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X chromosome Inactivation and the Pgk-1 Gene: Methylation and Mapping Studies Using Female Embryonal Carcinoma Cells

By

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ABSTRACT

In female mammalian cells all but one X chromosome is inactivated during embryogenesis in order to reduce the level of expression of X-linked genes to that in male cells. The inactive state is stable but reversible, and methylation of cytosines has been proposed to be involved. However, previous studies of the mouse phosphoglycerate kinase (pgk-1) gene in this lab (Adra,1988) did not reveal any evidence of this differential methylation in the promoter region on the inactive X.

Methylation of a cluster of sites for the restriction endonuclease AvaI in intron 1 of the pgk-1 gene was found to be correlated with inactivation in mouse cells and one female embryonal carcinoma cell line (C86) which has an inactive X.

A study of methylation of these AvaI sites in C86 cells whose X chromosomes had been "reactivated" did not show a clear correlation of methylation with inactivation. Of the two cell lines which showed dramatically elevated levels of pgk-1 expression following treatment of the parental cells with 5-azacytidine, one showed methylation of the intron 1 sites and one showed a clear absence of methylation.

Another female EC cell line (P10) which has two active X chromosomes did not show any methylation of these sites. P10 cells which had been differentiated to inactivate one X chromosome also showed no methylation of these sites. These results demonstrate that methylation does not play an important role in the initial controlling events of pgk-1 gene inactivation. That it may play a role in the maintenance of the inactive state remains a strong possibility.

The pgk-1 gene maps 5 map units from a region on the X chromosome which is thought to be important in inactivation, the X controlling element (Xce). The pgk-1a gene, which encodes an electrophoretic variant of the enzyme, is linked to a different Xce allele than that linked to the wild-type pgk-1b gene. Mapping the region immediately upstream of the pgk-1a and pgk-1b genes

revealed several polymorphisms extending to about 15 Kb 5' of the gene. Long-range mapping using field inversion gel electrophoresis around the gene allowed us to generate a map of 700 Kb of DNA surrounding the wild-type pgk-1^b gene.

RÉSUMÉ

Chez les cellules femelles de mammifères, tout qu'un seul chromosome X résiste à l'inactivation durant l'embryogénèse de façon à maintenir un niveau d'expression des gènes liés au chromosome X comparable à celui des cellules mâles. L'état d'inactivation est stable chez ces cellules mais réversible; il a été suggéré que la méthylation des cytosines serait impliquée dans le mécanisme d'inactivation. Toutefois, des études récentes portant sur le gène de la phosphoglycérate kinase (pgk-1) de souris (Adra, 1988) n'ont mis en évidence aucune méthylation différentielle dans la région du promoteur de ce gène chez l'allèle inactivé.

Il fut démontré que la méthylation d'un regroupement de sites de l'endonucléase de restriction AvaI dans l'intron 1 du gène pgk-1 est en corrélation avec l'inactivation chez les cellules de souris et chez les cellules femelles d'une lignée cellulaire de carcinome embryonnaire de souris (C86) (cette dernière lignée possède un chromosome X inactivé).

Une étude de l'état de méthylation de ces sites AvaI chez des cellules C86 dont les chromosomes X inactivés ont été réactivés n'a pas démontré de corrélation claire entre l'état de méthylation dt l'inactivation. Des deux lignées cellulaires ayant démontré une élévation marquée du niveau d'expression de pgk-1 après traitement des lignées parentales avec de la 5-azacytidine, une lignée démontrat une méthylation des sites AvaI de l'intron 1 alors que la seconde démontrat une absence totale de méthylation des mêmes sites.

Une autre lignée femelle EC (P10) qui dispose de deux chromosomes X actifs ne démontre pas de méthylation aux sites AvaI. Des cellules P10 induites à se différentier afin d'inactiver un chromosome X ne démontrent pas non plus de méthylation à ces sites AvaI. Ces résultats démontrent que la méthylation ne joue pas de rôle important dans la phase initiale d'inactivation du gène pgk-1. Il est toutefois très possible que ce mécanisme soit impliqué dans le maintien de l'état inactivé.

Le gène pgk-1 est localisé a 5 unités de carte d'une région du chromosome X connue sous le nom de Xce que l'on croit être impliqué dans le phénomène d'inactivation. Le gène pgk-1a qui, une fois tranduit, produit une enzyme au comportement électrophorétique différent est lié sur la carte à un allèle Xce différent de celui lié au gène de type-sauvage pgk-1b. La cartographie des régions proximales en amont des gènes pgk-1a et pgk-1b a révélé plusieurs polymorphismes s'étendant jusqu'à 15kb en amont des gènes. La technique de cartographie à longue distance utilisant l'électrophorèse sur gel où le champs électrique est périodiquement inversé à permis de générer une carte de 700kb de l'ADN environnant le gène type-sauvage pgk-1b.

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CHAPTER ONE

INTRODUCTION

1.1 Introduction

The regulation of gene expression during the development of an organism remains one of life's great mysteries. The mechanisms which turn a given gene on or off at the appropriate stage are still very poorly understood. The phenomenon of X chromosome inactivation in female mammalian cells, whereby almost all of the genes on an entire chromosome become genetically inert, would seem to be a good system for studying this regulation. This project represents an attempt to answer some of the questions posed by X inactivation, both by traditional approaches, and by a newer route.

1.2 X chromosome inactivation

X chromosome inactivation is thought to be a means of reducing the level of expression of X-linked genes in female cells to that found in males. Since female cells have two X chromosomes, compared to one in males, the amount of X-linked gene product could potentially be double that in males, a situation which might be lethal for the cell. However this problem is prevented by an inactivation event which silences almost all of the genes on one X chromosome.

Inactivation occurs in each female cell between 3.5 and 6.5 days of gestation in the mouse and is considered to be made up of an initiation event, a spreading of the inactivation signal, maintenance of the inactive state, and finally reactivation prior to meiosis.

1.2.1 Stages of X chromosome differentiation

X inactivation proceeds sequentially in different cells starting with the extra-embryonic lineages at about 3 days of gestation. The outside cells of the morula differentiate to form the trophectoderm in which the paternal X (derived from the sperm) is inactivated (Kratzer,1982). At about 4.5 days of gestation the primitive endoderm forms, and once again only the paternally derived X chromosome is inactivated (Takagi and Sasaki,1975). It is not until about 6.5 days of gestation, when the inner cell mass differentiates to form the primary germ layers, that X inactivation is completed. This last stage in the embryonic ectoderm cells consists of the random inactivation of one X chromosome in each cell (Monk and Harper,1979; Takagi et al.,1982). The presence of an inactive X has been verified at these stages by measuring enzyme activities (Monk and Harper,1978), and by cytogenetic detection of a late-replicating, heterochromatic X (Takagi,1974).

It is thought that the process of paternal X inactivation in extra-embryonic tissue (and embryonic tissue of marsupials) differs from the random inactivation in the eutherian embryo. When the paternal X is preferentially inactivated, it replicates earlier than the active X until about day 6 of gestation. After day 6 this inactive X replicates later than the active one. In embryonic lineages after random inactivation has occurred the inactive X always replicates later than the active X. In addition, the hprt gene from the inactive paternal X behaves differently in DNA mediated gene transfer experiments than the same gene from randomly inactivated chromosomes (Liskay and Evans,1980; Kratzer et al.,1983). These differences may be due to the effects of parental imprinting on the X chromosomes which they acquire during gametogenesis (Lyon and Rastan,1984). Most likely imprinting is not an essential part of X inactivation but rather

serves to alter the probability that a given X chromosome will inactivate (Lyon,1988).

Reactivation of the inactive X chromosome occurs in female germ cells prior to oogenesis. This reactivation event has been confirmed by cytogenetic studies, allozyme expression studies using Hprt heterozygotes, and gene dosage experiments (Gartler et al.,1975; Takagi,1974; McMahon and Monk,1983). It has been proposed by Monk (1988) that the reactivation event is a consequence of meiosis itself. She has equated reactivation with the "dedifferentiation" of the germ line accomplished by erasure of imprinting which results in developmental totipotency.

1.2.2 X controlling element and X inactivation center

It is thought that X inactivation in female cells originates from a single site on the X chromosome, called the X inactivation center (Xic). Experiments using X-autosome translocations in mice support the existence of this center. On these chromosomes only one of the two X chromosome segments shows inactivation characterized by late replication (Russell and Cacheiro,1978; Takagi,1980; Rastan,1983). It has also been observed that inactivation can spread into the autosome from only one of the X chromosome segments (Russell and Montgomery,1970). Studies using partial deletions of the X chromosome in female embryonal carcinoma cells have also supported the existence of a single site (Rastan and Robertson,1985). The Xic site appears to map near a locus on the X chromosome called the X controlling element (Xce) which has been genetically mapped close to Ta, a coat colour marker about 4-5 map units away from the pgk-1 gene in mice (Cattanach and Papworth,1981). The results using X-autosome translocations have not only helped define the location of this inactivation center, but also support the idea that X inactivation is an active process of transcriptional suppression.

There are three known alleles at the Xce locus which can alter the probability that an X chromosome will remain active. These alleles, Xce^a, Xce^b, and Xce^c, act in a cis dominant manner, with the "c" allele having the most extreme effect. However the probability varies only slightly, ranging from a random likelihood in homozygotes to a 70:30 likelihood in Xce^a/Xce^c heterozygotes (Cattanach et al.,1983).

1.3 DNA methylation

Methylation of the pyrimidine cytosine is the only known covalent DNA modification in mammals, and is present on 2-5% of all cytosines. Methylation occurs following DNA replication primarily on cytosines which exist in the dinucleotide CpG, which is symmetric on both strands of DNA (Bird,1978). Following replication, the hemimethylated DNA becomes fully methylated by a maintenance methylase (Bestor and Ingram,1983; Sano et al.,1983; Pfeifer et al.,1983; Razin and Szyf,1984). The methylation pattern on the DNA is therefore stably inherited (Wigler et al.,1981).

Even before the discovery of methylase activity, it was believed that methylation might be a mechanism for the regulation of genes or even of whole chromosomes. Riggs (1975) and Holiday and Pugh (1975) proposed that methylation could achieve this by affecting the binding of regulatory proteins on the DNA. Their models presumed that the interaction of these proteins with specific DNA sequences is responsible for gene regulation and X inactivation. A system of de novo and maintenance methylation would control these interactions by setting a pattern which dictated the extent of protein binding. The pattern could later be erased (by the absence of maintenance methylation during replication) in order to reactivate the X chromosome. Support for this model has grown in recent years as the relationship between gene activity and methylation has been examined (reviewed by

Monk,1986; Cedar,1988). However it is becoming evident that methylation of DNA must be at most a secondary mechanism in the regulation of gene expression.

1.3.1 DNA methylation and gene activity

The most convincing data which link methylation with the expression of individual genes result from gene transfer experiments. One example is the human γ-globin gene (Busslinger et al.,1983; Cedar,1988). This gene is normally heavily methylated and not expressed in fibroblasts. When an exogenous copy of the gene is methylated in vitro and then introduced into fibroblasts, it too is unexpressed. Yet unmethylated transfected copies of the gene are transcriptionally active in these experiments. Clearly methylation plays a role in the repression of this tissue specific gene. A recent paper, however, demonstrates that this methylation is probably a secondary control mechanism, which acts to maintain a gene in its inactive state, but is not involved in the initial inactivation event. Enver et al.(1988) examined the timing of methylation in globin gene switching to determine whether methylation is involved causally in γ-globin gene inactivation, or is a secondary event in this process. They found that cell hybrids which no longer express γ-globin still carry unmethylated copies of the gene. They concluded that the genes had been turned off by another mechanism, and that the elimination of methylation following this event had not yet occurred.

1.3.2 DNA methylation and X inactivation

Several classes of experiments have supported the view that methylation is involved in X inactivation. Transfection of cells with the hprt gene from the inactive X chromosome does not result in expression of the exogenous gene, whereas using a hprt gene

from the active chromosome does give expression (Liskay and Evans,1980). These results suggest that the DNA from the inactive X chromosome has been modified in some way which prevents its expression.

Although the above experiments cannot unequivocally point to DNA methylation as a cause of gene repression, in vivo studies of methylation patterns in these genes support this view. Methylation of certain CpG sequences associated with X-linked genes has been correlated with their inactivation state (Yen et al.,1984; Wolf et al.,1982; Lock et al.,1986; Toniolo et al.,1984; Wolf et al.,1984; Keith et al.,1986). In these experiments, certain CpG residues (usually in the 5' region of the gene which is rich in CpGs), were found which were hypermethylated on the inactive X chromosome. It was not until recently,however, that the timing of this methylation event was examined. Lock et al.(1987) found that methylation of the HPRT gene in EC cells and mouse embryos did not occur until several days after the inactivation of the X chromosome in these cells. This study supported the view, discussed in 1.3.1, that methylation is a secondary mechanism of control which probably serves to maintain genes in a repressed state.

An additional set of experiments which support the view that methylation is involved in gene repression involve the use of 5-azacytidine, a DNA demethylating agent, to reactivate genes. Using human/mouse hybrid cells, several housekeeping genes from the inactive (human) X chromosome were reactivated following treatment with this drug (reviewed in Monk,1986). In addition, DNA from a reactivated HPRT gene is functional in the DNA mediated transformation experiments described above (Venolia et al.,1982). For the most part, however, reactivation in the above cases did not involve the whole X chromosome, and reactivants show variable expression of the gene product. These 5-azacytidine experiments have drawn some criticism since this drug has been shown to induce activation of genes even in organisms which do not contain 5-methylcytosine (Tamame et al.,1983). More recent experiments have

shown that the changes in gene expression which follows treatment with 5-azacytidine might be mediated by the activation of a few regulatory loci (Davis et al.,1987).

Differences between the preferential inactivation of the paternal X chromosome and random inactivation are evident in some of these methylation studies. The GC-rich islands of the X-linked genes in marsupials (in which the paternal X is preferentially inactivated) do not show any correlation between methylation and inactivation (Kaslow and Migeon,1987). In addition the hprt gene from the preferentially inactivated paternal X chromosome from yolk sac endoderm functions as well as the active gene in transfection experiments (Kratzer et al.,1983). The inactive hprt gene from sperm DNA also functions in these experiments (Venolia et al.,1984). Subsequent studies comparing the methylation patterns of sperm and oocyte DNA revealed that MIF (mouse interspersed fragment) sequences are more methylated in sperm DNA (Sanford et al.,1984).

