using 5-azadC; TSA, histone deacetylation was inhibited.
*Although not tested in this study, Xi chromatin was presumed to be enriched in histone macroH2A in *Xist* wild type cells, and unenriched in *Xist* mutant cells.
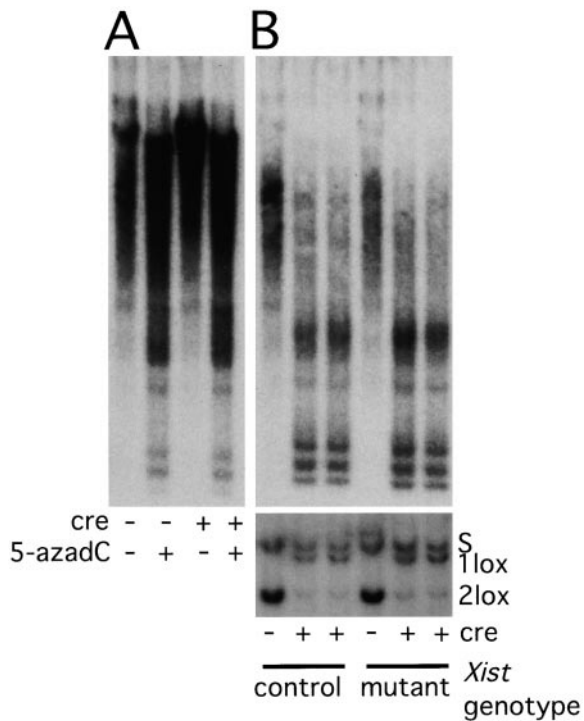
Rate of Hprt Reactivation in Xist-deficient Fibroblasts

To test whether the observed HAT-resistant colonies are the result of independent *Hprt* reactivation events or proliferation of a few reactivants, we calculated *Hprt* reactivation rates using the Luria-Delbrück fluctuation analysis (Luria and Delbrück, 1943). A large number of cultures of adenovirus-Cre–infected and –uninfected cells were expanded from a few cells (generally <100). During expansion, half of the cultures were treated with 5-azadC. After the desired culture size was reached, cells were selected using HAT, and the number of cultures that yielded HAT-resistant colonies and the reactivation rates were determined (Table II). The positive cultures ranged from those containing one clone to those containing confluent plates, indicating that the reactivation event took place at different times during cultivation. The spontaneous reactivation rate of *Hprt* was as low as previously reported mutation rates in wild-type cells (10^{-9}) (Chen et al., 1998), demonstrating the remarkable stability of X chromosome silencing. The reactivation rate increased by ~160-fold after deletion of *Xist* and 60-fold after 5-azadC treatment, indicating that both Xist RNA and DNA methylation contribute significantly to silencing. However, combining *Xist* deletion with 5-azadC treatment resulted in an almost 10,000-fold increase, confirming synergism of DNA methylation and Xist expression in the maintenance of the inactive state.

Discussion

We generated fibroblasts in which reactivation of two genes on the X chromosome, a *GFP* transgene and the endogenous *Hprt* gene, can be detected at a low frequency. We studied the effect of loss of Xist RNA, demethylation of genomic DNA and inhibition of histone deacetylation, on the maintenance of X inactivation. We observed that deletion of *Xist* leads to reactivation of *GFP* and *Hprt* in a very small proportion of cells and conclude that *Xist*, though not essential for the maintenance of X inactivation, contributes to the stability of the inactive state. We further showed that Xist RNA, histone deacetylation, and DNA methylation act synergistically to achieve the extraordinary stability of X chromosome silencing, with reactivation rates comparable to mutation rates.

Synergism of X Inactivation Mechanisms

It has been shown that during the early stages of cellular differentiation, an Xist RNA–mediated silencing mechanism initiates X inactivation (Penny et al., 1996; Marahrens et al., 1997; Wutz and Jaenisch, 2000), and that *Xist* becomes dispensable for the maintenance of X inactivation after subsequent differentiation (Brown and Willard, 1994; Rack et al., 1994, Csankovszki et al., 1999). However, we now present direct evidence that an Xist RNA–mediated maintenance mechanism contributes to silencing in somatic cells. Deletion of *Xist* reduces the histone macroH2A1 content of Xi chromatin (Csankovszki et al., 1999), and it is possible that this change in histone compo-

