

DNA Methylation and Gene-Expression in
Chinese Hamster Ovary cells

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ABSTRACT

DNA methylation in eukaryotes has been associated with the regulation of gene activity. A search for methylation deficient mutants from CHO cells was carried out, in order to further study the physiological role of 5mC modification. It was shown by HPLC chromatography, that treatment with 5-Aza-CR and 5-Aza-CdR introduces up to 46% demethylation in a CHO TK⁻ MT⁻ cell line, resulting in reactivation of the TK and MT genes at frequencies ranging from 1-59%. Southern blot analysis revealed that this reactivation was associated with specific demethylation of the TK and MT genes. Methylation changes in the TK gene was detected by a 200bp region near the third exon. It was also shown that TK⁺ or MT⁺ revertants have a significant increase in TK or MT mRNA levels.

A procedure for the isolation of temperature sensitive methylation deficient mutants was developed, which was based on the assumption that demethylation would result in reactivation of the TK and MT genes. A tsm strain was obtained by screening 7000 individual colonies from an EMS mutagenised CHO TK⁻ MT⁻ population, which showed temperature dependant (39⁰C) reactivation of TK and MT genes at a frequency of 10⁻³. tsm TK⁺ or MT⁺ reactivated clones show identical demethylation and increased transcriptional activity, as reactivated strains induced by demethylating agents. However, no measurable reduction in the 5mC levels was induced in the tsm strain by treatment at the high temperature. The tsm cells probably have a recessive functionally haploid mutation, or a dominant mutation involved in the regulation of DNA methylation. They are viable at 39⁰C and show no increased mutation rate.

TK⁺ reactivants from tsm or 5-Aza-CR treated cells reverted back to the TK⁻ phenotype at a frequency about three orders of magnitude greater than spontaneous mutation. These back-revertants were again reactivable with demethylating agents. Therefore de novo methylation is probably responsible for TK⁻ back-reversion. TK⁺ reactivants from tsm or 5-Aza-CR treated cells, were also de novo methylated, as they had gained, respectively, 60% and 23% higher 5mC than CHO TK⁻ MT⁻ about 150 cell generations after temperature or demethylating treatment.

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This thesis is dedicated to my parents

Γεωργιο and Μαρικα

and to Khash

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ABBREVIATIONS

Ad	Adenovirus
APRT	adenine-phosphoribosyl-transferase
5-Aza-CR	5-Azacytidine
5-Aza-CdR	5-Azadeoxycytidine
BUdR	bromodeoxyuridine
BHK	baby hamster kidney
Cd	cadmium
CHO	chinese hamster ovary
DHFR	dihydrofolate reductase
EMS	ethylmethansulfonate
HAT	hypoxanthine/aminopterin/thymidine
HPLC	high pressure liquid chromatography
HPRT	hypoxanthine-phosphoribosyl-transferase
HTF	HpaII tiny fragments
5mA	5-methyladenine
5mC	5-methylcytosine
MTI	metallothionein-I
PGK	phospho-glycerate-kinase
RSV	Rous sarcoma virus
SDS	sodium dodecyl sulphate
6-TG	6-thioguanine
TK	thymidine kinase

CHAPTER 1

INTRODUCTION

1.1.1. Postreplicative modification of DNA:

The genetic information consists of combinations of the four nucleotide bases. However, additional modified bases were first described in the DNA of living organisms by Hotchkiss (1948) and Wyatt (1951). DNA methylation is one ubiquitous form of DNA modification, which has been studied extensively in a variety of organisms. Two methylated bases have been identified in prokaryotic systems: 5-methylcytosine (5mC) and 6-methyladenine (6mA) (Dunn and Smith 1955, Doscosil and Sormova 1965). A fraction 0.5-2% of cytosines or adenines are methylated in bacterial chromosomes. The vertebrate genomic DNA contains 5-methylcytosine (5mC) as the only modified base (Grippe et al. 1968). The proportion of 5mC in eukaryotes varies from 30% in some plants (Shapiro 1968), to 0.01% in *Drosophila* (Achwal et al. 1984). In vertebrates between 2-7% of the cytosines are modified.

Chargaff and his colleagues (Chargaff et al. 1953) demonstrated that 5mC residues were not randomly distributed in different chromatin fractions prepared from calf thymus DNA. This led to suggestions that methylation is a postreplicative event, since incorporation of already modified cytosine should be random. Enzymatic activities (methyltransferases) were later detected capable of transferring methylgroups to nucleic acids (Gold and Hurwitz 1963, Srinivasan and Borek 1964, Sheid et al. 1968, Burdon and Adams 1969). The universal source of methylgroups used by methyltransferases is S-adenosylmethionine.

Evidence was obtained for prokaryotic systems, that DNA is

methyated soon after replication (Billen 1968, Lark 1968). However, in eukaryotes the methylation process can continue for several hours after completion of the S phase (Burdon and Adams 1969, Evans and Evans 1970, Sneider et al. 1975).

1.2.1. Restriction Modification systems:

One of the best documented functions of DNA methylation is that of host-controlled restriction modification in bacteria. This consists of two highly specific enzymatic activities: an endonuclease and a DNA methylase. The endonuclease enables a given strain to both recognise and destroy foreign DNA by cleaving the unmodified specific sequences (restriction). Methylation of the specific sequences protects the homologous DNA from restriction (modification).

The restriction modification systems have been classified in three types depending on their complexity and enzyme cofactor requirements for restriction or modification (reviewed by Yuan and Hamilton 1984). The most complicated of these are the type I systems, which consist of an enzyme complex with three nonidentical subunits coded by three contiguous genes on the E.coli chromosome. They require Mg^{+2} , ATP and S-adenosylmethionine for enzymatic activity and they can serve both restriction and modification functions. Separate enzymes in the type II systems confer restriction or modification of a specific sequence, and Mg^{2+} is their only requirement. The more recently characterised type III systems require ATP and Mg^{+2} for restriction, and contain two non-identical subunits. A separate modification enzyme consists of the smaller subunit of the restriction enzyme.

1.2.2. Methylation deficient mutants in *E.coli*:

One way of investigating the role of DNA methylation in bacteria was the isolation of methylation deficient mutants and the subsequent analysis of their phenotype in comparison to the wild type. The fact that viable *E.coli* mutants are available that lack either or both cytosine (dcm⁻) or adenine (dam⁻) methylation, suggests that, at least in *E.coli* methylation might not be essential for growth.

Mutagenic hotspots as a result of 5mC deamination, which yields thymine, are not found in dcm⁻ strains. Additionally partially deficient in 5mC mutants show accumulation of recombinogenic lesions (Korba and Hays 1982a,b), (although this is not a characteristic of fully deficient dcm⁻ mutants), suggesting that hemimethylated dcm sites might be the basis for the enhanced recombination.

Deficiency in adenine methylation has more obvious effects in the phenotype, since it results in: increased spontaneous mutation frequency, sensitivity to UV light and a hyperecombinogenic phenotype. Furthermore, dam⁻ recA⁻, dam⁻ recB⁻, dam⁻ recC⁻, dam⁻ recJ⁻, dam⁻ lexA⁻, and dam⁻ polA⁻ double mutants are not viable. These phenotypes suggest that there is an aberrant repair mechanism in dam⁻ strains which will be discussed below.

1.2.3. DNA Mismatch repair:

Errors in replication of DNA will lead to mismatched bases in daughter strands. Indirect evidence suggests involvement of adenine methylation with mismatch repair in the *E.coli* chromosome. Firstly,

dam⁻ mutants (defective in adenine methylation) are hypermutable (Marinus and Morris 1974). Secondly, overproduction of the dam methylase would lead to methylation of the daughter strand before DNA repair could occur. This would also result in hypermutability of the subsequent generations of cells (Herman and Modrich 1981). Thirdly, mismatch repair deficient mutants overcome the lethality of dam⁻ recA⁻ strains, suggesting that some action of the mismatch repair system is responsible for the killing of these mutants (Glickmann and Radman 1980, McGraw and Marinus 1980).

Evidence has recently been obtained for the direct involvement of adenine methylation in mismatch repair of lambda bacteriophage DNA in E.coli. Heteroduplexes containing mismatch were constructed with one strand methylated, both strands methylated, or neither strand methylated. Repair occurred on either DNA strand in the unmethylated constructs. The unmethylated strand was preferentially repaired in hemimethylated heteroduplexes. However, no repair was observed when both strands were methylated (Pukkila et al. 1983). Methyl-directed mismatch repair of artificially constructed heteroduplexes was also observed using E. coli extracts in an ATP requiring reaction (Lu et al. 1983). DNA adenine methylation therefore may determine strand selectivity for mismatch repair.

1.2.6. Regulation of gene activity in bacteria:

The presence or absence of methylgroups may be expected to alter DNA protein interactions. If such proteins were involved in regulating gene expression, DNA methylation might either interfere with or be required for gene activity. Thus, methylation of the dcm

site in the SOS box of the lexA gene prevents binding of the repressor (Brent 1984). Another example is the expression of the *mom* gene of phage Mu which requires intact adenine methylation (Toussaint 1977). In *dam*⁻ strains the level of *mom* mRNA decreases to 5% (Hattman 1982). It is now clear that the important methylated -GATC- sites lie upstream of the *mom* promoter (Plasterk et al. 1983).

Interestingly, inhibition of gene expression by methylation at -GATC- sequences in E.coli was shown for the transposable element IS10 (Roberts et al. 1985). Two regulatory -GATC- sites were identified, one within the promoter and one within the transposase binding region. Demethylation of these sites results in increased transposase activity and is responsible for induced transposition of IS10 in *dam*⁻ mutants.

1.3.1. DNA methylation in eukaryotes:

Vertebrates show a distinct preference for methylation at the CpG doublet (Daskocil and Sorm 1962, Grippo et al. 1968), although, unlike the prokaryotic systems many potentially methylatable sites are completely unmethylated. The predominance of CpG modification might be associated with the rarity of this doublet (Bird 1986). This occurs five times less frequently than expected in the DNA of vertebrates, probably due to failure of the DNA repair mechanism in recognizing deamination of 5mC which leads to thymine (Swartz et al. 1962, Russel et al. 1979).

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), while enzyme purified from rat liver shows no specificity for the methylation state of the substrate DNA (Simon et al. 1983). Despite the purification of maintenance or de novo enzymes from different tissues, there is no evidence for multiple species of DNA methylase within the same cells.

A family of DNA stretches between 600-1000 bp long that is devoid of methylation was recently detected in the genome of several vertebrates, (HpaII tiny fragments: HTF)(Bird et al. 1985). These contain more than 10 times higher levels of the CpG doublet than the rest of the genome. Undermethylation of HTF was suggested to be due to the presence of protein factors that make them inaccessible to methyltransferases. They are therefore likely to represent regulatory areas for gene expression (Bird 1986). Several HTFs have been

localized in promoters as well as downstream sequences of mammalian genes. Methylation of the HTF islands in the 5' promoter regions of HPRT (Yen et al. 1984) and APRT (Keshet et al. 1985) has been shown to inhibit expression of these genes. Additionally, high levels of CpG in the HTF islands may reflect protection due to demethylation, since in the absence of 5mC, C to T transition as a result of 5mC deamination would not occur (Bird 1986).

1.3.2. Possible roles for DNA methylation:

Ever since the discovery that cytosine methylation in eukaryotes is a post-replicative event, and that the level of 5mC remains stable over replication, interest developed in correlating this modification with biological functions. There is no doubt that DNA methylation adds an additional level of information to the DNA helix. Cytosine methylation has been postulated to affect chromatin structure, which is supported by the uneven distribution of 5mC between active and inactive chromatin fractions. For example, the heterochromatin fraction binds significantly higher amounts of anti-5mC antibodies than the euchromatin regions (Miller et al. 1974). In addition, it was also found that cytosine methylation may influence DNA conformation and topology (Behe and Felsenfeld 1981). The processes of folding and packaging of the daughter chromosomes at the end of mitosis might also be associated with DNA methylation (Razin and Cedar 1977).

By analogy to the prokaryotes it was proposed that DNA methylation may be involved in DNA mismatch repair (Holliday 1979). Information about mismatch repair in eukaryotes comes from studies on

gene conversion in fungi, and nothing is known about a possible post-replicative mechanism (reviewed by Whitehouse 1982).

The search of a role for DNA methylation in eukaryotes has focussed on hypotheses that 5mC residues in DNA may affect transcriptional activity of genes. Thus, it was initially suggested that deamination of 5mC to thymidine may generate specific mutational changes, which may regulate alterations in gene expression (Scarano 1971). Holliday and Pugh (1975) proposed that DNA methylation might provide the means of regulating gene expression during development, while Riggs (1975) suggested a role for DNA methylation in X chromosome inactivation. A major feature of these proposals is the sequence specificity of methylated sites, since CpG alone would be inadequate for the DNA-protein interactions necessary for the control of gene expression.

The mechanism by which DNA methylation could affect gene expression is still unknown. Methylated bases may regulate gene activity by modulating protein-DNA interactions, which is a feature observed in prokaryotic DNA methylation (Yuan and Meselson 1970, Boyer 1971). Such interactions can be effected either by direct recognition of the modified bases, or through the DNA conformation imposed by the presence of 5mC residues.

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. The finding that DNA methylation patterns are not identical in all the tissues of an organism, led to suggestions that de novo methylation may play an important role in cell determination during embryogenesis. This was further supported by recent observations that the totipotent

germ cells in the premeiotic stage have significantly reduced methylation levels (Monk and Lehnert in press).

The stability in level and the symmetry of CpG modification led to the concept of heritability in methylation patterns, which is coupled with the maintenance methylase (Holliday and Pugh 1975, Riggs 1975). This enzyme may act on hemimethylated DNA following the replication fork, and be responsible for the heritability of a methylation pattern. It was proposed that such an enzyme would not recognise non methylated DNA. Thus, the methylated or non methylated state could be faithfully transmitted through cell division. The ability of enzymes to distinguish between methylated and non methylated substrates is demonstrated by the restriction or modification systems in bacteria. The hypothetical action of a maintenance methyltransferase is shown in figure 1.

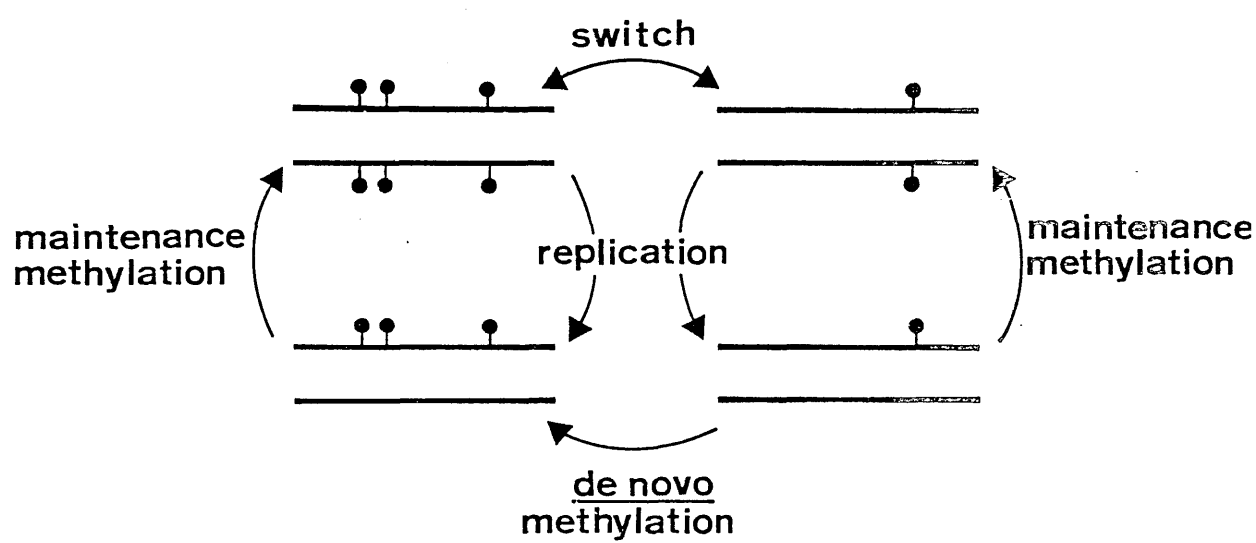
1.4.1. The role of DNA methylation in gene expression:

Early suggestions that methylation might control tissue specific gene expression were followed by accumulation of evidence associating DNA demethylation to gene activity. Initial studies involved careful observation of naturally occurring variations in the methylation pattern of a gene in different tissues of an organism. Thus, DNA from various tissues was digested using methylation inhibited enzymes and then screened with specific probes.

Such enzymes digest a CpG containing sequence when the C is not methylated, but they are inhibited by the presence of 5mC. The most commonly used methylation inhibited enzymes are HpaII which cleaves the unmodified CCGG palindrome and HhaI that recognises

Figure 1: Hypothetical action of maintenance methylation.

The (●) in the diagram indicate methylated sites. Maintenance methylation acts near the replication fork and methylates the newly synthesized hemimethylated DNA, and does not act on unmethylated sites. Specific switching could lead to alterations of methylation patterns, while non specific de novo methylation may create new methylation sites (section 1.3.2.).



unmethylated GCGC sites (Bird 1978, Bird and Southern 1978). The use of HpaII with MspI, an isochizomer that is not affected by methylation in the CpG doublet, provides a valuable control of unmethylated versus methylated sequences (Waalwijk and Flavell 1978). It is however,

important to point out that restriction enzymes can only detect a small percentage of methylatable sites (table 1). Therefore, failure to detect methylation alterations associated with expression using these enzymes, does not rule out the possibility that methylation is involved with gene expression.

Methylation patterns obtained following this approach were analysed in relation to expression of the gene in each cell type or tissue. Methylation maps of approximately 30 genes have already been reported (table 2). An inverse correlation between gene activity and methylation in the gene or flanking sequences was revealed in many cases. One of the most carefully studied gene families is the globin genes. Undermethylation of the adult globin genes was detected in the hemoglobin producing reticulocytes, while these genes are completely methylated in other tissues. Consistently, undermethylation of foetal globin genes was also observed in foetal liver where cells are actively producing hemoglobin (Van der Ploeg and Flavell 1980). In other studies, correlation between undermethylation and gene expression is less obvious. This is probably due to the sensitivity of the method used, since only about 16% of the CpG containing sites are detectable by restriction enzyme mapping (table 1).

More detailed analysis of the available data makes it clear that only a fraction of 5mC residues in the genome is likely to play an important role in regulation of gene expression. For example,

Table 1: Methylatable sites detected by 5mC inhibited enzymes.

		3'			
5'	CG	A	C	T	G
	A		AosII <u>GACGCC</u> *	AosII <u>GACGTC</u> *	
	C	AvaI <u>CCCGAG</u> *		AvaI <u>CCCGTG</u> *	HpaII + <u>CCGG</u>
	T	SalI <u>GTCGAC</u> * XhoI <u>CTCGAG</u> *			
	G	AvaI <u>CGCGAG</u> *	HhaI <u>GCGC</u>	AvaI <u>CGCGTG</u> * AosII <u>GGCGTC</u> *	

Table 1: Shows all the CpG containing tetranucleotide combinations and the most commonly used methylation inhibited restriction enzymes. Some of the enzymes cleave a six nucleotide sequence indicated in the corresponding box. It can be estimated that approximately 16% of the total CpG sequences in the genome are detectable.

* From all the tetranucleotide containing sequences, approximately 1/16 will be recognised by the 6 nucleotide cleaving enzymes.

+ MspI also recognises this sequence, but cleaves the DNA whether or not the CpG doublet is methylated.

Table 2: Methylation patterns.

Category	Species	Gene	Reference
General under-methylation when gene is expressed	Human	globins	Van der Ploeg & Flavell 1980
		HPRT	
		G6PD	Pai <u>et al.</u> 1984
	Mouse	Immunoglobulin heavy chain C γ , δ , κ	Rogers & Wall 1981
		IgK heavy chain	Mather & Perry 1983
		cHarvey-ras	Ramsden <u>et al.</u> 1985
	Rat	γ -casein	Johnson <u>et al.</u> 1983
		Insulin I	Cate <u>et al.</u> 1983
		Pepck	Benvenisty <u>et al.</u> 1985
	Chicken	α -globin cluster	Haigh <u>et al.</u> 1982
Some under-methylation when gene is expressed		β -globin cluster	Weintraub <u>et al.</u> 1981
			Ginder <u>et al.</u> 1983
	Xenopus	Globin	Gerber-Huber <u>et al.</u> 1983
	Human	h-metallothionein	Heguy <u>et al.</u> 1986
		c-myc	Cheah <u>et al.</u> 1984
	Rat	insulin II	Cate <u>et al.</u> 1983
	Rabbit	albumin	Orlofsky & Chasin 1985
		β -like globin gene cluster	Shen and Maniatis 1980
	Chicken	δ -crystallin I	Bower <u>et al.</u> 1983
	Xenopus	B I vitelogenin	Ng <u>et al.</u> 1984
under-methylated sites in all tissues	Mouse	HPRT	Lock <u>et al.</u> 1986
	Mouse	DHFR	Stein <u>et al.</u> 1983
	Chin. Ham.	APRT	Stein <u>et al.</u> 1983
	Chicken	α -2 (I) collagen	McKeon <u>et al.</u> 1982

Table 2: lists genes whose expression has been associated with demethylation in three categories depending on the extend of demethylation. The last category includes genes which are expressed in all tissues.

there was thought to be a discrepancy with the rat albumin gene expressed in foetal liver when partially methylated. There is however, one HpaII site that is consistently undermethylated in all the albumin producing cell lines and tissues studied, suggesting that changes in this particular site are responsible for expression (Orlofsky and Chasin 1974).

The evidence obtained from comparison of methylation patterns between expressing and non-expressing genes although informative is rather indirect. Thus, it cannot be established from these experiments that there is causal relationship between hypomethylation and gene expression, and it can still be argued that demethylation is the effect and not the cause of gene activation.

1.4.2. Specific areas in genes confer methylation control:

Initial studies on the methylation status of expressing versus non expressing genes indicated that specific sites in a gene confer methylation control. In an attempt to define methylation regulatory areas, cloned genes were fully or partially methylated in vitro. These constructs were then introduced into the cells and their expression was examined either in transient or after stable transformation. Studies were also performed on endogenous or introduced viral or retroviral genomes. Methylation in the promoter and 5' part of a gene generally led to inactivity, whereas methylation in the coding or 3' sequences had no effect on gene expression (table 3).

Methylation in the E1A promoter of Adenovirus 12 and E2A promoter of Adenovirus 2 was shown to inhibit the expression of early

Table 3: Regional methylation and gene expression.

Recipient	Species	Gene	Sites	Reference
Mouse L	Hamster	APRT	5' promoter	Stein <u>et al.</u> 1982
L	HSV	TK	5' & coding	Keshet <u>et al.</u> 1985
				Christy & Scangos 1984
L	Human	γ -globin	5' -210--+100	Murray & Grosfeld 1985
L	Ad12	E1A	promoter	Kruczek & Doerfler 1983
Hamster BHK ₂	RSV	src	5' region	Katala 1986
Xenopus oocytes	Ad2	E2A	promoter	Langner <u>et al.</u> 1984
Monkey COS7	Mouse	IAP	5' LTR	Feenstra <u>et al.</u> 1986
Rat	RSV	src	5' promoter	Searle <u>et al.</u> 1984

Table 3: Presents exogenous genes for which detailed studies have identified specific methylation regulatory regions.

or late viral functions (Sutter and Doerfler 1982, Vardimon et al. 1980, Kruczek and Doerfler 1983). Similarly the activity of human γ -globin gene was associated with the methylation status of the promoter sequences (Busslinger et al. 1983). In agreement with these, in vitro methylated Hamster APRT gene is active upon introduction into mouse L cells, if the promoter is unmethylated irrespective of the methylation status of the coding sequences (Stein et al. 1982, Keshet et al. 1985). Murray and Grosveld (1985) attempted to further localize methylation control to specific sites within the γ -globin promoter using selectively methylated DNA from M13 templates. They concluded that the level of methylation, rather than specific sites, in the -210 to +100 bp region conferred transcriptional regulation.

In other instances methylation of coding sequences was proved to be important for regulation of gene expression. One such example is the Herpes Virus thymidine kinase gene where methylation of either 5' or coding sequences is sufficient to prevent transcriptional activity (Keshet et al. 1985, Christy and Scangos 1984). Similarly demethylation of all 6 HpaII sites in the 5' and gene sequences of the mouse metallothionein-I gene was detected in cells induced by 5-Aza-CR to reactivate this locus. (Compere and Palmiter 1981).

Support for methylation affecting DNA-protein interactions was recently obtained. Thus, trans-activating functions of the E1 region of Ad2 or Ad5 can overcome the silencing effect of methylation in E2A promoter of Ad2 (Langner et al. 1986). Reactivation is due to a shift in the transcription initiation site. This suggests that methylation prevents interaction of the trans-acting factor with the correct sequences in the E2A promoter, leading to inaccurate transcription. Similarly, the enhancer of the Human cytomegalovirus compensates

inactivation of the E2A promoter due to methylation (Boshart et al. 1985).

In evaluating the expression of in vitro modified constructs it is important to bear in mind that severe structural alterations of the DNA in vitro may not faithfully reflect the in vivo situation. Thus, it was shown that N⁶-methyladenosine (a modification which does not normally occur in the mammalian genome) when introduced in the Herpes Virus thymidine kinase gene led to its inactivation (Waechter and Baserga 1982). In equivalent experiments N⁶-methyladenosine modification of the E1A promoter abolished its function (Knebel and Doerfler 1986).

1.4.3. Heritability of DNA methylation:

The discovery of cell-specific methylation patterns inherited through successive generations of somatic cells, provided additional evidence for the existence of maintenance methylation. DNA-mediated gene transfer was used in order to investigate the inheritance of DNA methylation (Wigler et al. 1981, Stein et al. 1981, Harland 1982). Thus, unmethylated or in vitro methylated cloned DNA was introduced to cells, and the methylation status of these sequences was studied several generations after integration in the host genome. The methylation pattern was stabilized in vivo early after integration, and it was thereafter faithfully conserved. To test the specificity of DNA methylation in vitro hemimethylated templates were synthesized and introduced into cells (Stein et al. 1982). This analysis indicated that maintenance methylation in vivo is specific to CpG sequences. Methylation at other sites is eliminated by dilution over

multiple cell divisions. The discovery that many silent genes can be activated by 5-Aza-CR (section 1.5.1.), also strongly indicates that the methylated state can be very stably inherited.

1.4.4. X-Chromosome inactivation:

Female cells in mammals have two X-chromosomes, one of which is inactive, which was first suggested by the mosaic appearance of heterozygotes for X-linked genes (Lyon 1961). The inactive X becomes condensed, late replicating and expresses minimal transcriptional activity after the epiblast stage (6day) of mouse development (Monk and Harper 1979). Inactivation is a random process in embryonic tissues, both maternal and paternal X-chromosomes have equal probabilities of becoming inactive. While inactivation is irreversible in somatic cells, an inactivation-reactivation cycle occurs in the germ line (Kratzer and Chapman 1981). Thus, condensed X-chromosome is not seen in the oocytes although present in the oogonia (Gartler et al. 1980). Additionally, XX oocytes have two fold higher levels of X-linked enzymes than XO or XY oocytes (Epstein et al. 1972, Monk and McLaren 1981). Therefore, the changes that led to inactivation, may be of epigenetic nature since they can be erased during meiosis. Riggs (1975) and Holliday and Pugh (1975) suggested that DNA methylation might be involved with the process of X-chromosome inactivation.

More recent evidence suggests that methylation may be associated with the inactivation-reactivation cycle. Analysis of the methylation status of foetal germ cells was performed during X chromosome inactivation and initiation of meiosis in female germ

cells. This revealed that germ cell DNA, was undermethylated further suggesting that methylation may provide the means for reprogramming the germ cells to totipotency (Monk and Lehnert in press).

Gene transfer experiments showed that transfected genes from the inactive X do not function, indicating that X chromosome inactivation results from direct DNA modification (Liskay and Evans 1980, Venolia and Gartler 1983). However reactivation of the HPRT gene using 5-Aza-CR led to efficient transformation of HPRT⁻ recipient cells to HPRT⁺ (Lester et al. 1982, Venolia et al. 1982).

The availability of DNA probes for the X linked HPRT, G6PD and PGK genes allowed molecular studies on the actual methylation pattern of these genes, in the active and inactive X chromosomes. Female specific restriction patterns were identified for both mouse and human HPRT genes. Hypomethylation of promoter and gene sequences was suggested to have functional significance (Wolf et al. 1984, Lock et al. 1986). Specific methylation differences were also detected between the active and inactive X chromosome for the 3' of the G6PD gene (Toniolo et al. 1984). Finally, specific pattern differences between the active and the inactive X chromosomes were revealed for parts of the PGK gene (Lindsay et al. 1985).

Reactivation of X linked genes following exposure to demethylating agents provided more specific evidence for the role of methylation in X chromosome inactivation (section 1.5.1.). Thus an HPRT⁻ mouse-human cell hybrid was isolated containing only one human inactive X chromosome (Mohandas et al. 1981, Graves 1982). Although the spontaneous HPRT⁺ incidence in these cells is 10^{-6} , treatment with 5-Aza-CR results in 10^{-3} reversion frequency. Reactivation of G6PD and PGK was additionally observed in one out of 14 HPRT⁺ revertants.

While independent observations provide very strong support for methylation control of X chromosome inactivation, the overall modification level is similar in both Xs. Additionally, experiments using random X specific probes failed to detect methylation differences in the DNA from male and female cells digested with MspI or HpaII (Wolf and Migeon 1982, Lindsay et al. 1985).

1.4.5. DNA methylation during development:

Methylation is likely to function as signal for long term inactivation of genes. This makes it a likely candidate, for the developmental regulation of gene expression. Such a role would involve alterations in methylation during embryogenesis in order to obtain lineage specific patterns. Since *de novo* methylation is required for alterations in modification patterns, developmental studies have focussed on the presence of de novo methylation in early embryonic stages.

There are two major limitations for studies on early embryonic development, the insufficient amounts of biological material, and the lack of molecular probes for genes that specifically express early in embryogenesis. One way to overcome these problems was the use of repetitive sequences as probes, which allows investigation of a greater part of the genome and requires less material (Chapman et al. 1984, Monk and Lehnert in press). Another widely used method is the introduction of a known probe in the embryo by means of microinjection or viral infection, and the subsequent investigation of the expression and methylation status of the introduced DNA (Jaenisch 1976, Jaenisch et al. 1981). Alternatively, studies can be performed on the

expression of endogenous retroviral genomes.

Using repetitive sequences it was shown that tissues of the embryo proper are methylated as early as 7.5 days of mouse development (Chapman et al. 1984). Interestingly, undermethylation was observed in extraembryonic trophectoderm and primary endoderm, as well as in gonads and germ cells, as late as the 16 1/2 day of gestation (Chapman et al. 1984, Monk and Lehnert in press). The difference in methylation level between the various tissues of the mouse embryo indicates severe methylation changes in the early stages of development.

Introduced retroviruses do not replicate in preimplantation mouse embryos, although reactivation was detected in some adult tissues (Harbers et al. 1981, Jahner et al. 1982). Additionally, no virus production was detected in infected undifferentiated teratocarcinoma cells, while low levels of activation occurred during differentiation (Stewart et al. 1982). In F9 cells the viral genomes were methylated immediately after integration, while the PCC4 teratocarcinoma cell line showed a delayed process of de novo methylation (Niwa et al. 1983, Gautsch and Wilson 1983). When infections were carried during or after differentiation of EC cells the retroviral genomes were maintained unmethylated. Finally, methylation was shown to be responsible for retroviral inactivity, since expression of inactive genomes was induced following treatment with demethylating agents (Stewart et al. 1982).

1.5.1 Activation of genes by 5-Azacytidine:

A. Incorporation into DNA and RNA:

In another experimental approach cells were treated with inhibitors of DNA methylation and the effects on the phenotype and gene expression were studied. One of the most widely used demethylating agents is 5-azacytidine (5-Aza-CR) which was first synthesized in Czechoslovakia for possible use in cancer chemotherapy (figure 2) (Piskala and Sorm 1964).

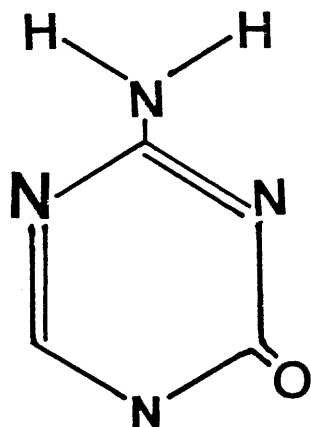
5-Aza-CR when introduced into the cells is converted by phosphorylation to 5-Azacytidine diphosphate (Lee et al. 1974, Liacouras and Aderson 1979). This can either integrate into RNA after phosphorylation to 5-Azacytidine triphosphate, or be reduced to 5-Azadeoxycytidine diphosphate and become incorporated into the DNA in the place of cytosine. The 5-Aza-2-deoxycytidine (5-Aza-CdR) is another widely used demethylating agent, which is simply phosphorylated and subsequently incorporated only into the DNA. The efficiency of 5-Aza-CR or 5-Aza-CdR incorporation into DNA is comparable to that of cytosine (Bouchard and Momparler 1983).

Inhibition of cytosine DNA methylation by 5-Aza-CR incorporation was first reported for E.coli K12 (Friedman 1979). 5-Aza-CR and 5-Aza-CdR have been directly shown to inhibit methylation of newly synthesized DNA in C3H 10T1/2 cells (Jones and Taylor 1980) and L1210 cells (Wilson et al. 1983). It was initially thought that the demethylating capacity of incorporated 5-Aza-CR and 5-Aza-CdR is due to the substitution of nitrogen in the 5th (methylacceptor) position of the pyrimidine ring, which cannot be methylated. However,

Figure 2: The molecular structure of 5-azacytosine.