1.3.3 Effects of methylation on protein binding

Recent studies of the effects of DNA methylation on protein binding suggest that the role of methylation in gene regulation might be more complicated than that proposed in 1975. It was found by Keshet et al.(1986) that the unmethylated DNA in chromatin is sensitive to DNAse I digestion, but fully methylated DNA adopts a configuration which is resistant to DNAse I. Since local DNA structure is likely to affect protein binding, this was seen as evidence that methylation was interfering with protein binding. Two other groups have found that methylation inhibits the binding of regulatory proteins to the liver specific gene tyrosine aminotransferase (Becker et al.,1987) and the adenovirus major late promoter (Watt and Molloy,1988).

In contrast to these results however, the Sp1 transcription factor appears to be completely insensitive to methylation of the

CpGs in its binding site on synthetic oligonucleotides (Holler et al.,1988). In fact this group proposes that Sp1 binding sites, which are usually present in methylation-free CpG islands, are maintained in an unmethylated state because Sp1 binds to them constitutively. Bird (1986) proposed that CpG islands are maintained methylation-free by the binding of specific proteins, and this sort of model is in agreement with the findings, described in 1.3.1, that methylation does not occur until after transcription has ceased. Another factor which binds the CpG island in the human pgk-1 gene is also unaffected by methylation of CpGs in its binding site (Yang et al.,1988).

The idea that some proteins must be able to function despite methylation of their binding sites is also evident from experiments which have shown that myoblasts can express a methylated skeletal α-actin gene which fibroblasts cannot (Yisraeli et al.,1986). The myoblasts specifically demethylate crucial sites in this gene. Although it is not known whether this demethylation occurs before or after transcription, some regulatory factors must be binding to the actin gene while it is still methylated.

The subsequent inhibition of other factors by methylation may reflect a second order of control. Experiments with the thymidine kinase (TK) gene (Buschhausen et al.,1987) found that repression of the methylated DNA is not immediate, but that this DNA becomes repressed more rapidly than unmethylated DNA, possibly due to the secondary chromatin structure it adopts. Methylation may be a mechanism for maintaining a gene's inactivity by altering the local chromatin configuration to prevent binding of subsequent transcription factors. It would be efficient for a cell to have a marking mechanism which also interferes with binding, probably by configurational changes, to distinguish genes which are transcriptionally active from those which are not.

1.4 Embryonal carcinoma cells

Embryonal carcinoma (EC) cells are the stem cells of malignant teratocarcinomas. EC cells resemble the cells of the early embryo; they are developmentally pluripotential cells and can be maintained in culture indefinitely without loss of potential as long as they are kept in exponential growth. They can be induced in vitro to differentiate into cell types normally seen in the embryo either by manipulating culture conditions or by exposure to chemical inducers (reviewed by Rudnicki and McBurney, 1987).

Embryonal carcinoma cells represent an ideal system for the study of X inactivation since they resemble embryonic cells at the time of X chromosome differentiation. The asynchrony of X inactivation and the small size of the embryo at these stages makes the study of this process in embryos difficult. Some female EC cell lines carry two fully active X chromosomes, and upon differentiation of these cells, one X inactivates (Martin et al., 1978; McBurney and Strutt, 1980). Other EC cell lines seem to represent embryonal cells which have been frozen at different stages of X chromosome differentiation, as determined by the presence of a late-replicating X and reactivatability with 5-azacytidine.

C86S1A1 is a mutant cell line which lacks activity of the X-linked hprt gene. These cells contain an active and an inactive, late-replicating X chromosome. Upon treatment with 5-azacytidine, the inactive X is at least partially reactivated, as measured by the increased levels of HPRT, PGK-1, G6PD, and α-GAL (Paterno et al., 1985).

The P10 cell line is a female embryonal cell line which has two active X chromosomes and is heterozygous for electrophoretic variants of pgk-1. The presence of two active X chromosomes in

these cells was confirmed by the presence of both PGK-1 isoenzymes and by the presence of two synchronously replicating X chromosomes (McBurney and Strutt,1980). Differentiation of P10 cells in retinoic acid results in rapid and synchronous inactivation of an X chromosome (McBurney and Strutt,1980; Paterno and McBurney,1985). Although these cells resembled extraembryonic endoderm following differentiation, this inactivation appeared to be random, rather than preferentially paternal as would normally occur in this cell type.

1.5 Pulsed field gel electrophoresis

There has been enormous interest lately in new techniques which allow molecular biologists to investigate long tracts of DNA. Until recently a gap existed between classical genetic linkage analyses and the physical mapping of small regions of DNA by cloning and mapping techniques. Schwartz and Cantor (1984) developed a technique, modified by Carle and Olson (1984), which allowed resolution of extremely large fragments of DNA (up to 2Mb) by electrophoresis in a periodically alternating electric field. Modifications to this technique have resulted in pulsed field gel electrophoresis systems with almost every imaginable electrode configuration. The original set-up made use of perpendicular fields, but since then orthogonal fields (Carle and Olson,1984), field inversion (Carle et al.,1986), and clamped homogeneous fields (Chu et al,1986) have been used. The impetus for these changes was that the lanes in the original orthogonal fields were badly distorted. Now electrophoresis with straight lanes is possible and DNA up to about 5-10Mb can be resolved.

The exact reasons for the separation of large molecules in an alternating field are still not clear. In ordinary electrophoresis DNA fragments up to about 30 Kb are effectively "sieved" by the gel matrix. DNA molecules larger than this, however, exhibit nearly size-independent mobilities. There is now general agreement that DNA molecules which are larger than the

pores in the gel matrix move through the pores with a one-dimensional, end to end motion called "reptation" (Lerman and Frisch, 1982). When this happens all DNA molecules move with the same net velocity because the gel is no longer acting as a sieve. PFG can effectively restore size dependent migration by forcing the DNA molecules to reorient themselves in the direction of the new field. The time required for relaxation from the initial field and reorientation in the new field is highly sensitive to molecular weight. The pulse times required to separate molecules of a given size are usually determined empirically. However a computer model to predict these has recently been developed (Lalonde et al.,1987).

The field inversion apparatus of Carle et al.(1986) uses a conventional horizontal gel box connected to a field inverter and is relatively easy to set up. The experiments described here made use of this system, which yields an almost linear separation of marker DNA in straight lanes. Genomic DNA used in these experiments must be prepared in such a way that it is left essentially intact prior to digestion with rare-cutting restriction enzymes. Most methods involve embedding the cells in low melting temperature agarose, either blocks (Schwartz and Cantor,1984) or beads (Heiter et al.,1985). Extraction of the DNA and restriction enzyme digests are then carried out in the agarose.

The enzymes used in these experiments cut the DNA infrequently, either because they recognize an 8 bp sequence, or because they have one or more CpGs in their recognition sequence. The dinucleotide CpG is under-represented in the mammalian genome, and usually exists in a methylated state which is resistant to cleavage. The exception is in "CpG islands", G+C rich regions which are maintained in an unmethylated state, and are often associated with genes. Brown and Bird (1986) have made use of these islands to map potential gene sequences over 1500 Kb of DNA. They used the CpG rare-cutting enzymes, and have calculated that

the sites for two or more of these enzymes usually exist in CpG islands.

Using PFG techniques, physical maps of the human muscular dystrophy gene (Van Ommen et al.,1986; Burmeister and Lehrach,1986; Kenwrick et al.,1987), the human pseudoautosomal region (Brown,1988; Petit et al.,1988), the human major histocompatability complex (Dunham et al.,1987; Lawrance et al.,1987), and the murine T-cell receptor gene family (Woolf et al.,1988) have been generated. In addition, these techniques make possible electrophoretic karyotyping of lower organisms such as yeast (Schwartz and Cantor,1984; Carle and Olson,1985), trypanosomes (Van der Ploeg et al.,1984), and parasitic protozoans (Giannini et al.,1986). In order to further expand the limits of PFG, techniques for obtaining clones from ever larger fragments of DNA have been developed. These include the generation of jumping libraries by circularizing large fragments to allow production of clones as far away as 50-2000Kb from an initial probe (Collins and Weissman,1984), and the cloning of fragments several hundred kilobases long as artificial chromosomes in yeast (Burke et al.,1987). These techniques clearly represent valuable tools in the efforts to further understand the mammalian genome.

1.6 Thesis project

In this thesis the molecular basis of X chromosome inactivation was studied using two different approaches: a study of methylation patterns in the pgk-1 gene and the physical mapping of the pgk-1 region. The pgk-1 gene is the closest cloned gene to the X controlling element (Xce) locus, which is thought to play an important role in the initiation of inactivation. The availability of the cloned mouse pgk-1 gene and several female embryonal carcinoma cell lines, which might be at different stages

of X inactivation, represented a unique opportunity to study the inactivation event.

Previous work in our laboratory examined the role of methylation in the promoter region of the pgk-1 gene. In this study, methylation of sites in the body of the gene was correlated with X chromosome activity. Following the location of important sites of methylation in mice, embryonal carcinoma cells were used to determine the importance of methylation in the early stages of X inactivation.

Methylation sensitive restriction enzymes were used to compare DNA from tissues of adult male and female mice, and from EC cells before and after differentiation and reactivation of the X chromosome. A cluster of sites in the first intron of the gene was found to be differentially methylated on the active and inactive X chromosomes in mice and a female embryonal carcinoma cell line. One reactivated clone showed demethylation of these sites. Another EC cell line which contains two active X chromosomes did not show any methylation, even after differentiation in retinoic acid, which was accompanied by X inactivation.

Because of its proximity to the Xce locus, rearrangements around the pgk-1 gene are of potential significance to the study of X inactivation. In the course of mapping the pgk-1a and pgk-1b alleles, several restriction fragment length polymorphisms were detected, in a region of about 15 Kb of DNA upstream of the gene. A 700 Kb restriction map of the pgk-1b gene in male embryonal carcinoma cells was generated by pulsed field gel electrophoresis. This map will hopefully provide a footing in this region of the X chromosome, from which the Xce locus might be approached.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Cell lines and cell culture conditions

The cell culture methods which were used are described in Rudnicki and McBurney (1987). All cell types, unless otherwise stated, were cultured in alpha minimal essential medium (Gibco, Mississauga, Canada) which contained 7.5% calf serum and 2.5% fetal calf serum (Bocknek Laboratories Inc., Rexdale, Ontario, Canada). The cells were maintained in plastic tissue culture dishes, which were placed at 37° C in a 5% CO_2 incubator. They were subcultured every 48 hr or less by first washing the monolayer in phosphate buffered saline (PBS) [0.8% NaCl, 0.02% KCl, 0.02% KH_2PO_4 and 0.115% $NaHPO_4$], and then incubating for 5 min in T-EDTA [1mM EDTA and 0.025% trypsin in PBS]. The cells detached from the dishes and were dispersed by vigorous pipetting with a pasteur pipette. They were then counted and replated at a concentration of 10^5 cells per ml into fresh medium.

The cell lines used are listed in Table 2.1, below. They are all derived from teratocarcinomas produced from transplantation of an embryo into an extra-uterine site in C3H/He mice.

Table 2.1. X chromosome characteristics of some EC cell lines.

EC Cell Line	Number of Active X Chromosomes	Late Replicating X Chromosomes
P19 (XY)	1	0
P10 (XO)	1	0
P10 (XX)	2	0
C86S1A1 (XO)	1	0
C86S1A1 (XX)	1	1

2.2 DNA Preparations

2.2.1 Genomic DNA preparation in solution

Genomic DNA was extracted from embryonal carcinoma cells by a modification of the procedure by Blin and Stafford (1976). Five confluent dishes of cells were rinsed in PBS and then lysed in 5 ml per 100 mm dish of Tris-buffered saline solution containing 0.5% SDS, 10 mM NaCl, 10 mM EDTA and 10 Mm Tris-Cl (pH 8.0) containing 100 ug/ml proteinase K. The cells were scraped from the dishes and incubated at 37° C for 2 hr with gentle shaking. The cell suspension was extracted with phenol/chloroform (1:1), treated with 50 ug/ml RNAse and incubated overnight at 37° C. The lysate was extracted again with phenol/chloroform and the DNA was precipitated in 2 volumes of 95% ethanol containing 2% potassium acetate. The DNA was spooled out with a pasteur pipette and re-dissolved in 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA.

DNA was extracted from mouse organs by placing the freshly dissected organs in ice-cold PBS, breaking the tissue up using a rubber spatula, and filtering to produce a single cell suspension. These cells were washed several times in PBS and then treated with the method described above.

2.2.2 Genomic DNA preparation in agarose

High molecular weight genomic DNA for use in pulsed gel electrophoresis was prepared by either of the following techniques. Mouse spleen DNA was prepared in agarose blocks essentially by the method described by Van Ommen and Verkerk (1986). The spleenocytes were washed several times in PBS. The cells were then resuspended at a concentration of 2×10^7 cells per ml of prewarmed PBS. They were then mixed with an equal volume of a 1% solution of LMT agarose (SeaPlaque lot 12276, FMC, Rockland, Me.,USA) at 45°C. This mixture was immediately

dispensed into the 10 x 6 x 1.5 mm slots of a mold which was covered on one side by tape, and placed on ice for 5-10 min. The blocks were then gently pushed out of the slots with a blunted pasteur pipette, into 5 volumes of 0.5 M EDTA (pH 8.0), 1% Sarkosyl. Proteinase K was added to 0.5 mg/ml and the blocks were incubated overnight at 50°C with gentle shaking. The blocks were then rinsed twice in 10 volumes of TE [10 mM Tris-HCl, 1 mM EDTA (pH 7.5)] with 0.1 mM phenylmethylsulphonyl fluoride (PMSF) at 50°C, and then twice more in TE at room temperature. The blocks were stored in 0.5 TE at 4°C.

