Gene reactivation: a tool for the isolation of mammalian DNA methylation mutants

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We report the isolation and characterization of a mammalian strain (*tsm*) that has a temperature-sensitive mutation in DNA methylation. The isolation procedure was based on the observation that treatment of a CHO TK⁻ MT⁻ cell line with demethylating agents introduces up to 46% demethylation, resulting in phenotypic reversion and transcriptional activation of the thymidine kinase (TK) and metallothionein (MT) genes at frequencies ranging from 1% to 59%. Seven thousand individual colonies from an EMS-mutagenized CHO TK⁻ MT⁻ population were screened for spontaneous reversion to TK⁺ phenotype after treatment at 39°C. Successful isolates were subsequently examined for MT⁺ reversion. A single clone (*tsm*) was obtained that showed temperature-dependent reactivation of both TK and MT genes at frequencies of 7.2×10^{-4} and 6×10^{-4} , respectively. The *tsm* cells were viable at 39°C and showed no increased mutation frequency. Reactivation correlated with transcriptional activation of the respective genes, whereas backreversion to the TK⁻ phenotype was associated with transcriptional inactivation. TK⁻ backrevertants were reactivable again with demethylating agents. Although demethylation in *tsm* cells was not detectable by HPLC, Southern blot analysis revealed that reactants, irrespective of their mode of generation, showed specific demethylation of both TK and MT genes. Also, after about 150 cell generations after treatment, reactants from both temperature-induced *tsm* and cells exposed to demethylating agents gained 60% and 23%, respectively, in 5-methylcytosine (5mC). It is proposed that the phenotype of *tsm* cells is due to a mutation involved in the regulation of DNA methylation. The further characterization of this and other mammalian mutants should help to clarify the physiological role of DNA methylation, as well as its regulation.

[Key Words: DNA methylation; temperature-sensitive mutant; CHO cells; gene reactivation; thymidine kinase; metallothionein]

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The search for a role for DNA methylation in eukaryotes has focused on hypotheses that 5-methylcytosine (5mC) residues in the DNA may affect the transcriptional activity of genes (Holliday and Pugh 1975; Riggs 1975). Methylated bases may regulate gene activity by modulating DNA-protein interactions, which are well known in the case of prokaryotic DNA methylation. The methylated or nonmethylated state could be faithfully transmitted through cell division by the action of a maintenance methylase, which may act on hemimethylated DNA at the replication fork, but not on nonmethylated DNA. Changes in methylation patterns, which represent either de novo methylation of previously unmethylated sites or loss of methyl groups, may introduce alterations in gene expression. Evidence that DNA methylation has a role in gene expression has come from a variety of sources. A general correlation between hypomethylation and gene activity has been demonstrated, particularly from transfection experiments (for reviews, see Doerfler 1983; Razin et al. 1984; Adams and Burdon 1985).

Although methyltransferase enzymes have been partially purified, the actual specificity and regulation of DNA methylation in eukaryotes is not yet understood. The mouse DNA methylase shows preference for hemimethylated substrate (Adams et al. 1979; Gruenbaum et al. 1982), whereas the enzyme purified from rat liver shows no specificity for the methylation state of the substrate DNA (Simon et al. 1983). The relationship of these activities to DNA maintenance or de novo methylation in vivo is far from clear.

Inhibitors of DNA methyltransferases such as 5-azacytidine (5-Aza-CR) or 5-deoxyazacytidine (5-Aza-CdR) have been used frequently to study the effect of DNA demethylation in mammalian systems. Treatment with micromolar concentrations of 5-Aza-CR induces differentiation of cells in culture and reactivation of previously inactive genes in a variety of systems (for review, see Jones 1985). Thus, growth in 5-Aza-CR has been reported to induce the reversion to normal phenotype of enzyme-deficient human (Ivarie and Morris 1986), hamster (Harris 1982, 1984a,b, 1986; Steglich et al. 1985; Jeggo and Holliday 1986; Stallings et al. 1986), rat (Ivarie and Morris 1982; Sugiyama et al. 1983; Delers et al. 1984; Goss 1984), and mouse (Compere and Palmiter

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1981; Christy and Scangos 1982, 1984; Clough et al. 1982; Nakamura and Okada 1983; Liteplo et al. 1984; DeVries et al. 1986; Hickey et al. 1986) cell lines. There are also reports of 5-Aza-CR-induced reactivation of silent retroviral genes (Groudine et al. 1981; Niwa and Suguhara 1981; Conklin et al. 1982; Searle et al. 1984). In most of these instances, phenotypic changes have been associated with detectable changes in the methylation patterns of the corresponding genes. However, since 5-Aza-CR may also affect other biological pathways (Lu and Randerath 1980; Tsao et al. 1984), it is possible that demethylation is not the only explanation for the observed effects.

In an attempt to define DNA sequences involved in regulation of gene expression by methylation, cloned genes have been fully or partially methylated *in vitro*. These constructs were then introduced into cells, and their expression was studied. Methylation in the promoter and 5' gene sequences generally led to inactivity, whereas methylation in the coding or 3' sequences had no effect on gene activity (Kruczek and Doerfler 1982; Stein et al. 1982; Busslinger et al. 1983; Langner et al. 1984; Keshet et al. 1985; Murray and Grosveld 1985). In at least two instances, the herpes simplex virus (HSV) thymidine kinase (TK) and the mouse metallothionein I (MTI) genes, methylation of the coding sequences has also been shown to be important for regulation of gene expression (Christy and Scangos 1984; Keshet et al. 1985). It was recently reported that reactivation of the TK locus in Chinese hamster fibroblasts by MNNG is associated with demethylation of 5' promoter sequences, which were, however, the only part of the gene studied in this report (Barr et al. 1986; Dobrovic, pers. comm.). Induction of MTI expression by UV irradiation is associated with extensive demethylation of the gene in mouse S49 lymphoma cells (Lieberman et al. 1983). However, it is difficult in studies using *in vitro* modified constructs to dissociate the direct effect of DNA methylation in gene expression from nonspecific effects.