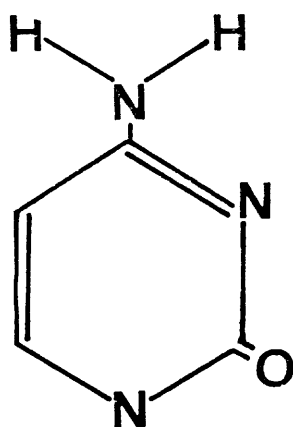
The structure of 5-Azacytosine is compared to that of cytosine and is indicated that methylation is inhibited by this analogue.

azacytosine

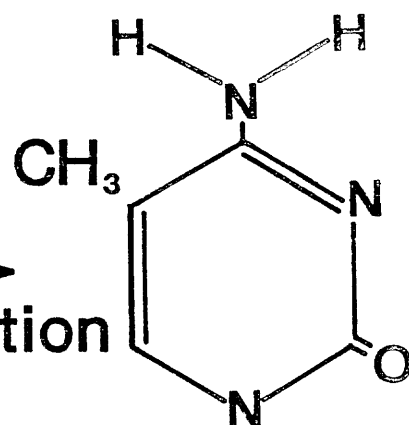


➡ **NO** methylation

cytosine



➡ methylation



the known level of substitution cannot explain the amount of demethylation (up to 85%). This led to suggestions that maintenance methyltransferase is directly inhibited by 5-Aza-CR or 5-Aza-CdR (Jones and Taylor 1980).

Inhibition of in vitro methylation was not detected in the presence of either 5-Aza-CR or 5-Aza-CdR (Taylor and Jones 1982), and it was directly shown that 5-Aza-CdR-triphosphate must be incorporated in the DNA in order to inhibit methyltransferase activity (Bouchard and Momparler 1983) . It was further reported that the DNA methyltransferase forms in vitro a tight complex with hemimethylated duplex DNA containing high levels of 5-Aza-CR. This cannot be dissociated even using high salt concentrations (Creusot et al. 1982, Taylor and Jones 1982). It is therefore possible that in vivo inhibition of methylation is due to covalent binding of methyltransferase to 5-Aza-CR containing DNA.

Demethylation introduced by 5-Aza-CR in the cells is heritable in the absence of further treatment (Jones and Taylor 1981). Incorporation of 5-Azacytosine into DNA leads to formation of hemimethylated DNA, which may result in demethylated DNA after the second round of replication if only maintenance methylation occurs. Alterations in DNA methylation were only detected using analogues with substitutions in the 5th position of the pyrimidine ring (Chambers and Taylor 1982).

B. Biological effects:

Treatment of cells with 5-Aza-CR or 5-Aza-CdR has dramatic physiological effects (table 4). Micromole concentrations of 5-Aza-CR

Table 4: Induction of differentiation and tumorigenicity by 5-Aza-CR.

System	Cell types induced	Reference
Mouse 10T1/2	Mesodermal stem cells	Konieczny & Emerson 1984
10T1/2	chondrocytes, adipocytes	
3T3	cartilage	Taylor & Jones 1979
C3H10T1/2	muscle cells	Constandinides <u>et al.</u> 1977
FriendEL	erythroid differentiation	Creusot <u>et al.</u> 1982
Human K-562	erythroid differentiation	Campari <u>et al.</u> 1984
Hamster CHEF/18	tumorigenesis	Harrison <u>et al.</u> 1983
CHEF/18	cartilage, adipocytes	Sager & Kovak 1982
BHK-21	transformation	Bouck <u>et al.</u> 1984

induce differentiation of the C3H 10T1/2 cell line to functional muscle cells, chondrocytes and adipocytes, several cell divisions after treatment (Constandinides et al. 1977, Taylor and Jones 1979). Similarly, multiple new phenotypes including muscle cartilage and adipocytes were induced by the analogue in CHEF/18 chinese hamster cells (Sager and Kovak 1982). Cartilage cells also arise after treatment of Swiss 3T3 cells (Taylor and Jones 1979).

5-Aza-CR and 5-Aza-CdR are weak inducers of differentiation in strains of erythropoietic origin such as the Friend Erythroleukemia cells (FEL), leading to hemoglobin production 5-7 days after treatment (Christman et al. 1977, Creusot et al. 1982). Additionally, in vivo treatment of baboons (DeSimone et al. 1982), and human patients suffering from thalasemia (Ley et al. 1982), or sickle cell anemia (Charache 1982), results in induction of foetal globin expression.

Interestingly, 5-Aza-CR was further shown to promote transformation in BHK-21/C1 13 cells (Bouk et al. 1984), and tumorigenicity in CHEF/18 cells (Harrison et al. 1983). The phenotypic changes induced by 5-Aza-CR and 5-Aza-CdR are not likely to be due to genetic mutations, since these two agents do not have significant mutagenic activity (Landolph and Jones 1982).

C. Activation of silent genes:

Differentiation induced by 5-Aza-CR treatment can be considered as an extensive case of alterations in gene expression. Indeed 5-Aza-CR induces expression of previously inactive loci in cultured cells from a variety of species (table 5). One of the first

Table 5: Induction of gene expression and by 5-Aza-CR.

Species & Cells	Gene	Reference
a) Endogenous		
Hamster CHO	pyrroline5carboxylate synthetase	Harris 1984a
CHO	ornithine carbamoyl transferase	
V79	glutamine synthetase	Harris 1984b
CHO	ornithine carbamoyl-transferase	Delers <u>et al</u> 1984
CHO	DNA repair gene (X-Ray sensit.)	Jeggo & Holliday 1986
CHO	ornithine decarboxylase	Steglich <u>et al.</u> 1985
Mouse W7	Metallothionein-1	Compere & Palmiter 1981
MEL	γ -globin	Ley <u>et al</u> 1984
T-lymph.	Glucocorticoid receptor	Gason <u>et al.</u> 1983
Rat sarcoma	Asparagine synthetase	Sugiyama <u>et al</u> 1984
GH ₂	prolactin	Ivarie & Morris 1982
HeLa-mouse myo-fibril hybrids	Muscle specific genes	Chiu & Blau 1985
Human HeLa	h-metallothionein I _B	Heguy <u>et al.</u> 1986
Nicotiana Tabacum	Ti (Tumor inducing plasmid)	Amasino <u>et al.</u> 1984
b) Exogenous		
Human	SV40 properties	Okada <u>et al.</u> 1984
Mouse germ cells	M-MuLV	Stewart <u>et al.</u> 1982
Chicken	endogenous retroviruses	Groudine <u>et al.</u> 1981
Rat NRK cells	AKR-M-MuLV	Hoffman <u>et al.</u> 1982

observations of 5-Aza-CR induced reactivation was that of the Rous Sarcoma Virus in 5-Aza-CR treated hamster cells transformed by the virus (Altanerova 1972). Exposure of chicken cells to 5-Aza-CR was shown to result in reactivation of endogenous retroviruses (Groudine et al. 1981). Reactivation of the mouse ecotropic retroviruses Balb virus 1 and Balb virus 2 was also observed upon treatment of AKR-2B cells (Niwa and Sugahara 1981). More recent reports have shown reactivation of silent viral properties by 5-Aza-CR treatment, such as the reactivation of the Herpes Virus TK gene (Clough et al. 1982).

Significant evidence on the role of methylation in X chromosome inactivation was obtained using 5-Aza-CR (section 1.4.4.). Up to 1% reactivation of the HPRT gene located in the inactive X-chromosome was induced by treatment of hybrid cells with 5-Aza-CR (Mohandas et al. 1981), and the highest reactivation was obtained when synchronized cells were treated during the S phase of the cell cycle (Jones et al. 1982). Additionally, transfection of HPRT⁻ cells with the inactive X chromosome failed in producing HPRT⁺ transformants (Liskay and Evans 1980), while DNA from reactivated clones induced by 5-Aza-CR treatment was capable in transforming HPRT⁻ cells (Venolia et al. 1982, Lester et al. 1982).

Tissue specific loci such as the glucocorticoid receptor (Gason et al. 1983), or the developmentally regulated γ -globin (Ley et al. 1984), as well as the inducible metallothionein genes (Compere and Palmiter 1981), were also reactivated by 5-Aza-CR. Finally, 5-Aza-CR was shown to induce expression of genes coding for enzymes involved with a wide range of metabolic pathways, like the ornithine decarboxylase (Steglich et al. 1985), or glutamine synthetase (Harris 1984a) (table 5 and 6). One of the best documented such genes is the

thymidine kinase which will be reviewed in section (1.5.2.).

In many of the above mentioned studies reactivation was followed by analysis of the methylation pattern of the reactivated gene as opposed to the inactive state. Change in the phenotype was always associated with detectable demethylation (Searle et al. 1984, Amassino et al. 1984, Campari et al. 1984). Although several lines of evidence relate phenotypic alterations with demethylation induced by 5-Aza-CR it should be considered that this agent is also involved with other biological pathways (Lu and Randerath 1980, Tsao et al. 1984). Therefore it is possible that other functions as well as demethylation account for the observed effects.

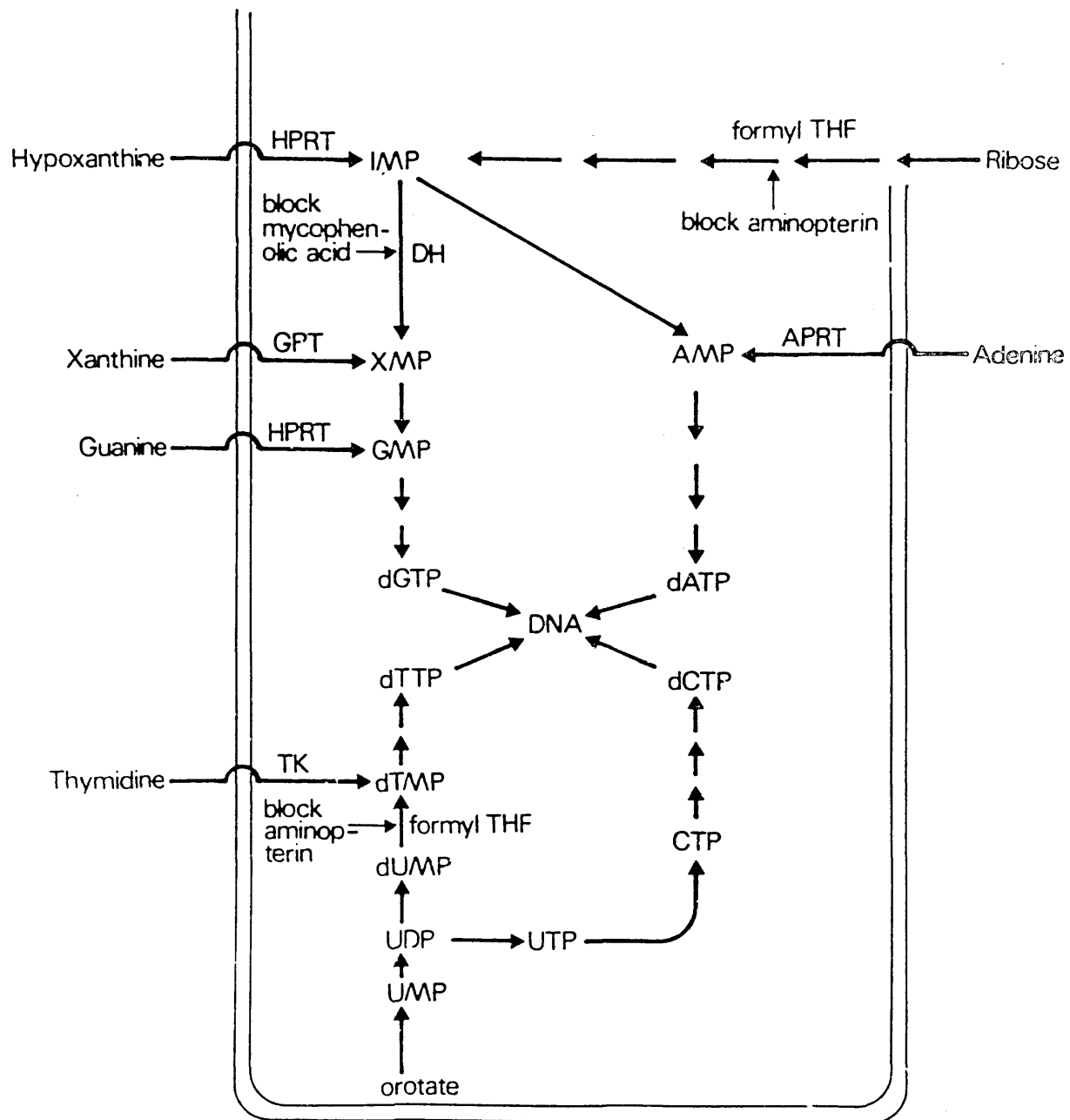
1.5.2. Methylation control of TK gene expression:

Thymidine kinase (TK) is an enzyme involved with the pyrimidine salvage pathway, which is coded by an autosomal gene. It catalyses an ATP and Mg^{2+} dependent phosphorylation of thymidine or its analogues to form deoxyribonucleotides (figure 3). This enzyme is responsible for recycling of endogenous thymidine via the pyrimidine salvage pathway. Thymidine kinase within mammalian cells is closely correlated with their proliferation (reviewed by Mitchison 1971), and enzyme activity is markedly elevated during S phase of the cell cycle. It was recently shown that cell cycle regulation of TK expression must be tightly associated with gene sequences. This was indicated by the finding that TK expression in mouse LTK⁻ cells transformed to TK⁺ with either total mouse genomic DNA, or a cloned human TK gene, is under cell cycle control (Schlosser et al. 1981, Bradshaw et al. 1984).

Table 6: Induction of thymidine kinase gene expression by 5-Aza-CR

Species & cells			reference
a) endogenous			
Hamster	V79	thymidine Kinase	Harris 1982
	CHO	" "	Gounari <u>et al</u> unp
Mouse	L5178Y	thymidine kinase	Nakamura & Okada 1983
	L-61-M	" "	Liteplo <u>et al.</u> 1984
	FEL	" "	Hikey <u>et al.</u> 1986
b) exogenous			
Mouse	LM	Herpes Thymidine Kinase	Clough, 1982
			Christy & Scangos 1982
			" " 1984

Figure 3: Diagram of the salvage pathways of nucleotide biosynthesis



Cells expressing TK can be selected by cultivation in medium containing a combination of hypoxanthine, thymidine, and aminopterin. Since aminopterin blocks the de novo pathway of nucleotide biosynthesis, cells can only survive through the salvage pathways (Littlefield 1966, Szybaska and Szybaski 1962). However, TK deficient cells cannot use externally supplied thymidine and get killed. Selection against TK expression is also possible, since TK activity would allow incorporation of bromodeoxyuridine (BUdR) in the DNA. This leads to increased radiation sensitivity, DNA damage and subsequent death of the cells expressing TK. The ability to select for and against TK enzyme activity has been used in a variety of studies. Initially in somatic cell genetics isolation of TK deficient mutants allowed studies on ploidy, or mutation frequency (reviewed by Siminovich 1976). Recently cloned TK genes were used as selectable controls to monitor DNA mediated gene transfer in TK deficient cells (Wigler et al. 1981).

Since TK was widely used as a selectable marker it was important to define the parameters affecting its expression. Evidence for methylation control of TK gene expression was obtained for the mouse and hamster genes (table 6). Thus, a chinese hamster V79 cell line which expresses stable TK deficiency shows up to 10% reversion to TK⁺ upon treatment with demethylating agents (Harris 1982). Mutagens like EMS have only a weak effect on TK⁺ reversion, approximately 5 orders of magnitude lower than the reversion induced by 5-Aza-CR. TK⁺ revertants show an elevation in TK activity, which is still only 10% of the activity detected in the TK⁺ wild type cells. Induction of TK gene expression by 5-Aza-CR was recently reported in a spontaneously occurring TK⁻ Friend Erythroleukemia clone (Hikey et al. 1985).

Additionally, a spontaneous TK deficient murine tumor cell line expresses up to 3% reversion to TK⁺ following exposure to 5-Aza-CR (Liteplo et al. 1984).

Clough et al. (1982) found that 5-Aza-CR induces activation of the TK gene in mouse cells transformed with HSV containing, inactive TK. Molecular studies associated reactivation with demethylation although it was not defined whether this occurred directly on TK sequences. In other studies Wigler et al. (1981) showed that partially methylated HSV TK gene does not efficiently transform LTK⁻ cells, suggesting that methylation inhibits expression of the gene. Finally Christy and Scangos (1982, 1984), successfully transformed mouse L cells with HSV TK gene. They then selected for TK⁻ spontaneous revertants, such isolates express no TK activity although they contain intact by restriction analysis TK genes. In a reverse process 5-Aza-CR induced TK⁺ revertants were obtained and studied at the molecular level. While the initially transformed L cells carried undermethylated HSV TK genes, these were found methylated in the derived TK⁻ clones in the 5' end and gene sequences. However, undermethylation was again detected in the reexpressing TK⁺ cell lines. Methylation control of HSV TK gene expression was further confirmed, using molecules methylated in vitro either at the 5' or 3' end of the gene. These molecules were inserted in LTK⁻ cells and their accurate transcription was studied. DNA methylation in both the 5' promoter and 3' structural region of the TK gene had a strong inhibitory effect at the transcriptional level (Keshet et al. 1985).

1.5.3. Methylation control of MT gene expression:

Metallothioneins were discovered by Margoshes and Vallee (1957) during their search for cadmium binding proteins. These are small (6000-7000 dalton) metal binding proteins which have a high cystein content and no aromatic aminoacids or histidine. They have been detected in a great variety of organisms and they can be classified in two major groups MTI and MTII according to their electrophoretic behaviour. The multiple MT forms in vertebrates are encoded by a multigene family (Griffith et al. 1983, Karin and Richards 1982, Searle et al. 1984). In the human genome in particular the MT family consists of at least 12 genes (Karin et al. 1984) about seven of these are functional and clustered with another 2 mutant MT sequences at 16q22 (Le Beau et al. 1985). Linkage of MTI and MTII genes was also detected for the mouse genome (Searle et al. 1984). Two cDNA species corresponding to MTI and MTII genes were further cloned and sequenced from chinese hamster cells. These show up to 90% homology with the equivalent mouse and human genes (Griffith et al. 1983). Hybridisation studies have suggested linkage between the chinese hamster MTI and MTII genes on the chromosome 3 (Crawford et al. 1985).

Induction of MTI expression by UV irradiation is associated with extensive demethylation of the gene in mouse S49 lymphoma cells (Lieberman et al. 1983). These cells have spontaneously lost the ability to induce MT expression and they are sensitive to the presence of heavy metals. Mouse thymoma W7 cells are another spontaneous cell line sensitive upon exposure to heavy metals. Treatment of these cells with the demethylating agent 5-Aza-CR results

in about 1500 times induction of MT expression above background (Comper and Palmiter 1981). These findings indicated that at least the mouse MT genes respond to methylation control of their expression. From the above studies it was also suggested that extensive demethylation throughout the gene is required for restoration of MT expression.

The human hMT-I_B gene was recently identified which was shown to express only in hepatoma and renal carcinoma cell lines. This specificity in expression is controlled by a cis acting mechanism which involves methylation. Thus, it was shown that the 5' flanking region of the hMT-I_B gene is highly methylated in HeLa cells, a nonexpressing cell type and undermethylated in a hepatoma (expressing) cell line. Incubation of HeLa cells hMT-I_B with 5-Aza-CR led to induction of hMT-I_B gene transcript (Heguy *et al.* 1986).

1.6.1. Chinese Hamster Ovary cells:

Chinese hamster ovary cells (CHO) were initially isolated by Puck Ciecura and Robinson (1958) and thereafter extensively applied in somatic cell genetic studies and the isolation of mutants, because they grow fast and they have high plating efficiency. During the evolution of the cell line the CHO genome has undergone severe chromosome and nucleotide rearrangements, although the total DNA content is close to that of normal chinese hamster cells. Further karyotypic studies have revealed that a number of single chromosomes remain unaltered (Deaven and Peterson 1973).

An interesting feature of the CHO phenotype is the large number of phenotypically recessive mutants that have been isolated from this cell line (for review see Siminovitch 1976). It seems

probable therefore that genes in CHO cells are not functionally diploid. Siminovitch (1976) suggested that hemizyosity might be a result of extensive chromosome rearrangements during the evolution of CHO cells. Studies performed on chromosome distribution of late replicating regions, revealed that even chromosomes with normal banding show abnormal labelling of late replicating regions (Deaven and Peterson 1973). Although hemizyosity seems to spread throughout the genome two steps were required for isolation of mutants at the APRT locus (Chasin 1974). Also, electrophoretically polymorphic mutations were detected in CHO cells after U.V. mutagenesis (Siciliano et al. 1983). Thus, it is apparent that a large number but not all the genes in this cell line are functionally hemizygous.

It has been recently demonstrated that several recessive mutations in CHO cells revert to the wild type upon treatment with demethylating agents. Thus, proline independence was shown to be induced in the spontaneously proline requiring CHO KI cell line (Harris 1984a). Relief of spontaneous arginine dependence due to reactivation of ornithine decarboxylase was also reported to result from exposure to 5-Aza-CR (Steglich et al. 1985). Additionally several X-ray sensitive CHO mutants revert to the X-ray resistant phenotype upon 5-Aza-CR treatment (Jeggo and Holliday 1986). These findings place functional hemizyosity under a different perspective, since they suggest that epigenetic events might underlie behind stable heritable variation. Irrespective of the mechanism responsible for functional hemizyosity it makes CHO cells a particularly suitable system for isolation of mammalian mutants.

The functional hemizyosity as well as spontaneously occurring deficiencies is a feature observed in tissue culture cell lines.

Relief of the deficiency has been shown to be induced by treatment with demethylating agents in several instances (Compere and Palmiter 1981, Liteplo et al. 1984, Harris 1984a,b). It is possible that tissue culture cells express defects in the regulation of DNA methylation, and spontaneously occurring alterations in methylation patterns may lead to inactivation of genes.

1.7.1 Aims of the investigation:

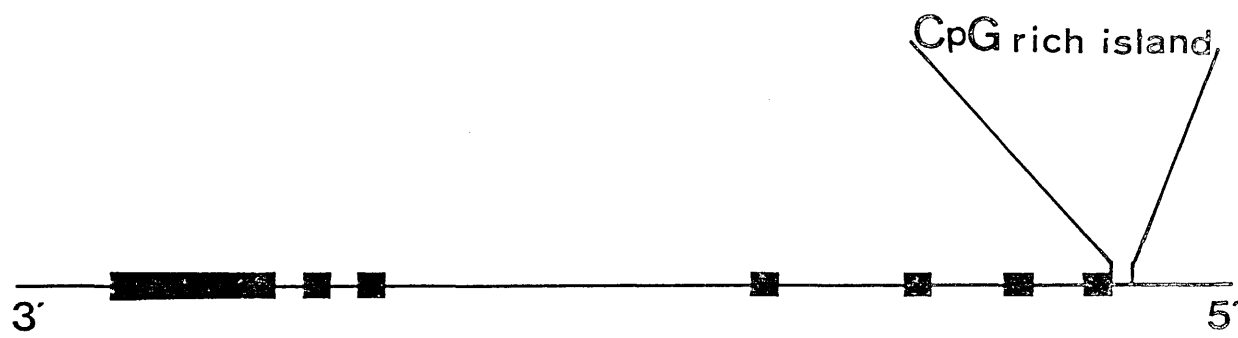
To better understand the biological function of DNA methylation in eukaryotes, the isolation of mutants is necessary. Isolation of methylation deficient mutants in bacteria has provided insight as to the function of cytosine and adenine modification in prokaryotes (Hattman et al. 1973, Marinus and Morris 1973, Marinus and Konrad 1976). From these studies it is possible that equivalent mammalian isolates may have increased mutability (analogy to the dam⁻ strains). If so, this could provide direct evidence that 5mC is important in directing mismatch repair. Alternatively, methylation deficient mutants may express alterations in gene expression as a result of changes in the methylation patterns.

The reversion of TK and MTI by 5-Aza-CR suggests that the expression of these genes is under methylation control and provides the means for selection of methylation deficient mutants. For this purpose Dr Jeggo used EMS mutagenesis, to isolate a TK deficient strain, from the already MT deficient CHO KI subline. TK deficiency occurs in CHO cells at the frequency expected for mutation in a haploid locus. Isolation of temperature sensitive methylation deficient mutants will be based on reactivation of these two genes

upon spontaneous demethylation. Since the viability of cells lacking DNA methylation is not certain it was decided to use a screening procedure to detect temperature sensitive DNA methylation deficient mutants. Another advantage of conditional mutants is that they allow reversible manipulation of the phenotype, and they provide an ideal internal control since both phenotypes can be studied in the same cells.

Successful isolates will be characterised in various ways. The biological phenotype of these strains and the conditions which result in the maximum level of TK^+ and MT^+ reversion will be investigated in order to gain insight to the nature of the mutation. The effect of treatment at the high temperature in the level of 5mC modification will also be examined biochemically. Finally, the availability of molecular probes for the hamster TK and MTI genes allows mapping of their methylation patterns and the possible changes that led to reactivation of these genes, as well as studies on their transcriptional status in the parent and reactivated strains. The 1219 bp of TK cDNA sequence are distributed through 11.2 kb of genomic DNA at the chromosome 7. This is coded by 7 exon segments and contains a 702 bp open reading frame (figure 4) (Lewis et al. 1983, Lewis 1986).

Figure 4: The map of the TK gene



CHAPTER 2

MATERIALS AND METHODS

2.1. Cell culturing techniques:

2.1.1. Cell Lines: The Chinese Hamster Ovary thymidine kinase deficient cell line was isolated by Dr P.A. Jeggo from CHO KI cells after mutagenesis by ethylmethansulphonate (EMS), followed by long term growth in bromodeoxyuridine (BUDR). Since CHO KI cells are already metallothionein deficient (Crawford et al. 1985), and because regulation of metallothionein expression was also studied, the parent cell line was termed CHO TK⁻ MT⁻. Thus, TK⁻ or MT⁻ indicates the deficient phenotype, while revertant cells are referred as TK⁺ or MT⁺ respectively. The CHO KI parent cell line was obtained from frozen stocks maintained at the Genetics Division of this Institute.

2.1.2. Tissue Culture Medium and selection conditions: Cells were routinely grown in minimal essential medium (MEM) (Gibco F15), from Gibco-Biocult Ltd, U.K., supplemented with 10% (v:v) foetal calf serum inactivated by one hour treatment at 56°C (FCS), antibiotics (penicillin 0.06mg/ml, streptomycin 0.1mg/ml), 0.11mg/ml sodium pyruvate, 0.25mg/ml glutamine and MEM non essential amino acids x100, 1% (Gibco-Biocult Ltd, U.K.). The FCS used during the isolation of the mutants was purchased from Gibco-Biocult Ltd, U.K., while all subsequent experiments were carried out using FCS obtained from Imperial Laboratories Ltd, U.K.. Cloning efficiency and growth were

essentially the same in either FCS.

TK⁺ revertants were selected in growth medium supplemented by 100 μ M hypoxanthine, 4 μ M aminopterin, 16 μ M thymidine (HAT medium). Medium containing 400 μ g/ml bromodeoxyuridine (BUdR) was used to select cells expressing the TK⁻ phenotype. Metallothionein expressing cells, are cadmium resistant (Cd^r) and were selected in 2 μ M CdCl₂. The frequency of 6-thioguanine resistant mutants (6-TG^r) due to inactivation of hypoxanthine-phosphoribosyltransferase, was measured by plating in medium containing 2 μ g/ml 6-thioguanine.

2.1.3. Tissue Culture Vessels: 25cm² and 80cm² plastic flasks, and 50mm and 35mm petri dishes were supplied by Nunc (U.K.) Ltd. The 24 flat bottom multiwell plates were obtained from Flow Laboratories Ltd, (U.K.).

2.1.4. Routine subculturing: 25cm² plastic flasks containing 5mls of medium were used for routine culture of cell stocks. Flasks were normally kept with loose tops in a LEEC incubator with humidified atmosphere of 5% CO₂ at 37°C. When cultures reached confluency they were washed with phosphate buffered saline (Dulbecco's PBS 'A'), followed by the addition of 0.5ml trypsin-versene (0.125% trypsin, 0.1% ethylenediaminetetracetic acid (EDTA)). After 1 minute incubation at 37°C in the presence of trypsin cells were detached by a sharp knock to the flask. Any clumps of cells formed were dispersed by careful pipetting up and down through the narrow bore of a Pasteur pipette. 5mls of medium were added to inactivate trypsin. Cells were then diluted in fresh medium at the desired densities.

2.1.5. Cell counting procedure: Cell numbers were estimated by counting a 0.2ml aliquot of the cell suspension diluted in 9.8mls of Isoton II (Coulter Electronics Ltd.) with a Coulter Counter (Model ZB1, Coulter Electronics Ltd, Harpenden, U.K.) that had been previously calibrated for CHO cells.

2.1.6. Cell storage and reconstitution: Cells to be frozen were harvested by trypsinisation, counted and transferred to Sterilin 25ml screw cap tubes, and centrifuged with a bench centrifuge at 2,000rpm for 10 minutes. Pellets were resuspended in fresh medium with 10% dimethylsulfoxide (BDH Chemical Ltd), and 1ml aliquots containing 5×10^5 - 10^6 cells were transferred to 2ml freezing ampoules (Nunc U.K., Ltd). An initial 2-4 hours time was allowed at -20°C before they were finally stored in liquid nitrogen.

Frozen cells were thawed out as quick as possible and added to 5mls of prewarmed medium in 25cm^2 flasks. After 4 hours incubation period at 37°C , when the cells had attached to the surface of the flask, the medium was changed.

2.1.7. Plating Efficiency: Approximately 200 cells were seeded into 50mm petri dishes with 5mls of medium, and incubated for 7-10 days until visible colonies had formed. The medium was removed, and the cells were first fixed in methanol:acetic acid (3:1 ratio) for 2 minutes, and then stained for 30 minutes in crystal violet. The dishes were washed with tap water, and colonies containing approximately 50 cells were counted. The plating efficiency is the ratio of colonies formed to cells plated.

2.1.9. Measuring the doubling time of CHO cells by a one step growth curve: The cell doubling time was estimated by seeding 2×10^4 cells into multiple 50mm petri dishes. 4 hours later and every 24 hours thereafter, duplicate dishes were trypsinised and counted, until cells stopped growing exponentially.

2.1.10. Survival after drug treatment: To examine cell survival after various drug treatments, 200 cells were plated into 50mm petri dishes, left to attach for 4 hours and then subjected to graded drug concentrations. 24 hours later plates were media changed with drug free medium and allowed to grow for 7 to 10 days until visible colonies were formed. Cells were fixed, stained, washed and counted.

2.1.11. Treatment with 5-Aza-CR and 5-Aza-CdR: 5×10^4 cells were seeded in 25cm^2 plastic flasks and incubated for 48 hours. Appropriate concentrations of freshly prepared 0.1mg/ml stocks of 5-Aza-CR or frozen at -70°C 1mg/ml stocks of 5-Aza-CdR (Sigma Chemical Company LTD) were added to the growth medium for 24 hours. Then cells were media changed with drug free medium, and they were either subcultured or allowed a recovery period before plating in selective medium, as specified in the text.

2.1.8. Mycoplasma contamination: 2×10^4 cells were seeded on a sterile coverslip (22mmx22mm) in a 35mm petri dish with 2mls of medium and left for 24-48 hours. Cells were fixed in situ with methanol:acetic acid (3:1) for two minutes and then fresh fixative was added for another 5 minutes before air drying the coverslip. The cells were stained for 20 minutes in $0.1\mu\text{g/ml}$ of Hoescht 33258 stain

(Reidal de Haeneg, Seelze, Hannover, Germany) in PBS and washed in distilled water. After drying, the coverslip was placed cell layer down onto a drop of glycerol:PBS (9:1), on a glass slide. The presence of mycoplasma was examined under the U.V. light using a BG-3 excitation filter with a Zeiss fluorescent microscope system. In the rare occasions of mycoplasma contamination, small bright green spots were detected in the black field. Contaminated cultures were immediately discarded.

2.1.13. Mutagenesis treatment About 5×10^5 CHO TK⁻ MT⁻ cells were seeded in 80cm² flasks and incubated between 24-48 hours before mutagenesis using ethylmethansulfonate (EMS) (Sigma Chemical Company LTD) or X-rays. After 24 hours cells were media changed with mutagen free medium. When cultures reached confluency, usually 2 days after treatment, they were split to aliquots containing 5×10^5 cells each and frozen in liquid nitrogen. The frequency of 6-thioguanine (6-TG) resistant mutations (TG^R) was estimated by plating 10^5 mutagenised cells into each of five 90mm petri dishes containing 2µg/ml 6-TG. After 7-10 days of incubation, colonies were fixed stained and counted. Only treatments yielding more than 10^{-3} 6-TG resistant colonies were further used.

For the X-ray mutagenesis of cells a Neuton Victor X-ray machine was used with an output of 23 Kev at a dose rate of 48rads/minute. The diffuser plate was not fitted.

2.1.14. Estimation of back-reversion 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

according to the methods by Luria and Delbruck (1943) and Lea and Coulson (1949).

100 cells were seeded into each of 10 25ml plastic flasks and cultivated to an approximate number of 10^5 for TK^+ revertant clones induced by demethylating agents and 5×10^5 for tsm TK^+ revertants (section 3.3.1. and 4.2.3.). Cultures were then trypsinised and approximately 3/5 of the total number of cells were seeded in each of three 90mm petri dishes in BUdR containing medium. After 7-10 days incubation plates were fixed stained and counted. In order to estimate cloning efficiency per population 200 cells were plated in each of two 50mm petri dishes and incubated until visible colonies were formed.

2.1.15. Procedure for the isolation of methylation deficient mutants:

Ampoules of mutagenised CHO TK^- cells were thawed and grown to confluency. 500 cells were seeded into 90mm petri dishes in medium containing $400 \mu\text{g/ml}$ 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 overlayed with 5mls of 0.5% soft agar (Jeggo and Kemp 1983). A further 2 days incubation period is required for the cells to become rounded and start growing into the agar. At this stage they could be easily transferred in two replicate multiwell plates containing 1.5mls 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 hours incubation at 34°C plates were examined under a low power microscope and if the average number of cells per well was

10^3 approximately one plate of each replicate was shifted to 39°C . 24 hours later $15\mu\text{l}$ HAT ($\times 100$) were added, and the plates were incubated at 34°C . Any isolate which produced HAT resistant clones could be recovered from the untreated replica plate.