The agarose bead method described by Overhauser and Radic (1987) was used to prepare high molecular weight DNA from embryonal carcinoma cells, as the block method described above failed to give good results with these cells. The cells were washed in PBS, trypsinized, and washed several times again in PBS before resuspending at a concentration of 1 x 10^8 cells in 5 ml PBS. This cell suspension was prewarmed to 45°C and added to an equal volume of 1% LMT agarose. Twenty ml of prewarmed mineral oil was added to this mixture, and then swirled vigorously for 30 sec to form a uniform emulsion. The emulsion was then poured immediately into a beaker containing 100 ml ice-cold PBS on a stir-plate set at medium speed. Beads of agarose of less than 1mm in diameter were formed. The total mixture was centrifuged for 10 min at 400-500 x g to pellet the beads. After several centrifuging steps the mineral oil and excess PBS were completely removed and the beads were resuspended in 20 ml of 1% SDS, 0.5 M EDTA (pH8.0). The bead suspension was rocked at room temperature for 10 min, re-centrifuged, and beads suspended in 20 ml 1% sarkosyl, 0.5 M EDTA (pH 8.0), and 50 ug/ml proteinase K. The beads were dispersed and incubated overnight at 50°C with gentle rocking. After proteinase K digestion, the beads were pelleted and resuspended in 20 ml TE containing 0.1 mM PMSF. After two more washes in TE, the beads were stored at 4°C.

2.2.3 Plasmid DNA Preparation

Small scale preparations of plasmid DNA were prepared by a modification of the method described by Birnboim and Doly (1979). Five ml of medium containing the appropriate antibiotic were innoculated with a single bacterial colony and then incubated overnight at 37°C with vigorous shaking. Bacteria from 1.5 ml of the overnight culture were centrifuged in an eppendorf centrifuge. The pellet was resuspended in 100 ul of an ice-cold solution of lysis buffer containing 50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl (pH 8.0) and 5 mg/ml lysozyme and incubated at room temperature for 5 min. The lysate was then mixed gently with 200 ul of denaturation solution containing 0.2 M NaOH and 1% SDS. After 5 min, a high salt solution containing 3 M potassium acetate and 11.5% glacial acetic acid was added to neutralize the bacterial lysate. After centrifuging the lysate for 5 min, the supernatant was removed and extracted with an equal colume of phenol/chloroform (1:1). Two volumes of 95% ethanol containing 2% potassium acetate were added and mixed by vortexing. The precipitated DNA was recovered by 5 min of centrifugation and resuspended in 50 ul of TE containing 10 ug/ml RNAse. These plasmid DNA preparations were stored at -20°C.

2.2.4 Isolation of restriction fragments of plasmid DNA

Restriction fragments of DNA were isolated by electrophoresis into troughs cut into the agarose gels (Maniatis,1982). The diluted DNA fragments were recovered and concentrated by passage through a NACS column (BRL, Gaithersburg, MD, USA).

2.3 Preparation of high molecular weight DNA markers

High molecular weight markers for use in pulsed field gel electrophoresis were prepared both from concatemers of lambda DNA molecules and from yeast chromosomes.

The lambda concatemers were prepared as described by Smith et al. (1986). Lambda phage particles, whose recombinant genome contained an 11.5 Kb insert derived from a pgk-1 pseudogene in the vector EMBL3 (Karn et al.,1983; Frischauf et al., 1983), were provided by C.Adra. A 400 ml overnight culture of phage was prepared as described by Maniatis et al. (1982). This culture was treated with 1 ug/ml of DNAse and RNAse for 30 min at room temperature, and then centrifuged at 11,000 x g for 10 min at 4°C to remove the cell debris. A solution of 10% polyethylene glycol and 1 M NaCl was added to the supernatant and mixed in by slow stirring. After 1 1/2 hr the phage particles formed a precipitate and were recovered by recentrifuging. The precipitate was suspended in 3 ml of 0.5% LMT agarose (Ultrapure, BRL, Gaithersburg, MD, USA) and poured into the slots of a mold. The blocks were left to set, and then were proteinase K treated as described for the blocks of mammalian genomic DNA in 2.2.2. After rinsing the blocks in TE several times (without PMSF), they were incubated for 48 hrs in 0.1 M EDTA (pH 8.0) at 50°C to allow concatemers to form.

Yeast chromosome markers were prepared by V. Seligy at the National Research Council, Ottawa, Canada. Haploid yeast (Saccharomyces cerevisiae strain C627-4B) were grown overnight to an O.D. of 1.0, centrifuged, and then resuspended in 4 volumes of SE [75 mM NaCl, 25 mM NaEDTA (pH 7.4)]. The cells were then mixed with an equal volume of 1% LMT agarose (BRL, Gaithersburg, MD, USA) made with SE. As the mixture was cooling, 20 ug/ul of Zymolase 60,000 and 20 mM DTT was added. This was then poured into the slots of the mold and chilled. The blocks were gently pushed out of the slots into the SE-DTT-Zymolase solution and incubated at 37°C for 1 1/2 hr. The blocks were then transferred

to 20 ml of 450 mM EDTA, 10 mM Tris (pH 9.0), 7.5% B-mercaptoethanol and incubated at 37°C for 4 hr with 3 changes. At the last change, 1% Sarkosyl and 1 mg/ml proteinase K were added and the blocks were incubated overnight at 50°C. The blocks were rinsed twice with 0.5 M EDTA (pH 9.0) and stored at 4°C.

2.4 Restriction endonuclease digestion of DNA

DNA in solution was digested with restriction endonucleases under the conditions recommended by the manufacturers. Plasmid DNA was mixed with 2 units/ug of enzyme in the appropriate digestion buffer and incubated at the optimum temperature for at least 2 hr. Eukaryotic genomic DNA was usually digested with 5-10 units of enzyme/ug of DNA for at least 6 hr.

To digest DNA in agarose blocks, the blocks were usually cut in half and washed several times with the appropriate digestion buffer and then equilibrated with the buffer for 1-2 hr. This buffer was then decanted and fresh buffer containing 20-100 units of endonuclease was added for each half block (5-10 ug of DNA). The digests were usually left for 6 hr to overnight. The agarose beads were also treated in this manner, using 100-200 ul of the bead suspension and centrifuging the beads in a microcentrifuge in order to decant the changes of buffer.

2.5 Agarose gel electrophoresis

2.5.1 Agarose gel electrophoresis

Double stranded DNA fragments up to 25 Kb were size fractionated by electrophoresis through horizontal agarose slab gels with a uniform electric field. Depending on the size range to be resolved, 0.8 - 1.2% agarose gels, made up with electrophoresis buffer were used. The electrophoresis buffer contained 0.08 M Tris-phosphate (pH 7.8), 0.002 M EDTA and 0.5 ug/ml ethidium bromide. Samples were loaded with 1/10 volume of

loading buffer containing 0.1% bromophenol blue, 0.1% xylene cyanol and 30% glycerol and run at about 8 V/cm for 2 hr to overnight.

2.5.2 Field inversion gel electrophoresis

To separate double stranded DNA fragments greater than 25 Kb field inversion gel electrophoresis (FIGE), described by Carle et al. (1986), was used. High molecular weight DNA which was prepared in agarose, as described in 2.2.2, was digested by restriction endonucleases which cleave infrequently, either due to an octomeric recognition sequence or to the presence of one or more C_pG dinucleotides in their recognition site. Following digestion with one or more of these enzymes, the DNA samples were melted with loading buffer at 65°C for 10 min and then loaded into sample wells using a cut off pipette tip. Marker DNA samples were loaded directly into the wells and then sealed with 5 ul of 1% LMT agarose. The gels used were 0.8 - 1% horizontal agarose gels which were made up in 0.5 x TBE [45 mM Tris, 45 mM boric acid, 0.5 mM EDTA, pH 8.3]. The gels were run in an ordinary horizontal electrophoresis tank with the 0.5 x TBE circulated, connected to a field inverter (PPI-100, MJ Research, Inc., Cambridge, MA, USA). Electrophoresis was for 8-48 hr, depending on the size range to be separated, at 150 V with a buffer temperature of 10-15°C.

2.6 Southern transfer

Prior to transfer of DNA fragments greater than 20 Kb, the DNA was either depurinated by treating twice for 15 min in 0.25 M HCl or by U.V. irradiating the gel for 7 min with 254 nm light. For fragments less than 20 Kb this step was ommitted. The gel was then immersed in several volumes of 1.5 M NaCl and 0.5 M NaOH for 1 hr at room temperature to denature the DNA. Neutralization of the gel was carried out in several volumes of 1 M Tris-Cl (pH 8.0) and 1.5 M NaCl for 30 min at room temperature. The gel was placed

over Whatman 3MM paper which had been prewet and was in contact with a resevoir containing 10 x SSC. Hybond-N nylon membrane was placed over the gel and then covered with 3 sheets of 3MM paper which had been wet with distilled water, and a layer of paper towels 10 cm thick. A 0.5 kg weight was placed on top of the towels and the DNA was left to transfer overnight. Following transfer, the nylon membrane was air dried and then exposed to 254 nm U.V. light for 1-5 min.

2.7 Southern hybridization

Prior to hybridization, nylon membranes were prehybridized for 1 hr to overnight in 5 x SSC, 5 x Denhardt's solution, 50% formamide, 0.3% SDS and 100 ug/ml denatured salmon sperm DNA at 42°C. The radiolabelled probe was then added to the above solution and the hybridization was carried out for at least 8 hr at 42°C. Following hybridization, the membranes were washed in several changes of 2 x SSC, 0.1% SDS at room temperature with vigorous shaking, and then twice for 15 min in 0.2 x SSC, 0.1% SDS at 65°C with shaking. The membrane was then sealed in plastic and placed on Kodak XAR-5 film with 2 Lanex regular intensifying screens and left for 1 to 10 days at -70°C.

2.8 Radioactive labelling of DNA

Plasmid DNA fragments were labelled for use as hybridization probes by the method of Feinberg and Vogelstein (1983). In a total volume of 50 ul, 25 ng of denatured, linearized plasmid DNA was incubated with 50 mM Tris-Cl (pH 7.8), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 20 uM each of dATP, dGTP and dTTP, 50 uCi of [alpha-^{32}P]dCTP (>3000 Ci/mM), random hexanucleotides and 2 units of the Klenow fragment of DNA polymerase I. The reaction was allowed to proceed for 3 hr at room temperature, and then monitored by TCA precipitation. Specific activities of over 10^9 cpm were obtained, and the labelled DNA fragments were used directly, without removal of unincorporated nucleotides.

CHAPTER THREE

ANALYSIS OF DNA METHYLATION AND X-INACTIVATION IN THE PGK-1 GENE

3.1 INTRODUCTION

Methylation of the cytosine in the dinucleotide CpG is the only known post-replicational modification of eukaryotic DNA. Although it remains to be discovered whether methylation in fact has an effect on gene expression through transcriptional regulation, it is known to stably alter the local structure of DNA, has been correlated with gene inactivity, and in the case of X chromosome inactivation could provide a mechanism for the stable inheritance of the inactive X.

A number of studies done on other X-linked housekeeping genes, including the human pgk-1 gene, seem to indicate that there is a correlation between the methylation of GC clusters in and around the genes, usually in the G+C rich promoter region, and the inactivation state of the gene (Wolf et al.,1984; Yen et al.,1984; Keith et al.,1986; Lock et al,1986; Toniolo et al.,1988). However, in contrast to the results obtained from the human pgk-1 (Keith et al.,1986), previous data from this laboratory suggested that there is no such methylation pattern in the promoter region of the mouse pgk-1 gene (Adra,1988). This result is interesting since conservation of the methylation pattern in mice and humans in the Hprt gene has been cited as evidence of its importance (Lock et al., 1986). However, in order to exclude the possibility that methylation is playing a role in the inactivation of the mouse pgk-1, it is necessary to examine other regions of the gene, as well as sites which are on the outskirts of the gene. In both the mouse and human hprt genes, the sites which show methylation on the inactive chromosome are in intron 1 (Wolf et al.,1984; Lock et al.,1984). And in the human G6PD gene, the sites which were differentially methylated on the inactive X were in GC clusters in the 3' region of the gene and in a region 100 Kb away

(Toniolo,1984). If the mouse pgk-1 gene shows differential methylation in another region than that found in the human pgk-1 gene, it is of potential significance both in defining the function of DNA methylation, and in adding to knowledge of the evolutionary changes between these two species.

It is also of interest to study the methylation patterns in the pgk-1a gene which is linked to the most extreme allele of the X chromosome controlling element, Xce^c. The pgk-1a is therefore more likely to remain active than its wildtype counterpart. It is possible that the region in and around this pgk-1 allele reflects the difference in Xce alleles, either by having altered GC clusters which serve as specific sites for methylation and/or DNA binding, or other structural differences. The opportunity to study the inactivation differences of the two alleles presents itself in the P10 embryonal carcinoma cell line which is heterozygous for the pgk-1 alleles. These female cells have two active X chromosomes but when they are induced to differentiate in culture, one of these X chromosomes is inactivated. It is possible that since the pgk-1a is more likely to stay active, it will show less methylation of important sites.

In order to study methylation differences which might be related to X chromosome inactivation, DNA from male and female mice was digested with methylation sensitive restriction enzymes and compared. Once sites which showed differential methylation on the active and inactive chromosomes were found, they were studied in embryonal carcinoma cells which had X chromosomes with different activation characteristics. Using a female EC cell line which had an active X which could be inactivated, and another cell line whose inactive X could be reactivated, the causal relationship between methylation and inactivation was investigated. Cells carrying the pgk-1a allele were also used in these experiments.