A powerful approach for studying the biological role and regulation of DNA methylation is the isolation of mutants. In the present report a CHO TK⁻ MT⁻ cell line has been used for this purpose. Chinese hamster ovary (CHO) cells were initially isolated by Puck et al. (1958) and thereafter used extensively in somatic cell genetic studies. Although chromosomal rearrangements have occurred in these cells, the total DNA content is close to that of normal Chinese hamster genome. The large number of recessive mutants isolated from CHO strains suggests that they are functionally hemizygous for a large number of their genes (Siminovitch 1976). It has been demonstrated recently that several recessive mutations in CHO cells revert to the wild type upon treatment with demethylating agents (Harris 1984a,b; Steglich et al. 1985; Jeggo and Holliday 1986). These findings place functional hemizygosity under a different perspective since they suggest that epigenetic events might underlie stable heritable variation. Irrespective of the mechanism responsible for functional hemizygosity, CHO cells are particularly suitable for the isolation of mutants of mammalian cells.

We report here the isolation of a CHO cell line that reverts to the TK⁺ MT⁺ phenotype upon induction at 39°C. The selection procedure was based on the observation that demethylating agents induce reversion to TK⁺ and MT⁺ phenotypes, suggesting that the expression of these genes is under methylation control. Reversion was associated with transcriptional activation and specific changes in the methylation patterns of the TK and MT genes. High-frequency spontaneous backreversion to the TK⁻ phenotype was associated with transcriptional inactivation and remethylation of the TK gene. These backrevertants could be induced to revert again to the TK⁺ phenotype by treatment with demethylating agents. Our observations suggest that the isolated cell line has a mutation in the regulation of DNA methylation.

Results

Reactivation of TK and MT genes in CHO cells by 5-Aza-CR and 5-Aza-CdR

The CHO TK⁻ MT⁻ subline is a derivative of the CHO KI (MT⁻) line. The spontaneous incidence of reversion to TK⁺ in these cells is about 10⁻⁷. 5-Aza-CdR was used at concentrations ranging from 0.05 to 0.4 µg/ml (Creusot et al. 1982) to examine whether demethylating agents revert these cells to TK⁺ and MT⁺ phenotypes. The frequencies of reversion to the TK⁺ phenotype, estimated by growth in HAT medium (Szybalski et al. 1962; Littlefield 1964), are shown in Figure 1a. Maximum TK⁺ reversion was observed using 0.4 µg/ml 5-Aza-CdR, with a recovery period of 2 days after treatment. Under these conditions less than 20% of cells survived the treatment, but up to 28% of the surviving cells expressed the TK⁺ phenotype. Cells treated with 5-Aza-CdR were also examined for reversion to MT⁺ phenotype. Up to 59% of the cells surviving 5-Aza-CdR treatment expressed the MT⁺ phenotype, as revealed by their resistance to 2 µM CdCl₂ (Fig. 1b).

Reversion to the TK⁺ phenotype was also examined after treatment with 5-Aza-CR, which is significantly less toxic. Treatment of cells for a period of 24 hr with 5-Aza-CR induced up to 1% reversion to the TK⁺ phenotype in a dose-dependent manner. Optimum frequencies of reversion were obtained in the range of 1–3 µg/ml. In this concentration range, more than 80% of cells survived the treatment (data not shown).

The massive induction of reversion to TK⁺ or MT⁺ by demethylating agents greatly exceeds the level expected from exposure to mutagens (Roufa et al. 1973; Adair et al. 1980). To confirm this, direct comparisons were made using the DNA-damaging agents EMS and X rays (Table 1). The mutagenesis experiments were performed using doses that allowed 10% survival (300 µg/ml EMS or 500 rads X rays). The mutation frequency was then assayed by selection in 6-thioguanine (6-TG) and compared to TK⁺ reversion induced by the same treatment. EMS and X rays increased the level of TK⁺ reversion by two orders of magnitude. However, this is still 10³–10⁴ times lower than reversion induced by demethylating agents.

Isolation of mammalian DNA methylation mutants

Figure 1. (A) TK⁺ reversion induced by 5-Aza-CdR treatment. CHO TK⁻ MT⁻ cells were treated with graded concentrations of 5-Aza-CdR from a 1 mg/ml stock solution kept in -70°C . After 24 hr the cultures were rinsed with PBS and allowed to recover for 2 ($\blacktriangle$), 5 ($\triangle$), and 7 ($\blacklozenge$) days before HAT selection. Each point represents the average colony count of three petri dishes seeded after treatment. (B) Reversion to the MT⁺ by 5-Aza-CdR treatment of CHO TK⁻ MT⁻ cells. CHO TK⁻ MT⁻ cells were treated with graded concentrations of 5-Aza-CdR as described above. Twenty-four hours later flasks were rinsed with PBS and cells were allowed to recover in normal medium for 2 ($\triangle$), 5 ($\diamond$), and 9 ($\blacklozenge$) days. After recovery cells were plated in medium containing 2 mM CdCl₂ and incubated for 7–9 days. Each point represents the average colony count of three petri dishes corrected for the fraction of cells that survived treatment. (C) TK⁺ reversion of the *tsm* mutant. *tsm* cells were treated for variable length of time at 39°C. Reversion frequency was estimated as a function of the cells surviving the treatment. Each point represents the average reversion of three plates seeded with 10^5 cells each. (D) MT⁺ reversion of the *tsm* mutant. *tsm* cells were treated for variable length of time at 39°C, and after 2 days recovery they were plated under Cd selection. Reversion frequency was estimated as a function of the cells surviving the treatment. Each point represents the average reversion of the three plates seeded with 10^5 cells each. The graph presents results of two experiments.

Isolation and characterization of temperature-sensitive mutants with altered DNA methylation

If demethylation is responsible for reversion to the TK⁺ and MT⁺ phenotypes, a mutant that spontaneously loses methyl groups should show high levels of rever-

sion to TK⁺ and MT⁺. On this basis a procedure was designed for the isolation of methylation-deficient mutants. It was decided to search for mutants that reverted in a temperature-sensitive manner to the TK⁺ phenotype and to subsequently examine these for reversion to MT⁺.