These isolates were plated in 80cm^2 flasks and incubated at 34°C until they reached confluency. An aliquot of these cells was then used for a quantitative secondary screening while the rest were stored frozen in liquid nitrogen. For the secondary screening from each isolate, 1×10^5 cells were seeded into each of five 90mm petri dishes, which were incubated at 39°C for 24 hours. HAT was added to the medium immediately after the plates were transferred back to 34°C . Cultures were allowed 7-10 days incubation to form visible colonies before they were fixed, stained, washed and counted.

2.2. Molecular Biological techniques:

2.2.1. Phenol/Chlorophorm extraction and ethanol precipitation of nucleic acids: Extraction of the DNA with 10mM Tris pH7.5, 1mM EDTA (TE) saturated-phenol : chloroform : isoamylalcohol (PCI, 25:24:1, v:v:v), containing 0.1% hydroxyquinoline was performed as in (Maniatis et al. 1982). When isolating high molecular weight genomic DNA, the DNA solution and PCI were mixed by gentle swirling, and large bore pipettes were used for transfer of the aqueous phase.

After adjusting the salt concentration to 0.5M NaCl, 2.5 volumes of ethanol were added and the solution was allowed to cool down at -20°C for 30 minutes. Nucleic acids were then recovered by centrifugation at 12,000g for 15-30 minutes at 0°C . High molecular weight DNA was recovered by gently mixing the DNA solution after the addition of salt and ethanol and lifting out the fibrous DNA precipitate with a sealed pasteur pipette. Ethanol precipitation was always followed by washing with 70% ethanol to remove the excess salt.

2.2.2. Bacterial strains and growth: Plasmids were propagated in the recA⁻ E.coli strain DH₁. Bacteria were grown in Luria Broth (LB) medium supplemented with 30 $\mu\text{g}/\text{ml}$ ampicillin or 15 $\mu\text{g}/\text{ml}$ tetracycline depending on the antibiotic resistance gene carried by the plasmid of interest.

2.2.3. Preparation of competent DH₁ cells and transformation: The methods used were modifications of those described by Mandel and Higa (1970) and by Cohen et al. (1973), in which the uptake of DNA by

bacteria was enhanced by their treatment with calcium chloride.

0.1ml of a bacterial overnight culture were inoculated into 20mls of LB medium and grown to OD_{600} 0.6. This preculture was diluted into 40mls of LB and grown to stationary phase. Cells were cooled on ice for 20 minutes and harvested by spinning with a bench centrifuge for 5 minutes at 4,000rpm, the pellet was resuspended in 1ml of 100mM $CaCl_2$, and 0.5 of the culture volume of $CaCl_2$ were added. The mixture was incubated in ice for 20 minutes. The cells were pelleted again by spinning with a bench centrifuge for 5 minutes at 4,000rpm, and resuspended in 400 μ l 100mM $CaCl_2$. Competent cells were either used immediately or stored in 30% glycerol at $-70^{\circ}C$.

50 μ l of frozen competent cells were thawed on ice. 1-10 μ l DNA (~ 100ng) and 100 μ l TCM buffer (10mM Tris pH7.5, 10mM $CaCl_2$, 10mM $MgCl_2$) were added and the mixture was left in ice for 30 minutes. The bacteria were subjected to heat shock for 90 seconds at $42^{\circ}C$. 1.2mls of LB was then added and the cells incubated at $37^{\circ}C$ for 45 minutes. Bacteria were pelleted by centrifugation with an Eppendorf microfuge for 1 minute, resuspended in 100 μ l LB medium spread on an agar plate containing the appropriate antibiotic and incubated at $37^{\circ}C$. Colonies of transformed bacteria which appeared after ~16 hours were picked inoculated into 10mls of LB and a miniculture grown.

2.2.4. Large scale preparation of plasmid DNA from bacterial cultures: E.coli transformed with recombinant plasmids were grown in LB medium and the plasmid was amplified as in (Maniatis et al. 1982). These bacteria were lysed by alkali and the plasmid DNA was purified by centrifugation to equilibrium in cesium chloride-ethidium bromide density gradients (Maniatis et al. 1982).

The plasmid DNA preparation was extracted repeatedly with water-saturated butanol until ethidium bromide was removed from the aqueous phase. DNA was diluted three times with TE, precipitated and resuspended in TE twice, and treated with 100 μ g/ml DNase free RNase for 30 minutes at 37 $^{\circ}$ C. The plasmid DNA was finally resuspended in TE and stored at -20 $^{\circ}$ C.

2.2.5. Preparation of plasmid DNA from bacterial minicultures: 10ml bacterial minicultures were inoculated from single colonies of bacterial transformation (2.2.3). Bacteria were pelleted by centrifugation with a bench centrifuge at 4,000rpm for 10 minutes resuspended in 200 μ l 50mM glucose, 10mM EDTA, 25mM Tris pH8 and transferred to a 1.5mls snapcap tube. Cells were lysed by alkali using a scaled down version of the method described in section 2.2.4.. This preparation was treated with 100 μ g/ml RNase for 30 minutes at 37 $^{\circ}$ C. The DNA was finally extracted with ethanol and resuspended in 100 μ l TE.

10ml bacterial cultures yielded 10-20 μ g plasmid DNA which was subsequently analysed by restriction enzyme digestion and agarose gel electrophoresis.

2.2.6. Isolation of high molecular weight DNA from tissue culture cells: The procedure was a modification of that described by Jeffreys and Flavell 1977. To avoid contamination of genomic DNA preparations with plasmid DNA, autoclaved solutions and disposable plasticware were used throughout.

1-5x10⁷ cells were pelleted by centrifugation with a bench centrifuge at 2,000rpm for 10 minutes, resuspended in 5-10mls of TNE

(100mM NaCl, 50mM Tris pH7.5, 5mM EDTA) and lysed by the addition of sodium dodecyl sulphate (SDS) to a final concentration of 1%. The solution was treated with 100 μ g/ml proteinase K (Boehringer Corporation LTD (BCL)) at 37°C for 4-16 hours, extracted with PCI and the DNA was precipitated with ethanol. DNA was dissolved in 0.5mls TE and digested with DNase free RNase for 1 hour at 37°C. A final extraction with PCI was followed by ethanol precipitation and the DNA, resuspended in 200-500 μ l of TE, was stored at 4°C. 10⁷ cells usually yielded 200 μ g DNA.

2.2.7. Isolation of RNA from tissue culture cells: RNA was prepared using guanidinium chloride to simultaneously disrupt the cells and inactivate nucleases (Chirgwin *et al.*, 1979, Cox 1968).

1-5x10⁷ cells were pelleted as in (section 2.2.6.) dispersed and lysed by addition of 500 μ l 6M guanidinium chloride. The lysate was mixed carefully and left on ice for 1-16 hours, extracted with PCI and the phases were separated by centrifugation at 12,000g for 30 minutes. The nucleic acids were precipitated with ethanol, pelleted by centrifugation, resuspended in 200 μ l, TMN (10mM Tris pH7.5, 10mM MgCl₂, 50mM NaCl) and treated with 1 μ g RNase free DNase (Amersham International PCL) for 20 minutes at 37°C. The solution was then extracted with PCI, the RNA was precipitated in 2 volumes ethanol and stored in -20°C. 10⁷ cells usually yielded 200-300 μ g RNA.

2.2.8. Isolation of Poly-A tailed RNA from tissue culture cells:

Poly-A tailed RNA was used for the Northern blot analysis of specific RNA species. To avoid degradation of the RNA by the action of ribonucleases all the procedures were performed under RNase free

conditions.

$3-5 \times 10^7$ cells in confluent 90mm petri dishes were washed in ice cold PBS. 2.5mls of ice cold STE (0.1M NaCl, 20mM Tris pH7.4, 10mM EDTA) were added, and the cells were scraped with a rubber policeman. The cell lysate was transferred in a cold homogenizing chamber and supplemented with proteinase K to 200 μ g/ml and SDS to 0.5%. The solution was homogenized with a Sorval Omnimixer at 3/4 full speed for 30 seconds. The homogenate was carefully transferred to disposable polyallomer tubes (Nunc (Ltd) U.K.) and incubated for 20 minutes at 37 $^{\circ}$ C. NaCl was added to 0.5M final concentration and 50-100mg Oligo (dT) cellulose (P.L. Biochemicals) per tube.

Tubes were put on a shaking platform for 2-16 hours at room temperature. Oligo (dT) cellulose was pelleted by centrifugation with a bench centrifuge at 2,500rpm for 30 seconds. The supernatant was removed and the cellulose was washed and pelleted again in binding buffer (0.5M NaCl, 20mM Tris pH7.4, 10mM EDTA, 0.2% SDS). 10mls of binding buffer were used to finally resuspend the oligo (dT) cellulose, which was poured into a disposable plastic column. Columns were washed with approximately 20mls of washing buffer (0.1M NaCl, 10mM Tris pH7.4, 1mM EDTA, 0.2% SDS) and eluted with elution buffer (1mM Tris pH7.8, 1mM EDTA, 0.2% SDS) prewarmed to 37 $^{\circ}$ C. The eluate which was collected in siliconized 15ml corex tubes, was mixed and 0.4mls were withdrawn for measurement of optical density.

NaAc pH5.5 was added to the solution to a concentration of 0.15M together with 20-40 μ gs carrier tRNA. RNA was precipitated with 2 volumes ethanol, stored at -70 $^{\circ}$ C overnight and pelleted by centrifugation at 10,000rpm for 20 minutes with a Sorval SS34 rotor. The precipitate was dissolved in 200 μ l TE with 0.2% SDS and

transferred to 1.5mls snapcap Eppendorf tubes. The samples were made up to 0.15M NaAc pH5.5 and precipitated again by 2 volumes ethanol, followed by cooling to -70°C for 15 minutes. RNA was recovered by centrifugation using a Sorval SS34 rotor at 10,000rpm for 5 minutes. Finally, the pellet was resuspended in TE with 0.2% SDS to give an RNA concentration of $500\mu\text{g/ml}$, and stored at -70°C .

2.2.9. Restriction enzyme digestion of DNA: Restriction endonucleases were purchased from Bethesda Research Laboratories. DNA was digested using the conditions recommended by the manufacturers for each enzyme. In order to examine completion of the digestion, 2 hours after the reaction had started $0.5\mu\text{g}$ of treated DNA were subjected to agarose electrophoresis. When genomic DNA was digested, 3units/ μg excess of enzyme was used, which was added in 2 fractions, 1.5units/ μg initiating the reaction and the rest 2 hours later. Digestion of the same sample with more than one enzyme was performed sequentially. The digestion conditions were corrected between each enzyme.

2.2.10. Nucleic acid gel electrophoresis:

a. Agarose gel electrophoresis of DNA: DNA fragments between 0.1-30kb were separated by electrophoresis through 0.7-1.5% horizontal agarose gels (Aaij and Borst 1972).

Electrophoresis was carried out with Tris Acetate buffer (Maniatis et al. 1982). After separation DNA was stained with ethidium bromide and visualised by ultraviolet (300nm) illumination (Sharp et al. 1973).

b. Formaldehyde:agarose gel electrophoresis of RNA: RNAs were electrophoresed through 1% agarose gels with 2.2M formaldehyde (35.7ml formaldehyde (37% stock) for 200ml agarose final volume). Electrophoresis was carried out with MAE buffer (20mM Mops pH7, 1mM EDTA, 5mM NaAc) adjusted to 2.2M formaldehyde. The buffering capacity of MAE is low and replacement of fractions of it with fresh buffer are required during electrophoresis.

a. Polyacrylamide gel electrophoresis of DNA: ^{32}P labelled DNA fragments which were products of S1 nuclease mapping (section 2.2.7) were analysed by electrophoresis through denaturing polyacrylamide: urea gels (Maniatis et al. 1982).

4-6% polyacrylamide gels of dimensions 15cm x15cm x0.3mm, were poured, loaded and electrophoresed in TBE buffer (0.089M Tris-Borate, 0.089M Boric acid, 0.002M EDTA) as in Maniatis et al. (1982). ^{32}P labelled restriction enzyme digests of PBR322 were used as DNA fragment molecular weight markers.

2.2.11. Recovery of DNA fragments from agarose gels: Agarose gels were prepared using "low temperature melting" agarose, when the electrophoresed DNA fragments were to be recovered. This melts at 37°C , i.e. below the melting temperature of DNA. DNA was recovered from melted agarose using a modification of the method described by Weisslander et al., (1979).

DNA was electrophoresed through 0.7-1.5% low melting agarose gels as described in Maniatis et al. (1982), and the appropriate DNA band was excised under long wave length ultraviolet illumination (300-360nm). The gel slice was melted at 65°C and extracted twice

with phenol saturated in 0.5M NaCl. The DNA solution was then extracted once with PCI precipitated with ethanol and resuspended in the desired volume of TE and stored at -20°C .

2.2.12. Dephosphorylation of DNA: The terminal 5' phosphates were removed from DNA fragments prior to labelling the 5' end with T4 polynucleotide kinase. Calf intestinal phosphatase (CIP)(Chakonas and Van De Sande 1980) was obtained from Boehringer Corporation LTD (BCL).

1 μl (0.5units) of CIP were added to the plasmid DNA, while this was digesting with the appropriate enzyme for isolation of the fragment to be used as a probe.

2.2.13. Preparation of ^{32}P radiolabelled probes:

a. Oligo-labelling of DNA fragments by hexanucleotide primers: Fragments or plasmid DNA to be used in Southern or Northern blot hybridisation were labelled by oligo-labelling using hexanucleotide primers (Feinberg and Vogelstein 1983).

50-200ng of DNA in 13 μl of H_2O were boiled for 3 minutes and transferred to 37°C for 10-60 minutes, before the addition of OLB buffer (Feinberg and Vogelstein 1983), 0.8 μl Bovine Serum Albumin (BSA) from a 10mg/ml stock solution, 2.5 units Klenow polymerase 1 (large fragment)(Pharmacia PLC) and 20 μCi of ^{32}P labelled dTTP. The solution was mixed and incubated at room temperature for 4-16 hours.

b. Labelling 5' ends using T4 polynucleotide kinase: T4 polynucleotide kinase (P.L.Biochemicals Inc.) was used to label the 5' termini of DNA fragments to be used as probes in S1 nuclease mapping

(section 2.2.16.).

0.1-1 μ g DNA was end labelled by treating with 10 units T4 polynucleotide kinase in 10 μ l, 50mM Tris pH9.5, 10mM MgCl₂, 5mM dithiothreitol, 1mM spermidine, 0.1mM EDTA, 20-60 μ Ci ³²P-ATP for 30 minutes at 37°C.

c. Labelling 3' ends using reverse transcriptase: Reverse transcriptase (Anglian Biotechnological) was used to label the 3' termini of DNA fragments to be used as probes in S1 nuclease mapping (section 2.2.16.).

0.1-1 μ g of DNA was treated with 5-15 units reverse transcriptase in 50 μ l, 50mM Tris pH8.3, 6mM MgCl₂, 40mM KCl, 100 μ g/ml BSA, 1mM dNTPs at 37°C for 1 hour. 20-60 μ Ci ³²P dNTP was included in the labelling reactions.

Unincorporated ³²P labelled nucleotides were separated from the probes by chromatography through 1 ml G50 sephadex spun columns (Maniatis et al. 1982).

2.2.14. Southern transfer and hybridisation:

Solutions:

Hybridisation	3xSSC
	10xDenhardt's
	0.1% w/v SDS
	200 μ g/ml denatured salmon sperm DNA
	10% w/v Dextran sulphate
	0.1% w/v Sodium pyrophosphate

Wash solutions	1st	3xSSC, 0.1% SDS
	2nd	1xSSC, 0.1% SDS
	3rd	0.3xSSC, 0.1% SDS
	4th	0.1xSSC, 0.1% SDS

Agarose gels to be blotted to nitrocellulose filters were soaked in 0.1M HCl for 20 minutes. The DNA was denatured by treatment with 0.2M NaOH 1.5M NaCl for 45 minutes and the gel was neutralised with 0.5M Tris pH7.5, 1.5M NaCl for 45 minutes, under constant agitation.

The gel was blotted onto a nitrocellulose filter as described in Maniatis *et al.* (1982). Transfer was allowed to proceed for 12-16 hours in the cold room, then the filter was washed in 3xSSC and baked for 2 hours in an 80°C oven.

Baked nitrocellulose filters were cut into strips (15x5cm) and wetted in 3xSSC. They were prehybridised by shaking in 15mls of hybridisation mixture for 4-6 hours at 65°C and then transferred individually into 15mls of hybridisation solution which contained oligo-labelled probe (specific activity > 10⁸ cpm).

When the probe contained repetitive sequences the labelled 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 DNA.

Hybridisation was for 16-24 hours at 65°C in plastic boxes. The filters were then washed to the desired stringency by shaking sequentially in the above wash solutions; each wash was for 30 minutes at 65°C in 350mls. Filters were then rinsed, air dried and autoradiographed

2.2.15. Northern transfer and hybridisation:

Hybridisation solution	3xSSC
	10xDenhardt's
	0.1% w/v SDS
	200µg/ml denatured salmon sperm DNA
	10% w/v dextran sulphate
	0.1% w/v sodium pyrophosphate
	50% v/v Formamide

2µg Poly-A⁺tailed RNA samples were electrophoresed through formaldehyde:agarose gels which were soaked for 60 minutes in 20xSSC, prior to blotting onto a nitrocellulose filter for 12-16 hours in the cold room. Filters were then washed in 3xSSC and baked for 2 hours at 80°C.

Baked nitrocellulose filters were cut into strips of 15x5cm and wetted in 3xSSC. They were prehybridised by shaking in hybridisation solution for 4-6 hours at 42°C, and then transferred to 25mls of preheated hybridisation solution containing 50-200ng oligolabelled probe (specific activity > 10⁸ cpm/µg DNA). Hybridisation was for 18-24 hours in plastic boxes. Filters were washed in reducing stringency as in section (2.2.14.). Washed filters were rinsed in 3xSSC, air dried and autoradiographed.

2.2.16. S1 nuclease mapping of RNA: Specific mRNAs were detected and mapped by S1 nuclease analysis (Berk and Sharp 1977), using end labelled probes (Weaver and Weissman 1979, Falavoro *et al.* 1980).

^{32}P labelled double stranded DNA probe (specific activity $\sim 10^7$ cpm) was resuspended in TE at concentrations of 1-2ng/ μl of probe were added in 30-50 μg of RNA previously resuspended in 30 μl , 40mM PIPES pH6.4, 1mM EDTA, 0.4M NaCl, 80% formamide, in a snapcap tube. The nucleic acids were then denatured by heating at 85 $^{\circ}\text{C}$ for 10 minutes. The tubes were quickly transferred to a water bath at 52-60 $^{\circ}\text{C}$ hybridisation temperature. The optimal hybridisation temperatures were chosen in order to minimize the formation of DNA-DNA hybrids while allowing RNA-DNA hybrids to form (Casey and Davidson 1977) and were determined empirically for each probe. Samples were hybridised between 12-16 hours and then digested for two hours at 20 $^{\circ}\text{C}$ with 300 units S1 nuclease in 300 μl , 200mM NaCl, 30mM NaAc pH4.8, 2mM ZnSO_4 . After digestion samples were extracted with PCI and the nucleic acids precipitated with ethanol. The products were electrophoresed on 4-6% denaturing polyacrylamide-urea gels

2.2.17. Preparation and hydrolysis of DNA to be used for HPLC

analysis: The level of 5mC in the CHO genome was determined by High Pressure Liquid Chromatography (HPLC) analysis of hydrolysed DNA. Appropriate amounts of DNA prepared as in section 2.2.6. were ethanol precipitated and resuspended in 500 μl formic acid (98% stock). The solution was transferred to glass ampoules, which were sealed using oxygen-city gas mixture flame. To prevent nucleotide deamination the ampoule air space was filled with nitrogen gas before sealing. Samples were then hydrolysed in an oven at 180 $^{\circ}\text{C}$ for 30 minutes. After cooling on dry ice the hydrolysed tubes were filed open.

At this stage the solution was dried under constant nitrogen flow by heating at 100 $^{\circ}\text{C}$. and DNA was resuspended in 200 μl 0.1M HCl

and transferred to 1.5ml eppendorf tubes. Any precipitates were pelleted by centrifugation with an eppendorf microfuge for 30 seconds. Supernatant was transferred to new 1.5ml tubes and used for determination of the nucleotide base composition.

2.2.18. HPLC system: For the HPLC a Watters Associates system was used which consisted of:

- a. Dual chromatograph pump
- b. Inject apparatus, Model U6K
- c. Cation column, Partisil SCx10u Type F
Female
- d. Programmer, Model 660
- e. Fixed absorbance detector, 280 nm, Model 440
- f. Variable absorbance detector, Model 450
- g. Pen recorder, red pen for variable detector
blue pen for fixed detector

Approximately 15-40 μ g hydrolysed DNA in 0.1M HCl were injected in the inject apparatus with a Hamilton microsyringe and eluted with 0.05M HCl through the column. The absorbance of the eluted samples was detected by the absorbance detectors and recorded with the pen recorder at two scales of sensitivity 1-0.01. This setting allowed the detection of both C and 5mC from the same injected sample.

CHAPTER 3REACTIVATION OF TK AND MTI GENES IN CHO CELLS BY 5-AZA-CR AND
5-AZA-CdR

3.1.1. Introduction:

It was necessary to demonstrate that methylation is responsible for the TK^- and MT^- phenotype in CHO TK^- MT^- cells. Thus, treatment with demethylating agents was used to induce reversion to the TK^+ and MT^+ phenotype. Reversion using demethylating agents was further compared to that induced by mutagenic treatment. In order to define whether methylation is involved with back-reversion to the TK^- phenotype, the stability of TK^+ revertants was also examined. Finally the ability of demethylating agents to again revert TK^- to TK^+ was investigated.

3.2.1. 5-Aza-CR and 5-Aza-CdR induce reversion to TK^+ in CHO TK^- MT^- cells:

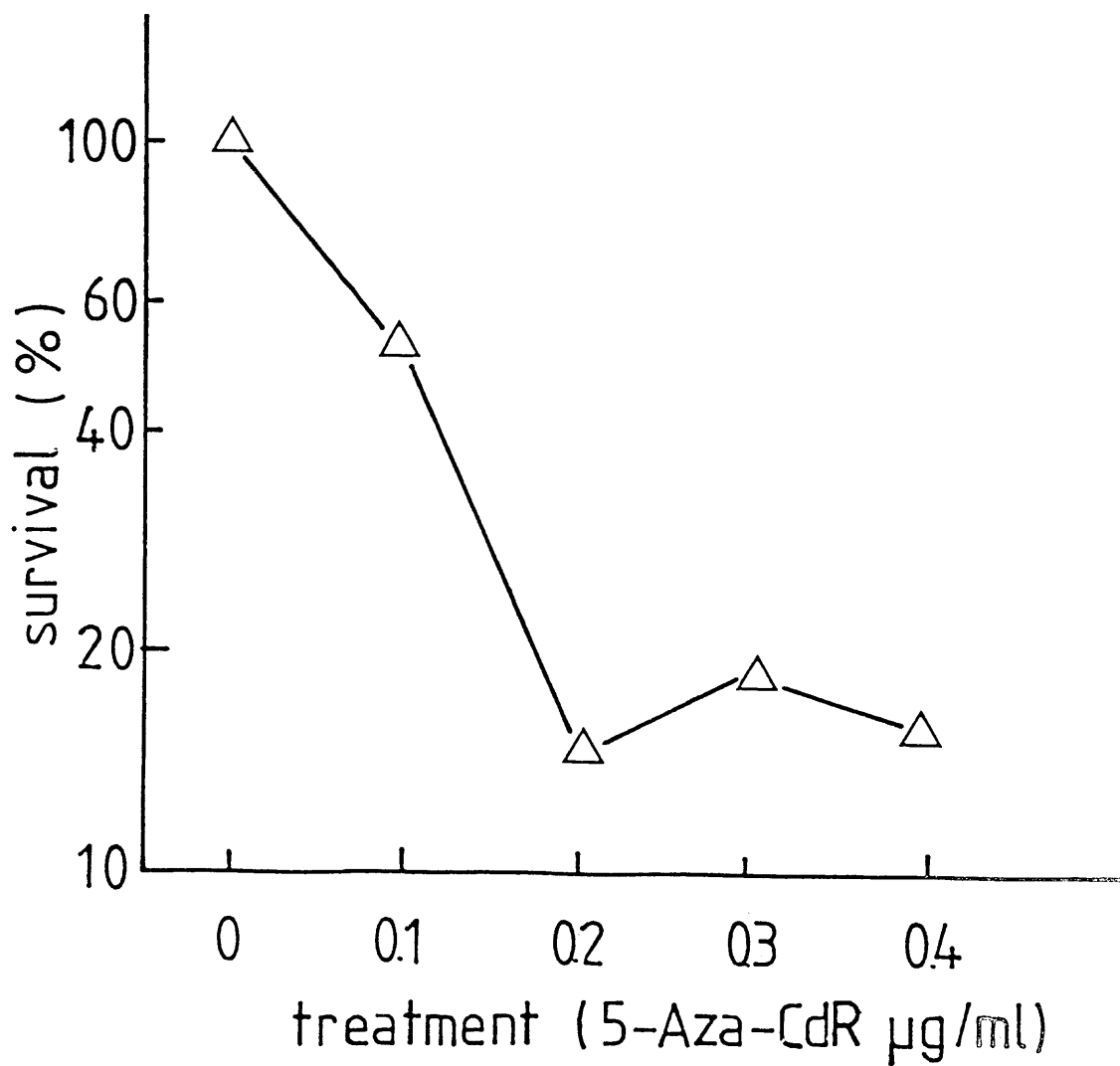
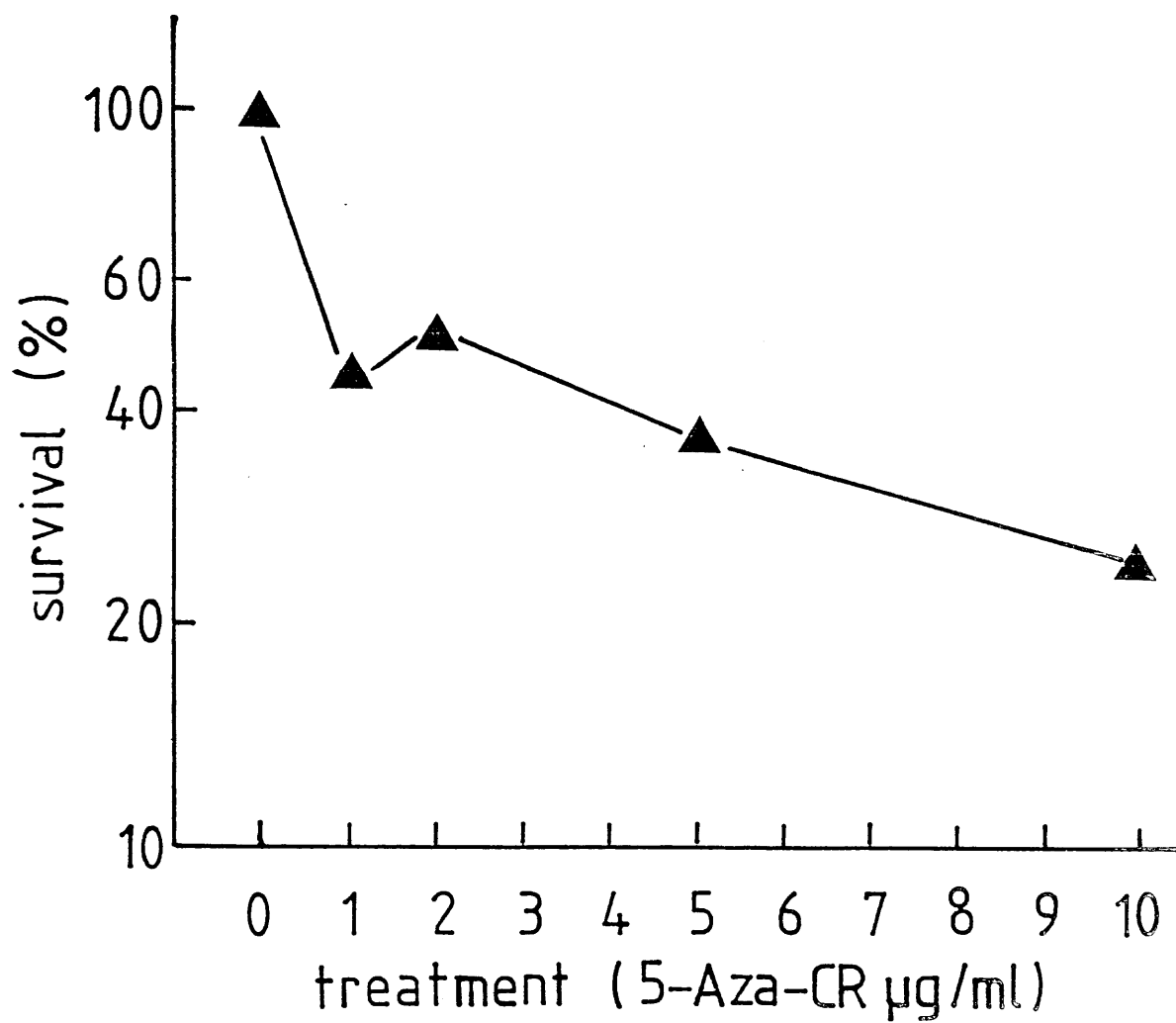
CHO TK^- MT^- cells were treated with 5-Aza-CR concentrations ranging from 0.3 to 10 μ g/ml. 24 hours later, the medium was replaced by drug free medium and the cells allowed to recover for periods between 2 to 7 days before HAT selection was applied and the survival measured. Treatment with increasing concentrations of 5-Aza-CR progressively reduces viability (figure 5). The frequencies of TK^+ reversion were corrected for the fraction of cells surviving the treatment. These frequencies are shown in figure 6.

Figure 5a: Effect of 5-Aza-CR treatment on plating efficiency of CHO TK⁻ MT⁻ cells.

Cells were inoculated into tissue culture dishes and treated with graded concentrations of 5-Aza-CR from freshly prepared stock. After 24 hours the cultures were rinsed and maintained in drug free medium for an additional 7-8 days to allow colony formation. The points represent the average colony count of three dishes, expressed as a percentage of the colony count of untreated cells

Figure 5b: Effect of 5-Aza-CdR treatment on plating efficiency of CHO TK⁻ MT⁻.

Cells were plated as in figure 5a and treated with graded concentrations of 5-Aza-CdR from stocks kept frozen (-70°C). The points represent the colony count of three plates, expressed as a percentage of the colony count of untreated cells.



The results obtained show that 5-Aza-CR treatment induces up to 1% reversion to TK^+ . This effect is dose dependant with an optimum in the range of 1 to $3\mu g/ml$. With higher concentrations of 5-Aza-CR a reduction in the TK^+ incidence is observed. The effect of expression time is also documented in figure 6. The maximum reversion is obtained when cells are allowed to recover for two days after treatment. Nevertheless up to $3.3 \times 10^{-4} TK^+$ can still be detected after 18 days cultivation of treated cells in normal medium.

TK^+ reversion in CHO $TK^- MT^-$ cells was also examined using 5-Aza-CdR, which is active in demethylating DNA at lower concentrations than 5-Aza-CR (Creusot *et al.* 1982). Thus, CHO $TK^- MT^-$ cells were treated with 5-Aza-CdR concentrations ranging from 0.05-0.4 $\mu g/ml$ for 24 hours. Cells were allowed to recover after treatment for 2-7 days and then plated in HAT medium and in normal medium to measure survival. 5-Aza-CdR is significantly more toxic to CHO $TK^- MT^-$ cells than 5-Aza-CR as shown in figure 5b.

The frequencies of TK^+ reversion induced by 5-Aza-CdR treatment are shown in figure 7. Maximum TK^+ reversion was observed with $0.4\mu g/ml$ 5-Aza-CdR concentration, but the survival in this concentration was less than 20%. Since $0.4\mu g/ml$ was the highest concentration used, it might be that increase in 5-Aza-CdR levels would induce greater reversion. This however could not be examined, because of the significant decrease in viability as a result of treatment. Figure 7 also shows the effect of expression time in the TK^+ incidence. Maximum reversion is obtained when 2 days recovery period after treatment is allowed. Under these conditions up to 28% of the cells surviving treatment express TK^+ phenotype (figure 7). This frequency is even higher than the one obtained using 5-Aza-CR.

Figure 6: TK^+ reversion induced by treatment of CHO $\text{TK}^- \text{MT}^-$ cells with 5-Aza-CR

CHO $\text{TK}^- \text{MT}^-$ cells were treated with graded concentrations from freshly prepared stocks of 5-Aza-CR. After 24 hours treatment the drug was removed and cultures were allowed to recover for 2 ($\blacktriangle$), 5 ($\triangle$) and 7 ($\diamond$) days in drug free medium before HAT selection. Each point represents the average colony count of three petri dishes seeded with 2×10^4 cells and corrected for the fraction of cells that survive treatment.