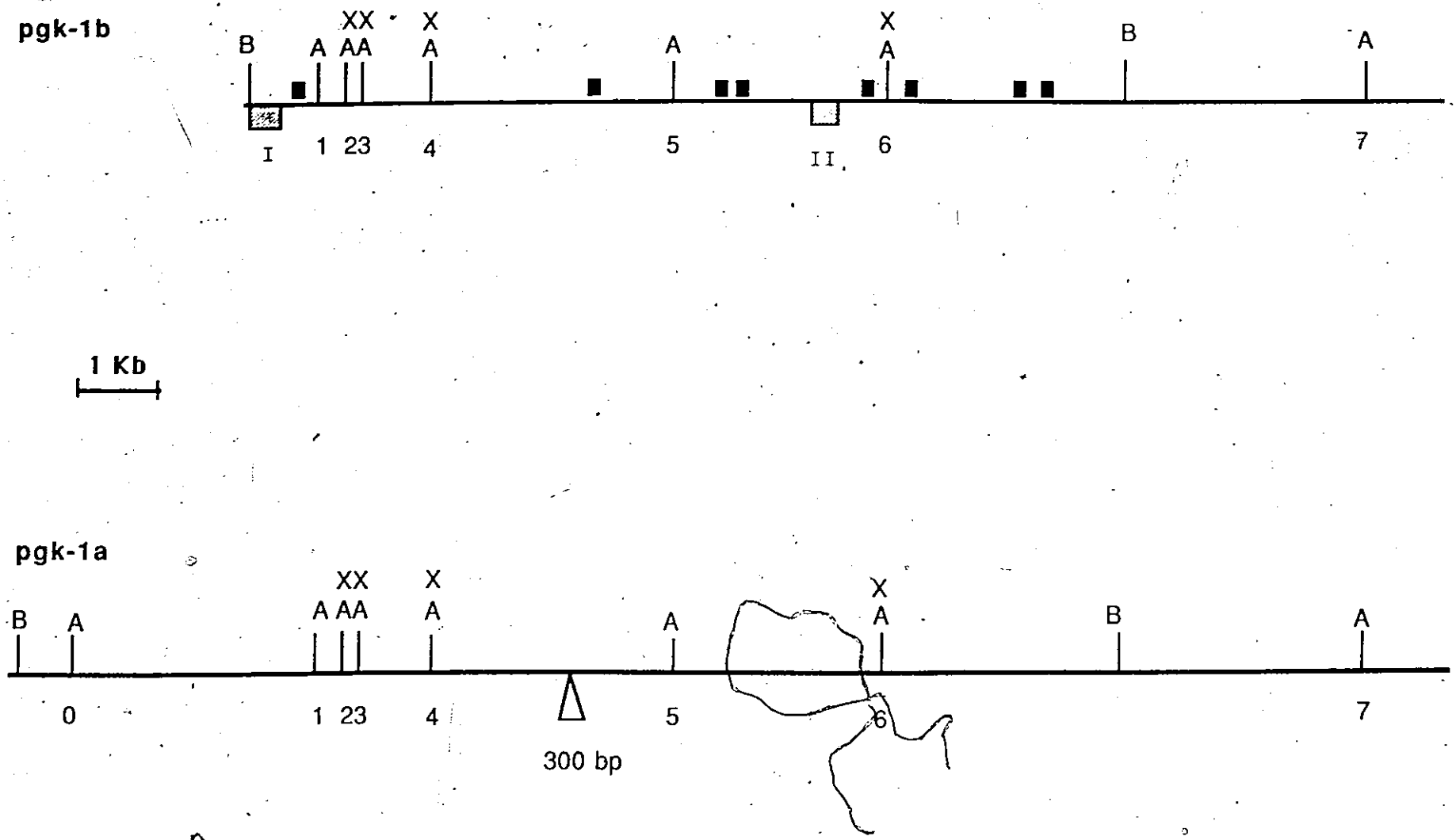
3.2 RESULTS

3.2.1 Restriction map of some methylation sensitive sites within the gene

A restriction map for both alleles of the mouse pgk-1 gene has previously been constructed, by C. Adra (1988), using restriction enzymes which are not methylation sensitive. Only the G+C rich region 5' of exon 1 was mapped for use in methylation studies. In order to get a picture of methylation in the rest of the gene, two enzymes which recognize a 6 base sequence containing the CpG dinucleotide were used, XhoI and AvaI. XhoI (which recognizes the sequence C^TCGAG) actually recognizes a subset of the sites cleaved by AvaI (C^PyCGPuG), and both enzymes show sensitivity to methylation of the internal cytosine. Both XhoI and AvaI were used in this study in order to detect cleavage at different sites. The restriction sites for these enzymes were mapped by performing double digests with BamHI on cloned fragments containing the pgk-1 gene, and with other enzymes which had been mapped by C.Adra (1988).

The maps in Figure 3.1, show the XhoI/AvaI sites spread throughout the gene, with a concentration of four AvaI sites in the first intron, indicating that this region is relatively rich in CpG sequences. The AvaI sites are numbered, and the speckled boxes I and II, shown below the gene, represent the fragments which were used as probes in these studies, to detect methylation in different regions of the gene. It was found that the restriction maps for these enzymes are essentially identical within the pgk-1 alleles, with differences only occurring upstream of the gene. The AvaI site 5' of the gene in pgk-1a is not present in the pgk-1b allele. A polymorphism for the 5' BamHI site is also indicated. The only other difference detected was an insertion downstream of exon 2 in pgk-1a.

Figure 3.1: A restriction map of the methylation sensitive XhoI and AvaI sites in a 20 kb region of the X chromosome which contains the pgk-1 gene. Fragments cloned by C.Adra (Adra et al.,1987) derived from the pgk-1a and the pgk-1b were used to map the XhoI and AvaI sites for these two alleles. The restriction sites are B, Bam HI, A, AvaI, and X, XhoI, and the AvaI sites are numbered. The approximate locations of the exons are indicated by black boxes and an insertion in the pgk-1a gene is shown. The fragments used as probes in these experiments are shown as speckled boxes, I and II.


pgk-1b
B
A
XX
AA
X
A
A
X
A
B
A
I
1 23
4
5
II
6
7
1 Kb
pgk-1a
B
A
XX
AA
X
A
A
X
A
B
A
0
1 23
4
5
6
7
300 bp

3.2.2 Methylation in various tissues of adult male and female mice

To determine whether the active and inactive chromosomes contain DNA methylated to different extents, DNA from adult male and female animals were compared, since males have an active X and the females have an active and an inactive X. Since DNA derived from different tissue types can show different patterns of methylation, it was also important to test for tissue, as well as sex, differences.

Figure 3.2 shows an autoradiogram resulting from digesting male and female DNA from different tissues with the methylation sensitive enzymes XhoI and AvaI and hybridizing the resulting Southern blots to probes I and II from the pgk-1 gene. After electrophoresis and transfer of the DNA, these filters were first hybridized with the 5' probe (I), shown in Figure 3.1, to detect methylation at the A_1 to A_4 sites, and then stripped and rehybridized with the probe from the middle of the gene (II), to detect methylation at the A_5 and A_6 sites. Completeness of the restriction digests was confirmed by increasing the amount of enzyme in the digestion mixture from a 5 fold to a 10-20 fold excess with no difference in bands resulting. The origins of the bands seen in this figure are depicted in Figure 3.3.

It will immediately be noticed in Figure 3.2 (A) that the banding patterns in the male and female lanes differs dramatically for all of the tissue types tested. The male DNA in lanes 1,3 and 5, which was digested with BamHI and XhoI, yields only the 1.5 kb fragment expected from cleavage at the first XhoI site, A2. In lanes 2,4 and 6, which contained female DNA there are additional, higher molecular weight bands present. As seen in Figure 3.3 these are 1.7, 3.5 and 11.0 kb and result from incomplete cleavage at sites A2, A3 and A4. For example, a 3.5 Kb fragment results from methylation of A2 and A3 but no methylation of A4.

Figure 3.2: Analysis of the DNA methylation of some sites in the pgk-1 gene in adult mouse tissue. Spleen, kidney or liver DNA was digested to completion with an excess of BamHI and either XhoI or AvaI. The DNA was electrophoresed overnight in 1% agarose gels, transferred to nylon membranes, hybridized to probe I, shown in Figure 3.1 (A), and then stripped and rehybridized with probe II (B). The filters were all washed at high stringency following hybridization. Lanes 11 and 12 are spleen and kidney DNA from female mice homozygous for the pgk-1a allele. The other lanes contain DNA from male or female mice carrying the wild-type pgk-1b allele.

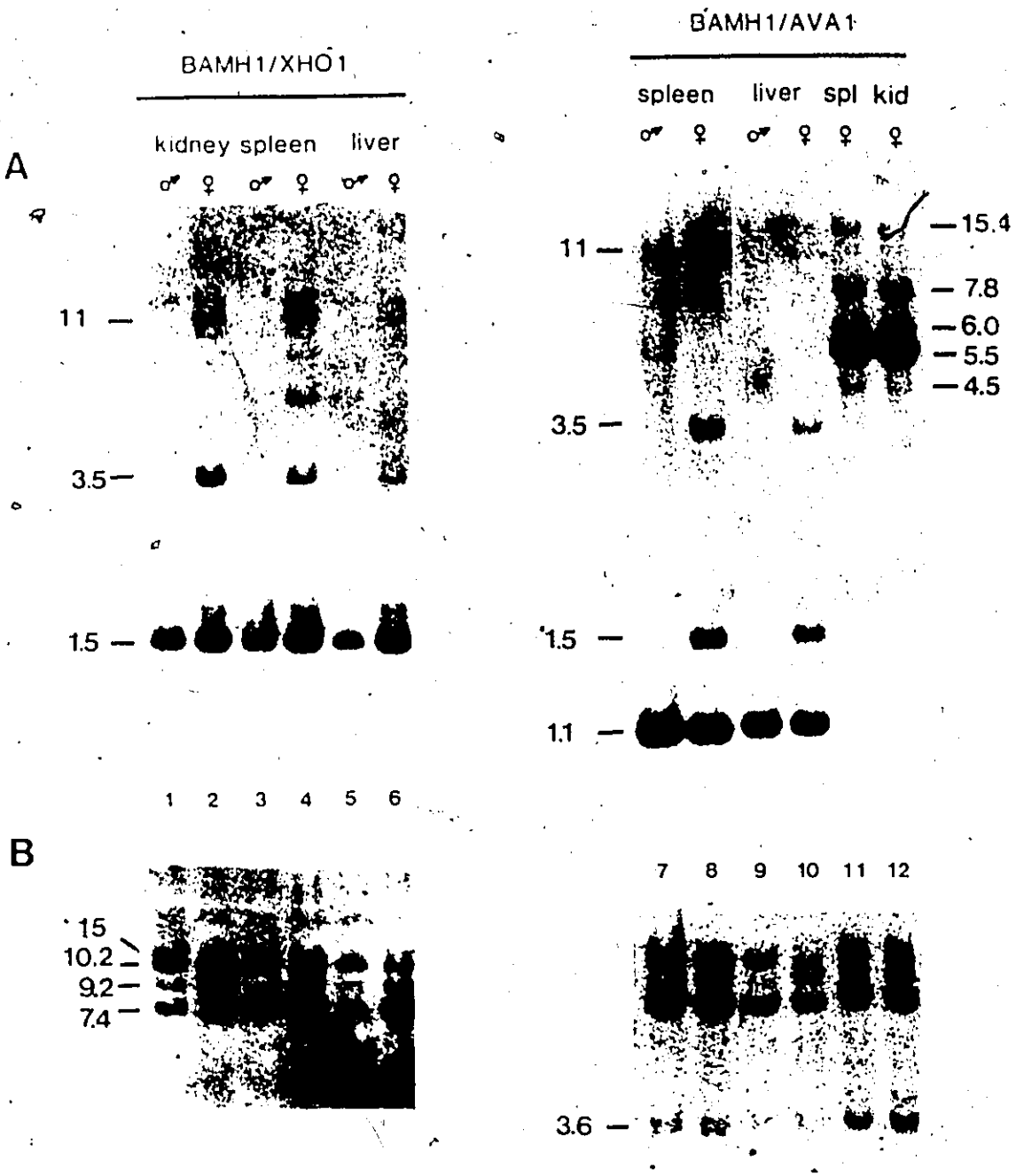
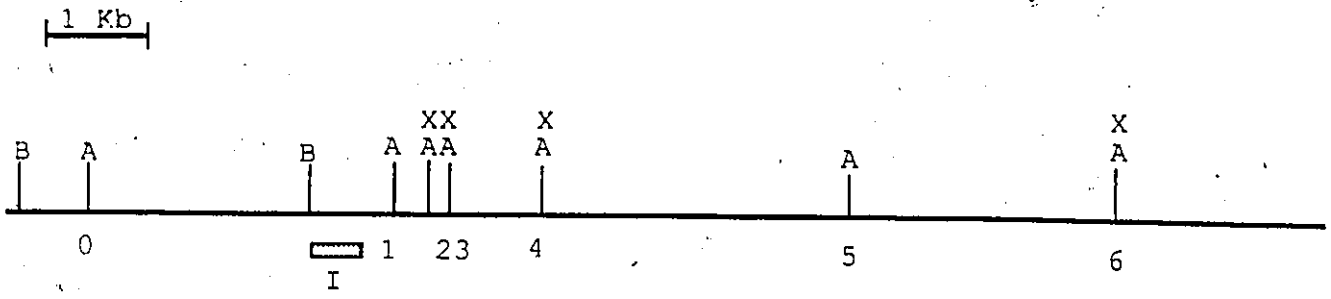

A
BAMH1/XHO1
kidney spleen liver
♂ ♀ ♂ ♀ ♂ ♀
11
3.5
1.5
BAMH1/AVA1
spleen liver spl kid
♂ ♀ ♂ ♀ ♀ ♀
11
3.5
1.5
1.1
15.4
7.8
6.0
5.5
4.5
B
1 2 3 4 5 6
15
10.2
9.2
7.4
7 8 9 10 11 12
3.6

Figure 3.3: A summary of the fragments which resulted from the experiments shown in Figure 3.2. At the top of the figure is a map of the BamHI, XhoI, AvaI and probe locations, for reference. The bands seen in Figure 3.2 are depicted as horizontal lines shown under the portion of the pgk-1 gene from which they originated. Panel A shows the results of Figure 3.2 which arose from probing digests of mouse DNA with probe I. Panel B shows the results using probe II.