Table 1. Reversion to the TK⁺ phenotype induced by mutagenic and nonmutagenic agents

Treatment	TK ⁺ frequency	Approximate increase in TK ⁺ frequency
Spontaneous rate	$<10^{-7}$	
EMS (300 $\mu\text{g/ml}$)	1×10^{-4} – 2.5×10^{-5}	10^2 – 10^3
X-rays (500 rads)	8×10^{-5} – 2.5×10^{-5}	10^2 – 10^3
5-Aza-Cr (2 $\mu\text{g/ml}$)	1.5×10^{-2} – 0.5×10^{-2}	10^5 – 10^6
5-Aza-CdR (0.2 $\mu\text{g/ml}$)	2.8×10^{-1} – 1×10^{-2}	10^6 – 10^7

Cells were mutagenized by treatment with EMS (see Materials and methods). To eliminate TK⁺ cells resulting directly from the mutagenesis, EMS-treated CHO TK[−] MT[−] populations were plated for single colonies in medium supplemented with BUdR. After 7–8 days of incubation at 34°C, medium was removed, and the colonies were overlaid with 0.5% soft agar containing normal growth medium. These were left to grow for a further 2 days. Individual colonies were then transferred to replica wells in two separate 24-well plates. When the average number of cells per well was about 10^3 , one of each replica plate was transferred to 39°C. After allowing 24 hr for the expression of temperature-sensitive mutations, the test plate was returned to 34°C and put under HAT selection. For any well where reversion to the TK⁺ phenotype was detected, the cells from the reference plate were isolated and subjected to quantitative studies.

Seven thousand individual colonies were screened with this procedure, and 11 preliminary isolates were obtained for the secondary screening. Two of the 11 clones proved to be true positives and were studied further. One of them showed constitutive reversion to TK⁺ at both temperatures with a frequency of 5×10^{-5} , but did not show detectable reversion to the MT⁺ phenotype. The other clone expressed temperature-dependent reversion to both TK⁺ and MT⁺ phenotypes at frequencies of 7.2×10^{-4} and 6×10^{-4} (Fig. 1c,d) and was designated *tsm* (temperature-sensitive methylation). Thus, the *tsm* isolate showed reversion frequencies about 10^3 – 10^4 times higher than the parental strain at 39°C, whereas at 34°C there was no significant difference.

Maximum TK⁺ or MT⁺ reversion was obtained after 24–48 hr treatment at 39°C, whereas no reversion was detected for treatments shorter than 16 hr (Fig. 1c,d). These results suggest that at least one S phase or cell division at 39°C is required for expression of the *tsm* phenotype. Longer exposure of the cells to high temperature did not increase the frequency of reversion.

tsm cells remained viable during 2 months of growth at 39°C, showing that the temperature-sensitive phenotype expressed in these cells is not lethal. The doubling time of the *tsm* strain was 23 hr at 34°C, which is 5 hr slower than the equivalent of the CHO TK[−] MT[−] cells. The doubling time decreased to 16 hr at 39°C, but it was

still 2 hr slower than that of CHO TK[−] MT[−] cells at 39°C. To define the initial growth response of *tsm* at 39°C, 10^4 cells were plated in each well of a multiwell plate and a growth curve was obtained at 34°C. Then the multiwell plate was transferred to 39°C, and estimation of the growth rate continued until cells reached confluency. The growth rate of *tsm* cells immediately increased at the high temperature, indicating that no significant growth interruption or cell killing is caused by this treatment.

The mutability of *tsm* cells at 39°C was also examined in comparison to that of the CHO TK[−] MT[−] cell line. The mutation frequency was estimated by resistance to 6-TG (loss of HPRT activity); it was found to be $<10^{-6}$ in both cell lines, indicating that *tsm* does not show a significantly elevated spontaneous mutation rate.

Stability of the TK⁺ revertant phenotype

The rate of spontaneous backreversion to the TK[−] phenotype by the TK⁺ revertant clones might indicate whether the inactivation of this gene is a result of genetic or epigenetic events. The frequency of backreversion was estimated by fluctuation analysis. Ten populations from each revertant were used, and estimation of the reversion rates was based on both the method of Luria and Delbruck (1943) and its modification by Lea and Coulson (1949). TK⁺ revertant cells induced by treatment with 5-Aza-CR and 5-Aza-CdR showed rates of spontaneous backreversion to TK[−] of 1.5×10^{-4} and 5.6×10^{-4} , respectively, whereas TK⁺ clones induced by heat treatment of the *tsm* strain backrevert to the TK[−] five times less frequently (6×10^{-5}). The rate of backreversion in all the clones studied was about three orders of magnitude higher than the reported spontaneous mutation frequency of the TK locus in Chinese hamster cells (Roufa et al. 1973; Adair et al. 1980). The instability of TK⁺ revertants suggests that backreversion might also be a consequence of epigenetic events. Indeed, treatment of TK[−] backrevertants with 1 $\mu\text{g/ml}$ 5-Aza-CR for 24 hr resulted again in high-frequency reversion (6.2×10^{-3} to 1.3×10^{-2}) to the TK⁺ phenotype. These results indicate that the phenotypic alterations studied are the result of epigenetic events.

Phenotypic alterations correlate with changes in transcriptional activity of TK and MT genes

Poly(rA)⁺ RNA was prepared from the parent CHO KI, CHO TK[−] MT[−] and *tsm* cell lines, TK⁺ revertant and TK[−] backrevertant clones, as well as from *tsm* MT⁺ revertant clones. The RNAs were separated by electrophoresis through agarose denaturing gels and blotted onto nitrocellulose filters. A Chinese hamster TK cDNA provided by Dr. J. Lewis (Lewis 1986) was used as a probe for detection of the TK transcript. A Chinese hamster cDNA MTI probe was used for detection of MT transcripts. This probe also recognizes coding sequences of the MTII transcript, which is expected to migrate with the same mobility (Griffith et al. 1983). To monitor the

levels of RNA loaded per lane, hybridization was also performed using a hamster APRT probe.

As shown in Figure 2a, TK⁺ revertants generated either by treatment at 39°C or with demethylating agents show manyfold increase in the levels of TK transcript compared with parent cells. Backreversion to the TK⁻ phenotype correlates with reduction in the level of TK transcripts to that of the parent CHO KI cells. The low levels of TK homologous mRNA in TK⁻ cells could be due either to mitochondrial TK transcripts or to a mutated cytosolic TK mRNA. The presence of a processed TK gene has also been reported in Chinese hamster cells (Barr and Lieberman 1987).

Similarly temperature-induced reversion of *tsm* to MT⁺ phenotype correlated with transcriptional induction of MT mRNA (Fig. 2b). The higher-molecular-weight band in Figure 2b may be an intermediate product of RNA processing. The lack of selective procedure for the MT⁻ phenotype prevented transcriptional analysis of backrevertants.