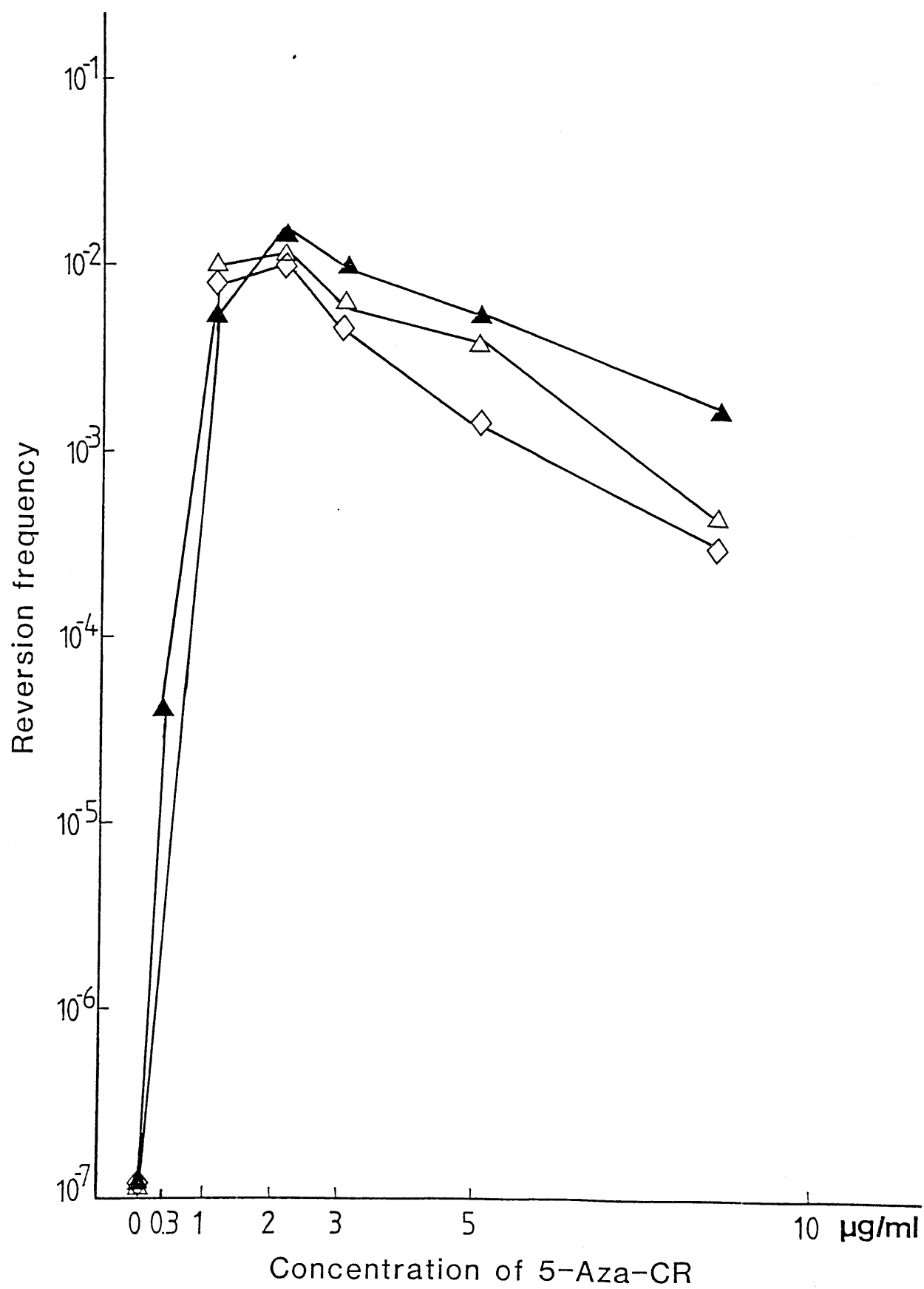
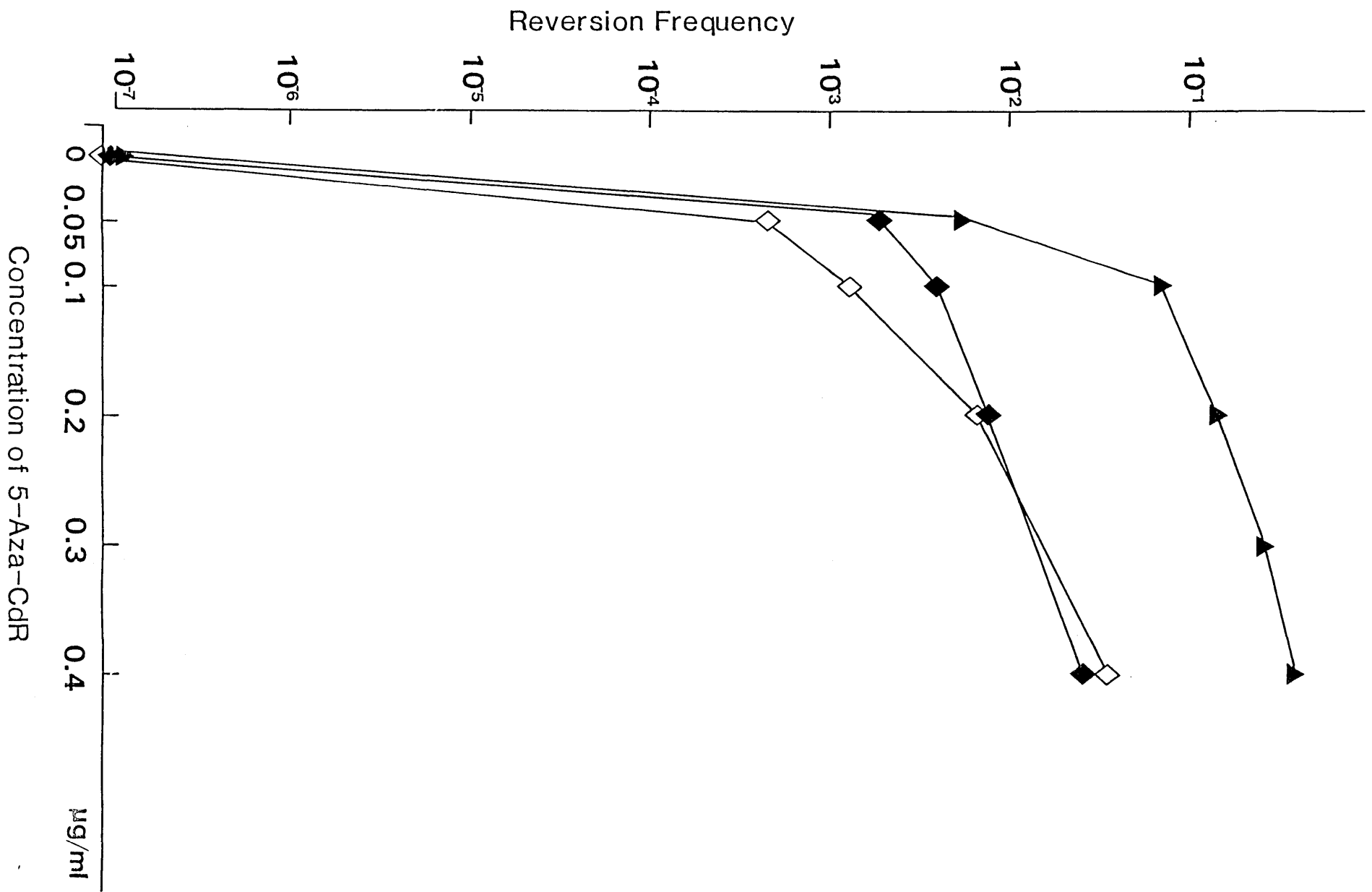


Figure 7: TK⁺ reversion induced by 5-Aza-CdR treatment.

CHO TK⁻ MT⁻ cells were treated with graded concentrations of 5-Aza-CdR from a 1 μ g/ml stock solution kept at -70⁰C. After 24 hours cultures were rinsed with PBS and allowed to recover for 2 ($\blacktriangle$), 5 ($\blacklozenge$) and 7 ($\blacklozenge$) days, before HAT selection was applied. Each point represents the average colony count of three petri dishes seeded with 2×10^4 cells each and corrected for the fraction of cells that survive treatment.



Interestingly the curve of TK^+ reversion produced by 5-Aza-CdR treatment differs from that of 5-Aza-CR since it does not show reduction in reversion at higher drug concentrations.

Five additional TK^- isolates were obtained in order to examine whether reversion following 5-Aza-CR treatment is a general feature of TK^- cells isolated from the CHO KI cell line. Thus, CHO KI cells were mutagenised by X-rays and subjected to BUdR selection while the mutation frequency to TG^r caused by the mutagenesis was estimated. This was once again shown to be in the level of mutation in a haploid gene (section 1.7.1.). The five clones isolated after BUdR selection were treated with $1\mu g/ml$ 5-Aza-CR for 24 hours. HAT selection was applied to the treated cells after one day recovery time. Three of the TK^- clones showed detectable TK^+ reversion, but the other two did not. TK^- strains may arise in at least two ways, one of which could be due to alterations in DNA methylation and the other to mutation, that is, a change in DNA sequence (section 8.5.).

3.2.2. TK^+ reversion of CHO TK^- MT^- cells by EMS and X-rays treatment:

The massive induction of reversion to TK^+ by demethylating agents greatly exceeds the level expected from exposure to mutagens (Adair et al. 1980, Roufa et al. 1973). To confirm this, direct comparisons were made using the DNA damaging agents EMS and X-rays. Mutagenesis experiments were performed using either $300\mu g/ml$ EMS for 24 hours or 500rads X-rays, doses which result in approximately 10% survival in both cases. After treatment, a recovery period of 5 days in normal medium was allowed. Mutation frequencies were then

assayed by selection in 6-TG and induction of TK^+ reversion was estimated by survival in HAT containing medium.

Table 7 summarizes the results obtained in comparison to those of demethylating agents. EMS and X-rays treatment increases the frequency of TK^+ reversion, approximately two orders of magnitude above the background. However this is still 10^3 - 10^4 times lower than reversion induced by demethylating agents. Nevertheless the significant induction of TK^+ reversion by treatment with EMS or X-rays raises questions about the mechanism of action of these mutagens which will be discussed in (section 8.3.)

3.3.1. Stability of the TK^+ revertant phenotype:

The rate of spontaneous back-reversion to the TK^- phenotype of TK^+ revertant clones might indicate whether the inactivation of this gene is the result of genetic or epigenetic events. The rate of back-reversion to TK^- in these cells was estimated by fluctuation analysis. This involves determination of the number of TK^- back-revertants that occur during a defined period of growth by plating a known number of cells into each of 10 replicate cultures. Each culture was grown up to a final cell number (Nt) of approximately 10^5 . At this stage cultures were trypsinised and $\sim 6 \times 10^4$ cells from each population were plated under BUdR selection. BUdR^r colonies were counted and the average number of TK^- was corrected for the cloning efficiency estimated for each population. Frequency of back-reversion was then calculated using the equation (Luria and Delbruck 1943, second method):

Table 7: Reversion to the TK^+ phenotype induced by mutagenic and non mutagenic agents.

Treatment	TK^+ frequency	Approximate increase in TK^+ frequency
Spontaneous rate	$<10^{-7}$	
EMS ($300\mu\text{g}/\text{ml}$)	$6.25 \pm 3.7 \times 10^{-5}$	$10^2 - 10^3$
X-rays (500 rads)	$5.72 \pm 2.42 \times 10^{-5}$	$10^2 - 10^3$
5-Aza-CR ($2\mu\text{g}/\text{ml}$)	$1 \pm 0.6 \times 10^{-2}$	$10^5 - 10^6$
5-Aza-CdR ($0.2\mu\text{g}/\text{ml}$)	$1.48 \pm 1.8 \times 10^{-1}$	$10^6 - 10^7$

$$y = G N t \log_e (N t C G)$$

Where y = mean number of back-revertants

Nt = number of cells examined for TK^- at time t

C = number of parallel cultures

G = back-reversion rate

As well as the method proposed by Lea and Coulson (1949):

$$\log_e m - y/m + 2.5/2.02 = 0$$

Where: y = median number of back-revertants

Nt = number of cells examined for TK^- at time t

m = $Nt \times$ mutation rate

In both these methods the cells examined must originate from small cell populations that initially contain no TK^- cells. Use of the mean number of back-revertants in the first method bases estimations on all the populations used in the experiment. However, use of the median eliminates all the extreme back-reversion values. This does not seem to severely affect the calculations since similar back-reversion frequencies were estimated by both methods (table 8). The TK^+ clone induced by 5-Aza-CR treatment shows 1.5×10^{-4} back-reversion to TK^- by method (1) and 1.52^{-4} by method (2) (table 8). Similarly TK^+ clones induced by 5-Aza-CdR treatment show back-reversion frequencies 5.2×10^{-4} (method 1) and 6×10^{-4} (method 2). Figure 8 shows the distribution of TK^- cells per population, for the two clones tested. Although the calculated frequencies for both clones

Table 8: Back-reversion to TK^- of HAT^{r} cells induced by 5-Aza-CR or 5-Aza-CdR.

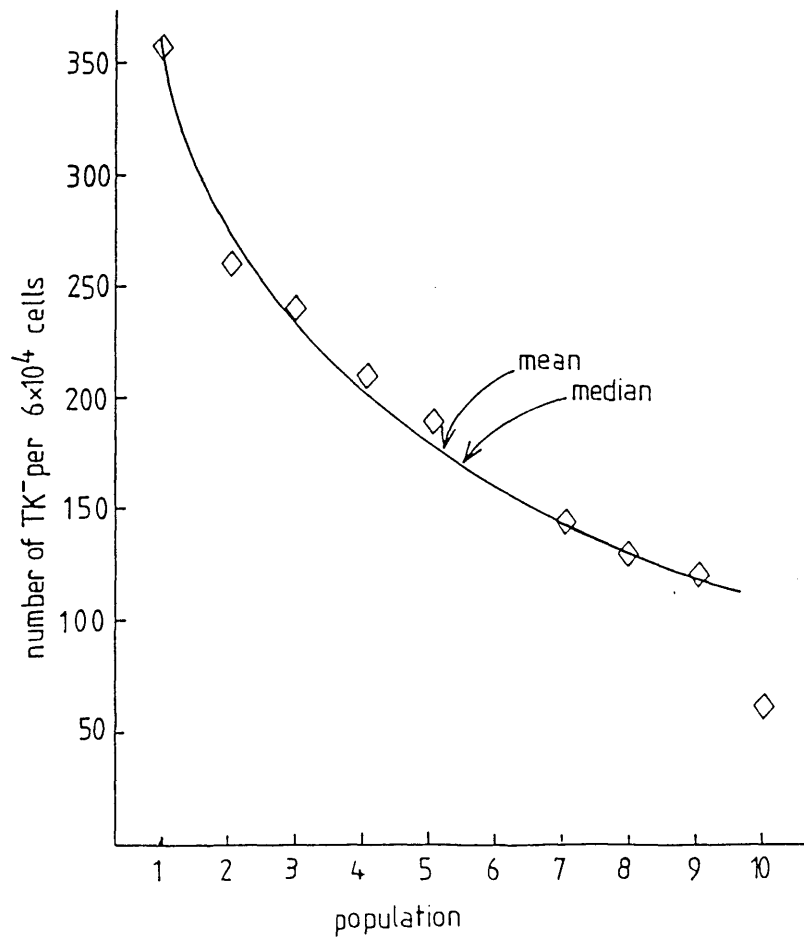
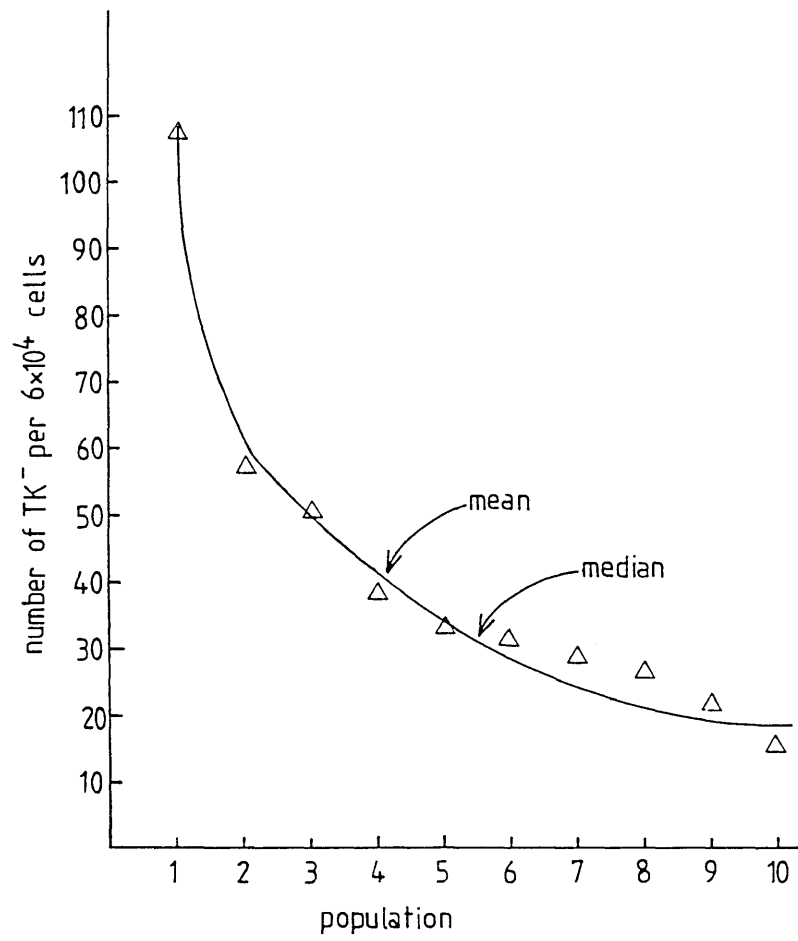
Variable	5-Aza-CR induced TK^+	5-Aza-CdR induced TK^+
N^0 of subcultures	10	10
Initial N^0	100	100
Total	10^5	10^5
Cells tested	6×10^4	6×10^4
Mean	41	177.8
Median	31.4	174.5
N^0 of BUdR^{r} colonies		
Range	(14.8-108.46)	(61.36-359.88)
Reversion rate/cell/generation		
Luria and Delbruck (1943)	1.5×10^{-4}	5.2×10^{-4}
Lea and Coulson (1949)	1.52×10^{-4}	6×10^{-4}

Figure 8a: Back-reversion to TK^- of a TK^+ strain induced by 5-Aza-CR treatment.

Ten populations of $100\ TK^+$ cells each were grown up $\sim 10^5$. 6×10^4 cells from each population were examined for the incidence of TK^- . The colony count was corrected for the plating efficiency estimated per population and the populations were ranked in order of frequency. The mean is the average number of colonies in the ten populations. The median is the average count between the 5th and 6th population.

Figure 8b: Back-reversion to TK^- of a TK^+ strain induced by 5-Aza-CdR treatment.

Ten TK^+ populations were initiated and tested for TK^- back-reversion as in figure 8a. The colony count was corrected for the plating efficiency estimated per population and the populations were ranked in order of frequency. The mean is the average number of colonies in the ten populations. The median is the average count between the 5th and the 6th population.



are quite similar, they are about three orders of magnitude higher than those expected by back-reversion of TK^+ by genetic mutation.

3.3.2. TK^+ reversion of TK^- back-revertants by 5-Aza-CR:

The instability of TK^+ revertants induced by demethylating agents suggests that back-reversion to TK^- might also be a consequence of epigenetic events. Treatment of these cells with 5-Aza-CR should establish whether de novo methylation has led to the TK^- phenotype. Thus TK^- back-revertant cells were treated with $1\mu g/ml$ 5-Aza-CR for 24 hours. Then, they were media changed and allowed to recover for 2 days before seeding in HAT medium. The observed reversion to the TK^+ phenotype in TK^- clones that originated from 5-Aza-CR and 5-Aza-CdR induced TK^+ revertants is 6.2×10^{-3} and 5.4×10^{-2} respectively (table 9). Reversion of the parent CHO TK^- MT^- cell line in the same experiment was 6×10^{-3} . These findings indicate that the TK^- back-revertants arise from de novo methylation and that they are again revertible by demethylation.

3.4.1. Reversion to the MT^+ phenotype induced by 5-Aza-CdR

treatment:

The ability of demethylating agents to induce reversion to MT^+ in the CHO TK^- MT^- cells was also examined. In these experiments only 5-Aza-CdR was used because it is known to induce higher reversion frequencies (section 3.2.1.). An average of 5×10^5 cells were treated with graded concentrations of 5-Aza-CdR for 24 hours. After media changing in drug free medium a period between 2-9 days was allowed

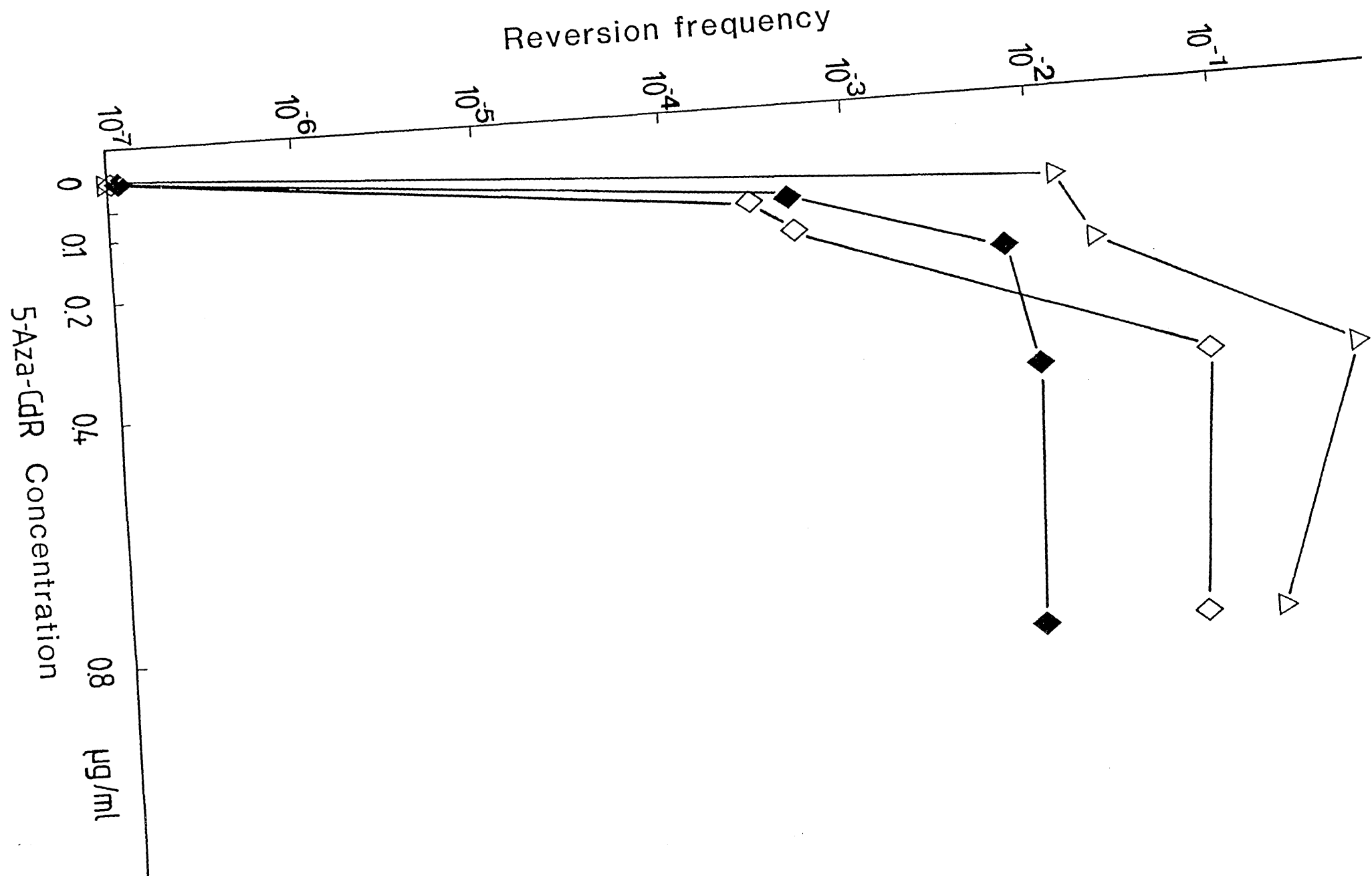
Table 9: Rereversion to TK^+ of TK^- back-revertants:

Derivation of Cell line	Treatment	Frequency of Reversion to TK^+
B	1 μ g/ml 5-Aza-CR	6.0x10 ⁻³
D (from 5-Aza-CR treatment)	" "	6.2x10 ⁻³
D (from 5-Aza-CdR treatment)	" "	5.4x10 ⁻²

The definitions for B and D are shown in figure 10.

Figure 9: Reversion to the MT^+ by 5-Aza-CdR treatment of CHO $TK^- MT^-$ cells.

CHO TK^- cells were treated with graded concentrations of 5-Aza-CdR from $1\mu g/ml$ frozen ($-70^{\circ}C$) stocks. Flasks were washed in PBS and cells were allowed to recover in normal medium for 2 (Δ), 5 ($\diamond$) and 9 ($\blacklozenge$) days. 2×10^4 cells were then plated in each of three petri dishes in Cd containing medium and incubated for 7-9 days. Each point represents the average colony count of these plates, corrected for the fraction of cells that survived treatment.



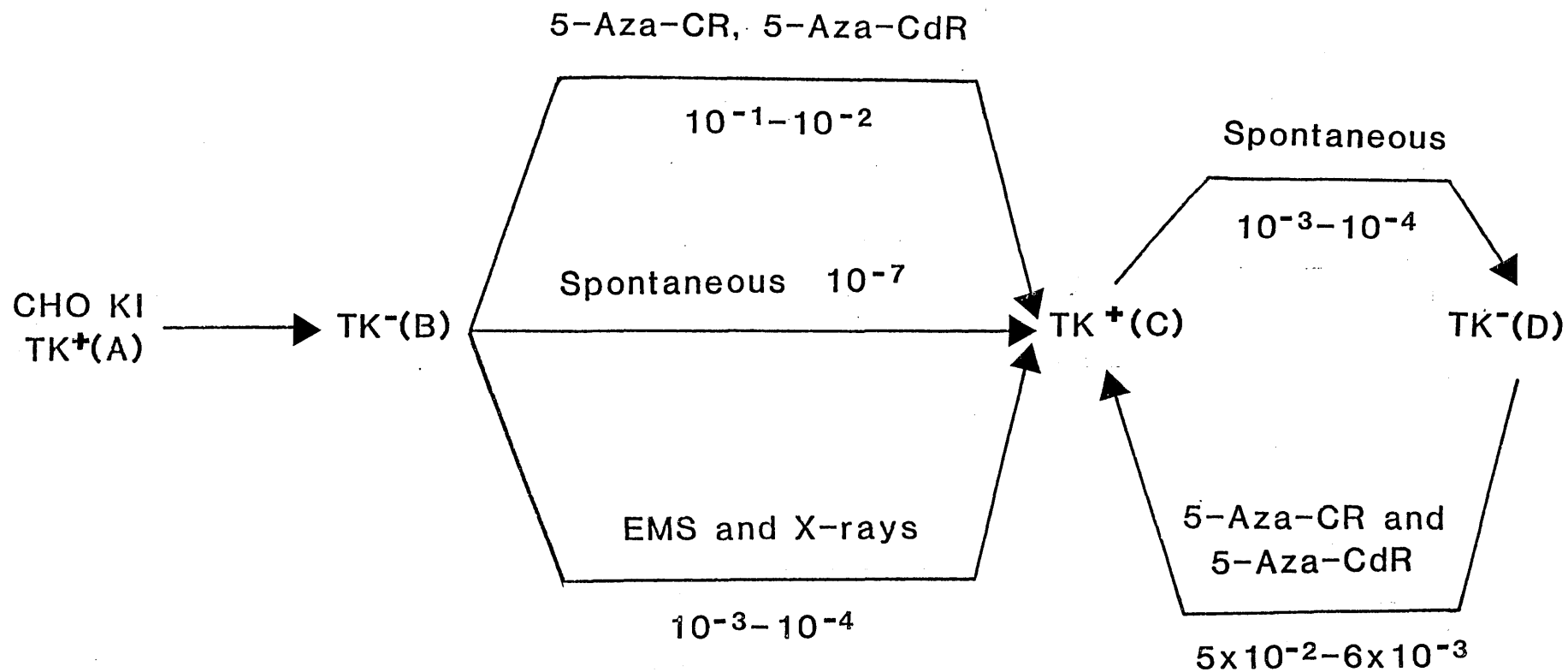
before any selection was applied. Cells were then seeded in $2\mu\text{M}$ Cd and the plating efficiency was estimated. The MT^+ reversion frequencies obtained were corrected by the plating efficiency. Up to 59% of the cells surviving the treatment expressed the MT^+ phenotype (figure 9). Optimum reversion was obtained when the shortest recovery period was allowed.

3.5.1. Conclusions:

The results presented in this chapter suggest that hypermethylation is responsible for the TK^- and MT^- phenotype in CHO TK^- MT^- cells. Treatment with demethylating agents increases spontaneous incidence of TK^+ or MT^+ by 10^6 fold. However, mutagenic treatment results in only 10^2 fold induction of the TK^+ reversion. TK^+ clones induced by demethylating agents are unstable and frequently back-revert to TK^- . These TK^- clones can again be reverted to the TK^+ by treatment with demethylating agents. It is therefore suggested that differences in DNA methylation control the TK^- and TK^+ phenotype in CHO cells. Thus demethylation is required for reversion to TK^+ , while remethylation is sufficient for back-reversion to the TK^- phenotype. Figure 10 summarizes the origin of the strains studied in this chapter.

Figure 10: Origin of the cell lines described.

Figure 10 presents the cell lines described in Chapter 3, as well as the original parent cells. Additionally, it indicates the frequency in which the new phenotype was obtained by various treatments or spontaneously.



CHAPTER 4ISOLATION AND PHENOTYPE OF A TEMPERATURE SENSITIVE STRAIN
AFFECTED IN DNA METHYLATION

4.1.1. Introduction:

The main problem in the isolation of methylation deficient mutants is that a method for the direct selection of the desired phenotype is not available. However, a selection procedure can be based on the reactivation of an appropriate selectable gene as a result of demethylation. The CHO TK⁻ MT⁻ cell line is a suitable system for isolation of such mutants, since evidence suggests that demethylation is sufficient for reversion to the TK⁺ or MT⁺ phenotype.

The development of a selection procedure was based on four criteria. It should be a reliable method for producing replica plates. Genetically identical cells should be transferred without contamination from other colonies, which would obscure reversion to the TK⁺ or MT⁺ phenotype. It must be possible to recover cells of the same genotype that have not reverted to the TK⁺ or MT⁺ when a potential mutant is observed. The whole procedure must be rapid enough to allow screening of large number of colonies in short time.

Initially, the conditions for optimum TK⁺ reversion were explored, and the isolated tsm strain reverting both to TK⁺ and MT⁺ in a temperature dependent manner was further studied. This was followed by studying the effect of treatment at 39°C on the growth of tsm cells immediately after shifting, or upon long term cultivation at this temperature. Experiments were done to find out whether heat

treatment induces higher mutation rates. Finally, the rate of TK^{-} back-reversion of tsm TK^{+} revertant cells was measured in order to determine whether back-reversion is likely to be the result of genetic or epigenetic events. tsm TK^{-} back-revertant cells were treated with demethylating agents in order to establish whether remethylation is responsible for TK inactivation.

4.2.1. Procedure for the isolation of temperature sensitive mutants with altered DNA methylation:

The selection procedure outlined in figure 11 was based on the hypothesis that methylation deficient CHO TK^{-} MT^{-} cells should revert to the TK^{+} and MT^{+} phenotype at high frequency. It was decided to search for a temperature sensitive mutation to allow for the possibility that DNA methylation deficiency may cause lethality. Also temperature sensitive mutants allow reversible manipulation and studies on both the defective and normal phenotype.

Cells from mutagenised populations were plated for single colonies in numbers sufficient to produce 100 colonies per 90mm dish in medium supplemented with BUdR. This selection was applied in order to eliminate TK^{+} cells resulting from the mutagenesis, since it was shown in chapter 3 that EMS induces low levels of TK^{+} reversion. Plates were incubated for 7-8 days and the medium was removed. At this stage colonies are visible by eye and can be ringed. The colonies were then overlayed with 0.5% soft agar and left to grow for a further 2 days. Agar overlay serves two purposes, it prevents formation of satellite colonies and it allows accurate and successful

Figure 11: Procedure for the isolation of temperature sensitive mutants deficient in DNA methylation.

CHO TK⁻ MT⁻ cells were treated with 300 μ g/ml EMS. 24 hours later the mutagenised cultures were rinsed in mutagen free medium and allowed to grow to confluency. Mutagenised cells were plated in plastic dishes (~500 per dish) and grown for 7-8 days to form visible colonies. Dishes were overlayed with 0.5% soft agar. After two days further incubation single colonies were picked with a yellow tip and inoculated in replica multiwell dishes. One of them was transferred for 24 hours at 39^oC, while the other was kept at 34^oC. HAT selection was applied to the heat treated cells and they were transferred back to 34^oC. For any clones that survived the selection the replica cells were isolated for quantitative studies.

CHO TK⁻ cell line

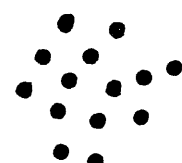


EMS

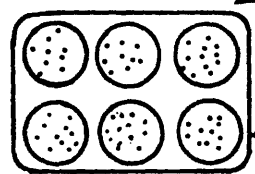
mutagenesis



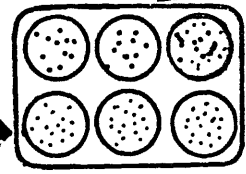
single colonies grown BUdR



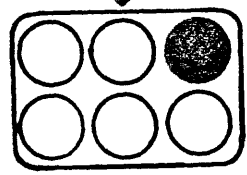
39°C
for 24h



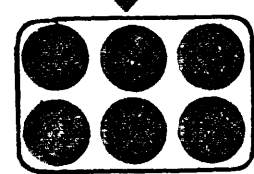
multiwell
plates



kept
at 34°C



selection
for TK⁺ (HAT)



isolate for
quantitative
studies

transfer of cells with the end of a yellow tip of a Gilson pipettor. The length of time for which the cells were overlayed was also important, as this should be a balance between allowing the colonies to grow as long as possible without reducing cell viability. For this reason it was necessary to add the agar just prior to the time of satelliting so that the colonies were as large as possible when picked.

Cell transfer was achieved by gentle stirring of the agar above a ringed colony with the end of a yellow tip. Cells trapped in the tip were transferred to replica 24 well plates containing medium by pipetting up and down vigorously. The efficiency of transfer with this procedure was high, averaging between 80-100%. This was estimated each time by scoring the cells transferred after four hours incubation of the multiwell plate.

When the average number of cells per well was about 10^3 , one of each replicas was transferred at 39°C . Exposure to the high temperature should induce expression of any temperature sensitive mutation. The length of treatment at 39°C is very important since it is not clear whether methylation deficiency causes lethality. This should be long enough to introduce phenotypic alterations without affecting survival. Thus, cells were induced at 39°C for 24 hours. The second plate of each doublet was kept and subcultured at the incubation temperature of 34°C . This plate served as a replica for any potential mutants that show the desired phenotype.