Panel A (probe I)

BamHI/XhoI

pgk-1b

1.5
1.7
3.5
11.0

pgl-1a

6.0
7.8
15.4

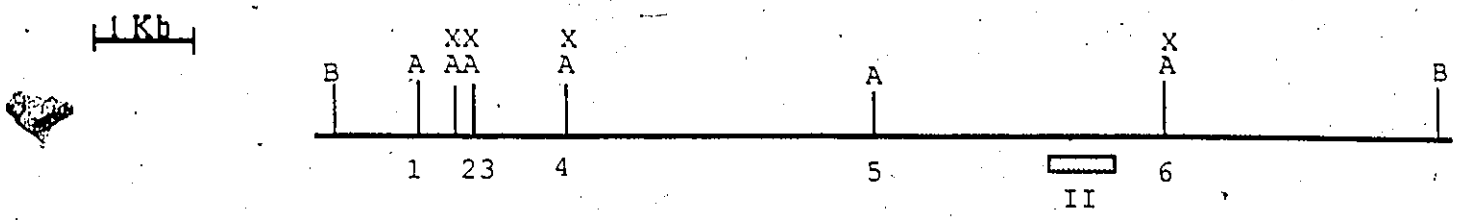
BamHI/AvaI

pgk-1b

1.1
1.5
1.7
3.5
11.0

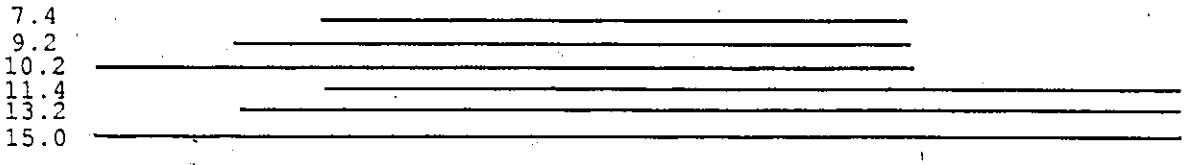
pgk-1a

4.5
5.5
6.0
7.8
15.4

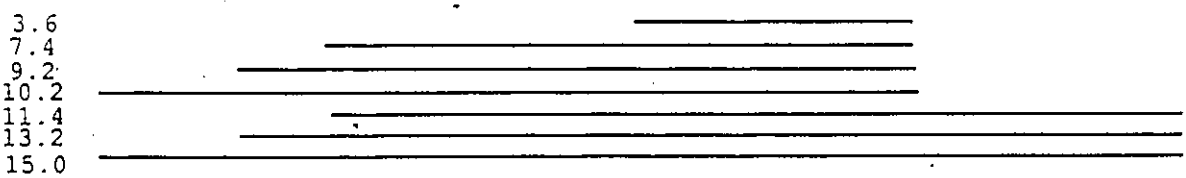


Panel B (probe II)

BamHI/XhoI



BamHI/AvaI



The panel of BamHI/AvaI digests in Figure 3.2(A) shows similar results. Lanes 7 and 9, which contained male DNA have only the 1.1 Kb fragment which results from complete cleavage at the first AvaI site, A1. In contrast, the female DNA in lanes 8 and 10 yielded several higher molecular weight fragments. These fragments were 1.5, 1.7, 3.5, and 11 kb, and result from incomplete cleavage at sites A1 through A5, as shown in Figure 3.3.

The incomplete cleavage by XhoI and AvaI in the female DNA is almost certainly caused by the presence of 5-methylcytosines at the enzyme sites. Increasing the amount of enzyme did not change the cleavage patterns seen, and both of these enzymes are sensitive to cytosine methylation. It can also be concluded with confidence that it is the inactive X chromosome in the females which is methylated, since the active, male X chromosome was readily cleaved. In addition, analysis of the autoradiograms with a scanning laser densitometer revealed that approximately half of the hybridization signal in the lanes which contained female DNA was in the female specific, higher molecular weight bands. This supports the conclusion that the female inactive X chromosomes were completely methylated at sites A1 and A2, which are in intron 1.

In lanes 4 and 7 of Figure 3.2 (A), there are two bands of intermediate size which cannot be explained by the restriction map of this region. It is possible that in these cases probe I is cross hybridizing with one of the pgk pseudogenes (Adra et al.,1988). It is also possible that the endonuclease BamHI is not cleaving the DNA completely in these digests. However this is not likely since repeated experiments with an excess of this enzyme yielded similar results for these DNA types.

DNA from different tissues was included in this study to discriminate between tissue specific and sex specific methylation. The banding patterns of spleen and kidney DNA in lanes 1 to 4 appear the same except for the unexpected bands mentioned above in the female spleen DNA. The female liver DNA appears to have a much fainter 11 Kb band than the kidney and spleen DNA in both the

AvaI and XhoI digests. It is therefore likely that the methylation sensitive sites A4 and A5 are showing tissue-specific rather than sex specific methylation. However probe I reveals that sites A1, A2 and A3 are heavily methylated in female kidney, liver, and spleen DNA which supports the existence of sex-specific methylation of these sites.

An analysis of the AvaI sites in female mice homozygous for pgk-1a appeared to show that sites A1 to A3 were heavily methylated on the inactive but not the active X. From Figure 3.3, a 4.5 Kb band corresponds to cleavage at A0 and A1. (The BamHI site is missing on this allele). The 5.5, 6.0, 7.8 , and 15.4 Kb bands correspond to incomplete cleavage (due to methylation) at sites A0 to A5 predicted in Figure 3.3. However sex specific methylation cannot be unequivocally asserted since male mice carrying this allele were not used for comparison in these experiments.

It can be seen from probing the pgk-1a DNA with the 5' probe that the AvaI site upstream of the gene, A0, is heavily methylated in both female spleen and kidney cells. Although, once again, it is not known whether this also occurs in male cells, it is not likely to be inactivation-related methylation, since it seems to occur on both the active and inactive chromosomes in female cells (far less than 50% of the signal is in the band corresponding to cleavage at A0).

Panel B of Figure 3.2 shows the results of rehybridizing the Southern filters used in panel A to probe II, which is from further downstream in the gene. This enabled some conclusions to be made about the methylation of XhoI and AvaI sites in the middle region of the pgk-1 gene. It is evident from this experiment that both the male and female, and thus active and inactive, DNA is partially methylated in this region. The BamHI/XhoI digests in lanes 1-6 reveal that sites A4 and A6 are partially methylated on the active X chromosome. The BamHI/AvaI digests show that the A5 site is heavily methylated in both active and inactive DNA. The main conclusion which can be drawn from these results is that

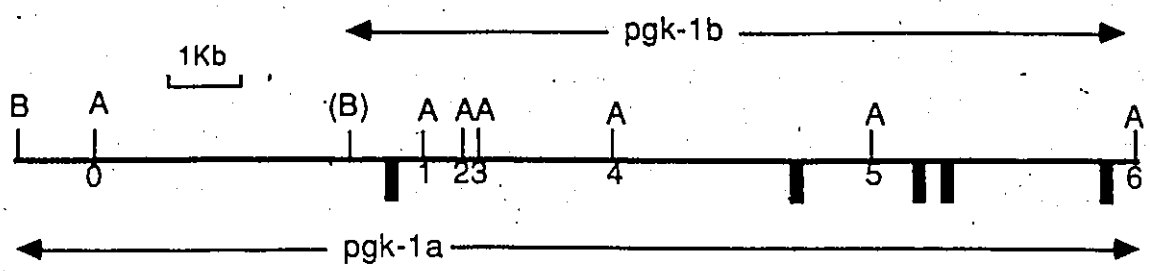
there is methylation of sites in the middle of the gene, and this is seen in both male and female, and thus active and inactive DNA.

Although there are no clear sex-specific differences in this region of the gene, there may be tissue specific differences. For example the male spleen and liver DNA show a different pattern when digested with XhoI from the male kidney DNA. In addition, when digested with AvaI, the male spleen and liver show different patterns of methylation.

The results using probe II from the middle of the gene are not as clear as the 5' results, since the AvaI fragment containing this sequence is bordered on both sides by methylation sensitive restriction sites, and the electrophoretic separation of this size range was not optimal in these experiments. Therefore it must be noted that it is possible that in the middle of the gene, at site A6 in particular, there is an inverse pattern of methylation similar to that found in this region of the hprt gene (Lock et al.,1986). For example, if A_6 were methylated on the active X-chromosome and unmethylated on the inactive one, and sites A4, A3, A2, and A1 were only methylated on the inactive chromosome, a similar band of about 11 Kb would be detected in both the male and female DNA. Since the results using probe I revealed that the intron I sites are unmethylated on the active X, this is likely to be the reason that the probe II results appear similar for male and female DNA.

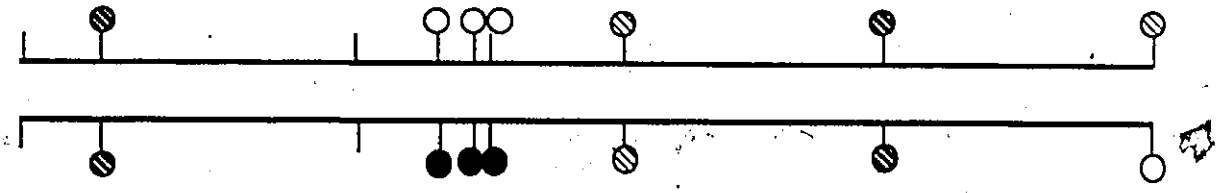
Figure 3.4 (A) summarizes the methylation status of the AvaI sites in the pgk-1 gene. The experiments described in Figures 3.2 and 3.5 were used to determine the extent of methylation on the active and inactive X chromosomes. Although the autoradiograms shown in Figure 3.2 and 3.5 were the ones used to quantitate the amount of signal in the higher molecular weight bands using the scanning laser densitometer, the relative proportion of signal in these bands was found to be reproducible in 1 subsequent trial using mouse DNA, and 2 subsequent trials using EC cell DNA. A portion of the BamHI/AvaI restriction map of the gene is shown in Figure 3.4 for reference. The presence of methylation at an AvaI site is depicted by a fully shaded circle and absence of

Figure 3.4: Summary of methylation of AvaI sites in the pgk-1 gene on the active and inactive X chromosomes. A portion of the BamHI and AvaI restriction map of the mouse pgk-1 gene is shown for reference. The experiments described in Figures 3.2 and 3.5 were used to determine the methylation state of the internal cytosine in the AvaI recognition sequence. Part A summarizes the difference in methylation patterns on the active and inactive X chromosomes in mice. The experiments shown in Figure 3.5 revealed that the EC cell lines P19 and C86 have similar patterns. Part B shows the methylation patterns observed on the inactivated X chromosome of the P10 EC cells, and the "reactivated" X chromosomes of reac 2a. The amount of signal in the methylation dependent bands seen on the autoradiograms was measured by scanning with a laser densitometer. Shaded circles at the sites indicate full methylation, and clear circles indicate lack of methylation (or cleavage) of the site. Varying degrees of partial methylation are indicated by circles which are lightly striped (<50% methylation) or heavily striped (>50% methylation).



A

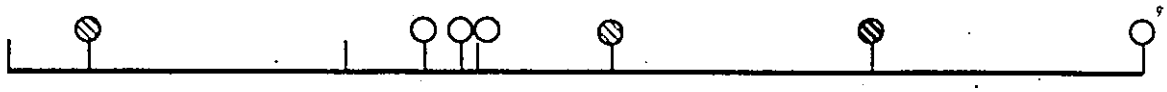
Active X



Inactive X

B

Inactive X *from reac 2a cells and differentiated P10 cells:*



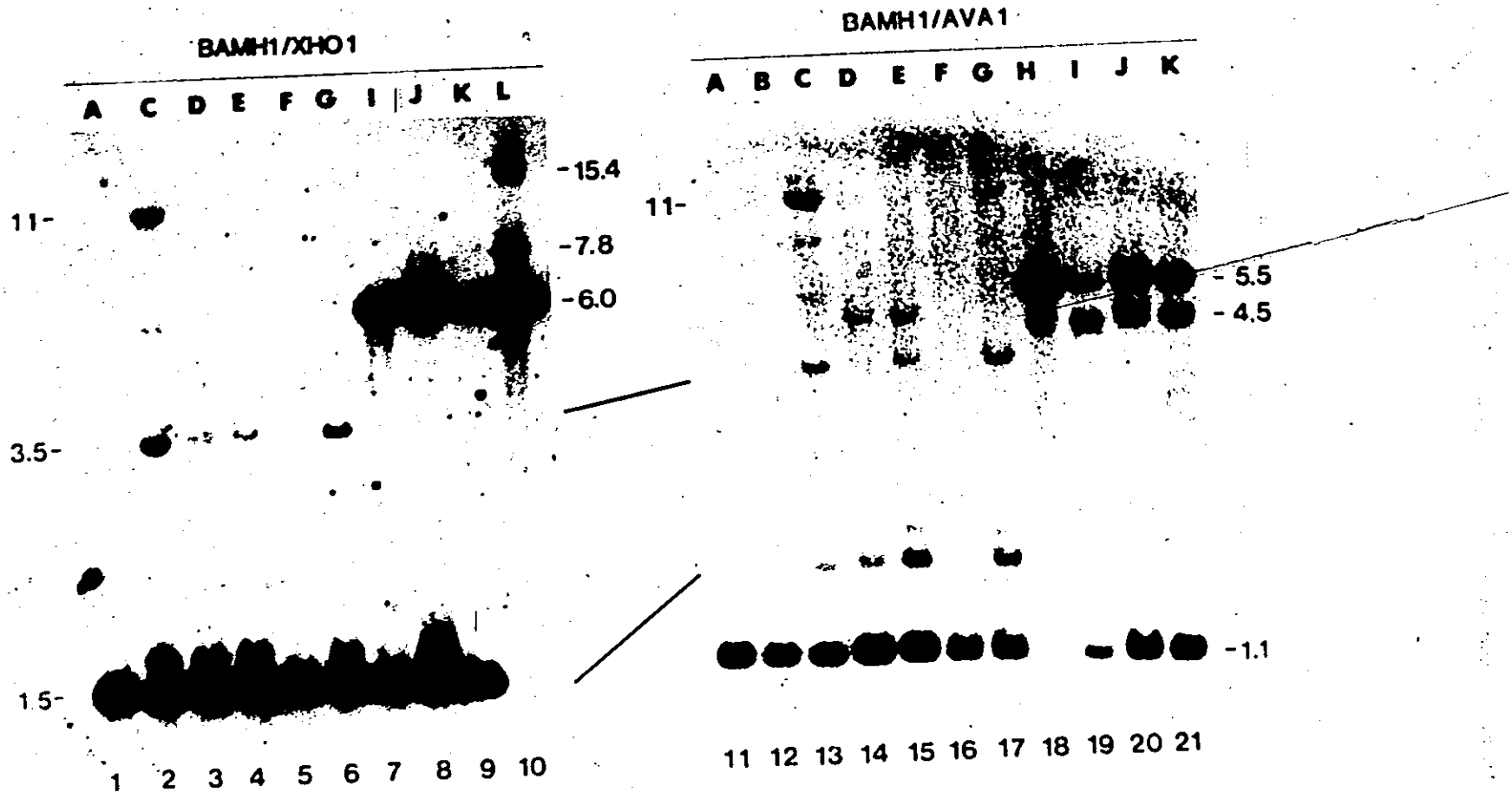
methylation (full cleavage of the site) an empty circle. Partial methylation of sites is indicated by lightly striped (<50% methylated) or heavily striped (>50% methylated) circles. It is clear from this figure that the sites which show inactivation-related methylation are in intron 1. The other sites tested show methylation to varying degrees, but with no clear relationship between methylation and inactivation.