Finally, the level of APRT transcript is not affected by the phenotype of the cells, indicating that transcriptional alterations are restricted to the TK and MT genes. These results suggest that there is a direct correlation between the phenotypic alterations described above and transcriptional activity of the TK and MT genes. Therefore, the *tsm* mutation is likely to affect the transcriptional regulation of at least two independent genes, TK and MT.

HPLC analysis of the 5mC content in the CHO genome

The results described above suggest that reversion of CHO TK⁻ MT⁻ and *tsm* cells to the TK⁺ or MT⁺ phenotype upon 5-Aza-CR, 5-Aza-CdR, or temperature treatment is associated with demethylation. This was examined directly by measuring the level of 5mC by HPLC chromatography. Up to 46% reduction in the 5mC level of CHO TK⁻ MT⁻ cells was detected immediately after treatment with 5-Aza-CR or 5-Aza-CdR (Table 2). No significant remethylation was detected for the first 10 cell generations (cgs), whereas after 50 cgs methylation nearly reached the levels before treatment. Surprisingly, TK⁺ clones isolated from 5-Aza-CR or 5-Aza-CdR-treated cells had significantly higher 5mC levels than the CHO TK⁻ MT⁻ strain 150 cgs after treatment (Table 2).

Demethylation was not detectable in DNA from *tsm* cells after 24–48 hr growth at 39°C, although because the sensitivity of the method is limited, a methylation change of only 5–10% could not be detected significantly. However, *tsm* TK⁺ revertant cells induced at 39°C also showed a gradual increase in their 5mC content compared to the parent *tsm* strain. Thus, up to 60% higher methylation was detected in these cells some 150 cgs after temperature treatment. This increase in the levels of 5mC could suggest that the mutation in the *tsm* strain might affect the overall control of DNA methylation.

Figure 2. (A) Analysis of the TK transcript. Two micrograms per lane of poly(rA)⁺ RNA from CHO KI (wt), CHO TK⁻ MT⁻ (tk⁻), *tsm*, their TK⁺ derivatives induced by either 5-Aza-CR (actk⁺) or heat treatment (tsmtk⁺), and a *tsm* spontaneous backrevertant (tsmtk⁺→⁻) were electrophoresed through a 1% agarose-formaldehyde gel. This was blotted onto nitrocellulose and hybridized against a ³²P-labeled TK cDNA probe. (B) Analysis of MT transcripts. Poly(rA)⁺ RNAs were prepared from CHO KI (wt), CHO TK⁻ MT⁻ (tk⁻) and *tsm*, and two *tsm* MT⁺ revertants (*tsmcd*r1, *tsmcd*r2). These were electrophoresed through a 1% denaturing agarose/formaldehyde gel and blotted onto nitrocellulose filters. The Chinese Hamster MTI cDNA was labeled with ³²P and used as a probe against these filters. The amount of RNA per lane was monitored by hybridization against APRT in both A and B.

Table 2. The proportion of methylated cytosine in CHO KI cells and various derivatives after 5-Aza-CR or 5-Aza-CdR treatment

Strain	Treatment	Number of determinations	5mC/5mC + C(%)	
			Range	Mean
CHO KI	—	5	2.32–2.68	2.5
CHO TK ⁻ MT ⁻	—	4	2.4–2.8	2.6
CHO TK ⁻ MT ⁻	immediately after 5-Aza-CR treatment	3	1.32–1.48	1.4
CHO TK ⁻ MT ⁻	immediately after 5-Aza-CdR treatment	4	1.41–1.45	1.43
CHO TK ⁺	5-Aza-CR followed by 150 cgs growth	4	2.83–3.23	3.03
CHO TK ⁺	5-Aza-CdR followed by 50 cgs growth	1	2.56	2.56
CHO TK ⁺	5-Aza-CdR followed by 150 cgs growth	2	2.96–3.46	3.21
<i>tsm</i>	—	6	2.6–3.0	2.8
<i>tsm</i>	24 hr at 39°C	3	2.55–3.05	2.8
<i>tsm</i>	48 hr at 39°C	3	2.51–3.01	2.76
<i>tsm</i> TK ⁺	24 hr at 39°C followed by 50 cgs growth	1	3.23	3.23
<i>tsm</i> TK ⁺	24 hr at 39°C followed by 150 cgs of growth	2	4.46–4.6	4.53

Demethylation is associated with reactivation of the TK and MTI genes

A sensitive method for detecting alterations in the methylation pattern of specific genes is restriction analysis using methylation-inhibited enzymes. Therefore, the methylation patterns of the TK gene were studied in the CHO KI cell line, the CHO TK⁻ MT⁻ subline, the *tsm* strain, and their derivatives. Genomic DNAs were digested with *Eco*RI or *Hind*III, in combination with *Hpa*II or *Hha*I which cleave the CCGG or GCGC sequences when the internal cytosine is unmethylated but not when methylated. Digested DNAs were electrophoresed, Southern blotted onto nitrocellulose filters, and hybridized to genomic probes covering the TK gene (Lewis et al. 1983; Lewis 1986).

The 5.5-kb fragment covering the first two exons, the promoter sequences, and approximately 2 kb of 5'-flanking sequences (Lewis 1986) was initially used as a probe (Fig. 3a,b). Methylation changes associated with the phenotype were not detected using the 5.5-kb probe (Fig. 4). However, this does not eliminate the possibility that specific changes might occur within this region, since DNA fragments smaller than 300 bp created by specific demethylation would not be detectable with the sensitivity of our screening procedure. The 5.2-kb fragment spanning the last three exons and nearly 2 kb of 3'-flanking sequences was completely methylated at *Hpa*II and *Hha*I restriction sites in both TK-expressing and nonexpressing cells. Similarly, the 2.6- and 1.8-kb intronic *Eco*RI fragments were found fully methylated for these sites both in TK⁻ and TK⁺ cells (results not shown).