After 24 hours at 39°C the test plate was put under HAT selection and returned to the growth temperature 34°C . In any well where reversion to the TK^+ phenotype was detected, the cells from the reference plate were isolated and subjected to quantitative studies

for temperature dependant TK^+ reversion. Following the above procedure, 7000 individual colonies were screened. This led to the isolation of 11 potential mutants, which were subjected to a secondary screening procedure.

4.2.2. The secondary screening procedure:

The secondary screening procedure was a quantitative analysis of TK^+ reversion in the 11 preliminary isolates. Cells from each of the 11 isolates were treated for 24 hours at $39^{\circ}C$. After heat treatment they were transferred at $34^{\circ}C$ and allowed to recover for two days. Cells were then plated under HAT selection to test for reversion to TK^+ . 9 of the 11 clones were proved to be false positives and were not studied further. The isolation of false positives could be due to the primary screening procedure. Alternatively, it might be that these cells have undergone reversion to the parent phenotype.

The clone number 93A6 showed constitutive reversion to TK^+ (i.e. at both $34^{\circ}C$ and $39^{\circ}C$) at a frequency of 5×10^{-5} , while the clone number 86A4 expressed temperature dependent reversion at a frequency of 7.2×10^{-4} . The frequencies of reversion to the TK^+ in 93A6 and 86A4 are between 50-1000 times lower than those obtained by treatment with demethylating agents. However, they are still $5 \times 10^2 - 10^4$ times higher than the spontaneous TK^+ reversion. These two isolates were further examined for reversion to the MT^+ phenotype.

4.2.3. Reversion of 93A6 and 86A4 to the MT^+ phenotype:

Cells from the clone 93A6 were plated in medium containing $2\mu M$ Cd to test for Cd resistance. It was not necessary to induce 93A6 cells at $39^{\circ}C$ prior to selection because they revert to the TK^+ in a constitutive manner. In 10^6 cells examined, no Cd resistant colonies were observed. No further studies were carried out for the 93A6 isolate, and the mutation that leads to TK^+ reversion in these cells remains uncharacterized.

86A4 cultures were treated at $39^{\circ}C$, allowed two days recovery period at $34^{\circ}C$, and they were then plated in $2\mu M$ Cd. Reversion to Cd resistance was observed at a frequency of 6.1×10^{-4} . Thus, MT^+ reversion in 86A4 cells occurs at a frequency equivalent to TK^+ reversion. The results obtained from 93A6 and 86A4 are summarized in table 10. Studies on the phenotype of the 86A4 isolate continued, and it was renamed tsm (temperature sensitive methylation).

The tsm strain was isolated at a frequency of 1/7000 since 7000 individual colonies were screened. This frequency was expected for recessive mutation in a single copy gene or monosomic. After EMS mutagenesis for example the mutation frequency in the HPRT haploid gene is 1/1000 in the population screened for mutants, which is comparable to 1/7000.

4.3.1. Maximum reversion to the TK^+ phenotype:

Maximum reversion to TK^+ was shown when cells were allowed two days recovery time after treatment (chapter 3). Therefore only the optimum length of exposure at $39^{\circ}C$ was estimated for the tsm mutant.

Table 10: Comparison of TK^+ and MT^+ reversion in CHO $TK^- MT^-$, 93A6 and 86A4 strains in $34^\circ C$, $39^\circ C$ and $37^\circ C$.

Strain	TK^+ reversion			MT^+ reversion		
	frequency			frequency		
	$34^\circ C$	$37^\circ C$	$39^\circ C$	$34^\circ C$	$37^\circ C$	$39^\circ C$
CHO $TK^- MT^-$	$<10^{-7}$	$<10^{-7}$	$<10^{-7}$	ND	$<10^{-7}$	ND
93A6	1.2×10^{-5}	ND	5×10^{-5}	$<10^{-6}$	ND	ND
86A4	$<10^{-7}$	$<10^{-6}$	7.2×10^{-4}	$<10^{-7}$	ND	6.1×10^{-4}

tsm cells were incubated for variable length of time at 39°C. Heat treated cells were transferred back at 34°C and after a recovery period of two days they were seeded in medium supplemented with HAT. Reversion frequency was estimated for the cells that survived heat treatment (figure 12).

No reversion was detected for shorter than 16 hours incubation at 39°C. Occasionally TK⁺ revertants were induced by 16 hours heat treatment, which can be therefore considered the minimum for any detectable phenotypic alteration. Maximum TK⁺ reversion (7.4×10^{-4}) was obtained when 24 hours treatment at 39°C was allowed and longer incubation at 39°C had no further effect. These results suggest that at least one doubling time at 39°C is required in order to obtain measurable reversion to TK⁺.

4.3.2. Maximum reversion to the MT⁺ phenotype:

Experiments similar to those in 4.3.2. were performed in order to investigate maximum reversion to MT⁺. tsm cells were treated at 39°C for variable length of time and allowed 2 days recovery period before plating in medium containing 2μM Cd. Reversion frequency to the MT⁺ phenotype was estimated as a function of the cells surviving exposure at the non permissive temperature (figure 13).

Consistent with TK⁺ reversion, MT⁺ colonies were not detected when tsm cells were allowed less than 24 hours at 39°C. Failure to detect any MT⁺ colonies for 16 hours heat treatment is probably associated with the experimental procedure. Optimum reversion was induced for 24-48 hours of heat treatment (1.6×10^{-4}). This practically plateaus for longer exposures.

Figure 12: TK⁺ reversion of the t_{sm} mutant.

t_{sm} cells were treated for variable length of time at 39°C, and after two days recovery they were plated under HAT selection. Reversion frequency was estimated as a function of the cells surviving the treatment. Each point represents the average reversion of three plates seeded with 1×10^5 cells each. The symbol $\leq$, indicates that reversion could not have been higher than 10^{-6} (accumulated results of experiments).

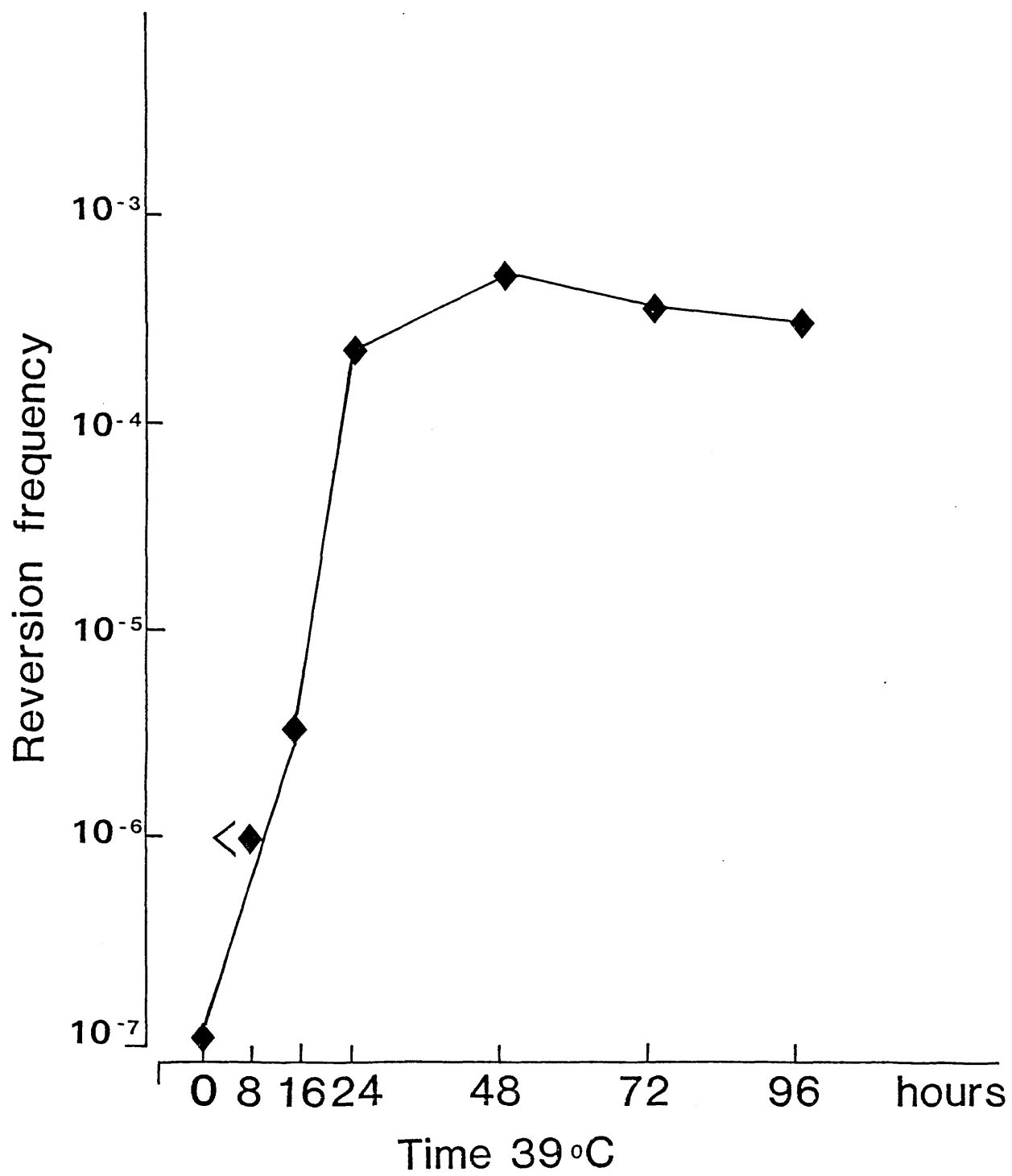
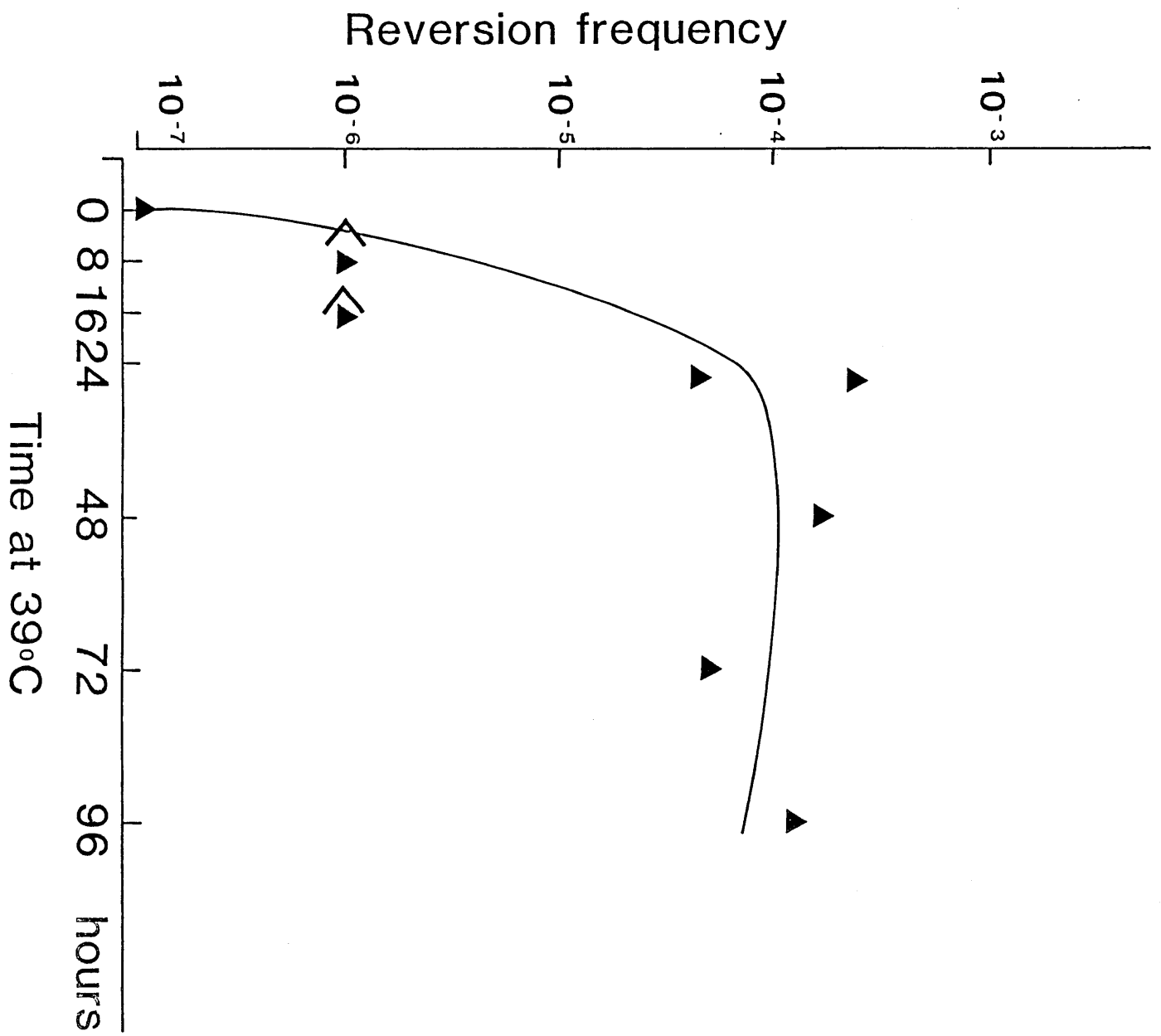


Figure 13: MT^+ reversion of the tsm mutant.

tsm cells were treated for variable length of time at $39^{\circ}C$, and after two 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 three plates seeded with 1×10^4 cells each. The symbol $<$, indicates that reversion could not have been higher than 10^6 (accumulated results of 4 experiments).



4.4.1. tsm cells are viable upon long term growth at 39°C:

Treatment of the tsm for 24-48 hours at 39°C is sufficient for reactivation of at least two selectable genes. It is possible that long term growth at the high temperature might lead to reactivation of other genes that respond to methylation control, and such an accumulation of phenotypic alterations might be incompatible with cell growth. For this purpose tsm cultures were cultivated for approximately two months at the high temperature. Interestingly, tsm cells remain viable after such treatment, showing that the temperature sensitive phenotype expressed in these cells is not lethal.

4.4.2. Growth rate of tsm at 34°C and 39°C:

Growth rates of the tsm strain were first compared to those of the parent CHO TK⁻ MT⁻ cells. The tsm cell line has a 23 hours doubling time at 34°C, which is 5 hours slower than the equivalent of CHO TK⁻ MT⁻ cells (figure 14a). The doubling time is decreased to 16 hours when tsm cells are cultivated at 39°C. This is still 2 hours slower than the growth rate of CHO TK⁻ MT⁻ cells at 39°C (figure 14b). Slower growth could be directly associated with a temperature sensitive mutation in DNA methylation. Alternatively, it might be a result of other mutations that occurred in the tsm strain during EMS mutagenesis.

4.4.3. The immediate effect of treatment at 39°C in tsm growth:

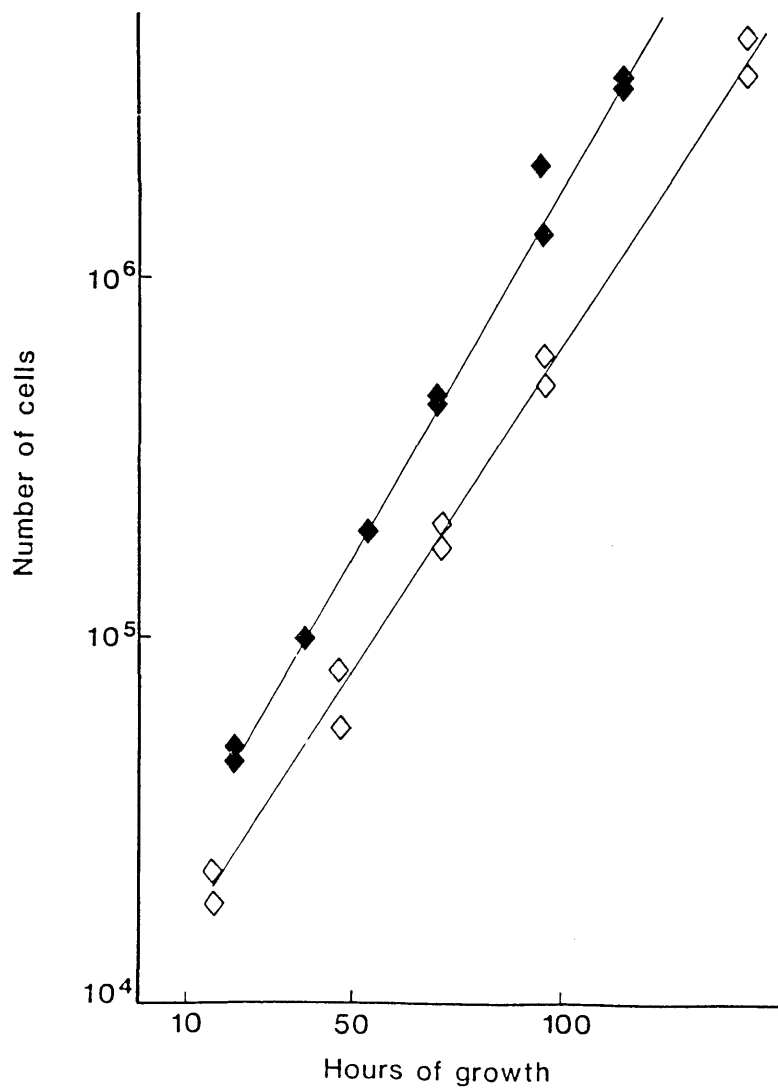
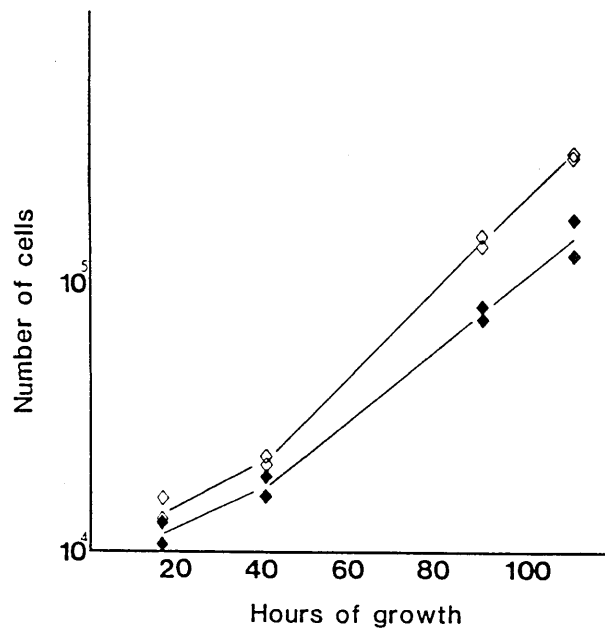
It was shown in 4.4.2. that the doubling time of tsm cells at

Figure 14a: Growth rate of tsm mutant at 34°C compared to CHO TK⁻ MT⁻ cells.

2×10^4 tsm or CHO TK⁻ MT⁻ cells were plated in 10 petri dishes. Doublicate plates were harvested and the cells counted in regular intervals. Each point represents the number of cells in one harvested well. The growth curve for tsm is defined by (◆) while the CHO TK⁻ MT⁻ growth curve is determined by (◇).

Figure 14b: Growth rate of tsm mutant at 39°C compared to CHO TK⁻ MT⁻ cells.

2×10^4 tsm or CHO TK⁻ MT⁻ cells were plated in 12 petri dishes. Doublicate plates were harvested and the cells counted in regular intervals. Each point represents the number of cells in one harvested well. The growth curve for tsm is defined by (◇) while the CHO TK⁻ MT⁻ growth curve is determined by (◆).



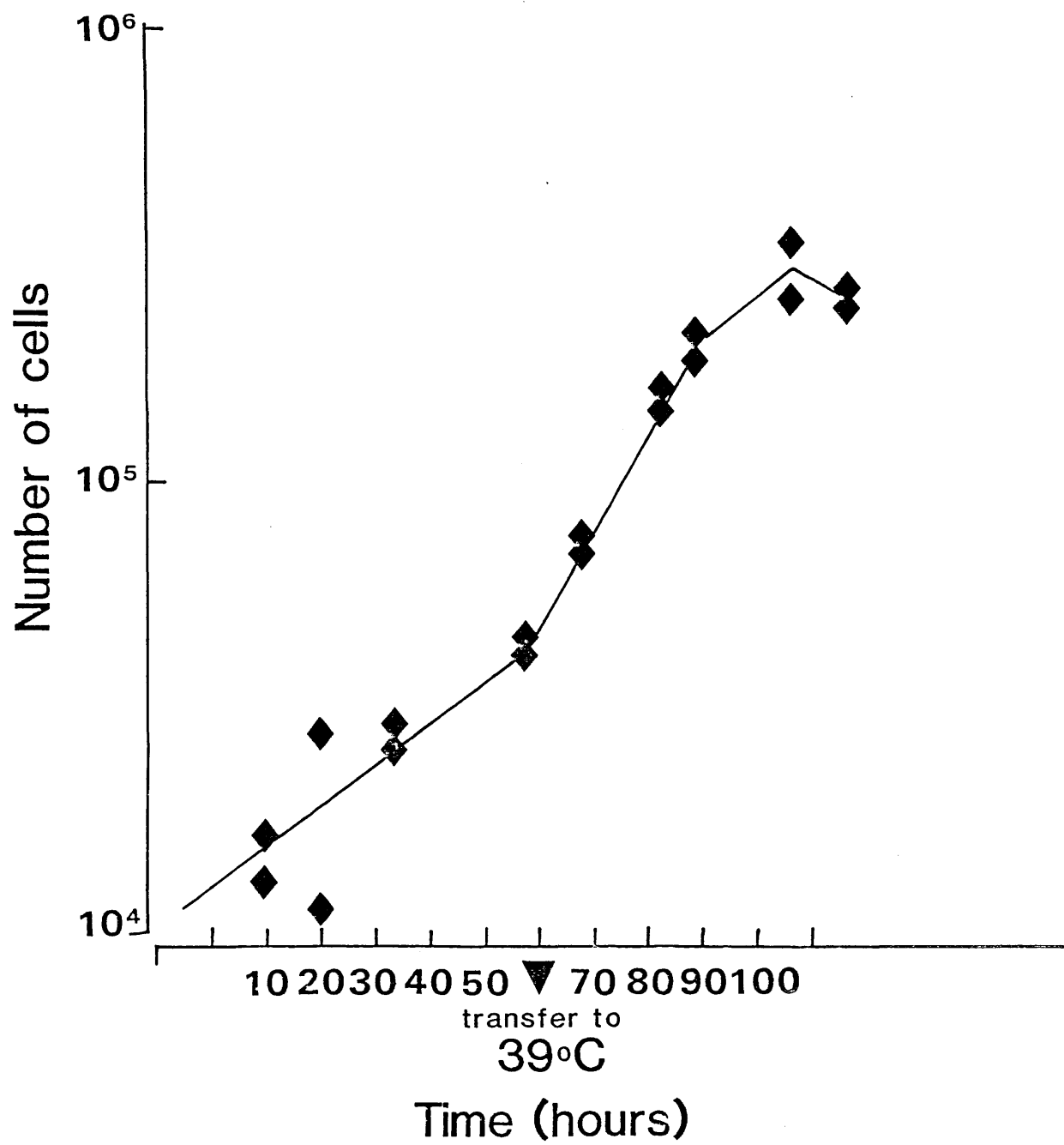
39°C is 7 hours faster than that at 34°C. It is important to examine the characteristics of this growth rate increase by studying the initial response of the tsm strain at this temperature. For this purpose, approximately 10^4 cells were plated in each well of a multiwell plate and a growth curve was obtained at 34°C. Then, the multiwell plate was transferred at 39°C and estimation of the growth rate continued until cells reached confluency. As shown in figure 15, tsm cells immediately responded by an increase in the growth rate at the higher temperature. This indicates that no significant growth interruption or killing is caused by treatment at the non permissive temperature.

4.5.1. The mutation rate of tsm after treatment at 39°C:

Studies on the mutation rate of tsm cells at 39°C can show whether the mutation expressed from these cells affects DNA metabolism and introduces a high mutation rate. The mutability of tsm cells at 39°C was examined in comparison to that of the CHO TK⁻ MT⁻ cell line. A total of 10^6 viable cells from each cell line were incubated for 24 hours at 39°C, and they were then transferred to 34°C. A further 5 days of incubation were allowed before plating in selective medium for recovery and expression of any mutations caused by heat treatment. Heat treated cells from each cell line were plated in medium containing 6-TG and the mutation rate was estimated from the fraction of viable cells. The mutation frequency for both cell lines was below 10^{-6} , since no TG^r mutants were detected. Thus, the tsm mutant does not appear to show a greatly elevated spontaneous mutation rate after treatment at 39°C. This makes it very unlikely that

Figure 15: Increase in the growth rate of tsm by shifting at 39°C.

10^4 tsm cells were seeded in each well of a 24 well plate. A growth curve for this temperature was obtained and the plate was transferred at 39°C, where estimation of the growth rate continued until cells stopped growing exponentially. Each point represents the cell number of a well at the time of harvesting. For every time point two wells were harvested and the number of cells estimated.



reactivation of TK and MT genes results from hypermutability.

4.6.1. The stability of TK⁺ revertants:

The rate of spontaneous back-reversion to tsm TK⁻ in tsm TK⁺ revertant clones was estimated, as described in chapter 3, using both the method proposed by Luria and Delbruck (1943) and that suggested by Lea and Coulson (1949). Ten populations of a tsm TK⁺ revertant cells were initiated and allowed to grow to approximately 5×10^5 cells each. 3×10^5 of these were plated under BUdR selection and the number of TK⁻ colonies obtained was corrected for the plating efficiency estimated per population.

The distribution of TK⁻ colonies in the ten populations studied is shown in figure 16. The TK⁻ incidence was similar for either method of calculation (table 11). Thus, tsm TK⁻ back-reversion in these cells occurs at a frequency of 5.2×10^{-5} (Luria and Delbruck 1943) and 6.9×10^{-5} (Lea and Coulson 1949). This is not significantly lower than the back-reversion rate observed in a TK⁺ revertant induced by treatment with demethylating agents (Chapter 3). In all three cases back-reversion frequencies were between two to three orders of magnitude higher than those expected for inactivation of the TK gene as a result of genetic mutation.

4.6.2. Reactivation of tsm TK⁻ back-revertants by 5-Aza-CR:

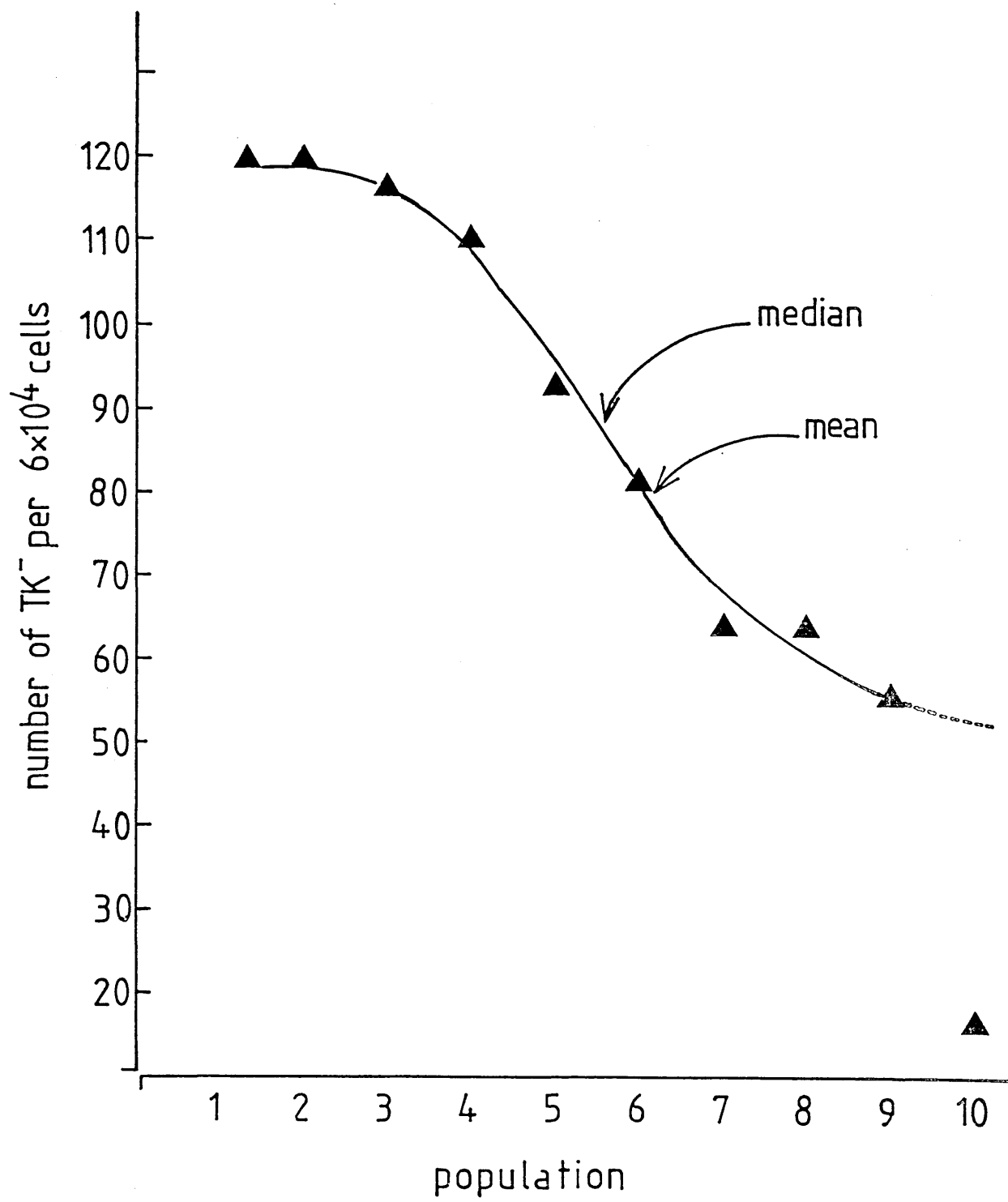
The association of DNA methylation with TK⁻ back-reversion in tsm cells was further examined. For this purpose tsm TK⁻

Table 11: Back-reversion to TK^- of tsm TK^+ revertant cells induced by treatment at 39°C .

Variable	<u>tsm</u> TK^+ revertant
N^0 of subcultures	10
Initial N^0	100
Total	5×10^5
Cells tested	3×10^5
Mean	79
Median	88
N^0 of BUdR^r colonies	
Range	(19.4 - 119.8)
Reversion rate/cell/generation	
Luria and Delbruck (1943)	5.2×10^{-5}
Lea and Coulson (1949)	6.9×10^{-5}

Figure 16: Distribution of the t_{sm} TK⁻ back-revertants.

10 populations of 100 cells each were grown to an average of 5×10^5 . 3×10^5 cells per population were seeded under BUdR selection. Each point represents the number of t_{sm} TK⁻ back-revertants corrected for the plating efficiency of t_{sm} cells per population and the populations were ranked in order of frequency. The mean is the average number of back-revertants between the ten populations. The median is the average number of back-revertants between the 5th and 6th populations.



back-revertants were treated with $1\mu\text{g/ml}$ 5-Aza-CR for 24 hours. Cells were allowed to recover for two days and then plated in medium supplemented with HAT. Reversion to the TK^+ was corrected for the plating efficiency of treated cells. As shown in table 12 tsm TK^- back-revertant cells are reactivated again in the TK gene at a frequency of 1.3×10^{-2} , which is comparable to that of the tsm parent cell line (1.4×10^{-2}).

4.7.1. Conclusions:

The temperature sensitive (tsm) isolate shows reversion to both TK^+ and MT^+ at frequencies up to 1/1000. The spontaneous incidence of TK^+ or MT^+ reversion is at least 10^3 fold lower than that observed in the tsm mutant. Maximum levels of reversion were obtained after 24 hours of heat treatment. The parallel effect of heat treatment in tsm cells to chemical demethylation suggests that they may be defective in DNA methylation.

tsm TK^+ revertants show approximately the same levels of back-reversion to the TK^- phenotype as TK^+ revertants induced by treatment with demethylating agents. In both cases the high frequency of back-reversion and the fact that these TK^- cells revert again to the TK^+ upon treatment with demethylating agents suggest that inactivation or reactivation of the TK gene is due to epigenetic events.

While exposure to 39°C is sufficient for induction of TK^+ and MT^+ reversion, no significant increase in the mutation to TG^r was observed by heat treatment. tsm is not a conditional lethal strain, since long term cultures at 39°C are viable. Additionally, the tsm mutation can be provisionally assigned to a gene present in single

Table 12: 5-Aza-CR induces TK^+ reactivation in tsm TK^- back-revertants.

Cell line	treatment	reversion frequency to TK^-
<u>tsm</u> TK^- (primary)	1 μ g/ml 5-Aza-CR	1.4×10^{-2}
<u>tsm</u> TK^- (secondary)	" "	1.3×10^{-2}

The primary TK^- is the original tsm strain and the secondary TK^- is derived from a TK^+ induced by treatment at 39°C .

copy or dominant, since it has been isolated at frequencies (1/7000) comparable to the mutation at the haploid HPRT (1/1000) gene.

CHAPTER 5

HPLC ANALYSIS OF THE 5mC CONTENT OF THE CHO GENOME

5.1.1. Introduction:

The experimental results described in chapter 3 suggest that the reversion of CHO TK⁻ MT⁻ cells to TK⁺ by 5-Aza-CR or 5-Aza-CdR treatment is associated with demethylation. This was examined directly by measuring the levels of 5mC by HPLC chromatography. The same method was used to measure the level of 5mC in the tsm strain at 39°C, or at the growth temperature 34°C.