3.2.3 Methylation in female embryonal carcinoma cells

The results of restricting the DNA from the EC cell lines described in Table 2.1, with the methylation sensitive XhoI or AvaI enzymes, in combination with BamHI, are shown in Figure 3.5. Only the probe I results are shown in these experiments, since the results using male and female DNA in 3.2.2 indicated that the methylation correlated with inactivation occurred at sites in intron 1. The P19 cells which were used in lanes 1 and 11 (A) have a normal male karyotype (XY). Since only the 1.5 Kb and 1.1 Kb fragments which were seen in the male mouse DNA in Figure 3.2, it is clear that these cells are not methylated at the A_1 and A_2 sites in intron 1.

The female C86(XO) cells (Fig. 3.5 lane 12), which have just one active X chromosome, also show just the 1.1 Kb band resulting from full cleavage at A_1. In contrast, the C86(XX) cells, (C), which contain 1 active and 1 inactive X chromosome show cleavage patterns similar to the adult female DNA in Figure 3.2. The 1.5 Kb band in lane 2 and the 1.1 Kb band in lane 13 contain approximately half of the hybridization signal in these lanes. The remaining signal is in the higher molecular weight bands predicted in Figure 3.3. The unexplained bands presumably caused by hybridization to a pseudogene which were seen in Figure 3.2 lanes 4 and 7 are also seen in these digests. The 11 kb band in these lanes is more prominent than it was in the female mice, indicating increased methylation in the middle region of the gene.

Figure 3.5: Analysis of the DNA methylation of some sites in the pgk-1 gene in undifferentiated, differentiated, and reactivated EC cells. Genomic DNA was digested to completion with BamHI and either XhoI (left panel) or AvaI (right panel), electrophoresed overnight in 1% agarose gels, transferred to nylon membranes, hybridized to the 5' probe and washed at high stringency. The cell lines used were: A, P19(XY); B, C86(X^bO); C, C86(X^bX^b); D, reac 1a; E, reac 1b; F, reac 2a; G, reac 2b; H, P10(X^aO); I, P10(X^aX^b); J, P10(X^aX^b) cells which had been differentiated in retinoic acid for 10 days; K, P10(X^aX^b) cells which had been differentiated for 13 days, and L, spleen DNA from a female mouse homozygous for pgk-1a. The "reac" cells are C86 derivatives which had been treated with 5-azacytidine which resulted in varying degrees of pgk-1 reactivation (Hockey, A., Adra, C.N., and McBurney, M.W., manuscript submitted).


BAMH1/XHO1
A C D E F G I J K L
11-
3.5-
1.5-
-15.4
-7.8
-6.0
1 2 3 4 5 6 7 8 9 10
BAMH1/AVA1
A B C D E F G H I J K
11-
- 5.5
- 4.5
-1.1
11 12 13 14 15 16 17 18 19 20 21

The reactivated C86 derivatives (D, E, and G), which had been treated with 5-azacytidine and have varying levels of pgk-1 activity (A. Hockey, unpublished results), also show some methylation at these sites. They appear to be less methylated than the C86 cells in the 3' region of the gene, since the 11 Kb band is not seen in these cells. The striking exception to the methylation of intron 1 sites is in the reac 2a cells, (F), which were measured to have pgk-1 mRNA levels at least twice as high as those in the parental derivative, and in reac 1a and 1b. There is no detectable methylation in these cells at site A_1 or A_2. However, when these filters were rehybridized with the probe II, from the middle of the gene (data not shown), the reac 2a cells showed the same fairly extensive methylation in this region as the C86 and mouse cells. This would appear to be good evidence that only methylation of the sites in intron 1 is correlated with X-chromosome activity. There is one inconsistency with these reactivated clones, however, in that reac 2b (G in lanes 6 and 17) showed even higher levels of pgk-1 mRNA than reac 2a. And yet this clone shows evidence of methylation of the first two AvaI sites which is comparable to that in C86(XX).

P10 cells are female cells which are heterozygous for the pgk-1a allele. They contain two active X chromosomes, but following differentiation in retinoic acid, one X is inactivated. It is clear, however, from Figure 3.3 that the P10 cells, lanes 7 to 10 and lanes 18 to 21, do not show methylation of the intron 1 sites on either chromosome, even after differentiation and X-inactivation. The only bands which are present in the P10 lanes digested with BamHI and XhoI are the 1.5 Kb band, corresponding to full cleavage at the first XhoI site in pgk-1b (B-A2), and a 6.0 Kb band corresponding to full cleavage at the first XhoI site (B-A2) in pgk-1a. In the P10 lanes digested with BamHI and AvaI, the only bands are the 1.1 Kb band from full cleavage at B-A1 in the "b" allele, a 4.5 Kb band resulting from cleavage at A0-A1 in the "a" allele, and a 5.5 Kb band resulting from incomplete cleavage at A0 and complete cleavage at B-A1.

Following treatment with retinoic acid, a two-fold decrease in pgk-1 enzyme activity is seen after 2-3 days, and a late replicating X is seen after 3-4 days. The P10 cells shown in lanes 18-21 had been differentiated for up to 13 days. There appears to be no methylation of the intron 1 sites even after X inactivation. In addition to the lack of methylation seen within the gene in P10 cells, the 5' AvaI site (A0) which was shown to be almost completely methylated on both chromosomes in mice homozygous for pgk-1a (described in 3.2.2), is less methylated in the P10 cells.

Figure 3.4 (B) summarizes the methylation patterns on the "reactivated" X chromosome of the clone reac 2a, and the inactivated X chromosome in the P10 cell line.

3.3 DISCUSSION

It has long been hoped that studies of DNA methylation would at last provide some concrete answers to the puzzles of gene expression. As early as 1975 it was proposed by Riggs (1975) and Holliday and Pugh (1975) that methylation of cytosines might be the basis for the stable and heritable inactivation of the X-chromosome and the control of tissue specific genes during development. Since then evidence has accumulated which links methylation to the regulation of activity of autosomal as well as X-linked genes (Cedar,1988).

In the present study, methylation in the body of the mouse pgk-1 gene was examined, in order to add to the results obtained from the promoter region of the gene. Somewhat surprisingly, the region which shows apparent inactivation-related methylation is in the first intron, not in the promoter region, as it is in the human pgk-1 gene (Keith et al.,1986; Hansen et al.,1988). The G+C rich sequences in the promoter region of the pgk-1 gene are conserved in mice and humans (Adra et al.,1987), but this region revealed little methylation in the mouse (C.Adra,1988).

From the cluster of AvaI sites which were mapped to intron 1, it is likely that this region is also G+C rich. The CpG dinucleotide, which is the target for methylation in the AvaI recognition sequence, is under-represented in the mammalian genome and exists mainly in a methylated state. The presence of a cluster of unmethylated CpGs, such as those seen on the active mouse X-chromosome in intron 1, may represent a CpG island which has some functional significance in the regulation of the gene (Bird,1986). The intron 1 cluster of AvaI sites shows a consistent inverse relationship between methylation and activity in all of the adult mouse tissues tested. The female mice carrying the pgk-1a allele show a similar pattern and degree of methylation of these sites, and although male mice carrying this allele were not tested, it is likely that they are undermethylated at these sites.

In the C86 embryonal carcinoma cells, the intron 1 sites showed the same sort of relationship between methylation and X chromosome activity as the adult mice. These cells show what appears to be methylation of these sites only on the inactive X. However one of the 5-azacytidine reactivants which showed increases in pgk-1 message showed methylation at these sites, while another showed that these sites had been exclusively "demethylated". The demethylation in these cells by 5-azacytidine seems to have been only at the cluster of sites in intron 1, since the middle of the gene (as revealed by hybridization to probe II) shows similar methylation to that found in C86(XX). This finding seems to support the idea that 5-azacytidine affects specific regulatory loci which then cause demethylation of only a subset of possible sites. The discrepancies between methylation and activity in the reac 2a and reac 2b cells may serve as a reminder of the dangers of over-interpreting 5-azacytidine results, or may call into question the significance of the differential methylation observed at these particular sites. It is also possible that the reac 2b cells represent an earlier stage of X reactivation than the reac 2a cells. Perhaps in 2b, the gene has

been transcriptionally reactivated, but the methyl groups of specific stabilizing sites have not yet been removed.

The only female cells which did not show methylation of the intron 1 sites on the inactive X chromosome were the P10 cells, embryonal carcinoma cells which are heterozygous for the pgk-1 alleles. These cells have two active X chromosomes, but can be induced to differentiate in the presence of retinoic acid, resulting in the appearance of an inactive, late replicating X chromosome (Paterno and McBurney,1985). The lack of methylation in these cells is probably widespread, since even the AvaI site 5' of the pgk-1a gene, which was heavily methylated on both chromosomes in mice carrying this allele, is less methylated in P10 cells. It is likely that this cell line is at a "pre-methylation" stage, as was found at sites studied in the hprt gene in LT-1 cells (Lock et al.,1987). The LT-1 cells also contain two active X chromosomes, but failed to show significant methylation of intron 1 sites even after differentiation and concomitant X-inactivation. The P10 and LT-1 cells may represent those cells of the female embryo at about 6.5-7.5 days of gestation, when inactivation has occurred but methylation has not yet set in. The pgk-1 gene on the inactive X in these cells is probably being held inactive by some other means until methylation takes over this role.

Alternatively, the retinoic acid treated P10 cells may represent extra-embryonic cells under the control mechanism used in the preferential inactivation of the paternal X chromosome. Although the P10 cells resemble extra-embryonic cells when differentiated, the paternal X is not preferentially inactivated (Paterno and McBurney, 1985). It is possible that the imprinting which is required for preferential inactivation has been lost during the passaging of these cells in tissue culture, but that the molecular mechanisms used to initiate inactivation in these cells still differ from those in the random inactivation seen in embryonic lineages.

The 5-azacytidine treatment of C86 cells, whose X chromosomes are perhaps at a later stage of inactivation, may work by causing local destabilization due to the removal of specific methyl groups. The inactive X chromosome in differentiated P10 cells, on the other hand, cannot be reactivated by 5-azacytidine (Paterno,1985), supporting the idea that these cells are at some earlier stage of inactivation, which does not yet involve methylation, or are ruled by a different mechanism of control altogether.

In summary, these results give fairly strong evidence that the CpG cluster located in intron 1 in the mouse pgk-1 gene might be involved in X inactivation. Since clusters which show differential methylation with X inactivation have been found for all other X-linked housekeeping genes studied, the localization of this site is reassuring. However, a clear role for methylation in the regulation of this gene cannot be presumed. The lack of conservation of methylated sites in the 5' region of the mouse and human pgk-1 genes is rather puzzling, given the sequence conservation in the promoter regions of these two species. This might be taken as evidence against a major role for methylation in X chromosome inactivation. In addition, the results of this study, using one cell line which can be inactivated (P10) and another which can be reactivated (C86), seem to indicate that significant methylation of sites in the pgk-1 gene must occur well after transcriptional regulation.

It has lately been shown by other groups, both for an X-linked housekeeping gene (Lock et al.,1987), and an autosomal tissue specific gene (Enver et al.,1988), that methylation occurs after transcriptional suppression. It is likely that methylation, if causally involved in the silencing of genes, works in concert with a complex network of DNA binding proteins, perhaps altering the configuration of binding sites to encourage the binding of some factors and inhibit the binding of others. Methylation may play a role in stabilizing and perhaps marking the inactive state, but it is probably not involved in initiating it.

CHAPTER FOUR

A PHYSICAL MAP AROUND THE PGK-1 GENE

4.1 INTRODUCTION

The X-linked pgk-1 gene has been genetically mapped to a region 3-5 map units away from the X controlling element (Xce) in the mouse (Cattanach and Papworth,1981). This is the closest cloned gene to the Xce locus, which is probably the location of the X inactivation center, or Xic. The inactivation center is thought to be the position on the X chromosome from which X inactivation initiates and spreads in both directions along the chromosome.

A rare variant allele of the pgk-1 gene was discovered by Nielsen and Chapman (1977) existing in a Danish population of Mus musculus. This variant allele, called pgk-1a, is linked to the extreme allele of the X controlling element, Xce^c, which can confer upon its X chromosome a 70% likelihood of remaining active in cells which are heterozygous for Xce^a/Xce^c (Johnston and Cattanach, 1980). Both the pgk-1a and the pgk-1b genes have been cloned in this lab by Chaker Adra (Adra,1987). The restriction map of the cloned pgk-1a gene was found to be homologous to that of the pgk-1b gene, except in intron 3 where a site for HindIII which is present in pgk-1b is absent in pgk-1a. In this study, the comparison of the two alleles is extended using both long range mapping techniques and conventional electrophoresis. According to some theories of the mechanism of X inactivation, there are local sequences in or near X-linked genes which play a role in the spreading and maintenance of inactivation. The Xce locus may be a region of the X chromosome which is especially rich in these sequences (McBurney,1988). In addition to approaching the Xce in this study, it was hoped that the discovery of

polymorphic segments in this region close to inactivation center might reveal sequences of importance either to the initiation, spreading, or maintenance of inactivation.