Although no changes in the methylation status of the TK gene were detected using the above probes, hybridization with the 4.2-kb internal fragment covering the third and fourth exons of the gene (Fig. 3a–c) revealed

Figure 3. (A) *Eco*RI subclones derived from recombinant phages containing the cloned Chinese Hamster TK gene (kindly provided by Dr. John Lewis; Lewis 1986). The 3' and 5' shaded boxes represent approximately 80 bp and 2.4 kb of phage DNA sequences, respectively. The entire *Eco*RI fragments were used as probes for analysis of the methylation pattern of the TK gene. (B) Restriction map of the TK gene for *Eco*RI (○) and *Hind*III (▽) sites. Position of the outermost *Eco*RI sites was estimated by Barr et al. (1986). Numbered solid boxes (1–7) indicate the approximate position of the exons. (C) Expanded map of the *Eco*RI-*Hind*III fragment covering the third and fourth exons as well as the putative methylation regulatory region. (a–h) Fragments detected by hybridization of the 4.2-kb *Eco*RI-*Eco*RI probe in *Eco*RI/*Hpa*II, *Eco*RI/*Hha*I, *Hind*III/*Hpa*II, and *Hind*III/*Hha*I blots of CHO DNA (Figs. 5 and 6).

Figure 4. Methylation analysis of the 5' TK region in derivatives of the CHO KI cell line. DNA samples (15 μ g per lane) are designated as: (1) TK⁺ CHO KI cell line; (2) CHO TK⁻ MT⁻ derivative; (3) TK⁻ *tsm* strain; (4) and (5) TK⁻ cells selected after treatment of (2) with 5-Aza-CR and 5-Aza-CdR, respectively; (6) and (7) TK⁺ cells selected after treatment of (2) with 5-Aza-CR and 5-Aza-CdR, respectively; (8) TK⁺ derivative selected after treatment of (3) at 39°C; (9), (10), and (11) TK⁻ clones spontaneously deriving from (6), (7), and (8), respectively. Samples were digested with *Hind*III/*Hha*I and electrophoresed in a 0.8% agarose gel and blotted. This blot was hybridized to the ³²P-labeled 5.5-kb genomic fragment (Fig. 3a).

specific demethylation in TK⁺ revertant clones (Figs. 5 and 6, lanes 6–8). In all the parent strains, a 4.2-kb *Eco*RI DNA fragment was detected for both *Hpa*II (Fig. 5, band a, lanes 1–3) and *Hha*I (Fig. 6, band a, lanes 1–3) digestions. This fragment is of identical size to the band detected in CHO KI DNA digested only with *Eco*RI (Fig. 5, band a, lane 1E), and it probably represents a copy of the TK gene completely methylated in this region. A similar size restriction fragment was detected in *Hind*III-digested DNA (Fig. 5, band e, lanes 1–3; Fig. 6, band e, lanes 1–3). The additional lower-molecular-weight bands (Fig. 5, bands d and h) in the *Hpa*II blots may then be an undermethylated allele. However, complete demethylation of the 4.2-kb fragment should create much smaller DNA fragments as revealed by *Msp*I digestion (Fig. 5 and Lewis 1986), suggesting that the second TK copy is not completely demethylated (see Discussion). Similarly, the two lower bands in the *Hha*I blots (Fig. 6,

bands b, c, f, and h) may represent either two undermethylated copies of the 4.2-kb fragment or population variability in the methylated state of one copy.

Reversion to the TK⁺ phenotype is correlated with the loss of the 4.2-kb fully methylated fragments (Figs. 5 and 6, bands a and e, lanes 6–8), indicating that demethylation has occurred. However, 5-Aza-CR- and 5-Aza-CdR-treated cells that retained the TK⁻ phenotype show no such demethylation (Figs. 5 and 6, lanes 4 and 5), suggesting that a correlation exists between TK⁻ to TK⁺ reversion and cytosine demethylation in this region of the gene. The detection of two partially demethylated *Hha*I–*Eco*RI or *Hha*I–*Hind*III restriction fragments (Fig. 6, bands b, c or f, h) suggests the presence of at least two *Hha*I sites susceptible to demethylation (Fig. 3c). Partial remethylation of the *Hha*I sites (Fig. 6, lanes 9, 10, 11, bands indicated with solid arrows; Fig. 3c, bands b, c or f, g) was detected upon backreversion from TK⁺ to TK⁻ phenotype, indicating that changes in the level of methylation might be important for reversion of the phenotype. Additionally, the *Hpa*II partially demethylated bands reproducibly showed size fluctuation in revertants to TK⁺ phenotype (Fig. 5, bands d and h, lanes 6–8) and backrevertants to TK⁻ phenotype (Fig. 5, bands d and h, lanes 9–11). One explanation for this is the presence of more than one *Hpa*II site in this region. Alternatively, fluctuation could be due to genetic mutations. The later suggestion is unlikely, since both TK⁺ revertant and parent cell lines show the same *Msp*I digestion patterns (Fig. 5).

Methylation changes were detected in the 4.2-kb *Eco*RI fragment at both the *Hha*I and *Hpa*II recognition sites. The bands, b–d in the *Eco*RI panels of Figures 5 and 6, lanes 6–8, range between 3.5 and 3.7 kb; the equivalent bands f–h in lanes 6–8 of the *Hind*III panels are approximately 200 bp longer. The difference in the size of these bands between *Eco*RI and *Hind*III blots suggests that they result from *Hpa*II or *Hha*I restriction of one of the ends of the *Eco*RI or *Hind*III fragments. Additionally, the detection of higher molecular weight fragments in the *Hind*III blots of TK⁺ revertants (Figs. 5 and 6, lanes 6–8) suggests that the demethylation may be located in an approximately 200-bp region some 500 bp downstream from the 5' end of the 4.2-kb fragment (diagrammatically presented in Fig. 3c).

The methylation status of the MTI gene was also studied in MT⁻ (CHO TK⁻ MT⁻ and *tsm* cells) and MT⁺ clones induced either by 5-Aza-CR or treatment of the *tsm* strain at 39°C. These DNAs were initially digested with *Eco*RI and then with either of the methylation-inhibited enzymes *Hpa*II and *Hha*I or the methylation-insensitive isoschizomer of *Hpa*II, *Msp*I. The products were electrophoresed and transferred to nitrocellulose filters. Specific MTI fragments were then detected by hybridization of the filter with a 64-bp-long MTI cDNA probe covering the 3' untranslated part of the cDNA of the gene. This probe has only 37% homology to the MTII counterpart and would only detect MTI homologous sequences (Griffith et al. 1983; Crawford et al. 1985). Figure 7 shows that the probe hybrid-

Figure 5. Methylation analysis of the *HpaII* sites of the 4.2-kb *EcoRI*–*EcoRI* fragment in the CHO KI cell line and its derivatives. DNA samples (15 µg per lane) were digested with either *EcoRI*/*HpaII* or *HindIII*/*HpaII*, electrophoresed through a 0.8% agarose gel in the numerical order described in Fig. 4, and blotted. Control digestion with *MspI*/*EcoRI* was also performed for the parent (lanes 1–3) and the TK⁺ revertant clones (lanes 6–8). These blots were hybridized to the 4.2-kb *EcoRI*–*EcoRI* genomic probe. Lane 1E in the *HpaII*/*EcoRI* panel is CHO KI DNA digested only with *EcoRI*. The origin of the bands a, d, e, and h is interpreted in Fig. 3.