5.2.1. The effect of 5-Aza-CR or 5-Aza-CdR treatment on CHO cells:

Genomic DNAs were prepared from CHO KI parent cells and the CHO TK⁻ MT⁻ derivative. In order to define the initial levels of demethylation induced in CHO TK⁻ MT⁻ cells by demethylating agents, they were treated with 5-Aza-CR or 5-Aza-CdR for 24 hours. DNA was prepared immediately after treatment. HPLC chromatography was performed on hydrolysed DNAs and the ratio of 5mC over the total 5mC+C was calculated from the profiles of nucleotide base content obtained (figure 17 and table 13). The 2.5% 5mC levels calculated for the CHO KI cells is in agreement with previous reports for CHO cells (Nyce et al. 1986).

Treatment with 5-Aza-CR and 5-Aza-CdR causes approximately the same levels of demethylation. Thus, up to 50% decrease in 5mC content

Table 13: The effect of 5-Aza-CR and 5-Aza-CdR treatment on 5mC level of CHO cells.

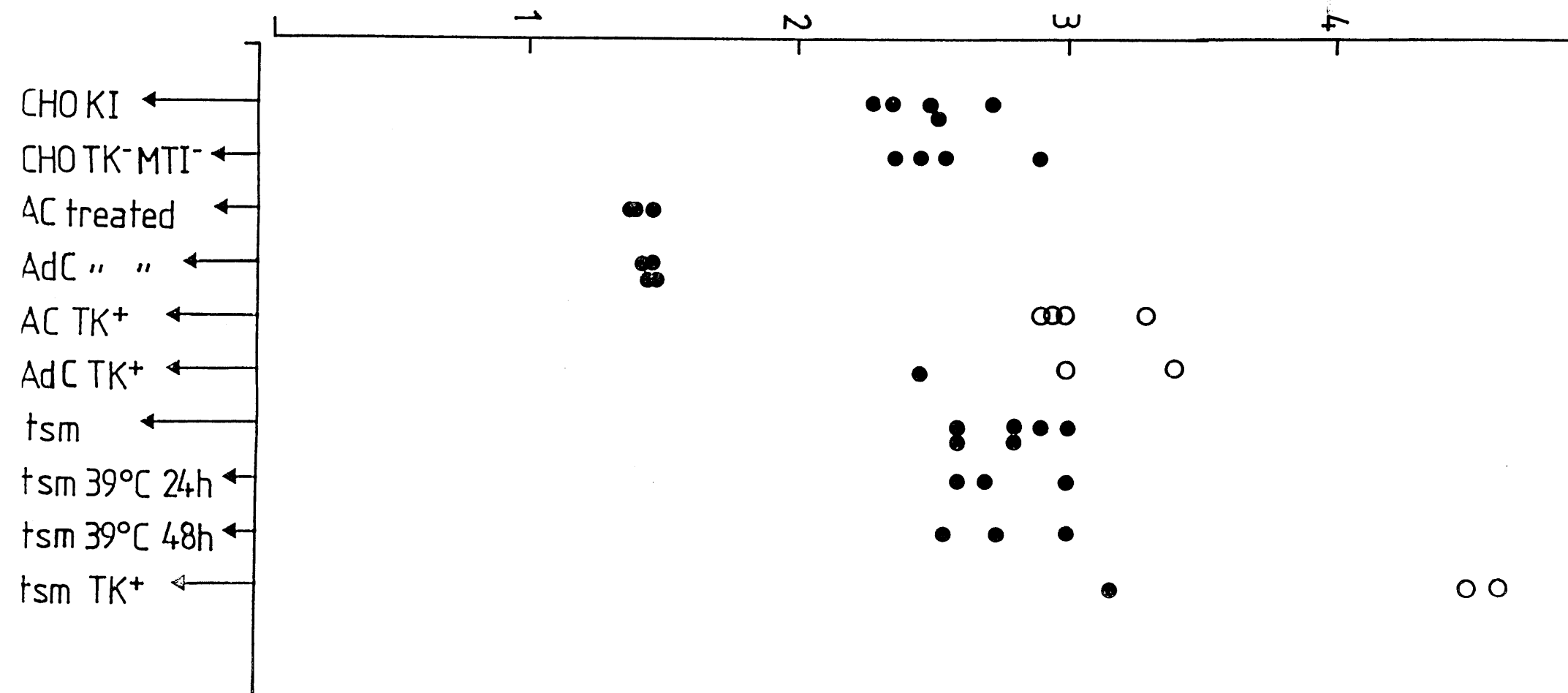
Cell line	% 5mC/C+5mC	% change over		
		the CHO	TK ⁻	MT ⁻
CHO KI (MTI)	2.5 ± 0.18			
CHO TK ⁻ MT ⁻	2.6 ± 0.2			
immediately after				
5-Aza-CR treatment	1.4 ± 0.08	-	46	
immediately after				
5-Aza-CdR treatment	1.43 ± 0.02	-	45	
5-Aza-CR TK ⁺	3.03 ± 0.2	+	16.5	
5-Aza-CdR TK ⁺	3.21 ± 0.25	+	23.5	

Table 13 demonstrates demethylation induced immediately after treatment with 5-Aza-CR or 5-Aza-CdR and remethylation in TK⁺ clones induced by demethylating agents that were subcultured for approximately 150 cgs after treatment.

Figure 17: Estimation of the 5mC content of the CHO genome.

All the estimations performed on the 5mC content of the CHO strains studied are presented. The open circles indicate estimations performed in TK^+ revertant cells ~150 cgs after treatment inducing demethylation. The determinations in TK^+ revertants presented by close circles were performed ~50 cgs after treatment

% 5mC / C + 5mC



was observed in cells harvested immediately after treatment (figure 17 and table 13). Estimation of the 5mC content was performed using unlabelled DNA, as such preparations would include DNA from cells that have not survived the treatment. Thus, the above levels represent average demethylation of all the treated cells. Estimation of the 5mC content in cells that were allowed to recover after treatment should define whether there is a selective death of heavily demethylated cells.

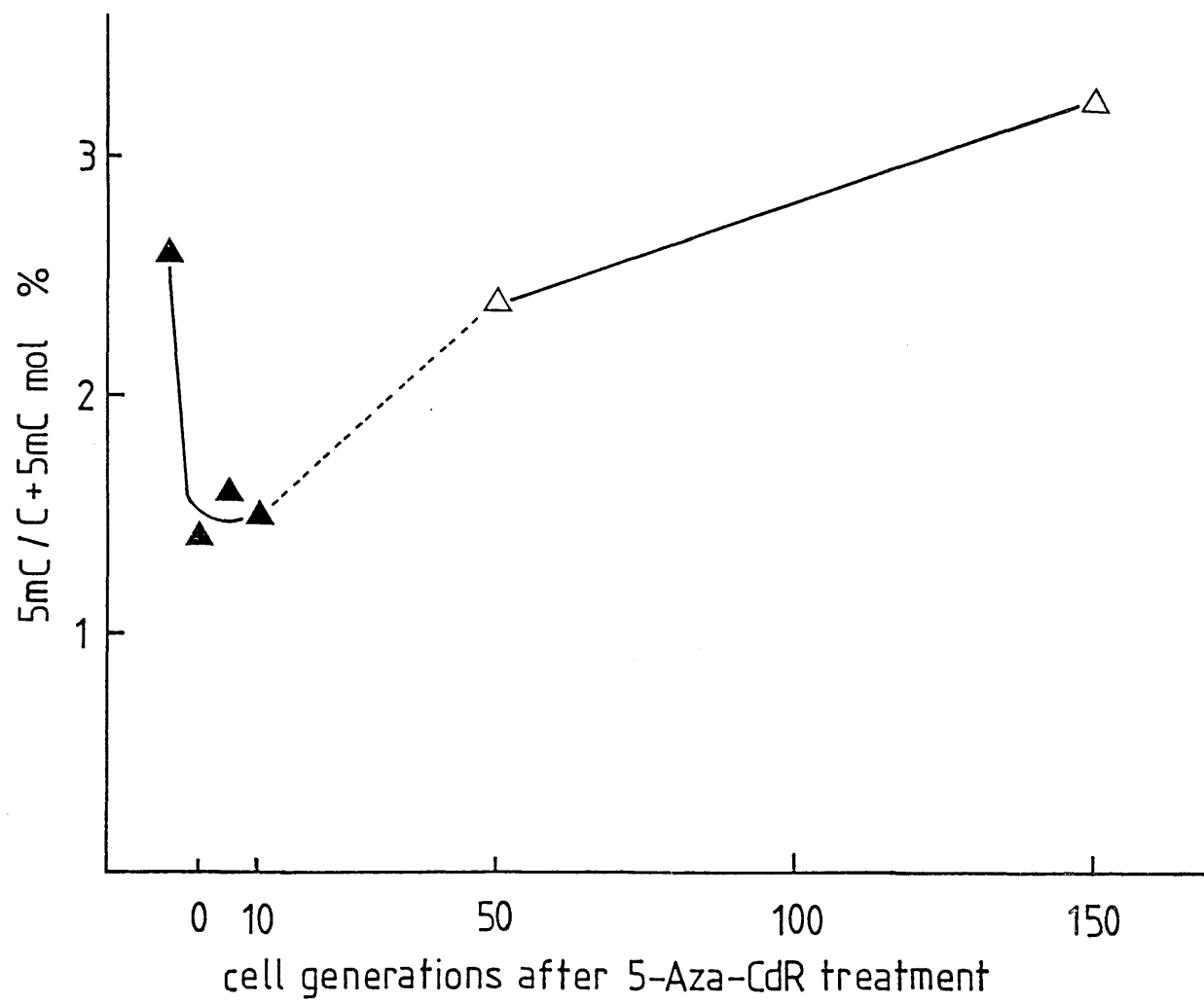
5.2.2. The process of de novo methylation after chemical demethylation:

TK⁺ clones from 5-Aza-CR or 5-Aza-CdR treated cells which had been subcultured for approximately 150 generations, had regained methylation and surprisingly they had significantly higher levels than the CHO TK⁻ MT⁻ strain (figure 17 and table 13). Since the revertants induced by 5-Aza-CR or 5-Aza-CdR treatment have originated from populations that had undergone up to 50% demethylation, de novo methylation activities must be responsible for restoring high methylation levels.

Examination of remethylation in cells previously demethylated by 5-Aza-CR treatment would reveal the characteristics of de novo methylation in the CHO TK⁻ MT⁻ cell line. The 5mC content of treated CHO TK⁻ MT⁻ cells decreases to 1.43% as a result of the 5-Aza-CdR treatment (figure 18), and no significant increase in the levels of DNA methylation was observed for the first 10 cgs of cultivation in drug free medium. This suggests that any de novo methylation proceeds very slowly, and also it indicates that there is no selective killing

Figure 18: The remethylation process of 5-Aza-CdR treated cells.

The 5mC content of CHO TK⁻ MT⁻ cells was estimated before they were treated with 5-Aza-CdR for 24 hours. Subsequent estimations followed immediately after treatment and 5 and 10 cgs thereafter. The values obtained are indicated by (▲). In another experimental set-up shown as (△) cells were treated with 5-Aza-CdR and selected for TK⁺ revertants. Such revertants were subcultured in normal medium in this case the 5mC content was estimated approximately 50 and 150 cgs after treatment.



in cells that show the highest demethylation. Estimation of the 5mC levels 40 cgs later (50cgs after treatment) shows remethylation to approximately the levels of the parent cells. However, this process of de novo methylation continues, since the methylation level 150 cgs after demethylation is up to 23% higher than that of untreated CHO TK⁻ MT⁻ cells, which is a significant increase.

5.2.3. The 5mC content of tsm cells:

In order to estimate the levels of cytosine methylation in tsm cells, DNA was prepared from cultures at 34⁰C and immediately after treatment for 24 or 48 hours at 39⁰C. Two times of exposure at 39⁰C were used in order to define whether any demethylation at this temperature is progressive. The estimations obtained for tsm cells were compared to those of the CHO KI and CHO TK⁻ MT⁻ parent cell lines. A slight increase in the methylation level is observed in tsm cells in comparison to the CHO KI cell line (figure 17 and table 14). No demethylation was detected in DNA from tsm cells after 24 or 48 hours of heat treatment.

Surprisingly, up to 60% higher methylation was revealed in tsm TK⁺ revertant cells as compared to the parent tsm cell line (table 14). The 5mC content in tsm TK⁺ cells was estimated approximately 150 cell generations (cgs) after treatment at 39⁰C. The number of cgs was calculated from the doubling time of cells and the period of subculturing after treatment. Increase in the levels of 5mC could suggest that mutation in the tsm cells might be associated with control on DNA methylation mechanisms.

Table 14: DNA methylation levels in the tsm mutant.

Cell line	% 5mC/C+5mC
CHO KI	2.5 $\pm$ 0.18
<u>tsm</u>	2.8 $\pm$ 0.2
<u>tsm</u> (24 hours at 39 ⁰ C)	2.8 $\pm$ 0.25
<u>tsm</u> (48 hours at 39 ⁰ C)	2.76 $\pm$ 0.25
<u>tsm</u> TK ⁺	4.53 $\pm$ 0.07

Table 14 shows the methylation levels in tsm cells, as well as, comparison to the 5mC content of CHO KI cells. The tsm TK⁺ is a revertant clone that was subcultured at 34⁰C for approximately 150 cgs after treatment at 39⁰C.

5.3.1. Conclusions:

From HPLC analysis it was revealed that 5-Aza-CR or 5-Aza-CdR treatment is associated with a significant decrease of the 5mC content of the CHO genome. De novo methylation activities present in TK⁺ cells are not only responsible for restoring the initial DNA methylation levels over a long period of subculturing, but they can also lead to hypermethylation. Demethylation was not detectable in tsm cells treated at 39^oC, demonstrating that this mutant is clearly not deficient in DNA methylation. The temperature dependant phenotypic alterations in tsm cells, have probably resulted from alterations in the DNA methylation patterns

CHAPTER 6

REGULATION OF THE THYMIDINE KINASE GENE EXPRESSION

6.1.1. Introduction:

Analysis of the 5mC content of DNA in CHO cells treated with demethylating agents revealed a correlation between demethylation and reversion to the TK^+ phenotype (chapter 5). Molecular studies were performed in order to identify changes in the methylation pattern of the TK gene which are associated with reversion to TK^+ .

It is, however, not certain whether reversion to TK^+ reflects transcriptional or post-transcriptional changes. In order to define this, studies were carried out on the levels of TK gene expression.

6.2.1. The methylation pattern of the TK gene:

High molecular weight DNAs were prepared from the CHO KI cell line and the derivatives CHO TK^- MT^- and tsm in order to examine the methylation pattern of the TK gene. Equivalent preparations of genomic DNAs were carried out for TK^+ revertant clones induced by 5-Aza-CR, 5-Aza-CdR or treatment at 39°C. To ensure that any alterations observed are associated with changes in the phenotype, CHO TK^- MT^- cultures were treated with demethylating agents and DNA was isolated from cells which still have the TK^- phenotype despite reduced methylation. Finally, the methylation pattern of the TK gene in back-reverted clones was examined (i.e. $TK^+ > TK^-$), since results in

sections 3.3.2. and 4.6.2. suggest that epigenetic events might be responsible for back-reversion to the TK⁻ phenotype.

DNAs were digested either with EcoRI or with HindIII restriction endonucleases. These enzymes cleave the sequences GAATTC and AAGCTT respectively and restrict the DNA to an average size of approximately 5kb. EcoRI and HindIII digested DNAs were subsequently cleaved with either of the methylation inhibited enzymes HhaI or HpaII. HhaI cleaves the CGCG and HpaII the CCGG sequences when the internal C is not methylated. Double digested DNAs were electrophoresed through a 0.7% agarose gel and blotted onto nitrocellulose filters, which were sequentially hybridised to genomic probes covering the TK gene.

Five EcoRI fragments were provided by Dr J. Lewis (figure 19) spanning the entire chinese hamster TK gene and 5' as well as 3' flanking sequences, subcloned individually into PBR322. These were used as probes in analysing the methylation pattern of TK genes.

6.2.2. Methylation pattern of 5' and 3' sequences of the TK gene:

The 5.5kb fragment covering the first two exons and approximately 1.4kb of 5' flanking sequences was initially used as a probe, because it contains a CpG rich island overlapping with the promoter sequences. Interestingly the CpG rich island was shown to be hypomethylated irrespective of the cell phenotype (figure 20). This does not rule out the possibility that critical changes might occur within this fragment since DNA pieces smaller than 300bp created from specific demethylation would not be detectable. In contrast to hypomethylation detected at the 5', the 5.2kb fragment spanning the

Figure 19: Organisation of the Chinese Hamster TK gene and the probes available.

Upper part : The thin line represents the TK gene while blocks indicate position of the exons.

Lower part : The genomic probes are EcoRI fragments provided by Dr J. Lewis to screen the methylation pattern of the TK gene. The order of probes in the figure represent their actual place in the gene.

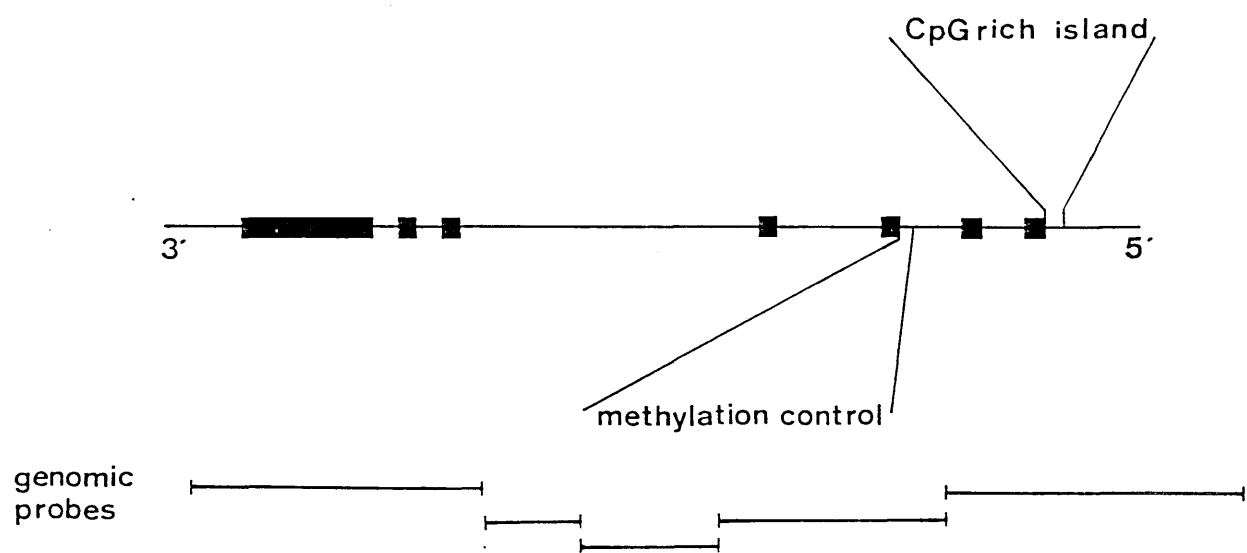
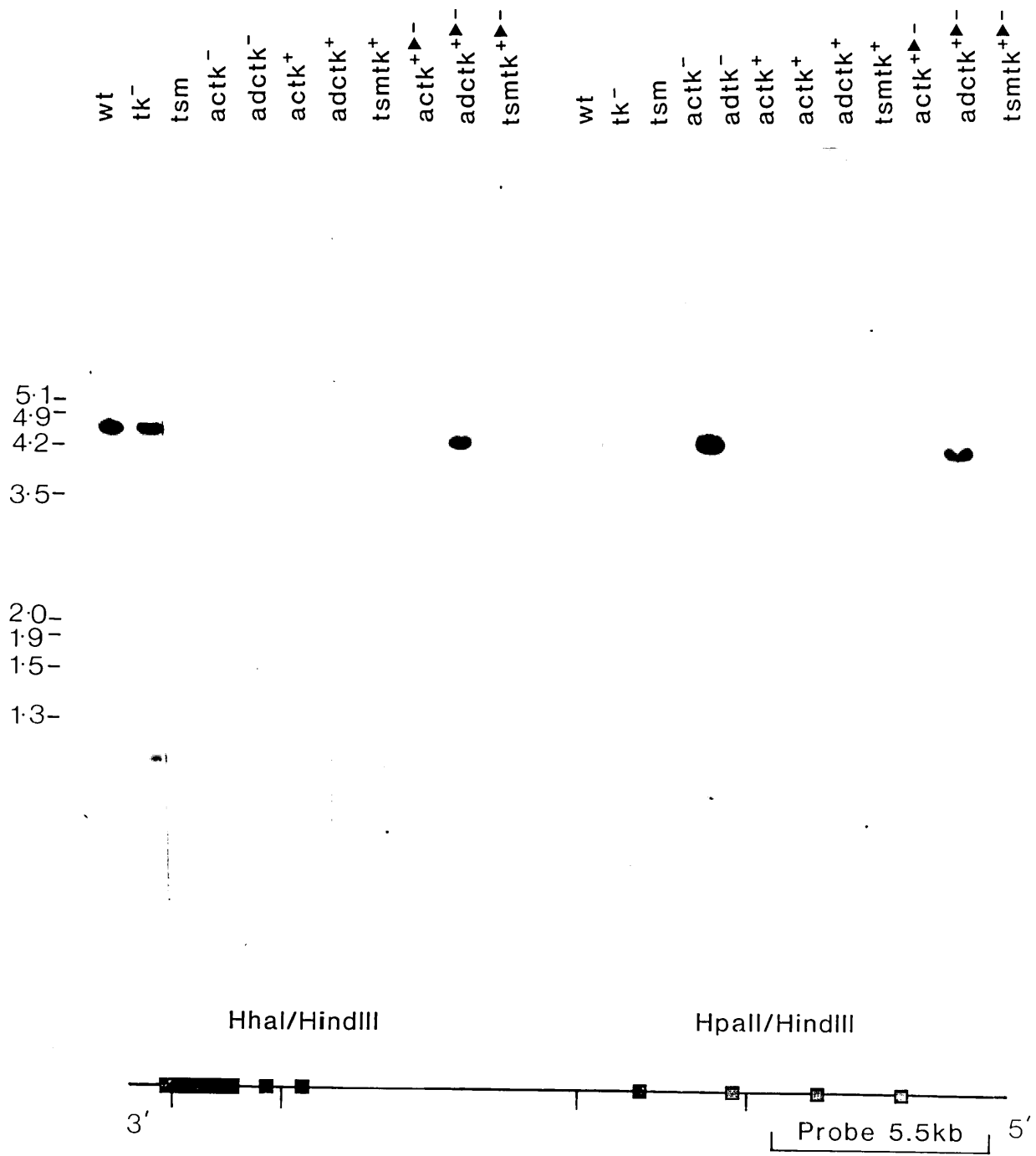


Figure 20: Methylation status of the 5' promoter sequences of the TK gene.

DNA from CHO TK⁻ MT⁻ (tk⁻), tsm (tsm), 5-Aza-CR or 5-Aza-CdR treated CHO TK⁻ MT⁻ that have retained the TK⁻ phenotype (actk⁻, adctk⁻), 5-Aza-CR or 5-Aza-CdR or temperature induced TK⁺ (actk⁺, adctk⁺, tsmtk⁺) and spontaneous TK⁻ back-revertants from the actk⁺, adctk⁺, or tsmtk⁺ cells, was digested as indicated with HpaII/HindIII and HhaI/HindIII. Cleaved DNAs were subjected to electrophoresis through a 0.8% agarose gel, blotted onto nitrocellulose and hybridised with ³²P labelled 5.5kb EcoRI fragment covering the 5' promoter sequences and the first two exons of the TK gene. A 6Kb Hind III fragment was expected. The bars indicate Hind III sites.



last three exons and nearly 2kb of 3' flanking sequences was completely methylated in HpaII and HhaI restriction sites in both expressing and non expressing cells (figure 21). Similarly, the 2.6kb and 1.8kb intronic EcoRI pieces were found fully methylated for these sites both in TK⁻ and TK⁺ cells (figure 22). Thus, methylation control of TK gene expression is probably not conferred from 3' sequences, or the intronic 2.6kb and 1.8kb EcoRI fragments.

6.2.3. Specific demethylation is associated with reversion to the TK⁺ phenotype:

While no alterations in the methylation status were detected using the above described probes, hybridisation with the 4.2kb internal fragment covering the 3rd and 4th exons of the TK gene revealed specific demethylation in TK⁺ revertant clones (figure 23). In all of the parent strains a 4.2kb DNA fragment is detected for both HpaII and HhaI digestions. This fragment is of identical size to the band detected in cells digested only with EcoRI endonuclease, and it probably represents a copy of the TK gene completely methylated in the 4.2kb EcoRI fragment. The additional lower molecular weight band in the HpaII blot may be an undermethylated TK allele. However, complete demethylation of the 4.2kb fragment should create much smaller DNA fragments as revealed by MspI digestion (Lewis 1986, and data not shown), suggesting that the second TK copy is not completely demethylated. Similarly, the two lower bands in the HhaI blot may represent either two undermethylated copies of the 4.2 kb fragment, or population variability in the methylated state of one copy. The first suggestion is unlikely since these two bands show different

Figure 21: Methylation status of the 3' sequences of the TK gene.

DNA from the cell lines described in figure 20 except from back-revertant TK⁻ clones, was digested with EcoRI/HpaII or EcoRI/HhaI. Cleaved DNAs were electrophoresed through 1% agarose gel and blotted onto nitrocellulose. Filters were hybridised against ³²P labelled 5.2 kb EcoRI fragment spanning the last two exons and approximately 2 kb 3' flanking sequences.

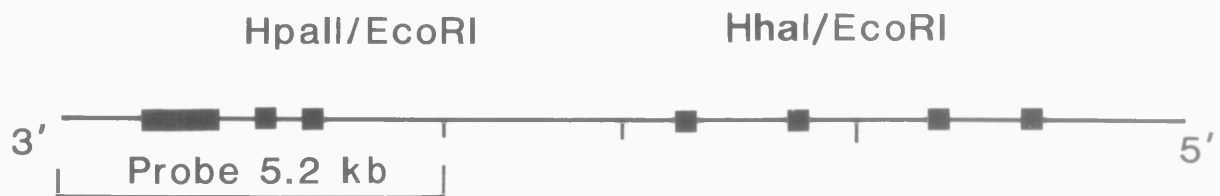
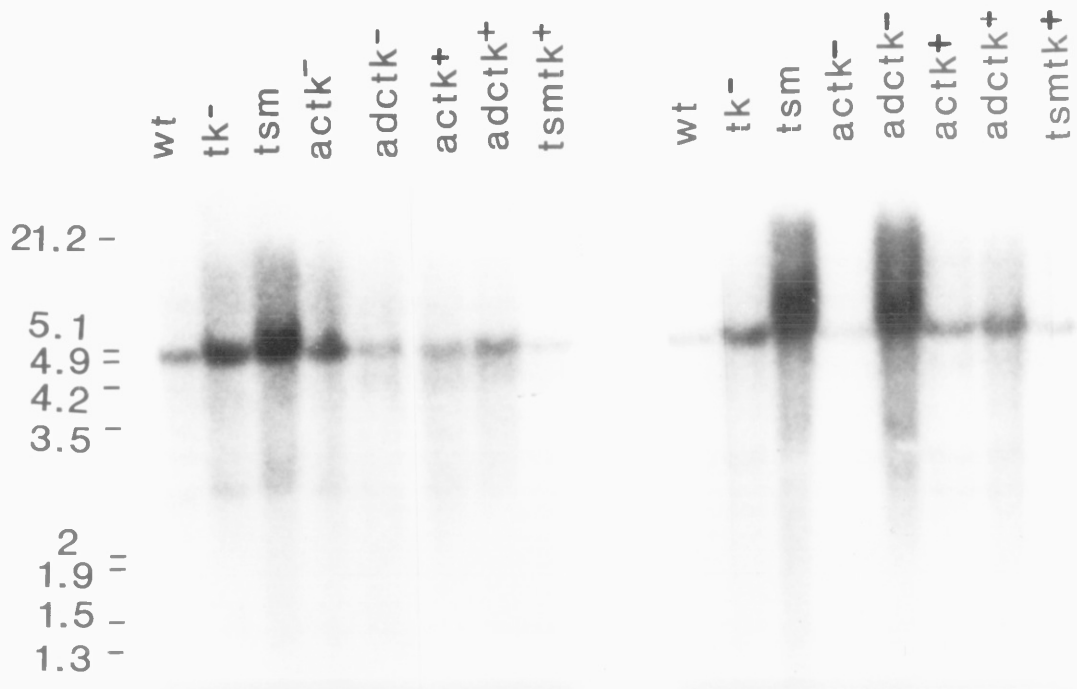


Figure 22: Methylation status of the 2.6 kb intronic EcoRI fragment of the TK gene.

The blot described in figure 21 was washed and hybridised to ^{32}P labelled 2.6 kb intronic EcoRI fragment.

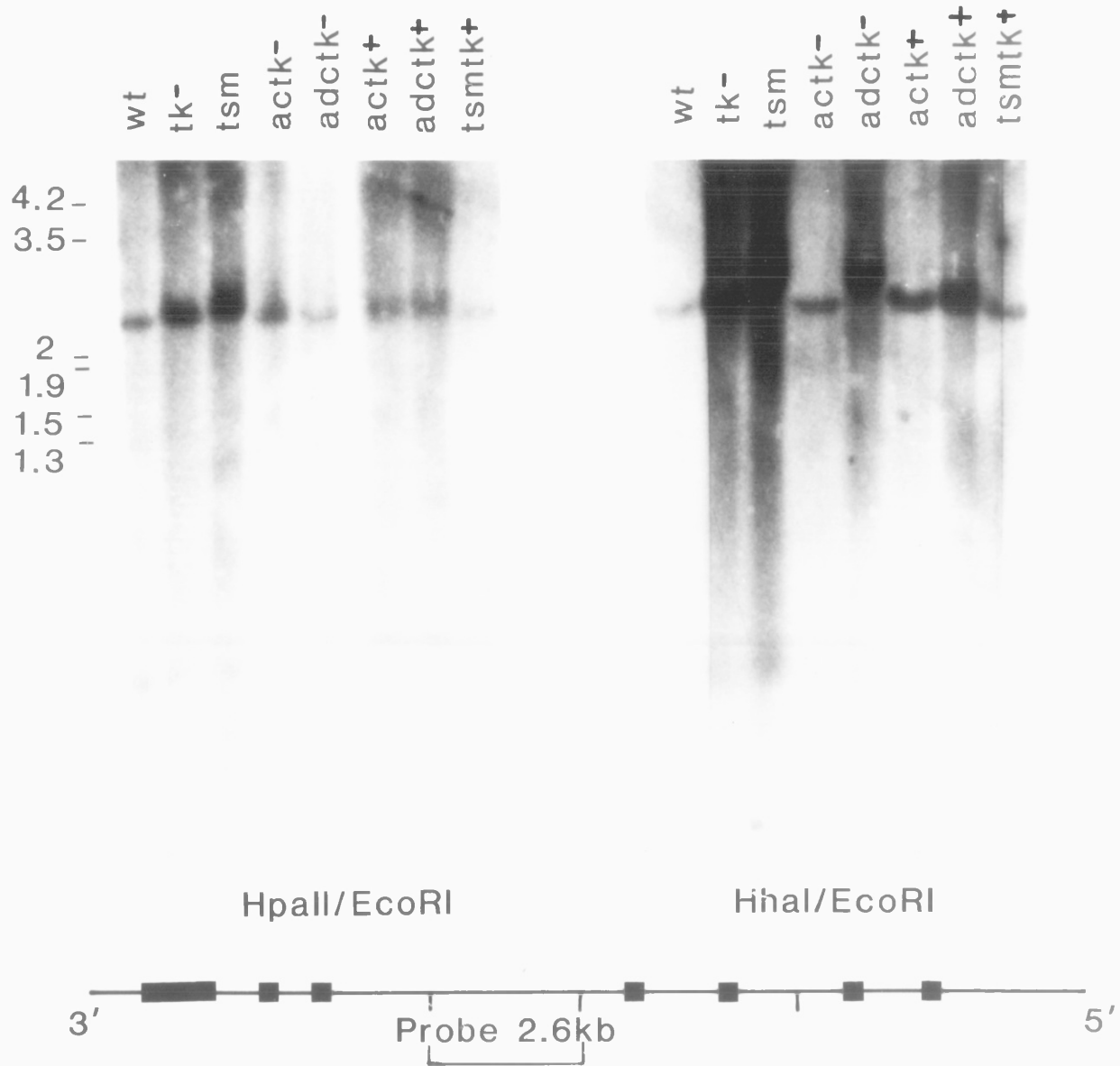
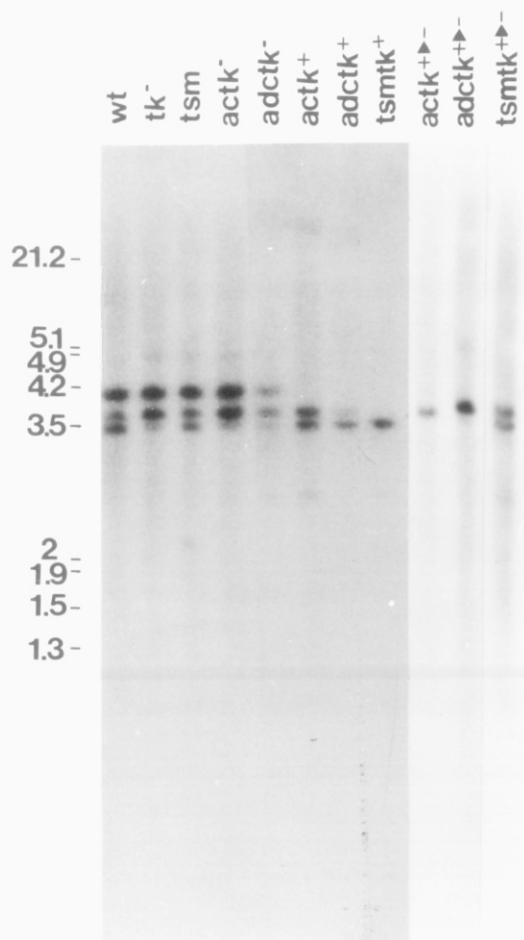
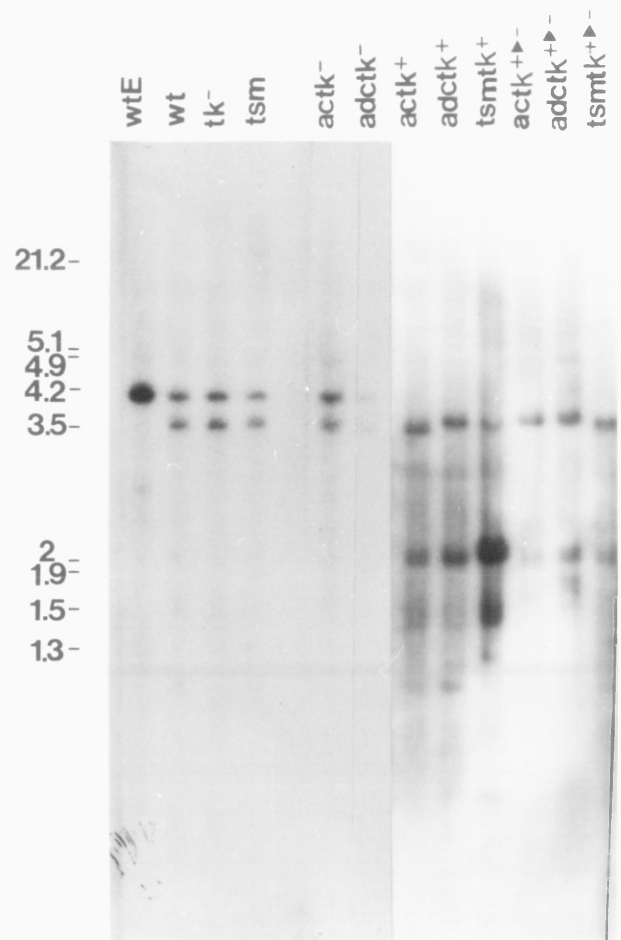


Figure 23: Methylation status of the 4.2 kb EcoRI fragment of the TK gene.