Figure 7. Demethylation of genomic DNA. (A) Demethylation of genomic DNA after 5-azadC treatment of cells. Adenovirus-Cre–infected or –uninfected *Xist* conditional mutant cells were treated with 5-azadC or were left untreated. Genomic DNA was isolated and digested with the methylation-sensitive restriction enzyme HpaII. The blotted DNA was hybridized with an IAP probe to analyze demethylation of bulk genomic DNA. Demethylation due to 5-azadC treatment is indicated by the appearance of low molecular weight bands. (B) Adenovirus-Cre–mediated recombination in *Dnmt1²ˡᵒˣ/ˢ; Xist⁺/ᐞ* (control) and *Dnmt1²ˡᵒˣ/ˢ; Xist²ˡᵒˣ/ᐞ* (mutant) cells. Only *Dnmt1* recombination in SV-40 T-antigen–immortalized cells is shown on Southern blots of SpeI-digested DNA hybridized with the HV probe (lower blot). Nearly 100% recombination is seen in both controls and mutants. The same genomic DNA samples were also digested with HpaII and hybridized with the IAP probe (upper blot). The appearance of more low molecular weight bands and the disappearance of high molecular weight bands indicate that the genomic DNA in these samples is more extensively demethylated than in 5-azadC–treated cultures.

over uninfected (*Xist* wild type) cells. 5-azadC treatment of *Xist* wild-type cells resulted in a less significant 10-fold increase in the number colonies per plate. However, 5-azadC treatment of *Xist* mutant cells resulted in a 50-fold increase in HAT-resistant cells over untreated *Xist* mutant cells and an almost 5,000-fold increase over untreated *Xist* wild-type cells (Table I). The combined effect of *Xist* deletion and demethylation is more significant than either treatment alone, implying a synergistic interaction of Xist RNA and DNA methylation in keeping *Hprt* silent. It is interesting to note that although the absolute numbers of GFP- and Hprt-positive cells are different, 5-azadC treatment led to comparable enrichment in reactivants for both genes (19- and 12-fold, respectively). However, Xist RNA seems to play a more major role in *Hprt* silencing than in *GFP* silencing (100-fold enrichment compared with two- to threefold enrichment). The *GFP* transgene may not be

Table II. Hprt Reactivation Rates

Xist RNA	DNA methylation	No. independent cultures	No. positive cultures	Average no. cells/culture	Reactivation rate
+	+	46	1	3.55×10^6	6.2×10^{-9}
+	5-azadC	46	33	3.13×10^6	4.0×10^{-7}
Δ	+	49	36	1.32×10^6	1.0×10^{-6}
Δ	5-azadC	90	54	1.77×10^4	5.2×10^{-5}

Hprt reactivation rates were determined using the P0 method of Luria and Delbrück (1943). A number of independent cultures of *Xist* conditional mutant fibroblasts infected with adenovirus-Cre (Δ) or left uninfected (+) were expanded to the appropriate size. When appropriate, cultures were treated with 5-azadC to inhibit DNA methylation. After HAT selection, the number of cultures with HAT-resistant clones was determined, and *Hprt* reactivation rates were calculated.

sition leads to compromised efficiency of silencing. However, to directly assess the role of histone macroH2A1 in X chromosome inactivation, analysis of a targeted mutation of the gene will be necessary.

DNA methylation is another significant contributor to silencing. Demethylation by 5-azadC has been used before to reactivate genes on Xi (Mohandas et al., 1981; Graves, 1982). In this study, we also introduced a *Dnmt1* mutation that proved to be ~100 times as effective as 5-azadC treatment in achieving reactivation of *GFP*. Demethylation combined with *Xist* deletion increased reactivation rates to a greater extent than either did alone, indicating synergism of silencing mechanisms in keeping Xi silent. In the case of the *GFP* transgene, we accomplished ≥30% reactivation in *Xist/Dnmt1* double mutant fibroblasts. However, at least in the case of *Hprt*, the majority of the cells maintained silencing even after deletion of *Xist* and demethylation, indicating the presence of additional stabilizing mechanisms, such as late replication.

Partial compensation for reduced methylation on Xi by other silencing mechanisms occurs in ICF patients. Xi in these patients lacks methylation on CpG islands (Hansen et al., 2000) and, therefore, presumably histone deacetylation is also compromised. However, Xist RNA localizes normally to Xi and, together with late replication, is able to maintain X inactivation, although with reduced efficiency (Hansen et al., 2000).

Inhibition of histone deacetylation by TSA led to a small increase in the number of *GFP* reactivants. One possible explanation for the modest effect of histone deacetylase inhibitors is the slow rate of histone acetate turnover on the Xi of differentiated cells leading to limited enrichment in acetylated histones after TSA treatment (Keohane et al., 1998). Indeed, treatment of cells with another histone deacetylase inhibitor, sodium butyrate, did not increase antiacetylated histone staining on Xi (Jeppesen and Turner, 1993). Furthermore, TSA only inhibits *GFP* silencing in demethylated cells. A similar dependence of reactivation of genes by TSA on demethylation has been observed by others studying genes silenced in cancer (Cameron et al., 1999), implying that in these cases DNA methylation is the primary mechanism that recruits histone deacetylases to the silenced loci, possibly via binding of methyl-DNA binding proteins (Jones et al., 1998; Nan et al., 1998). In other cases, histone deacetylase inhibitors alone were sufficient to achieve reactivation, such as reexpression of the FMR1 gene in cells derived from fragile X syndrome patients, whereas synergism of histone deacetylation and DNA methylation was still observed (Chiurazzi et al., 1999).