Figure 6. Methylation analysis of the *HhaI* sites of the 4.2-kb *EcoRI*–*EcoRI* fragment in the CHO KI cell line and its derivatives. DNA samples (15 µg per lane) were digested with either *EcoRI*/*HhaI* or *HindIII*/*HhaI*, electrophoresed through a 0.8% agarose gel in the numerical order described in Fig. 4, and blotted. These blots were hybridized to the 4.2-kb *EcoRI*–*EcoRI* genomic probe. The origin of the bands a, b, c, e, f, and g is interpreted in Fig. 3.

Figure 7. Methylation pattern of the MTI gene. DNAs from CHO TK⁻ MT⁻, *tsm*, and four MT⁺ revertant clones were digested with *EcoRI/MspI*, *EcoRI/HpaII*, or *EcoRI/HhaI* and electrophoresed through a 1% agarose gel. This was blotted onto a nitrocellulose filter that was hybridized against a 64-bp Chinese Hamster MTI-specific cDNA probe. Lanes 1 and 2 are CHO TK⁻ MT⁻ and *tsm* DNA, respectively. Lanes 3 are DNA from an MT⁺ clone induced by 5-Aza-CR treatment, and lanes 4, 5, and 6 are DNAs from three different *tsm* MT⁺ revertant clones. Lane 1E is CHO TK⁻ MT⁻ DNA digested only with *EcoRI*.

ized to a 5.2-kb band in all the TK⁻ cells (lanes 1 and 2 in *EcoRI/HpaII* and *EcoRI/HhaI* panels), which was approximately 3 kb shorter than the MTI homologous fragment resulting from single *EcoRI* digestion (lane 1E in *EcoRI/HpaII* panel). This indicates that *HpaII* and *HhaI* sites are already demethylated in the MT⁻ clones.

None of the MT⁺ revertants showed the 5.2-kb band, indicating that demethylation had occurred (Fig. 7, panels *EcoRI/HpaII*, *EcoRI/HhaI*, lanes 3–6). An 0.8-kb fragment was detectable in *HhaI/EcoRI* digests of the DNA from all MT⁺ clones examined but not from MT⁻ cells (Fig. 7, panel *EcoRI/HhaI*, lanes 3–6).

Discussion

This report describes the application of a novel method for the isolation of a temperature-sensitive strain expressing alterations in DNA methylation. The CHO TK⁻ MT⁻ cell line was used for this purpose because evidence was available that the TK and MT genes in mouse, hamster, or human cells are under methylation control (Compere and Palmiter 1981; Harris 1982; Heguy et al. 1986; Stallings et al. 1986). The finding that 5-Aza-CR or 5-Aza-CdR caused 1–59% reversion to the TK⁺ and MT⁺ phenotype provided important evidence for such methylation control of TK and MT expression in CHO TK⁻ MT⁻ cells. This evidence was supported

by the specific demethylation of the genes detected by Southern blot analysis of reactivated clones and the demethylation observed by HPLC chromatography immediately after treatment. Our findings are in agreement with earlier observations on the demethylating effect of these agents (Jones and Taylor 1980, 1981; Wilson et al. 1983; Flatau et al. 1984).

5-Aza-CdR-treated cells that had lost 46% of their methylated cytosines showed no detectable remethylation for the first 10 cgs following treatment. Subsequently (by approximately 50 cgs), the fraction of methylated cytosines had significantly increased, demonstrating the presence of de novo methylation activity. Surprisingly, some 150 cgs after treatment, the DNA in CHO cells not only reached normal levels, but also gained up to 23% more methylation than that of untreated cells. Several previous reports showed that remethylation occurred in cells treated with 5-Aza-CdR, but the efficiency of recovery varied between different cell lines (Compere and Palmiter 1981; Creusot et al. 1982; Gasson et al. 1983). In other studies it was shown that unmethylated cloned DNA introduced to cells in culture was eventually methylated (Clough et al. 1982; Hardies et al. 1983; Christy and Scangos 1984). However, none of these studies examined DNA remethylation for such an extensive period of growth as this report.

The isolation of *tsm* was based on the assumption that temperature-dependent demethylation would result in reactivation of the TK and MT genes. The decision to search for a temperature-sensitive phenotype was based on the possibility that methylation deficiency might be lethal. The *tsm* strain described here may have a recessive mutation at a functionally haploid locus (Siminovitch 1976; Jeggo and Holliday 1986) or possibly a dominant mutation. The phenotype is stable, and the strain was isolated at a frequency (1/7000), comparable to mutations in the X-linked HPRT gene (1/1000) after similar mutagenesis. The possibility that *tsm* is itself due to a change in DNA methylation is unlikely, since the temperature-sensitive phenotype indicates that it has an altered gene product rather than loss of gene expression. From the characteristics of *tsm* we assume it is due to a heritable mutation. Several observations defined the nature of the *tsm* mutation. Thus, induction at 39°C for at least one cell cycle resulted in reactivation of silent TK or MT genes at frequencies up to 1/1000. Longer treatment at this temperature did not increase the level of phenotypic changes. *tsm* did not show increased mutation frequency at the HPRT locus. *tsm* TK⁺ revertants showed high frequency of backreversion to TK⁻, these TK⁻ backrevertants being reactivable by 5-Aza-CR, suggesting that TK⁻ backreversion is probably due to de novo remethylation. Although our isolation procedure would allow the recovery of a conditional lethal mutation, *tsm* is in fact fully viable after long-term growth at 39°C.