New blots were prepared as described in figure 21. Filters were also prepared from EcoRI/HhaI and EcoRI/HpaII digested DNA isolated from TK⁻ back-revertant clones as specified in figure . An additional lane was included of CHO KI DNA digested only with EcoRI (wtE). Filter were hybridised with ³²P labelled 4.2 probe which covers the 3rd and fourth exons.



HhaI/EcoRI



HpaII/EcoRI



intensities within the same lane.

All the TK^+ revertant clones lose the completely methylated copy in both HhaI and HpaII digested DNA (figure 23, lanes $actk^+$, $adctk^+$, $tsmtk^+$). However 5-Aza-CR and 5-Aza-CdR treated cells that retain the TK^- phenotype (figure 23, lanes $actk^-$, $adctk^-$), show no specific demethylation, which suggests a requirement of demethylation for TK^+ reversion. The detection of variable demethylation between different revertant clones in HhaI digested DNA can be explained if demethylation in either of the two HhaI sites detected is sufficient for TK^+ reversion. Additionally, the band detected in the HpaII digestion of the revertant TK^+ clones shows size fluctuation suggesting the existence of more than one HpaII sites in this area demethylation of any of which is sufficient for TK^+ reversion. Although the TK^- back-revertant clones show no significant remethylation in the HpaII blot, the HhaI blots revealed remethylation in all the TK^- back-revertants in comparison to the TK^+ cells they have originated from (figure 23, lanes $actk^+$ with $actk^{+>-}$, $adctk^+$ with $adctk^{+>-}$ and $tsmtk^+$ with $tsmtk^{+>-}$). Nevertheless since these back-revertants do not recover the completely methylated band they must have undergone only partial remethylation, which is sufficient for the change in the phenotype (reversion to TK^-).

6.2.4. Methylation regulation is conferred by a restricted area covering the 3rd exon of the TK gene:

Since methylation changes were only detected in the 4.2kb EcoRI fragment for both HhaI or HpaII digestions, it is likely that a small area within this fragment confers regulation of the TK gene

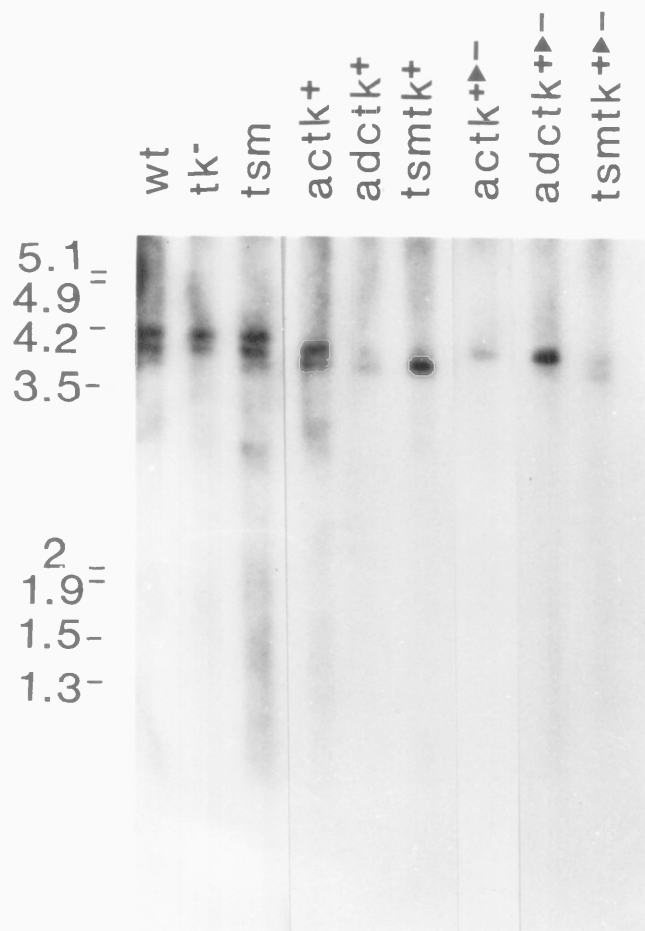
expression. Therefore further restriction of the methylation regulatory area was attempted. For this purpose DNA digested with the HindIII restriction endonuclease in combination with HhaI or HpaII, and was hybridised against the 4.2kb probe. The fully methylated fragment is defined by two HindIII sites approximately 300bp downstream of the equivalent EcoRI. If the detected demethylation lies towards the 5' EcoRI site the demethylated bands would approach in size the completely methylated 4.2kb fragment. The opposite is expected if the demethylated sequences are towards the 3' EcoRI site. Since in this Southern blot (figure 24) the lower molecular weight undermethylated fragments approach in size the complete 4.2kb piece for both HpaII and HhaI digestions, it can be concluded that HpaII are adjacent to the HhaI sites, and that they all form a cluster towards the 5' of the 4.2kb EcoRI fragment (figure 25).

6.3.1. Induction in the levels of TK transcript in TK⁺ revertant cells:

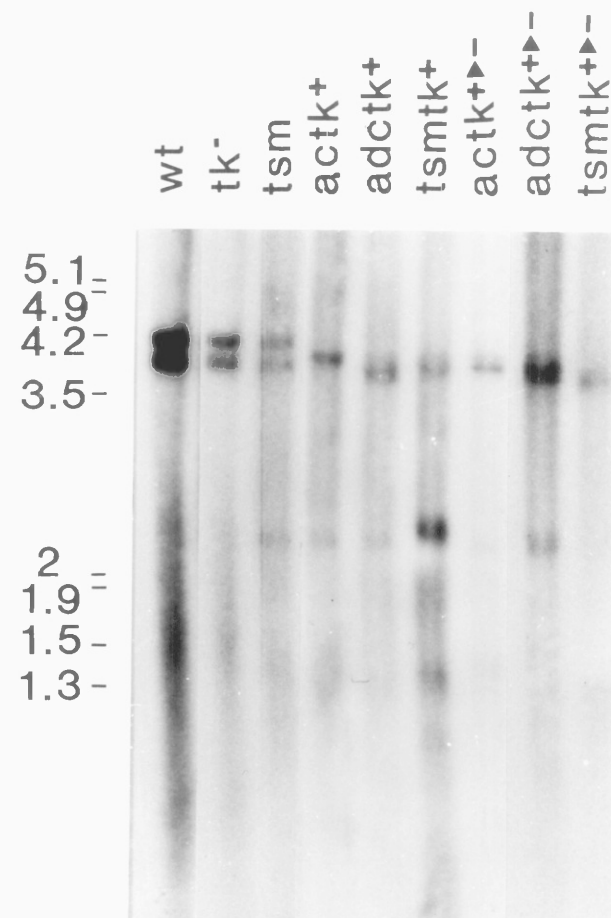
Quantitative analysis of the TK transcript was performed by Northern blot hybridisation. Thus, poly-A tailed RNA was prepared from the parent CHO KI, CHO TK⁻ MT⁻ and tsm cell lines. Poly-A tailed RNA was also isolated from TK⁺ revertant clones induced by 5-Aza-CR or treatment at 39°C and the back-revertant TK⁻ clones that derived from these cells. RNAs were separated by electrophoresis through 1% denaturing agarose/formaldehyde gels and blotted onto nitrocellulose filters. A chinese hamster TK cDNA provided by Dr J. Lewis was used as a probe for detection of the TK transcript. In order to monitor the levels of RNA per lane hybridisation was also performed using a

Figure 24: Detectable methylation changes are restricted in 200bp
internally of the TK gene.

The blot described in figure 20 was washed and hybridised against the
4.2 EcoRI fragment labelled with ^{32}P



HhaI/HindIII

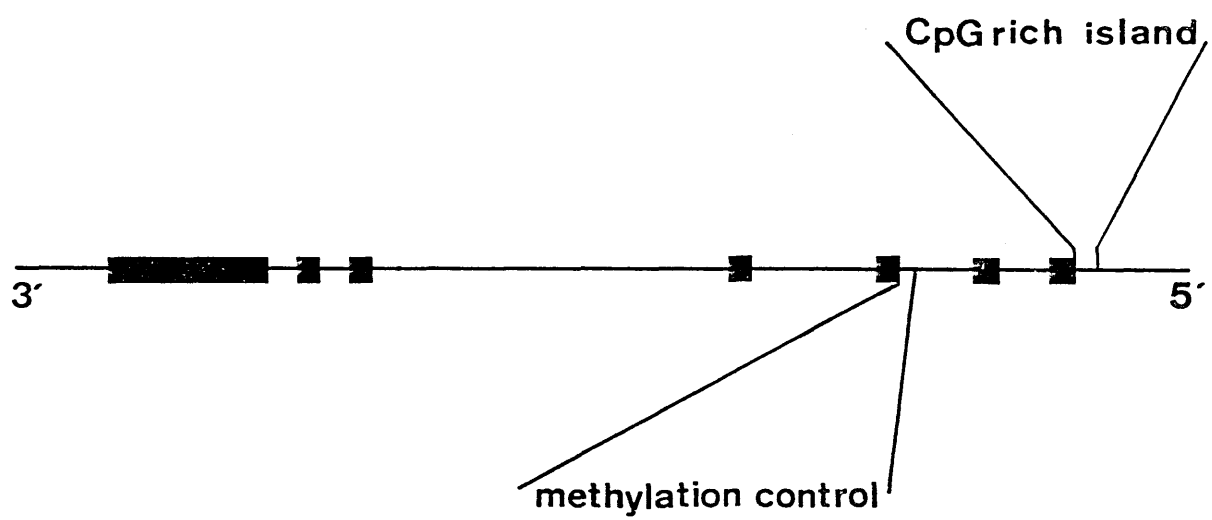


HpaII/HindIII



Figure 25: Localisation of the methylation regulatory area in the TK gene.

The 200 bp area which confers methylation control of TK expression, is localized within the gene. The position of the CpG rich island is also outlined in this drawing.



chinese hamster APRT probe.

Revertants induced by either genetic or chemical demethylation show several fold increase in the levels of TK transcript as opposed to parent cells (figure 26). Back-revertant clones return to almost the transcriptional state detected in the parent CHO KI cells. Surprisingly, the CHO TK⁻ MT⁻ cells show low levels of TK mRNA. The possibility of this being the mitochondrial TK transcript was tested by qualitative analysis using S1 nuclease mapping. This procedure requires very high homology between the probe and the RNA under detection in order to obtain protection of the probe from the nuclease digestion.

6.3.2. Qualitative analysis of the detected TK transcripts:

Total mRNA from the TK⁻ parent and TK⁺ revertant cells as well as mRNA from CHO KI cells were prepared and hybridised with ³²P labelled probes. The PvuII-PvuII 900bp cDNA 5' probe overpassed 5' flanking sequences, the first 6 exons and part of the last one. It was labelled using T4 polynucleotide kinase. The 3' probe, an MspI-MstII 1,200bp fragment compassed the rest of the last exon and 3' flanking sequences. It was labelled using reverse transcriptase (figure 27).

It was expected that the mitochondrial TK mRNA, would not be identical with the cytosolic TK transcript. Therefore the non homologous parts of the DNA-RNA hybrid would be single stranded, and subject to S1 nuclease digestion. This would result in smaller protected fragments or complete degradation of the probe. However, no difference was seen in the size of TK transcript by this method (figure 28). Additionally, the level of TK mRNA was reduced in TK⁻

Figure 26: Northern blot analysis of the TK transcript.

Poly-A tailed RNA from CHO KI (wt), CHO TK⁻ MT⁻ (tk⁻), tsm (tsm) their TK⁺ derivatives induced either by 5-Aza-CR (actk⁺) or by heat treatment (tsmtk⁺), and the spontaneous back-revertants from , and tsmtk⁻ (tsmtk^{+>-}) was prepared. This was electrophoresed through 1% denaturing agarose/formaldehyde gel and blotted onto nitrocellulose filters. Filters were hybridised against a ³²P labelled TK cDNA probe.

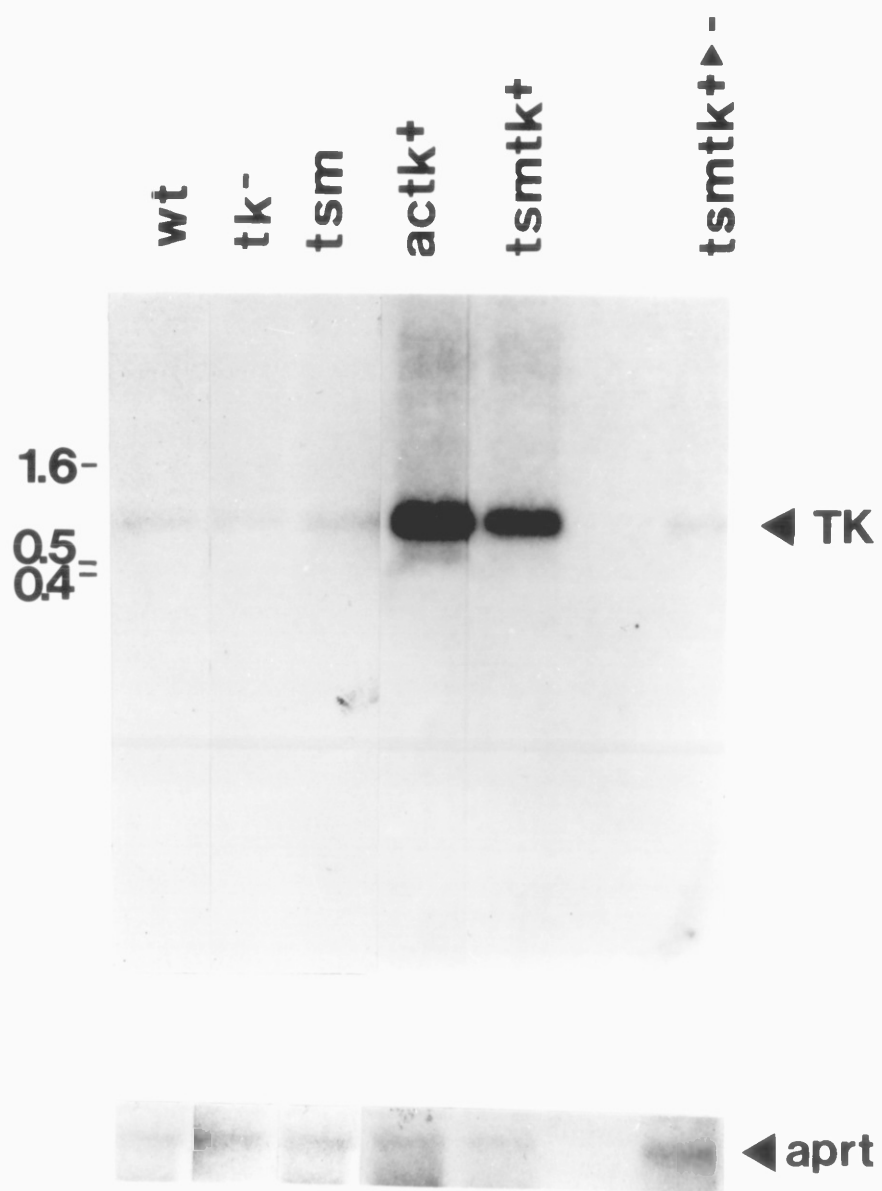


Figure 27: Probes used for S1 analysis.

The upper map represents the TK gene and the lower map shows the position of the probes in relation with the gene, as well as the protected fragments expected after S1 nuclease digestion.

S1 probes

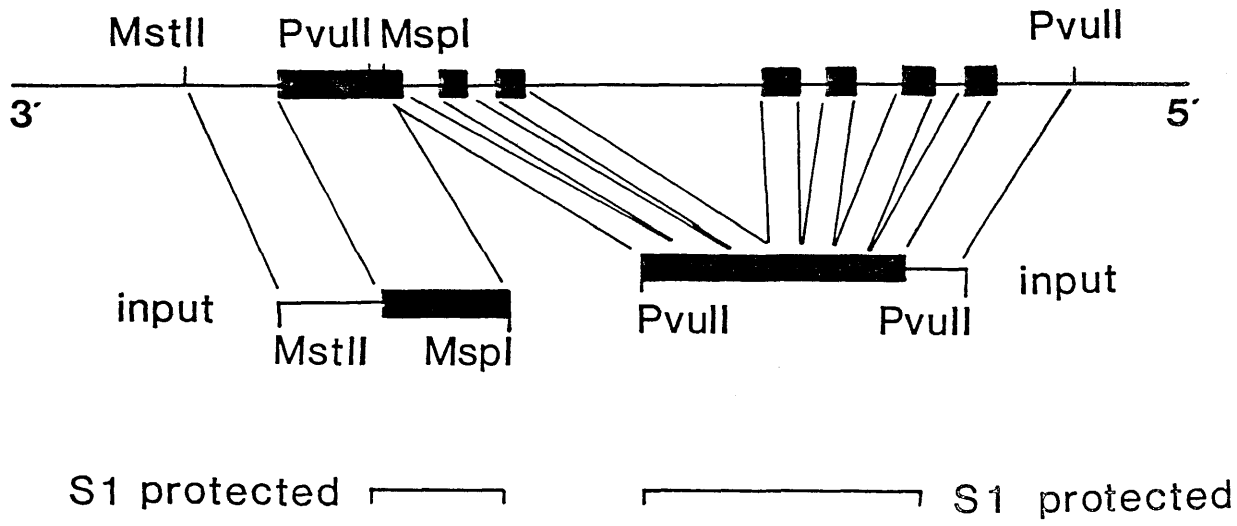
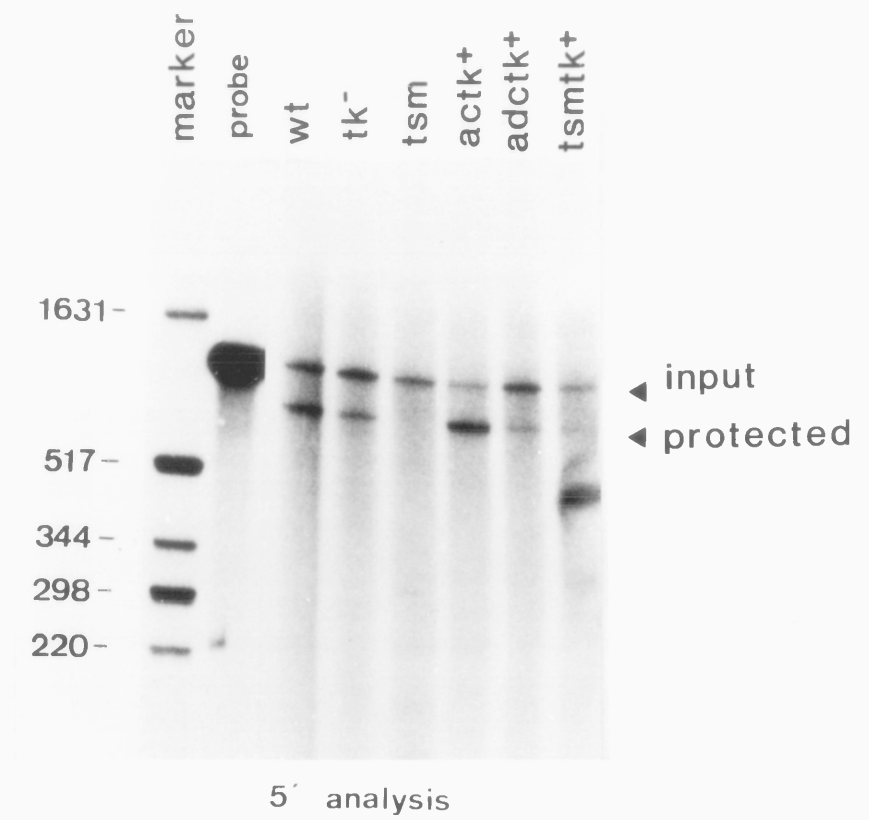
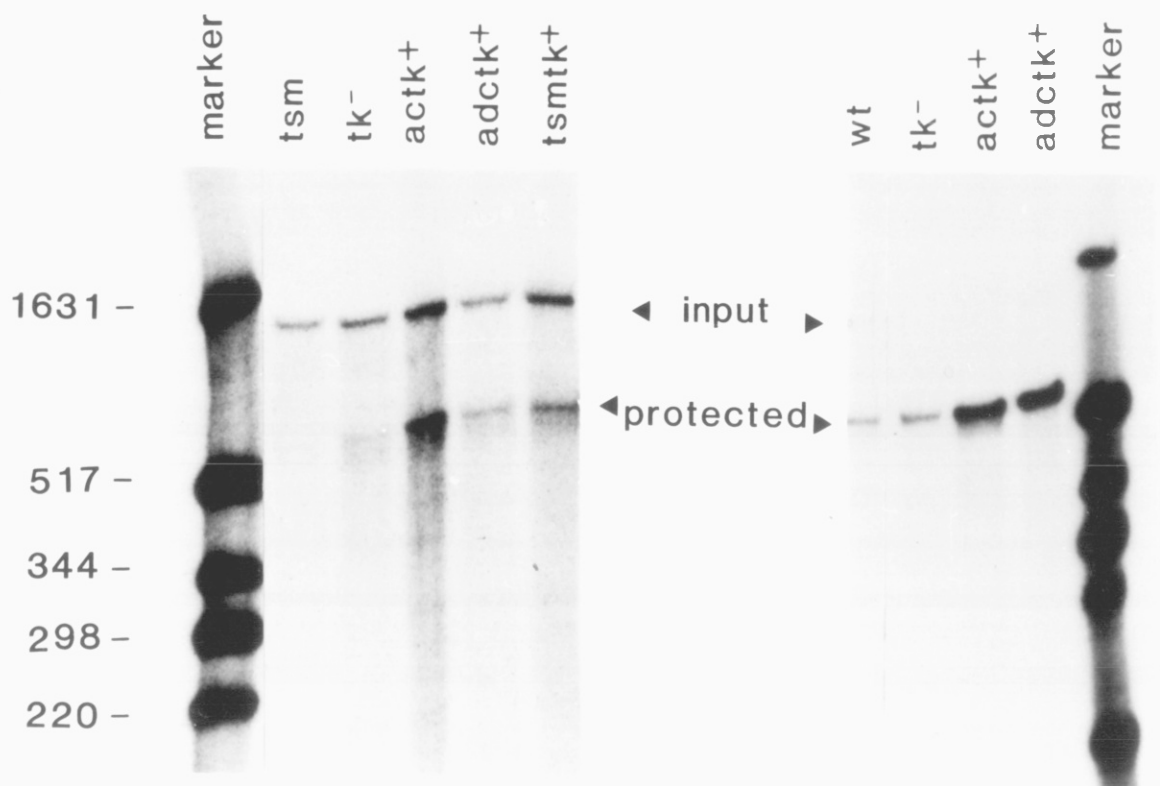


Figure 28: S1 mapping of the TK transcript.

Total RNA from CHO KI (wt) CHO TK⁻ MT⁻ (tk⁻) and tsm (tsm), and their TK⁺ derivatives actk⁺, adctk⁺ and tsmtk⁺, were hybridised to ³²P end labelled double stranded probes and subjected to S1 nuclease digestion. After digestion they were denatured, electrophoresed through denaturing 4% polyacrylamide/urea gels and autoradiographed. The upper picture is 5' analysis with the same RNAs hybridised to a control APRT probe. The lower part is 3' analysis of the TK transcript.



aprt ▶



cells which were harvested immediately after BUdR selection, suggesting that residual TK activity may be coded from the detected transcript (figure 29). Although TK mRNA was detected in the tsm mutant cell line by Northern Hybridisation no protected fragment was revealed by S1 mapping. This suggests that a similar but not identical to the TK mRNA is present in these cells.

Finally, experiments were done to find out whether the presence of a TK transcript in CHO TK⁻ MT⁻ cells is a characteristic shared between all the TK⁻ cell lines isolated from CHO KI cells. Thus, S1 nuclease analysis was applied on RNA from TK⁻ clones (section 3.2.1.), isolated by X-ray mutagenesis of the CHO KI cell line. None of these clones showed detectable TK transcript (figure 30). This suggests that the presence of TK transcript in the CHO TK⁻ MT⁻ strain is an exceptional case. The mutation in CHO TK⁻ MT⁻ cells could be a frameshift or a single base substitution or a small deletion, that produces an inactive or partially active TK transcript (section 8.1.).

6.4.1. Conclusions:

The results presented in this chapter demonstrate that reversion to the TK⁺ phenotype induced either by demethylating agents or in the tsm by heat treatment is associated with specific demethylation. An area of approximately 200bp near the 3rd exon of this gene was identified where the levels of demethylation appear to confer regulation of TK gene expression.

As a result of demethylation, significant transcriptional induction was observed specifically for the TK gene. This is in agreement with previous studies suggesting that methylation controls

Figure 29: Comparison of the level of TK mRNA in selected and non selected TK⁻ cells.

S1 analysis was performed using RNAs prepared either from selected in BudR TK⁻ cells, or harvested after culture in normal medium, hybridised with ³²P labelled 5' probe (see figure 28).

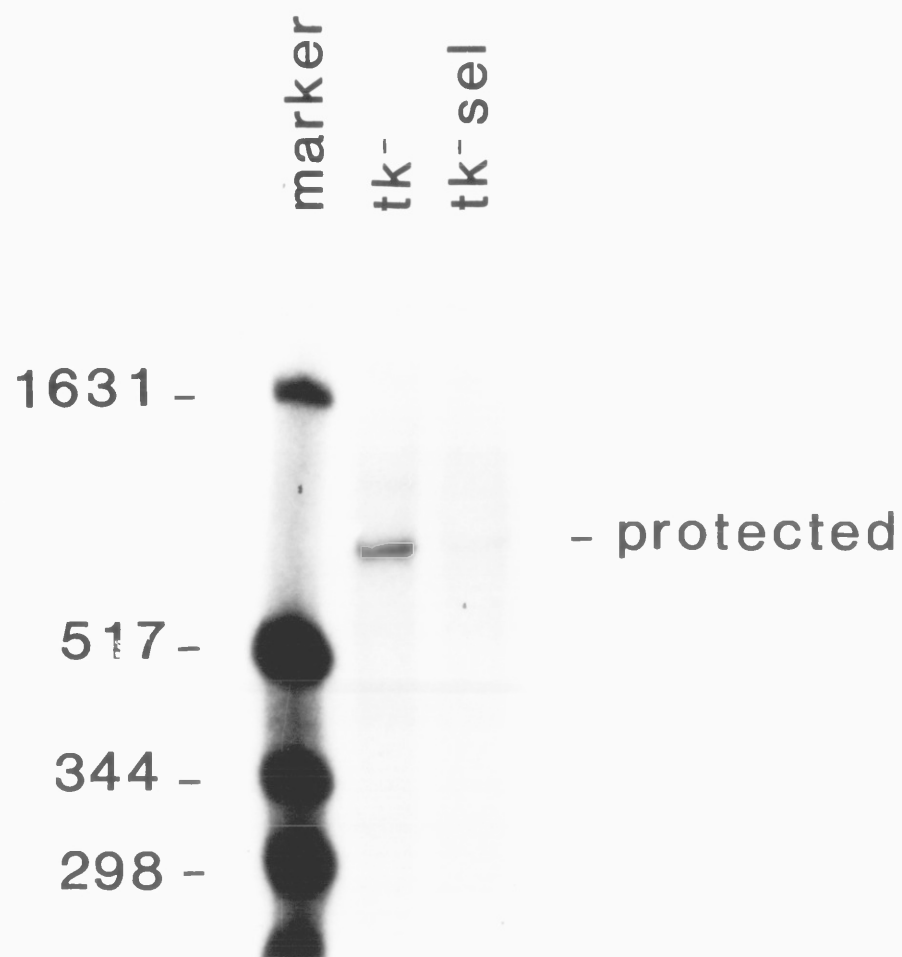


Figure 30: S1 mapping of five additional TK⁻ clones.

Total RNA was prepared from five additional TK⁻ clones isolated from CHO KI cells after X-Rays mutagenesis. These were hybridised to the 5' probe described in figure and digested with S1 nuclease. Digested RNAs were denatured, electrophoresed through 4% denaturing polyacrylamide/urea gel and autoradiographed.

CHO XR Tk⁻1
CHO XR Tk⁻2
CHO XR Tk⁻3
CHO XR Tk⁻4
CHO XR Tk⁻5
Input Probe
Marker



5' Analysis

gene expression at the transcriptional level.

CHAPTER 7

REGULATION OF METALLOTHIONEIN GENE EXPRESSION

7.1.1. Introduction:

Evidence presented in chapter 3 suggests that methylation might be responsible for the control of MT gene expression. Thus, the methylation status of the MT genes was studied in MT⁻ and MT⁺ clones in order to relate reversion to MT⁺ phenotype with alterations in the methylation pattern of the genes.

Transcriptional analysis of MT⁺ clones and parent MT⁻ cell lines was also performed in an attempt to define whether the MT⁺ phenotype is due to transcriptional activation of MT genes.

7.2.1. Demethylation is associated with reversion to the MT⁺ phenotype:

The methylation patterns of MT genes were examined in genomic DNAs prepared from the MT⁻ parent cell lines (CHO KI, CHO TK⁻ MT⁻, tsm) and MT⁺ revertant clones induced either by 5-Aza-CR or treatment of the tsm strain at 39°C. These DNAs were initially digested with EcoRI restriction endonuclease and then with either of the methylation inhibited enzymes HpaII and HhaI, or the methylation insensitive isoschizomer of HpaII, MspI. The products were electrophoresed and transferred to nitrocellulose filters. Specific MT fragments were then detected by hybridisation of the nitrocellulose

filters with a 350bp long MTI cDNA probe (Griffith *et al.* 1983).

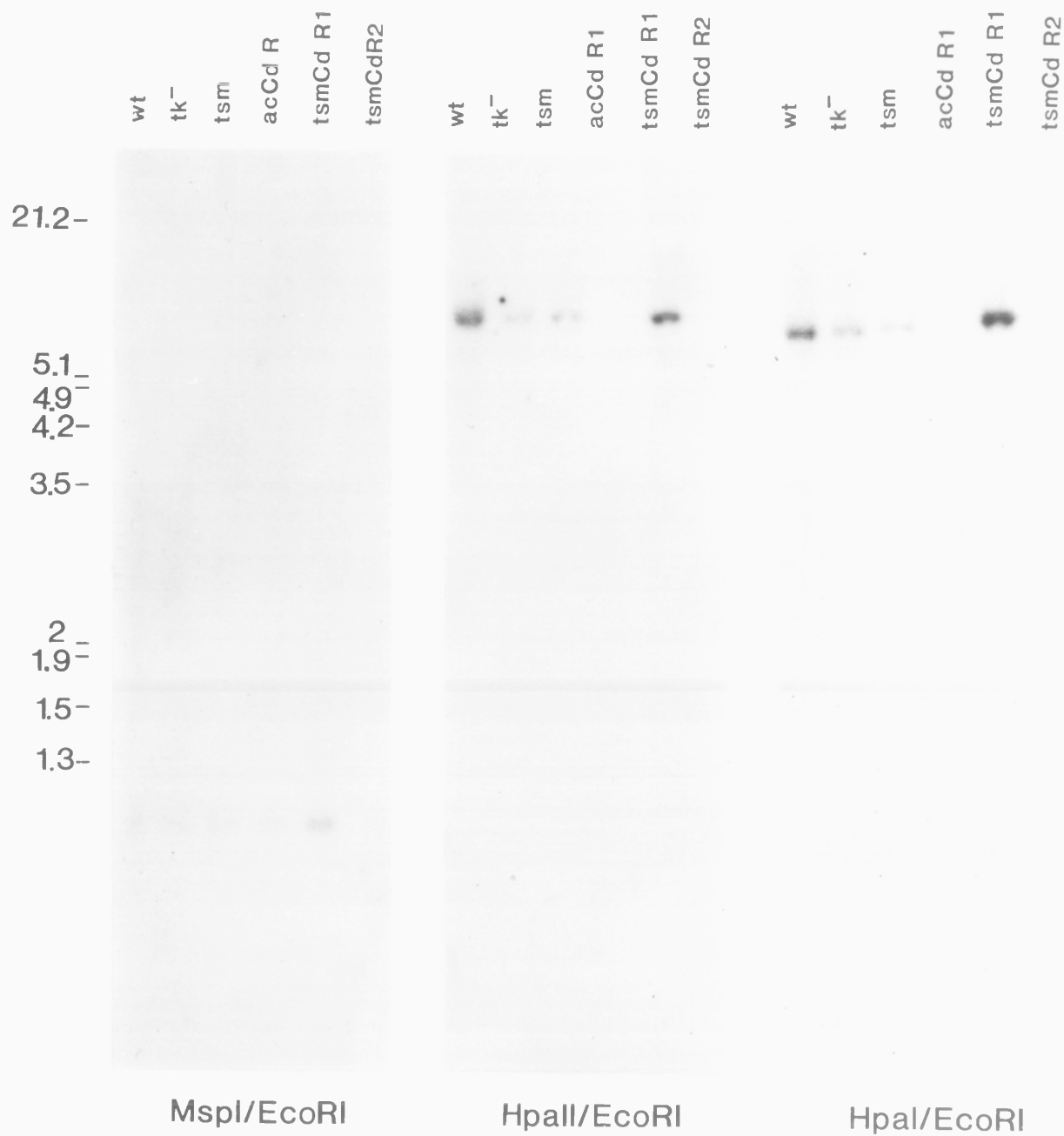
The initial hybridisation experiments were performed under the high stringency washing conditions of 0.1xSSC at 65°C. These revealed a faint band of approximately 8.5kb in all the parent cells. The probe used in these hybridisations is 80% homologous to the MTII coding sequences. It was therefore expected to detect both MTI and MTII containing DNA fragments under these stringency conditions. However, only one band was revealed suggesting that either the EcoRI fragments containing the MTI and MTII genes are of the same approximate size and detected as a unique band, or that the two genes are linked with no EcoRI restriction site between them. The 8.5kb band was not present in the 5-Aza-CR induced MT⁺ (Cd^r) revertant clone and in one of the two tsm MT⁺ revertants (figure 31, lanes acCdR, tsmCdR2). This change in the hybridisation patterns of Cd^r clones is probably not due genetic mutation since both parent and revertant strains show the same MspI pattern (figure 31). Surprisingly, the 8.5kb band is present in one of the tsm MT⁺ clones examined (figure 31, lane tsm CdR1). In order to resolve the discrepancy, new blots were prepared and were hybridised using the low stringency washing conditions of 1xSSC at 65°C (figure 32).