4.2 RESULTS

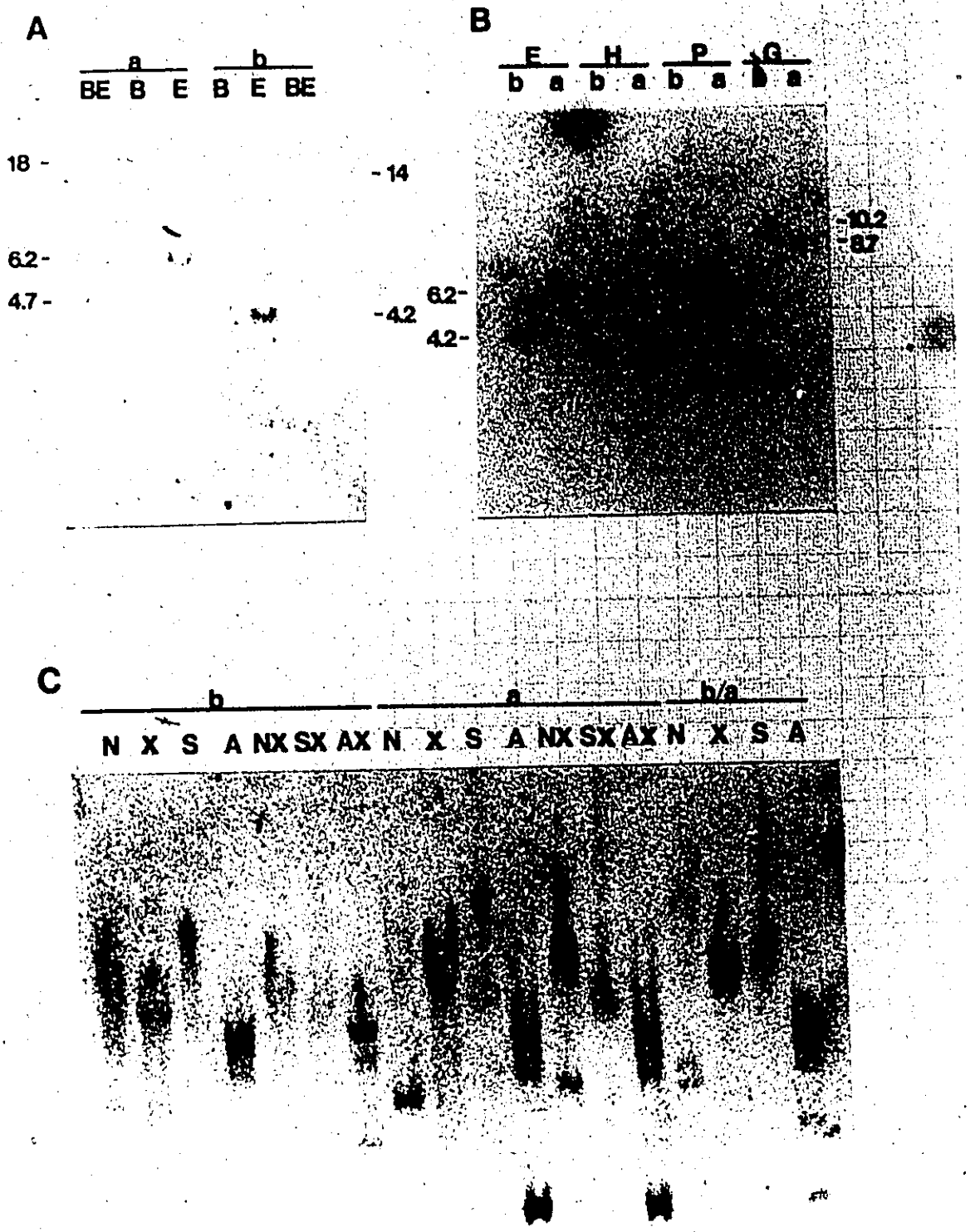
4.2.1 A comparison of the upstream region of the pgk-1b and pgk-1a alleles

In this study the mapping of the mouse pgk-1 alleles was extended to the region immediately upstream of the gene, where a BamHI polymorphism had previously been found. Using both conventional and pulsed field gel electrophoresis, DNA from cells carrying either the pgk-1a or the pgk-1b allele was hybridized to probe I from the cloned pgk-1 gene.

Figure 4.1 (C) shows the results of a pulsed field gel using DNA from the EC cells P19(XY), P10(XO), carrying the pgk-1a allele, and P10(XX), heterozygous for the pgk-1 alleles. This DNA had been prepared in solution rather than in agarose but was of sufficiently high molecular weight to yield detectable bands up to about 25 Kb. This allowed analysis of the NarI, XhoI and AvaI restriction sites in the region immediately upstream of the gene, using probe I, which was shown in Figure 3.1. It will be noticed from Figure 4.1 (C) that the DNA in the higher molecular weight range, when prepared in solution and run on a pulsed field gel shows quite remarkable streaking back in the direction of the wells. This did not occur when the same amount of DNA was restricted with more frequently cutting enzymes and run on an ordinary gel. Clearly less DNA needs to be used when it is being digested with rare-cutting enzymes and run in an alternating electric field.

The important result from this gel is that it clearly indicates allelic differences in the region immediately upstream

Figure 4.1: Southern analysis of the upstream region of pgk-1a and pgk-1b. DNA from P10 (XO) (pgk-1a), labelled "a" in panels A and C, P10(XX) (pgk-1a/pgk-1b), labelled "a/b" in panel C, P19(XY) (pgk-1b), labelled "b" in panels A and C, and female mouse spleens (homozygous for either pgk-1a or pgk-1b), labelled "a" or "b" in panel B, was digested with the enzymes indicated to determine whether the two alleles are polymorphic for these enzyme sites. The enzymes used were B, Bam HI, E, Eco RI, H, HindIII, P, PstI, G, BglII, N, NarI, X, XhoI, S, SfiI, and A, AvaI. In panels A and B, the DNA was electrophoresed in 1.2% agarose gels, transferred to nylon membrane and hybridized to probe I, shown in Figure 3.1. In panel C, the DNA was prepared in solution, digested and fractionated by FIGE in a 1% agarose gel, exposed to U.V. light for 5 min, transferred to nylon membrane, and hybridized to the same probe.

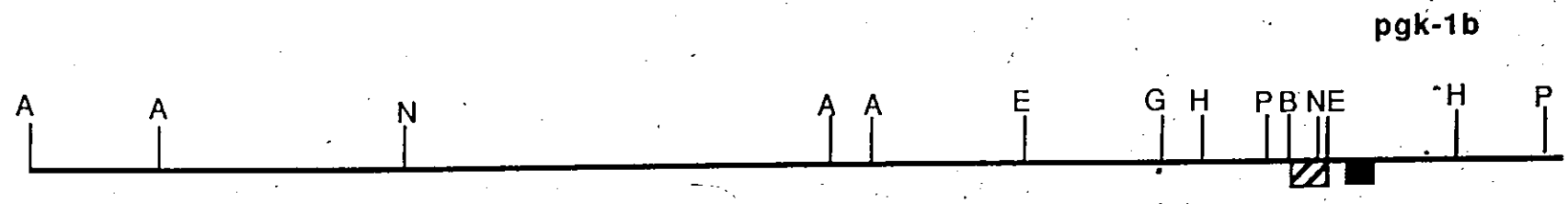

A
a
b
BE B E B E BE
18 -
6.2 -
4.7 -
14 -
4.2 -
B
E H P G
b a b a b a b a
10.2
8.7
6.2
4.2
C
b
a
b/a
N X S A NX SX AX N X S A NX SX AX N X S A

strong hybridization signal at about 10 Kb from the 5' end of the gene which is not present in the pgk-1b digests. When this experiment was repeated (data not shown), a faint signal in the P19 NarI digest could be seen at about 13.5 Kb. This band is visible in the P10(XX) NarI digest in Figure 4.1 (C). An analysis of the AvaI sites upstream of the pgk-1, also showed allelic differences spread over a 15-20 Kb region.

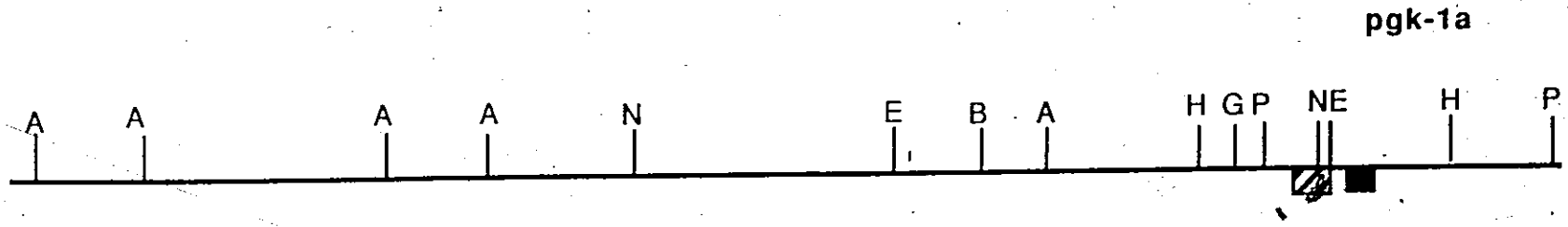
Both NarI and AvaI have potential sites for methylation in their recognition sequences, and this may be responsible for the detection of more than one hybridizing fragment. It should be noted that the multiple AvaI bands are probably not due to methylation of sites within the gene, since in the experiments described in Chapter 3, P19 and P10 DNA did not show any methylation of the intron 1 sites. It can be concluded that these AvaI fragments extend into the region upstream from the gene. The NarI results are less certain, due to the site preferences of this endonuclease, discussed in 4.2.2.

Because of these ambiguities, it was necessary to examine the upstream region for RFLPs using endonucleases which do not have methylation sensitivity or site preferences. The recognition sites of the restriction enzymes EcoRI, BamHI, HindIII, PstI, and BglII do not contain cytosines which are in the CpG configuration normally methylated by mammalian DNA methylases. In panels A and B, pgk-1a and pgk-1b DNA was restricted with EcoRI, BamHI, HindIII, PstI, and BglII. The autoradiogram in A clearly shows an EcoRI polymorphism in this region, in addition to the BamHI polymorphism which was found by C. Adra. To ensure that these differences were not peculiarities of the EC cell lines examined here, spleen DNA from female mice carrying either the pgk-1a or the pgk-1b allele was digested with these enzymes, shown in panel B. The BamHI polymorphism had been previously detected in these mice, the EcoRI polymorphism can be seen in Figure 4.1 (B), and the pgk-1a DNA appears to have a BglII site about 1.5 Kb upstream of the gene which is not present in the pgk-1b DNA. Figure 4.2 shows the restriction site polymorphisms detected in this study,

Figure 4.2: Restriction maps upstream of the pgk-1b and pgk-1a genes. These maps were constructed using the data in Figure 4.1 and other similar experiments. The enzymes used were A, AvaI, N, NarI, E, EcoRI, B, BamHI, G, BglII, H, HindIII, and P, PstI. AvaI and NarI are methylation sensitive enzymes, the rest are not. The sites for these enzymes within the gene are also shown, with the exception of BglII, which maps 8 Kb downstream of probe I. The black box shown in this figure is the first exon in the pgk-1 gene. The striped box indicates the location of probe I which was used in these experiments.


pgk-1b
A
A
N
A
A
E
G
H
P
B
N
E
H
P


1 Kb


pgk-1a
A
A
A
A
N
E
B
A
H
G
P
NE
H
P

as well as the upstream HindIII and PstI sites which were found to be conserved.

4.2.2 Long range mapping around the pgk-1 gene using pulsed field gel electrophoresis

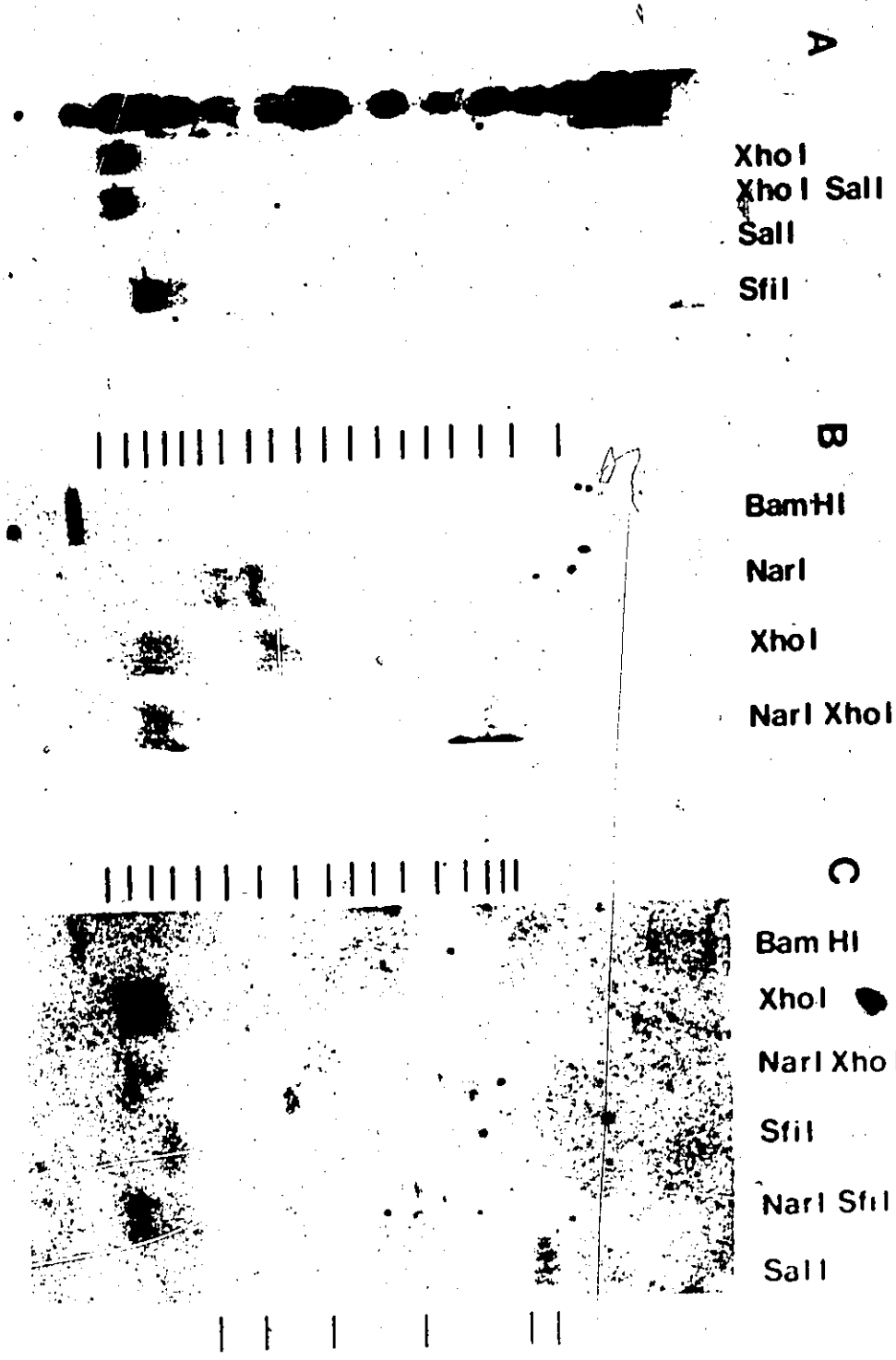
To generate a long-range physical map around the pgk-1 gene pulsed field gel electrophoresis was used, in combination with cloned fragments from the pgk-1 gene. High molecular weight genomic DNA was prepared in agarose blocks or beads and digested with various "rare-cutting" restriction enzymes. These enzymes digest genomic DNA infrequently either because of an 8 bp recognition sequence, as in the case of SfiI and NotI, or because of one or more CpGs in their recognition sequences. The CpG dinucleotide is under-represented in the mammalian genome, and is normally

methylated. The presence of this dinucleotide in a recognition sequence makes even a 6 bp sequence rare. Several different enzymes were tested, including NotI, MluI, SmaI, ClaI, SacII and PvuII, but the only enzymes which yielded one or more discernable bands with the probes used were XhoI, NarI, SfiI, and SalI. This sort of phenomenon has been cited by other investigators (Van Ommen and Verkerk,1986), and positive results appear to depend on the combination of enzyme and probe used.