Phenotypic alterations resulting from temperature or from demethylation treatment were associated with changes in the transcriptional activity of the TK and MT genes. TK homologous RNA was detected by Northern hybridization in all cell lines. TK⁺ revertant cells showed severalfold increase in the abundance of TK mRNA in comparison to the original TK⁺ CHO KI cells. This indicates that demethylation of the TK⁻ strain results in not only reactivation, but also induction of the TK gene over and above the wild-type level. The presence of some TK mRNA in TK⁻ cells could be explained by the detection of either the mitochondrial or a mutated cytosolic TK transcript. Northern hybridization revealed two MT homologous mRNA species in all cells, one of which corresponds in size to mature MT mRNA, and a larger molecule which may be either the product of an unidentified yet MT-like gene or an intermediate in the RNA processing of the MT transcript. The detection of MT transcripts even in MT⁻ strains is probably due to the expression of an MT gene homologous to MTI but not inducible. The transcriptional activity of this gene may not be sufficient for survival in the concentration of Cd in the medium used in these experiments. Low levels of MT mRNA were also detected in MT⁻ HeLa cells (Heguy et al. 1986). The MT⁺ clones showed severalfold increase in the levels of MT transcript, probably due to activation of an inducible MT gene.

Although demethylation in the *tsm* was below the sensitivity of detection by HPLC analysis, specific

changes in the pattern of DNA methylation were demonstrated by restriction analysis in *tsm* revertant clones. Additionally, like TK⁺ cells resulting from 5-Aza-CdR treatment, *tsm* TK⁺ revertant cells induced at 39°C showed a gradual increase in their methylation levels resulting in 60% higher methylation than the parent *tsm*. TK⁻ cells when grown for about 150 cgs after incubation at 39°C. These *tsm* TK⁺ cells showed 4.5% 5mC content, which implies that over 90% of their CpG doublets are methylated since CpG occurs in the mammalian genome five times less frequently than expected (Russell et al. 1976).

The availability of a series of CHO TK genomic probes (Lewis et al. 1983; Lewis 1986) facilitated a detailed analysis of the role of methylation in the expression of this gene. Demethylation in a region near the third exon was consistently observed in all TK⁺ cell lines analyzed irrespective of their mode of generation from TK⁻ cells. Thus, at least one *Hpa*II and two *Hha*I sites at the upstream end of the 4.2-kb *Eco*RI fragment were always unmethylated in TK⁺ cells, but methylated in the parental TK⁻ cell line. The detection of a 2-kb band in TK⁺ revertants of the *Hpa*II blots (Fig. 5, lanes 6–8) may also indicate demethylation of a *Hpa*II site in the middle of the 4.2-kb fragment. Interestingly, the 5' promoter region did not show any detectable epigenetic changes associated with the phenotype. This may result from the screening procedure, which would not detect DNA fragments smaller than 300 bp, or it may be that methylation in this area does not influence expression in our cell line. *Hpa*II and *Hha*I restriction sites in the rest of the gene are completely methylated in all cell lines, suggesting that they are not involved in the control of TK gene expression.

The sites whose methylation status is correlated with TK gene activity may, however, cooperate with other sites that are not detectable in the present experiments and form a cluster within the 4.2-kb *Eco*RI fragment. Support for this suggestion arises from the observed fluctuation in the size of the *Hpa*II band detected in TK⁺ revertants. Additionally, it should be borne in mind that *Hpa*II and *Hha*I sites can detect less than 16% of the methylatable CpG doublets in the genome. Thus, these other sites either may be too close to be defined clearly or may not be detectable by *Hpa*II or *Hha*I. However, the fact that detected epigenetic alterations associated with TK gene activation are identical when brought about by 5-Aza-CR, 5-Aza-CdR, and temperature increase in the *tsm* strain suggests that they are important in regulating TK gene expression.

Remethylation of *Hha*I sequences within the region identified above was found in all the TK⁺ to TK⁻ backrevertants. These TK⁻ backrevertants, in turn, generated TK⁺ clones at high frequencies with 5-Aza-CR, an observation consistent with methylation being important in the generation of backrevertants. Remethylation, however, was never 100%. Thus, the relative level of methylation may determine the phenotype, rather than the absolute modification status of specific nucleotides. Such a situation has been reported for the regula-

tion of γ -globin gene expression (Murray and Grosveld 1985).

Although promoter sequences have been shown to confer methylation control on several eukaryotic and viral genes (Busslinger et al. 1983; Kruczek and Doerfler 1982; Stein et al. 1982; Langner et al. 1984), the present work strongly indicates that methylation of specific internal sequences can also control TK gene expression in CHO cells. This agrees with reports that methylation of either promoter or internal gene sequences is sufficient for inactivation of the herpes TK gene expression (Christy and Scangos 1984; Keshet et al. 1985).

The demethylation of DNA observed in all MT⁺ clones could not be studied in the same detail as for the TK gene because genomic probes were not available, and only a 64-bp MTI-specific cDNA probe could be used. However, demethylation must have occurred in these cells because the detectable *HpaII* and *HhaI* sites were found to be unmethylated after, but not before reversion to the MT⁺ phenotype. These findings agree with previous reports on the regulation of the mouse MTI gene in which demethylation of *HpaII* sites was associated with its expression (Compere and Palmiter 1981). The 5.2-kb band in the MT⁻ cells is an MTI-specific fragment not completely methylated, since it is smaller than the fragment detected in DNA digested only with *EcoRI*.

The phenotypic alterations and subsequent increase in the levels of methylation in the *tsm* strain parallel those observed with demethylating agents and suggest that the *tsm* mutation affects DNA methylation. It is unlikely that *tsm* is affected in general DNA methylation since if this were the case, 5mC would be expected to continually decline during growth at 39°C. Instead, it is possible that it affects the methylation of sites critical for gene expression, at least for some loci. Recently, evidence has been published that some methylation sites at the HPRT locus are maintained more faithfully than others (Yen et al. 1986), and it is possible that *tsm* loses the ability to maintain similar sites at the TK and MT loci. However, the effect of temperature on the reactivation of these genes appears to operate over one generation, not continuously at 39°C. *tsm* also has the surprising property of increasing de novo methylation during long-term growth after a 24-hr treatment at 39°C. This suggests that *tsm* may be defective in the regulation of DNA methylation. A regulatory mutation might alter the dynamic state between maintenance and de novo methylation or possibly feedback between de novo and maintenance activities.

It was shown here, that it is feasible to isolate temperature-sensitive mutants affected in DNA methylation. The same procedure should lead to the isolation of a family of mutants affected in different steps of the DNA methylation pathway. The availability of such mutants would provide a powerful approach in studying both the regulation and the biological role of DNA methylation. Studies might be extended to a wide range of biological functions without the use of inhibitors such as demethylating agents or in vitro modified DNA molecules.