In these blots, a low molecular weight hybridisation band was detected in both HhaI and HpaII digested DNA from all tsm MT⁺ clones. This was not present in similar digests of DNA from MT⁻ cells. It should be noted that the lower 1kb band which was absent in the HpaII digest of CHO TK⁻ MT⁻ genomic DNA, was present in methylation-insensitive MspI digests. This observation eliminates the possibility that failure to detect the low molecular weight band in the HpaII digest is due to genetic mutation. It is therefore

Figure 31: Methylation pattern of MT genes under high stringency

hybridisation

DNAs from CHO KI (wt), CHO TK⁻ MT⁻ (tk⁻) and tsm (tsm) cells and their MT⁺ derivatives either after 5-Aza-CR or treatment 39^oC, were digested with EcoRI/MspI, EcoRI/HpaII or EcoRI/HhaI. Cleaved DNAs were electrophoresed through a 1% agarose gel and blotted onto nitrocellulose filters, which were hybridised to a ³²P labelled chinese hamster MTI cDNA probe. The blots were washed to a stringency of 0.1xSSC at 65^oC.

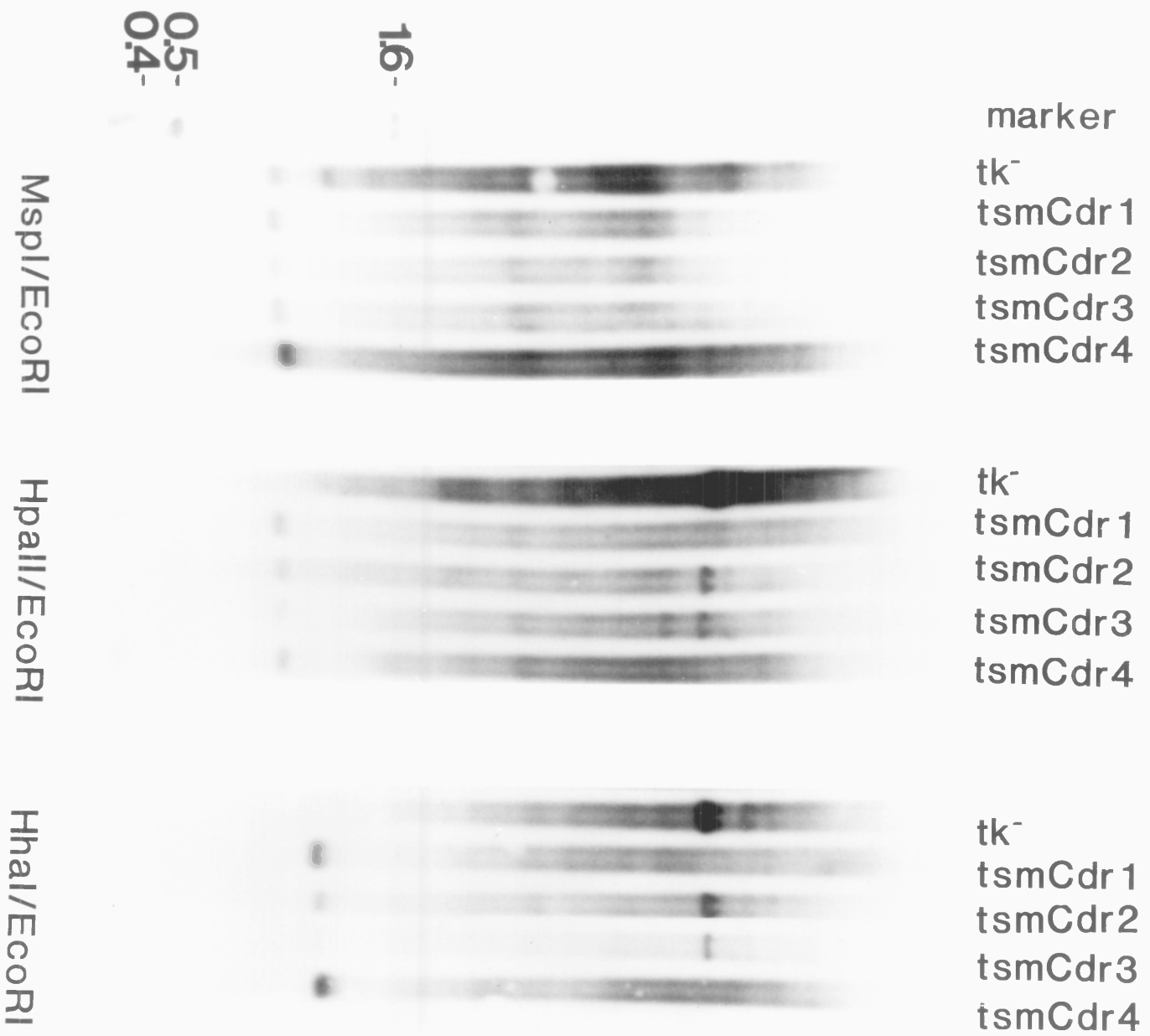


CH MTI cDNA probe

Figure 32: Methylation pattern of MT genes.

New blots were prepared using DNA from CHO TK⁻ MT⁻ (tk⁻), and four tsm MT⁺ clones, digested with EcoRI/MspI, EcoRI/HpaII or EcoRI/HhaI and electrophoresed through 1% agarose gel. This was blotted onto nitrocellulose filter which was hybridised against a ³²P labelled chinese hamster MTI cDNA probe. These blots were washed to a stringency of 1xSSC at 65⁰C.

CHO MTI cDNA probe



suggested that demethylation accompanies reversion to the MT^+ phenotype. In addition, the fact that for tsm MT^+ DNA, the HpaII and MspI hybridisation patterns are identical, suggests that their methylation patterns are similar and that demethylation at all detectable HpaII sites have occurred during generations of these clones.

Interestingly, two of the four revertant clones shown in figure 32 retain an upper band which is equivalent in size to the methylated gene sequences detected in MT^- parent cells. This may be due to activation of only one of the MTI alleles in these revertant clones. Alternatively it might be a result of detection of a methylated MTII gene.

7.3.1. The MT^+ phenotype results from activation of the MT gene:

The MT transcript was studied in MT^- and MT^+ cells by Northern blot hybridisation. Poly-A RNA was prepared for this purpose from the parent CHO KI, CHO TK⁻ MT^- and tsm strains and two tsm MT^+ revertant clones. A chinese hamster cDNA MTI probe was used for detection of the MTI transcript immobilized on nitrocellulose filters. This probe would also detect the 80% homologous MTII transcript which is expected to migrate at the same molecular weight (Griffith et al. 1983). The higher molecular weight band in figure 33 may be an intermediate product of RNA processing. The level of Poly-A RNA loaded per lane was monitored by hybridisation using an APRT probe.

MT transcripts were detected even in cells expressing MT^- phenotype (figure 33). Thus, although MT genes are transcriptionally active the cells are still sensitive to Cd selection. A possible

Figure 33: Transcriptional analysis of MT genes.

Poly-A RNAs were prepared from CHO KI (wt), CHO TK⁻ MT⁻ (tk⁻) and tsm (tsm), and two tsm MT⁺ revertants (tsmcd1, tsmcd2). These were electrophoresed through a 1% denaturing agarose/formaldehyde gel and blotted onto nitrocellulose filters. Chinese hamster MTI cDNA was labelled with ³²P and used as a probe against these filters

▲aprt

▲MT

0.0
2.2
0.4
9.0
=

wt
tk
tsm
tsmcd r1
tsmcd r2

explanation for these observations is that cells phenotypically MT^{-} contain methylated but expressed MTII alleles in addition to methylated unexpressed MTI alleles. MT^{+} alleles would contain demethylated MTII alleles expressed at the same level as in the MT^{-} cells, and demethylated, highly expressed MTI alleles. There is in fact some evidence that the MTII gene is not regulated by DNA methylation (Heguy *et al.* 1986).

It is important to note that all MT^{+} revertant strains examined showed many fold higher levels of MT transcripts compared with those in parental MT^{-} cells, probably as a result of the strong activation of the previously silent methylated MTI alleles. The lack of selective procedure for an absence of MTI gene expression, however, prevented more detailed investigations into the role of MT mRNA in MT^{-} (Cd sensitive) cell lines.

7.4.1. Conclusions:

In this chapter it was shown that DNA methylation regulates the expression of MT gene. Thus, demethylation of the gene is observed in all the MT^{+} revertant clones suggesting that loss of methylgroups is required for reactivation. Extensive demethylation is probably necessary since all the detectable HpaII sites were found demethylated in the MT^{+} revertants.

DNA methylation regulates MT gene expression at the transcriptional level as indicated by the increase in MT mRNA observed in MT^{+} clones. Although the parent lines cannot survive in the presence of Cd, they express low levels of MT transcripts.

CHAPTER 8

DISCUSSION

Isolation of methylation deficient mutants of eukaryotic cells would allow detailed studies on the biological function of 5mC to be carried out. This thesis describes the successful application of a novel method for the isolation of temperature sensitive mutants expressing alterations in their DNA methylation patterns. The method was based on the assumption that mutations affecting the methylation mechanism, thus altering DNA methylation patterns, would result in reactivation of previously inactive genes subject to methylation control. However, screening of a potentially large number of individual colonies would be a limiting factor and it would be only feasible to isolate dominant mutations, or recessive mutations in regions of the genome which are haploid. Additionally, it was necessary to use a procedure which would allow for the possibility that the required mutation would be lethal.

The use of the CHO TK⁻ MT⁻ cell line in the search for mutants, overcame some of these limitations. Evidence was already available suggesting that the expression of the TK gene in Chinese Hamster V79 cells and the MTI gene in mouse cells were under methylation control (Harris 1982, Compere and Palmiter 1981). The finding that 5-Aza-CR and 5-Aza-CdR caused 1-59% reactivation of TK and MT genes, provided important evidence for such methylation control of TK and MT expression in CHO TK⁻ MT⁻ cells. This evidence was supported by the observed loss of methylated cytosine residues in CHO TK⁻ MT⁻ cells

following treatment with 5-Aza-CR or 5-Aza-CdR, in agreement with earlier observations on the demethylating effect of these agents (Jones and Taylor 1980, Wilson et al. 1983, Flatau et al. 1984). Thus, the epigenetic change due to demethylation is probably sufficient to induce reactivation of TK and MTI genes in CHO TK⁻ MT⁻ cells, providing the required phenotypic alterations for the screening procedure. Additionally, the frequency with which a variety of recessive mutations have been isolated from CHO cells led to suggestions that a large number of CHO genes are functionally haploid (Siminovich 1976). It is therefore possible that the gene of interest is monosomic in the CHO TK⁻ MT⁻ strain. Finally, the selection of conditional mutants in combination with the maintenance of replicate cultures at the low temperature would permit rescue of even a lethal temperature sensitive mutation.

In the subsequent discussion, the molecular studies on the methylation of the TK and MT genes will be considered in progenitor cells, before the isolation of the putative methylation deficient mutant.

8.1. Methylation and TK expression:

It is already well documented that methylation regulates the expression of the Herpes Virus TK gene (Christy and Scangos 1984, Clough et al. 1982, Keshet et al. 1985). Additionally, molecular studies presented in this thesis provide evidence for methylation control of TK gene expression in CHO cells. The availability of a series of Chinese Hamster TK genomic probes allowed further, more detailed analysis of the role of methylation control in the expression

of this gene (Lewis et al. 1983, Lewis 1986). Demethylation was detected in all of the TK^+ revertant clones irrespective of their origin, suggesting that a specific ~200bp area near the third exon of the gene confers regulation. Thus, at least one HpaII and two HhaI sites at the upstream end of the 4.2kb EcoRI fragment, were always demethylated in TK^+ clones, irrespective of their mode of generation from the TK^- parent. Interestingly, the CpG rich island found in the 5' promoter region did not show any detectable epigenetic changes associated with the phenotype. This may be due either to the screening procedure that would not detect DNA fragments smaller than 300bp, or it may be that methylation in this area does not influence expression. HpaII and HhaI restriction sites in the rest of the gene were completely methylated in all cells examined, suggesting that this is probably not involved in the regulation of TK expression.

It is suggested that the three methylatable sites associated with methylation control of TK gene expression, might cooperate with others whose detection borders on the sensitivity of the analysis, and form a cluster within the 4.2kb DNA fragment. Support for this suggestion arises from the observed fluctuation in the size of the HpaII band detected in TK^+ revertants. Additionally, it should be borne in mind that HpaII and HhaI can detect less than 16% of the methylatable CpG doublets in the genome. Thus, these other sites may be either too close to be clearly defined or may not be detectable by HpaII or HhaI.

The remethylation detected in all TK^- back-revertants could either be a requirement for TK suppression, or a passive process following suppression. The TK^+ reversion of all these secondary TK^- cells by 5-Aza-CR suggests, however, that the detected remethylation

is important for back-reversion. Although all the TK^{-} back-revertant clones showed remethylation within the suggested cluster in comparison with the parent strains, this was never 100%. It is proposed, therefore, that the relative level of methylation within the 200bp region determines the phenotype, rather than the absolute modification status of specific sites. Such a situation has been reported for the regulation of γ -globin gene expression (Murray and Grosveld 1985).

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

Attempts were also made to associate the observed phenotypic alterations with changes in transcriptional activity of the TK gene. TK homologous RNA was detected by Northern hybridisation in all cell lines. TK^{+} revertant cells showed a several fold 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 in reactivation, but also its induction 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. The first suggestion is unlikely since S1 analysis did not detect qualitative differences between transcripts in the TK^{-} and TK^{+} strains. It is therefore proposed that the TK mRNA

detected in TK^{-} cells is mutated, probably due to a small deletion, insertion, or base substitution which may terminate or otherwise interfere with translation, or give rise to an inactive or partially active protein. Since selection in BUdR depends on the cumulative effects of the drug in the presence of an active and competing de novo pathway, residual TK activity may not cause lethality. However, growth in selective medium may suppress expression of a partially active TK enzyme because of growth disadvantage. Indeed, S1 analysis showed lower levels of TK transcript in TK^{-} cells harvested from selective than from non-selective medium. The presence of TK transcripts in TK^{-} cells is a specific feature of the CHO TK^{-} MT^{-} strain, since all five additional TK^{-} clones isolated from CHO KI cells by X-ray mutagenesis showed no TK detectable specific transcripts. This may be related to the nature of mutagenesis that gave rise to the particular CHO TK^{-} MT^{-} strain, since EMS mainly induces small deletions or single base substitutions.

8.2. Methylation and MT expression:

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 an MTI cDNA probe could be used. However, extensive demethylation after 5-Aza-CR treatment must have occurred in these cells because the detectable HpaII site were found to be unmethylated, indicating that reversion to the MT^{+} phenotype may be associated with extensive demethylation of the MTI gene. 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 MTI cDNA probe used in the studies described in this thesis has 80% homology with the MTII gene and the detection of more than one DNA fragments by hybridisation methods would have been expected. (Griffith *et al.* 1983). The detection of only one DNA species, therefore using the MTI probe may reflect a clustering of the two MT genes within the same EcoRI fragment, a situation found in the mouse and human genome (Searle *et al.* 1984, Karin *et al.* 1984). Crawford *et al.* (1985) have suggested that the MTI and MTII genes are also clustered in the CHO genome in this way. An EcoRI site was detected, however, between the two gene sequences in the experiments described above. It is possible that the difference in the EcoRI pattern reported by Crawford *et al.* (1985), and that found here is a result of restriction site polymorphism.

Northern hybridisation revealed two RNA species in all cells, one of which is MT mRNA, and a larger molecule which may either be the product of a unidentified yet MT like gene, or an intermediate in the RNA processing of the MT transcript. The detection of MT transcripts even in MT⁻ (Cd^S) 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 expression levels of MT mRNA were also detected in MT⁻ HeLa cells (Heguy 1986). The MT⁺ (Cd^R) clones showed several fold increase in the levels of MT transcript, probably due to demethylation and activation of an inducible MTI gene. Increase of the MT transcript in mouse cells has been reported to occur immediately after treatment with demethylating agents, reaching a maximum some eight hours later

(Compere and Palmiter 1981). Transcriptional activity then drops to a steady state level which is still higher than that before treatment. In the present study MT transcript was analysed after selection of MT⁺ clones, and further growth in order to obtain a sufficient number of cells for RNA preparation. Therefore by analogy to the mouse system, increased steady state MT transcript levels measured in these MT⁺ clones may well be less than those found immediately after treatment.

8.3. De novo methylation in CHO cells:

5-Aza-CdR treated cells which had suffered 46% demethylation of their methylated cytosine bases showed no detectable remethylation for the first 10 cell generations (cgs) following treatment. Subsequently (by 50cgs), however, the fraction of cytosine bases which were methylated had significantly increased, demonstrating the presence of de novo methylation activity. Several previous reports have shown that remethylation occurs in cells treated with 5-Aza-CR or 5-Aza-CdR, but the efficiency of recovery varies between different systems (Flatau et al. 1984, 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 (Christy and Scangos 1982, Hardies et al. 1983, Clough et al. 1982). However, none of these studies followed the kinetics of remethylation for such extensive periods of time as in these studies. Interestingly, some ~150 cgs after the treatment, the DNA in the CHO cells not only reached normal methylation levels, but it gained up to 23% more methylation.

De novo methylation activity would be expected to result in the inactivation of expression of some genes subject to methylation

control. Such a mechanism may, in fact, explain the spontaneous generation of TK^- cells from TK^+ clones (originating from 5-Aza-CR and 5-Aza-CdR treatment of TK^- cells), at frequencies three orders of magnitude greater than the spontaneous mutation frequency (Roufa et al. 1973, Adair et al. 1980). That this is most probably a result of remethylation of TK gene sequences is shown by the fact that treatment of these secondary TK^- cells with demethylating agents again generates TK^+ cells at frequencies comparable to those found for the original parental TK^- cell line. Thus, demethylation appears to be sufficient to activate TK gene expression and methylation to inactivate it, providing evidence for the existence of de novo DNA methylation in CHO cell lines, and suggesting a mechanism to generate functional hemizygosity (Jeggo and Holliday 1986). Thus, DNA methylation may stably inactivate one of the two alleles of a diploid locus, resulting to a functionally haploid phenotype. Further methylation or genetic mutation of the second allele may generate a recessive phenotype. This is supported by recent reports that several spontaneously occurring recessive phenotypes observed in CHO cells, are revertible by demethylating agents (table 5 and 6), and by the findings presented in this thesis that methylation is responsible for the spontaneous MT^- , and the TK^- phenotype.

Finally, treatment with EMS may produce epigenetic alterations as well as mutations. Evidence for the epigenetic effect of EMS has so far been controversial, both demethylation (Jones and Taylor 1980), and hypermethylation (Farrance and Ivary 1985, Ivary and Morris 1982) being proposed. The studies presented in this thesis favour the second suggestion. If demethylation results in reactivation of genes under methylation control, it may be that the reverse process of

remethylation produced as a result of EMS treatment can inactivate gene expression.

8.4. Properties of the t_{sm} phenotype:

The t_{sm} phenotype can be aligned with a functionally haploid locus, or a dominant one. This is because it has been obtained at a frequency (1/7000) comparable to that for a haploid gene, such as the X-linked HPRT gene (1/1000 by identical mutagenesis). Alternatively, it may be that alteration in the level or the pattern of expression in one allele of a diploid gene, changes the regulation of DNA methylation. The heritability, stability and temperature sensitivity of the t_{sm} phenotype, as well as the frequency of isolation (comparable to that of a haploid locus) indicate that it is probably a result of genetic mutation.

Several observations define the nature of the t_{sm} mutation. Thus, induction at 39°C for at least one cell cycle results in reactivation of silent TK or MT gene expression. Longer treatment at this temperature did not increase the level of phenotypic changes. Reactivation of these genes, is associated with demethylation and the same methylation changes were detected by Southern hybridisation analyses for t_{sm} and 5-Aza-CR induced revertants. The high back-reversion frequency to TK⁻ of t_{sm} TK⁺ revertants, and the reactivation of these back-revertants to TK⁺ by 5-Aza-CR, suggest that TK⁻ back-reversion is probably due to remethylation. The survival of t_{sm} cells upon long term growth at 39°C may be due to their low level of demethylation, and indicates that the normal locus is not essential for growth. Also, the low spontaneous mutation rate

of tsm cells at 39°C, suggests that the tsm mutation does not result in hypermutability.

Although specific demethylation was detectable by the restriction- hybridisation methods in the TK or MT genes of tsm TK⁺ or MT⁺ clones, no overall reduction in the level of 5mC was measured by HPLC chromatography in these tsm cells. Surprisingly, a tsm TK⁺ revertant clone induced at the high temperature, showed 60% higher methylation than TK⁻ tsm cells when grown for ~150cgs after exposure at 39°C. These tsm TK⁺ cells show 4.5% 5mC content, which implies that over 90% of their CpG doublets are methylated, since CpG occurs in the mammalian genome 5 times less frequently than expected (Swartz *et al.* 1962, Russel *et al.* 1976). Hypermethylation was also detected, but to a lower extent, after long term growth of cells treated with demethylating agents.

TK homologous mRNA was detected by Northern blotting in tsm TK⁻ cells, although S1 nuclease analysis failed to reveal a protected fragment in this strain. Thus, a TK like mRNA species is present in tsm cells which may not be homologous enough to be detected by S1 mapping. The mitochondrial TK gene transcript might well be a candidate for this mRNA. The failure to detect TK transcript in tsm cells might conceivably be due to increased methylation in these cells in comparison with the parent CHO TK⁻ MT⁻ strain, leading to inactivation of the proposed mutated TK allele (see section 5.3.1). As expected, the tsm TK⁺ cells express TK mRNA, but this is surprisingly maintained in the spontaneously derived TK⁻ back-revertants. It could be suggested that reactivation of both alleles occurs upon treatment at 39°C followed by selection in HAT. BUdR tolerant tsm back-revertant cells may retain a detectable level

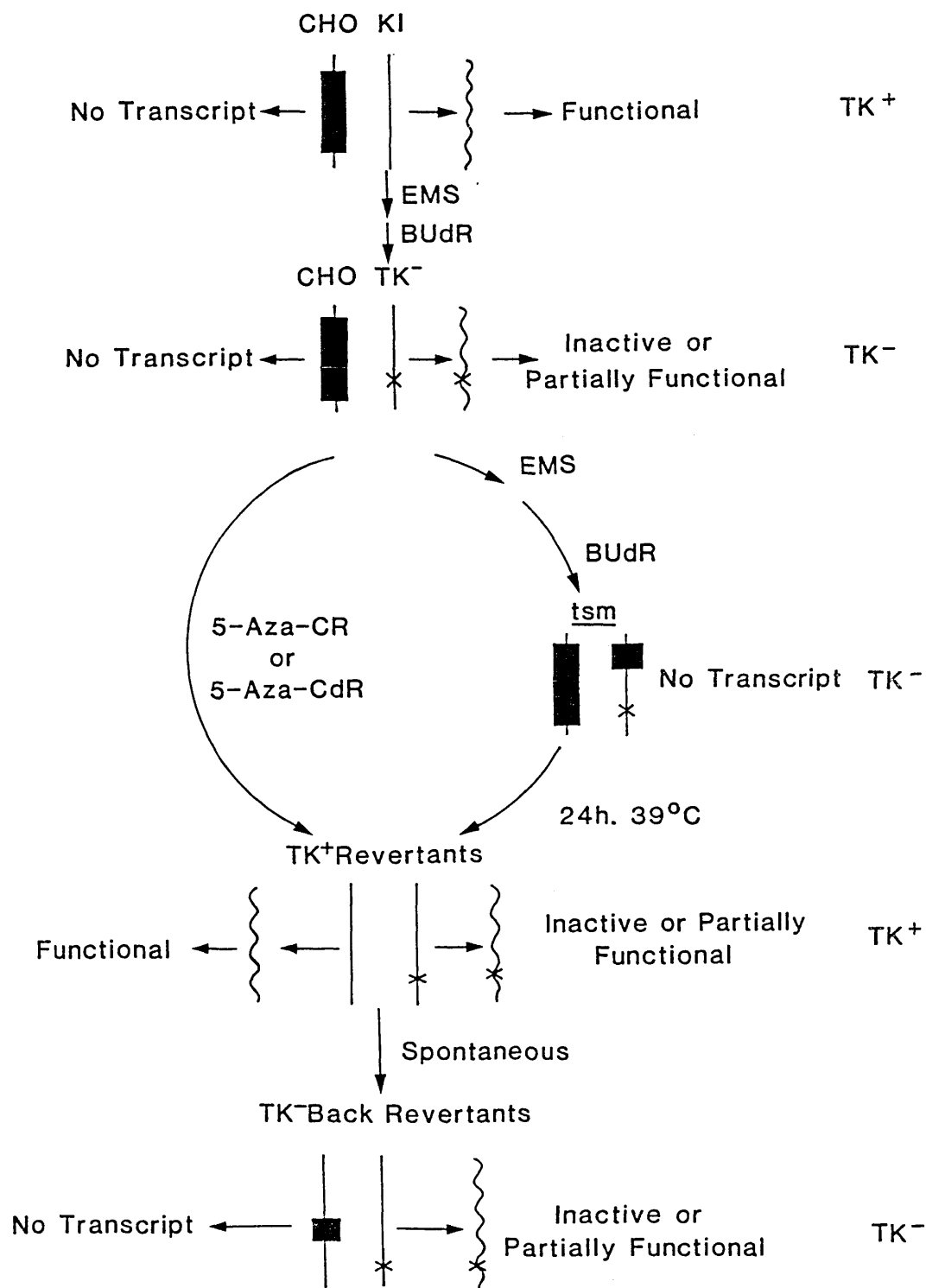
of a mutated partially active TK transcript.

8.5. Interpretations of the t_{sm} phenotype:

TK and MTI gene expression in CHO cells has been shown to be associated with changes in DNA methylation. Evidence was also obtained suggesting that de novo methylation activities in these cells may be responsible for the spontaneous inactivation of genes. These results suggest a model which is presented in figure 34. It is proposed that a fully methylated and a partially methylated copy of the TK gene are present in CHO KI cells as indicated by the detection of two bands homologous to the 4.2kb fragment upon HpaII/EcoRI digestion, while only one was revealed by cleavage with EcoRI. The frequency of isolation of TK⁻ cell from the CHO KI cells line suggests that only one copy of the TK gene is active in the CHO KI strain. It is proposed that the partially methylated TK copy is functional, whereas the completely methylated is inactive. EMS introduces mutation in the functional copy, which leads to the expression of an inactive or partially functional TK protein, while the second allele remains hypermethylated and inactive. EMS mutagenesis of CHO TK⁻ MT⁻ cells in order to select for the t_{sm} strain results in increased methylation, which may possibly inactivate the mutated TK copy. Treatment of CHO TK⁻ MT⁻ cells with 5-Aza-CR and 5-Aza-CdR demethylates the inactive TK allele resulting in reactivation to the TK⁺ phenotype. Treatment at 39°C introduces alterations in the methylation pattern of the t_{sm} cells leading to reactivation of the TK gene in about 0.01% of the cells. In both chemical or genetical demethylation, reactivation as well as increased expression of the TK gene is detected. Finally, the

Figure 34: The status of the TK gene in the CHO strains studied.

This flow diagram proposes a model on the status of the TK gene in the CHO-KI cell line, and its possible evolution through the studied strains that derived from these cells (discussed in section 8.6.).



tsm TK⁻ back-revertants show detectable TK transcript which suggests that treatment at 39⁰C and selection in HAT may have reactivated both the functional and the mutated copies of the TK gene. A discrepancy in this model could be the isolation of TK⁻ cells from the CHO KI strain that do not revert to TK⁺ upon treatment with demethylating agents (section 3.2.1.). However, the TK gene probably responds to multiple transcriptional regulation and it may be that the TK⁻ phenotype in some cases is due to regulatory mutations indirectly influencing TK gene expression. Alternatively, it is possible that one TK copy is lost from these cells while the other is inactive due to genetic mutation.

The phenotypic alterations and methylation changes in tsm cells suggest that the mutation affects DNA methylation. Two basic suggestions can explain the low level of demethylation detected after induction at 39⁰C: a) the mutation in tsm cells is in the maintenance methylase but the effect is not sufficient to introduce detectable demethylation; b) the mutation affects the regulation of DNA methylation.

Reduced general maintenance activity would result in general demethylation. Therefore the activation frequency of the TK and MT genes should be associated to the level of demethylation. However, demethylation in CHO cells is too low to explain the level of phenotypic alterations. It is more likely that tsm has a regulatory mutation which alters the sequence or conformation specificity of maintenance methylation. It could be proposed that maintenance DNA methylases which modify a specific sequence or a certain DNA conformation are present in the cells apart from the documented general methylase. Trans-acting factors might also exist that

coordinate sequence specific or conformation specific action of the general maintenance enzyme. Alternatively a regulatory mutation in tsm cells might alter the dynamic state between maintenance and de novo methylation, or a possible feed-back between de novo and maintenance activities.

It was suggested in section 8.5. that CHO cells may already be defective in de novo methylation. The tsm strain may be even more defective in the regulation of de novo methylation, as indicated by the high level of TK inactivation and the increased 5mC content of tsm cells ~150cgs after treatment at 39⁰C. Alternatively it might be that the temperature sensitive mutation in tsm cells which affects the maintenance methylation, directly or via trans-acting factors stimulates the de novo methylation pathway. The hypothesis that the tsm is a regulatory mutation is favoured, since the evidence suggests that both the maintenance and the de novo pathways are affected in the tsm strain. Induction of the de novo methylation pathway should be heritable, since it leads to a slow process of de novo methylation detected ~150cgs after treatment at 39⁰C, or with demethylating agents. DNA methylation is likely to play a role in long term regulation of gene expression, it is possible that inhibition of maintenance methylation triggers a pathway for demethylation and activation of de novo methylation.

8.6. Proposals for future investigations:

A number of interesting observations were made in the process of this study, and it would be valuable to examine them in more depth. Although the likely regulatory methylation area of the TK gene was narrowed down to ~200bp near the third exon of the gene, lack of sequencing information restricts further analysis on the number and organisation of the methylatable sites. Genomic DNA sequencing (Church and Gilbert 1984), could be applied for this specific area in TK⁻ and TK⁺ cells in order to specify the level of demethylation required for expression of the gene. The same approach should define whether methylation changes within the CpG rich island of the TK promoter are important for expression. The lack of genomic clones for the Chinese Hamster MT genes prevented detailed investigation on methylation control of MT expression. The availability of such clones may also allow the choice of probes that can distinguish between MTI and MTII mRNA and resolve the nature of transcript in MT⁻ cells. The surprising hypermethylation detected in TK⁺ revertant cell lines, upon long term growth after interruption of their DNA methylation patterns, led to the suggestion that there may be a feed-back interaction between maintenance and de novo methylation. This can be examined by measuring the kinetics of a de novo methylation process, or studying methylation activities in these cells biochemically.

It was shown in this thesis, that it is feasible to isolate temperature sensitive mutants affected in DNA methylation. The same screening 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 biological role and the regulation of DNA methylation. Thus, studies might be extended to a wide range of biological functions and overcome the restricted analysis of the reactivation of individual genes. Additionally, these investigations can be carried out without the use of inhibitors such as demethylating agents, or in vitro modified DNA molecules.

Finally, the screening procedure could be modified to allow the molecular cloning of genes involved with the methylation of DNA in CHO cells. While alterations in the expression of TK and MT genes can still be used as selectable markers, EMS could be replaced by an insertional mutagen which would permit identification of the mutagenised site. Insertional mutagenesis is a well known phenomenon in prokaryotes (Bukhari et al. 1977), yeast (Roeder and Fink 1980), *Drosophila* (Green 1980) and maize (McClintock 1956). In vertebrates retroviruses can act as insertion mutagens, interrupting genetic units by the integration of the provirus into the host DNA. Up to 50 copies of the retroviral genome have been shown to integrate per cell, which is a sufficient level to allow distinction between the spontaneous mutation in a haploid locus, and that induced by the retroviral insertion (Varmus et al. 1981, Frankel et al. 1985). Finally, the use of retroviruses as insertion mutagens in cultured mammalian cells is a particularly powerful tool, because they can be used as probes in the recovery of genes which are responsible for the mutant phenotype (King et al. 1985, Cepko et al. 1985).

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