The synergism of multiple silencing mechanisms to assure a highly stable repressed state possibly reflects the importance of dosage compensation for the proper functioning of the organism. It has been demonstrated that gene dosage imbalance in the embryo (Takagi and Abe, 1990), or in the extraembryonic tissues (Marahrens et al., 1997), causes lethality. However, partial reactivation of the chromosome appears to be tolerated in vitro, and it is possible to isolate and culture cells with reactivated X-linked genes. Yet, our fibroblast clones with reactivated Xi-linked genes grew more poorly than fibroblasts of the same genotype that did not reactivate any gene. These results argue that gene dosage imbalance is detrimental to cell growth even in vitro and that clones that reactivated the entire chromosome may not be viable. Therefore, our calculations of reactivation frequencies might be an underestimation, as cells with reactivated chromosomes may die before they can be detected.

Xist RNA and DNA Methylation Contribute Differently to Silencing of Two X-linked Genes

The two genes analyzed, *GFP* and *Hprt*, are both subject to X inactivation, but the reactivation rates are different. In the presence of Xist RNA and wild-type levels of methylation and histone H4 acetylation, *Hprt* is almost never reactivated, whereas *GFP* reactivation can be readily observed. The difference can be at least partially attributed to the difference in assays. Although reactivation of *GFP* can be observed almost instantly on FACS®, detection of *Hprt* reactivants by HAT selection requires that the cells survive reactivation and maintain their proliferative capacity. The smallest detectable HAT-resistant colony contained ~30 cells, the result of about five cell divisions.

The transgenic nature of *GFP* might also influence the way the gene responds to silencing mechanisms. Xist RNA may not be able to exert the same level of control over a transgene as over endogenous genes, therefore deletion of *Xist* has a smaller effect on *GFP* reactivation than on *Hprt* reactivation. On the other hand, DNA methylation contributes significantly to the silencing of both genes. Randomly integrated transgenes are frequently methylated (Hertz et al., 1999), which may contribute to their variable expression. *GFP* is integrated near the centromere and therefore might also be subject to silencing by centric heterochromatin. Nonspecific silencing of GFP by mechanisms unrelated to X inactivation (Eggan et al., 2000) may explain the rapid disappearance of GFP reactivants from the cultures. The observed influence of different mechanisms on *Hprt* may more faithfully represent silencing of endogenous X-inactivated genes.

Mechanism of X Chromosome Silencing

In recent years, significant progress has been made in deciphering how DNA and histone modifications regulate transcription. Methylation of DNA is thought to change accessibility of chromatin via binding of methylated DNA binding proteins that in turn can recruit histone deacetylases (Jones et al., 1998; Nan et al., 1998). Acetylated NH_2-terminal lysines of histones, aside from affecting compactness of chromatin packaging, can bind the bromodomain module present in a wide range of chromatin remodeling proteins (Dhalluin et al., 1999; Jacobson et al., 2000).

The mechanism of Xist RNA–mediated silencing is not as well understood. Xist RNA is involved in preferential localization of histone macroH2A1 to Xi (Csankovszki et al., 1999). However, we do not know whether histone macroH2A1 and Xist RNA interact directly and to what extent histone macroH2A1 participates in silencing. It has been shown that Xist RNA can silence in the absence of DNA methylation (Panning and Jaenisch, 1996), or even before cellular differentiation (Wutz and Jaenisch, 2000). Xist RNA can also accomplish transcriptional silencing without the chromosome becoming late replicating or hypoacetylated (Wutz and Jaenisch, 2000). This study provides evidence that the Xist RNA–mediated silencing mechanism acts synergistically with other silencing factors. Xist RNA, similarly to DNA methylation, appears to act in a localized manner, not as a master switch regulating the entire chromosome. Our observations on the effects of deletion of *Xist* and earlier studies on 5-azadC reactivated clones (Mohandas et al., 1981; Graves, 1982) indicate that reactivation of genes on Xi is neither coordinate nor independent, as reactivation of one gene correlates with reactivation of other linked genes only to a limited extent. *Xist* mutant clones with reactivated genes, in which the lack of Xist RNA–mediated silencing has phenotypic consequences, provide a useful reagent for further dissecting out how Xist RNA might accomplish silencing.

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