Materials and methods

Cell lines, medium, and selection conditions

The CHO TK⁻ cell line was isolated from CHO KI cells after mutagenesis by ethylmethanesulfonate (EMS), followed by long-term growth in bromodeoxyuridine (BUdR). The CHO KI cells are spontaneously MT⁻. All cells were routinely grown in MEM (Gibco F15) from Gibco-Biocult Ltd, UK, supplemented with 10% (vol:vol) fetal calf serum inactivated by 1 hr treatment at 56°C, antibiotics (penicillin, 0.25 μ g/ml glutamine, and MEM nonessential amino acids $\times 100$, 1% (Gibco Biocult Ltd, UK). TK⁺ cells were selected in HAT medium (100 μ M hypoxanthine, 4 μ M aminopterin, 16 μ M thymidine). Medium containing 400 μ g/ml BUdR was used to select cells expressing the TK⁻ phenotype. Metallothionein-expressing cells were selected in 2 μ M CdCl₂. Hypoxanthine phosphoribosyltransferase (HPRT) inactivation was measured by plating in medium containing 2 μ g/ml 6-thioguanine.

Treatment with 5-Aza-CdR and 5-Aza-CR

Cells (5×10^4) were seeded in 25-cm² plastic flasks and incubated for 48 hr. Appropriate concentrations of 1-mg/ml stocks of 5-Aza-CdR frozen at -70°C (Sigma Chemical Company Ltd) or freshly prepared 0.1-mg/ml stocks of 5-Aza-CR were added to the growth medium for 24 hr. Then cells were media changed with drug-free medium, and allowed a recovery period before plating in selective medium, or they were subcultured in drug-free medium.

Estimation of backreversion to TK⁻ in TK⁺ revertant cells

The rate of spontaneous back reversion to the TK⁻ phenotype was determined in TK⁺ revertant clones using fluctuation analysis (Luria and Delbruck 1943; Lea and Coulson 1949). One hundred cells were seeded into each of 10 25-ml plastic flasks and incubated to an approximate number of 10^5 cells per flask for TK⁺ revertant clones induced by demethylating agents and of 5×10^5 cells per flask for *tsm* TK⁺ revertants. Three-fifths of the total number of cells were seeded in each of three petri dishes in BUdR-containing medium. After 7–10 days, incubation plates were fixed, stained, and counted.

Procedure for the isolation of methylation-deficient mutants

CHO TK⁻ MT⁻ cells were mutagenized with 300 μ g/ml EMS, which allows approximately 10% survival. Ampules of frozen mutagenized cells were thawed and grown to confluency. Five hundred cells were seeded into 90-mm petri dishes in medium containing 400 μ g/ml BUdR and incubated for 7–8 days at 34°C. This plating density gave approximately 100 colonies per dish, which were ringed with a marker pen at the underside of the plate. The growth medium was removed, and the cells were overlaid with 5 ml of 0.5% soft agar as described by Jeggo and Kemp (1983). A further 1-day incubation period is required for the cells to become rounded and start growing into the agar. At this stage they could be transferred easily to two replicate multiwell plates containing 1.5 ml of medium per well using the plastic tip of an automatic Gilson pipettor. This procedure resulted in the transfer of approximately 10^3 cells per well. After 4 hr incubation at 34°C, plates were examined under a low-power microscope, and if the average number of cells per well was approximately 10^3 , one plate of each replicate was shifted to 39°C. Twenty-four hours later 15 μ l HAT ($\times 100$) was added, and the plates were incubated at 34°C. Any isolate that pro-

duced HAT-resistant clones could be recovered from the untreated replica plate.

Northern blot

Two micrograms per lane poly(A)-tailed RNA, prepared as in Vennstrom and Bishop (1982), was electrophoresed through formaldehyde/agarose gels which were soaked for 60 min in $20\times$ SSC, prior to blotting onto a nitrocellulose filter for 12–16 hr in the cold room. Filters were prehybridized for 4–6 hr at 42°C ($3\times$ SSC, 50% formamide, 0.1% SDS, $10\times$ Denhardt's solution), and then transferred to 25 ml of preheated hybridization solution containing probe labeled as in Feinberg and Vogelstein (1983) and 10% dextran sulfate. Hybridization was for 18–24 hr in plastic boxes. Filters were then washed to the desired stringency, air-dried, and autoradiographed.

HPLC analysis

The HPLC procedure for nucleotide analysis was developed by Dr. T. Matsumura in the Genetics Division. A Waters Associates system with a cation column (Partisil SC $\times$ 10u Type F) was used. Approximately 15–40 μg hydrolyzed DNA in 0.1 M HCl was injected in the injection apparatus with a Hamilton microsyringe and eluted with 0.05 M HCl through the column. The absorbance of the eluted samples was detected by the absorbance detectors and recorded with a pen recorder at two scales of sensitivity 1–0.01. This setting allowed detection of both C and 5mC from the same injected sample.

Isolation and digestion of genomic DNA

High-molecular-weight DNA from CHO cells was isolated as described by Jeffreys and Flavell (1977). Restriction enzymes were bought from Bethesda Research Laboratories, and the digestions were performed with the conditions recommended by the manufacturer, at a ratio of 3–4 units of enzyme per microgram of DNA.

Southern blot

Agarose gels to be blotted onto nitrocellulose filters were soaked in 0.1 M HCl for 20 min. The DNA was denatured by treatment with 0.2 M NaOH, 1.5 M NaCl for 45 min, and the gel was neutralized with 0.5 M Tris (pH 7.5), 1.5 M NaCl for 45 min under constant agitation. The gel was blotted onto a nitrocellulose filter. Transfer was allowed to proceed for 12–16 hr in the cold room. Nitrocellulose filters were prehybridized for 4–6 hr at 65°C ($3\times$ SSC, 0.1% SDS, $10\times$ Denhardt's solution), then they were transferred individually in hybridization solution that contained probe labeled as described in Feinberg and Vogelstein (1983) and 10% dextran sulfate. When the probe contained repetitive sequences, the labeled fragment was pretreated as described in Sealey et al. (1985). Similarly, when plasmid DNA was used, the probe was pretreated as above using sonicated vector. Hybridization was for 16–24 hr at 65°C in plastic boxes. The filters were then washed to the desired stringency, rinsed, air-dried, and autoradiographed.

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