The molecular weight markers used in these experiments were multimers of a 40 Kb lambda molecule, prepared in agarose according to the method of Smith et al.(1987), and chromosomes of the yeast Saccharomyces cerevisiae, prepared by V.Seligy at the National research council. These markers allowed size approximations up to about 800 Kb. With a ramped field inversion program, the migration distance of a DNA molecule is an almost linear function of its molecular weight.

Figure 4.3 (A), which shows the results of hybridizing a Southern filter of male mouse spleen DNA to probe I from the pgk-1 gene (shown in Figure 3.1), shows an 80 Kb XhoI band, which is also present in the XhoI/SalI double digest, and a 100 Kb SfiI

Figure 4.3: Long range restriction site mapping of the pgk-1 region of the X-chromosome. High molecular weight DNA from male mouse spleen cells (A), or from P19 cells (B and C) was restricted with the indicated enzyme(s), size fractionated by pulsed field gel electrophoresis, treated either in 0.25 M HCl or with U.V. light, transferred to nylon membrane and hybridized to a probe I (shown in Figure 3.1). Electrophoresis was for 24 hr in a gel of 1% agarose with an electric field of 7 V/cm, a buffer temperature of 15^{0}C and pulse times ramped from 0.3 s forward/0.1 s reverse to 60.81 s forward/20.27 s reverse. The markers shown in panel A and drawn at the left of panels B and C are lambda oligomers corresponding to multiples of 40 kb. In A the markers cross-hybridized with a pgk-1 probe which was used in a subsequent hybridization. In panel C yeast chromosome markers are also drawn on the right. The bands which were resolved on this gel, and on several other occasions correspond to 250, 290, 350, 450, 600 and 710 kb (Carle and Olson, 1986).


A
XhoI
XhoI SalI
SalI
SfiI
B
BamHI
NarI
XhoI
NarI XhoI
C
Bam HI
XhoI
NarI XhoI
SfiI
NarI SfiI
SalI

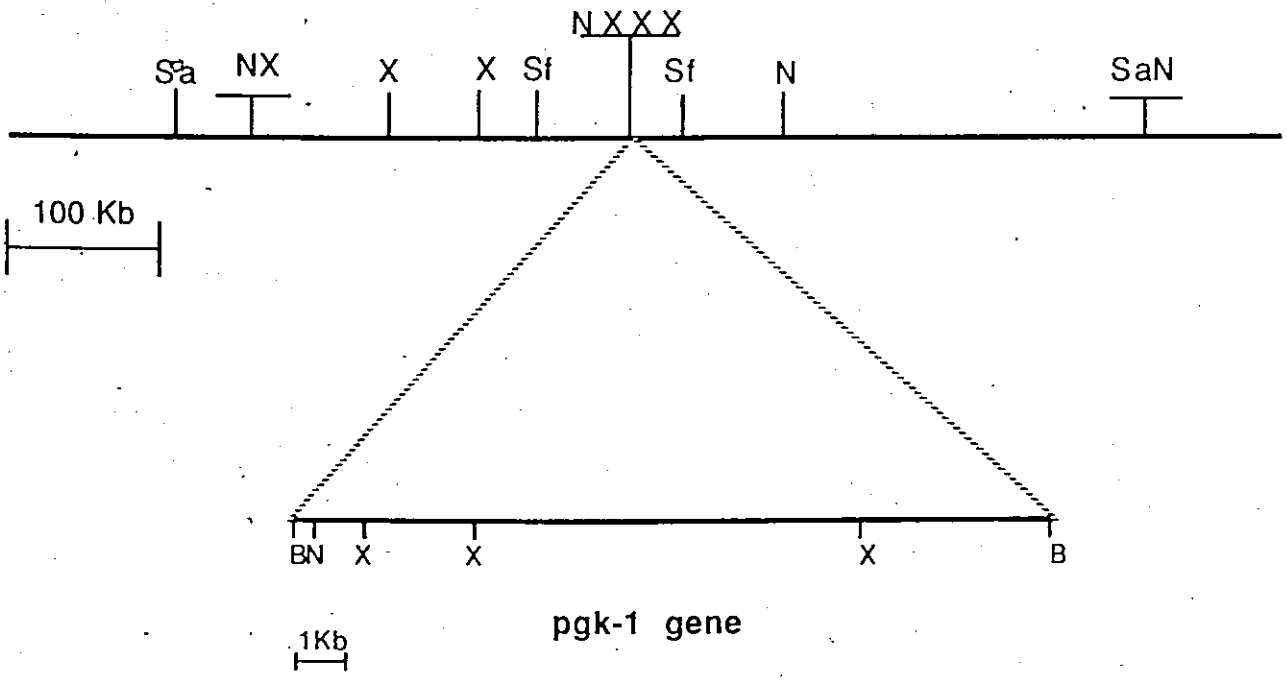
band. No SalI band can be seen on this Southern. An additional faint XhoI fragment at about 150 Kb can be seen, and this was detected more strongly in other experiments. Possibly this second XhoI band was generated by incomplete cleavage at the first XhoI site.

In (B), P19 DNA digested with XhoI gives a fuzzy hybridization signal in the 80-150 Kb range, which is probably due to a poor resolution of the 80 and 150 kb bands, as well as an upper band of about 350 Kb. It would seem that the two upstream XhoI sites which yielded the 80 and 150 kb bands seen in (A) are uncleaved in this instance to yield the larger 350 kb fragment. Since other experiments, described in chapter 3, have determined that the XhoI site immediately 3' of this probe is unmethylated in P19 cells, it can be assumed that the sites seen in these partial digests are 5' of the gene. The NarI lane in (B) gives two bands of about 250 and 350 Kb. From the sequence of the 5' end of the pgk-1 (Adra et al., 1987), there is a NarI recognition site about 500 bp upstream of the transcription initiation site. From this and other experiments, it is likely that NarI is not cleaving at the site which is in the pgk-1 gene itself. In experiments using genomic DNA, and also the cloned fragments from this region, digestions with an excess of NarI in combination with other enzymes consistently failed to give complete cleavage at this site. Since even the cloned DNA showed this problem, this is not likely to be a methylation effect. According to its manufacturers (New England Biolabs), NarI also exhibits strong site preferences so it is likely that in Figure 4.3 (B), the 350 Kb band is generated by lack of cleavage at the site in the pgk-1 gene. In Figure 4.3 (C), the 250 kb NarI band (seen in the NarI/SfiI double digest) and the 80 kb XhoI band are seen, without the upper bands, which would indicate a more complete digestion. In addition, the NarI/SfiI double digest shows a 60 Kb band, which locates the SfiI site at 60 Kb from the 5' end of the pgk-1 gene. The 650 Kb SalI fragment seen here spans the whole region being mapped.

The BamHI digests were included in these figures to demonstrate the hybridization of this probe to a known fragment. This digest also demonstrates that the fuzziness of the bands in these experiments is not caused by the FIGE apparatus or the encapsulation of DNA in agarose. The BamHI bands in panels B and C are relatively sharp compared to the bands of higher molecular weight, so perhaps these larger fragments cannot migrate uniformly due to tangling. Smith et al. (1987) have proposed that the poor resolution of most bands on pulsed field gels is due to the presence of restriction enzymes in the agarose at the start of electrophoresis. However, proteinase treatment prior to electrophoresis, according to their method, did not improve the sharpness of these bands.

These and other experiments using probe II, from the middle of the gene, were used to generate a map for a 700 Kb segment of the pgk-1 region, shown in Figure 4.4. The enzyme sites shown in Figure 4.4 were confirmed by the appearance of the expected band on two or more Southern autoradiograms. Restriction digests of cloned fragments to exactly map the sites within the gene, also shown in Figure 4.4, were extremely useful in orienting the map. This work should provide a general footing from which to proceed with future studies of this part of the X chromosome.

Figure 4.4: Long range restriction map around the pgk-1 gene in P19 cells. A physical map spanning about 700 Kb around the pgk-1 gene was constructed by analysis of the data in Figure 4.3 and other experiments using a probe II, from the gene, shown in Figure 3.1. The restriction endonucleases used were X, XhoI, N, NarI, Sf, SfiI, and Sa, SalI. The location of BamHI, B, NarI, N, and XhoI, X, sites in a cloned portion of the pgk-1 gene are indicated on a finer scale.



pgk-1 gene

4.3 DISCUSSION

The original objective of this work was to map the junction between the X chromosomes carrying the two different pgk-1 alleles, since they are known to be linked to different alleles of the X controlling element. However it happened that the enzymes which were most useful in mapping the wild-type pgk-1b region mapped very close to the pgk-1a gene. To distinguish between restriction site differences and methylation differences in this region, I also used several enzymes which do not show obvious sensitivity to cytosine methylation. In these experiments restriction fragment length polymorphisms for the enzymes NarI and AvaI were found as far away as 10 Kb upstream of the gene. BamHI, EcoRI and BglII, which are not methylation sensitive, showed RFLPs within a region 5 Kb upstream of the gene, in both EC cells and mice. Partial digests using these enzymes, and/or walking experiments, should demonstrate whether or not these differences extend further upstream. The enzyme SfiI, which maps outside of this area and is not sensitive to methylation or sequence peculiarities, appears to yield a fragment of approximately the same size (100Kb) for both alleles (data not shown). However, given the low resolving power of the pulsed field gel techniques, it is not known how small a difference within this fragment would be detected. Therefore it cannot be concluded from these experiments that the upstream polymorphisms arise from a rearrangement in this region rather than multiple single base mutations, or a smaller rearrangement in this region. Mice, rather than P10 cells, should be used in future experiments concerned with the mapping of the pgk-1a allele since this EC cell line seems to show undermethylation of possible DNA sites, in comparison to mouse cells (see section 3.2.3). Most of the enzymes used in long range mapping cleave DNA infrequently due to methylation sensitivity, so this sort of overall difference in methylation can lead to confusing results.

With these experiments a physical map of a 700 Kb region around the pgk-1 gene has been constructed. This map could provide a foothold in this region to allow chromosome walking or jumping to the Xce locus. If the region immediately surrounding the Xce locus is rich in X specific sequences, or perhaps GC islands for the binding of a specific protein, these sequences should reveal themselves as more of this DNA is mapped and cloned. Two candidates for CpG island status were found in the course of this mapping. These were sites distant from the gene which contained recognition sequences for two or more of the rare-cutting CpG restriction enzymes used in these analyses. One, about 250 Kb upstream of the gene contains sites for both XhoI and NarI, and another, about 350 Kb downstream of the gene contains sites for both NarI and SalI. These "islands" of unmethylated CpG dinucleotides could be associated with neighboring genes (Bird,1986) or could be local controlling elements for the spreading and/or maintenance of inactivation (Riggs et al.,1985). Clearly if methylation is implicated in some aspect of inactivation, these CpG islands, whether associated with genes or not, could be important.

Pulsed field gel electrophoresis has enabled the analysis of huge tracts of DNA, such as the human muscular dystrophy gene (Van Ommen et al.,1986; Kenwrick et al.,1987; Burmeister and Lehrach,1986) and pseudoautosomal region (Dunham et al.,1987; Lawrance et al.,1987), thus closing the gap between classical genetic analyses and previous molecular biology methods. It should be remembered, however, that this technique has some limitations. The use of "rare-cutting" enzymes, usually six-cutters which recognize one or more CpG dinucleotides, leaves open the possibility that a given restriction fragment length difference is due to methylation differences in the samples tested. The generation of multiple bands in a digest due, perhaps, to partial methylation of the DNA, while useful for the detection of multiple sites, can also complicate the analysis of results. The future availability of more methylation-insensitive

8-cutter restriction enzymes, such as SfiI, will enable clearer mapping results. It has been found here and by other investigators (Van Ommen and Verkerk,1986) that some restriction enzymes fail to give detectable signals with particular probes. Possibly the fragments generated are too large or too many to be detected on the average pulsed field gel. Another important limitation of these gels is that fragment size differences of about 20 Kb or less are usually not detected on the average gel. This means that small insertions or deletions would not be noticed. Differences in the enzyme recognition sequences themselves might be rare, since the recognition sites occur primarily in CpG islands, and these islands could have some regulatory function and be conserved. It is important therefore, that long range physical mapping is accompanied by cloning and walking techniques, particularly in regions of interest, such as around genes and CpG islands.

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