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**AN INVESTIGATION INTO THE
SIGNIFICANCE OF HISTONE
METHYLATION IN VARIOUS MOUSE
TISSUE**

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ABSTRACT

Histone lysine methylation is a post synthetic modification that occurs on the N-terminal tails of histones H3 and H4. This modification occurs at very specific residues, which have been highly conserved throughout evolution. It has been postulated that histone methylation may be involved in the pre-mitotic condensation of chromatin. Others have speculated that it may be involved in transcription. This modification seems to be a general feature of chromatin and occurs in all eukaryotic organisms ranging from mammals to yeast. The functional significance of histone methylation and of proteins in general is still at this stage not very well understood

In the first part of this project the levels of histone lysine methylation in various tissues were investigated in order to establish a correlation between the methyl content and cell division, age and differentiation. It was found that the levels of histone lysine methylation were low in the two tissue culture lines that were investigated correlating with the rapid growth rate of these cells. An increase in the histone lysine methylation content was observed when these cells were induced to differentiate. The highest levels of histone lysine methylation were found in brain tissue. Methylation levels in the histones isolated from the mouse and bovine brain tissue are very similar. It was concluded from these results that histone lysine methylation may be linked to the cessation of replication and differentiation of cells.

Previous studies on the rate of methylation in rat brain (Lee and Duerre, 1974) have shown that the rate of histone methylation decreases with age of the tissue. It was therefore decided to investigate the rate of histone methylation in nuclei isolated from mouse erythroleukemia (MEL) cells at different stages of differentiation. These cells can be induced to differentiate upon which they cease to replicate and mature into cells that are analogous to red blood cells. The rate of incorporation of radioactive methyl groups into the histones was determined in nuclei isolated from rapidly growing cells and cells that had been induced to differentiate for one and four days. It was established that only histones H3 and H4 are methylated at significant levels. The highest rate of histone methylation occurred in histone H3, and to a much lower degree, H4. This rate was maintained for the first day and decreased significantly

after the fourth day after induction. Even after the fourth day after induction, the rate of histone H3 methylation remained significantly higher than that of the other histones. It was concluded that histone methylation proceeds for a substantial amount of time after all DNA replication has ceased.

In the final part of this project the effect of the level of histone methylation on the stability of core particles, chromatin and nuclei was investigated. The core particles and chromatin, isolated from tissues with different levels of histone lysine methylation, were 'melted' using UV thermal denaturation. No differences were found. It was therefore concluded that histone lysine methylation did not increase the stability of the DNA of core particles or of soluble chromatin from these samples.

Differential scanning calorimetry was used to study the denaturation of nuclei isolated from mouse brain and MEL cells that have high and low levels of histone lysine methylation respectively. The "melting profiles" of the two types of nuclei seem to indicate that structural differences exist. Due to the complex structure of a nucleus it is uncertain whether these differences could be ascribed to the differing levels of histone lysine methylation.

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1 INTRODUCTION

The cell is the most basic building block of all life forms. All cells contain deoxyribonucleic acid (DNA). DNA is an informational macromolecule that possesses the capacity for self-replication. It regulates the transcription of defined subsections of its sequence (genes) into RNA, which can be translated into proteins. More complex organisms (eukaryotes) contain a cell nucleus into which vast quantities of this DNA is packaged in such a manner that it remains condensed, yet remains accessible to relatively large transcription factors and RNA and DNA polymerases. Over the past few decades, researchers have gradually begun to piece together the puzzle of how the cell has managed to accomplish this extremely complex task.

The DNA is condensed into the nucleus via several orders of folding. The first order of folding is the nucleosome, which constitutes the basic repeat unit of chromatin. The nucleosome consists of up to 200 base pairs of double stranded DNA wrapped around a core of four histone proteins; H2A, H2B, H3 and H4. At present the basic structure of the nucleosome is fairly well understood. This is partly due to the recent determination of the x-ray structure of the nucleosome at 2.8Å resolution (Luger *et al*, 1997).

The next order of structure or folding is still under debate. The majority view is that the nucleosomes are folded to form a solenoidal 300Å fibre under physiological conditions. The 300Å fibre consists of six to eight nucleosomes per turn with a pitch of 110Å and a diameter of 300Å. This 300Å fibre is only formed when a fifth histone type, the lysine rich histones, H1/H5, is present, indicating that they play a crucial role in condensation. However there have been some reports that condensation can occur without H1, for instance in high salt concentrations (Thoma *et al*, 1979).

In addition to H1, the tails of the core histones seem to be involved in the stabilisation of this condensed fibre. This was clearly demonstrated in

experiments by Allan *et al* (1982) in which H1 was removed from chicken erythrocyte chromatin followed by trypsin digestion that removed the tails of the core histones. This trypsinised chromatin was unable to condense into the 300Å fibre after the addition of histone H1 or H5. In the control experiment the untrypsinised chromatin yielded the correct fibre.

The 300Å fibre is further folded or coiled into thick fibres present in chromosomes. At low ionic strength these thicker fibres of the chromosome unfold to form extended loops of the 300Å fibres. These loops are probably attached to nuclear scaffold structures that consist of non-histone chromosomal proteins and may be involved in regulating condensation of chromosomal domains.

The nucleosome in more detail

As the nucleosome is the basic structural unit of chromatin, knowledge of its structure is important in the understanding of its dynamic role in the structure and function of chromatin. Many researchers have attempted to elucidate the structure of the nucleosome. The recent determination of the crystal structure of the nucleosome core particle at 2.8Å resolution (Luger *et al*, 1997) greatly increased our knowledge of the structure to near atomic detail. This has facilitated our understanding of how the nucleosome core is assembled (Rhodes, 1997).

The nucleosome consists of 146 base pairs of DNA wrapped around the histone octamer (two of each of the four core histones) in 1.65 turns of a flat, left-handed superhelix (Luger *et al*, 1997). The histone octamer consists of a (H3-H4)₂ tetramer and two H2A-H2B dimers. H2A and H2B are lysine rich histones whilst H3 and H4 are arginine rich histones. The overall charge on the histones is positive at physiological pH with a concentration of basic amino acid residues at the N-terminal tails.

The amino acid sequence of all the histones is highly conserved. The degree of conservation is extremely high in histones H3 and H4, somewhat less in H2A and H2B and the least in H1. These differences in the sequence conservation of the histones may suggest different functional roles for the different histones.

Histones are synthesised in the cytoplasm and transported to the nucleus where they are assembled into nucleosomes. The histones undergo several post-synthetic modifications, which include acetylation, phosphorylation, ubiquitination, ADP-ribosylation and methylation. The modification of histones may alter the structure of chromatin by altering the weak protein-protein and protein-DNA interactions at strategic sites. These modifications may thus trigger important structural and functional changes in the chromatin. It is also conceivable that the various modifications could act in a synergistic or antagonistic manner in modulating the structure of the genetic material. For example methylation of a lysine residue would prevent acetylation at the same site. Acetylation and methylation predominantly occurs at the N-terminal tails of the histones. The crystal structure of core particles at 2.8 Å resolution has shown that the N-terminal tails of the histones extend from the nucleosome. Therefore, they are not in contact with the DNA of the core particle as previously postulated. This finding together with experimental evidence that the tails were shown to be involved in formation of the 300Å fibre (Allan *et al*, 1982), seem to suggest that the N-terminal histone tails may be involved in higher order structure of nucleosomes.

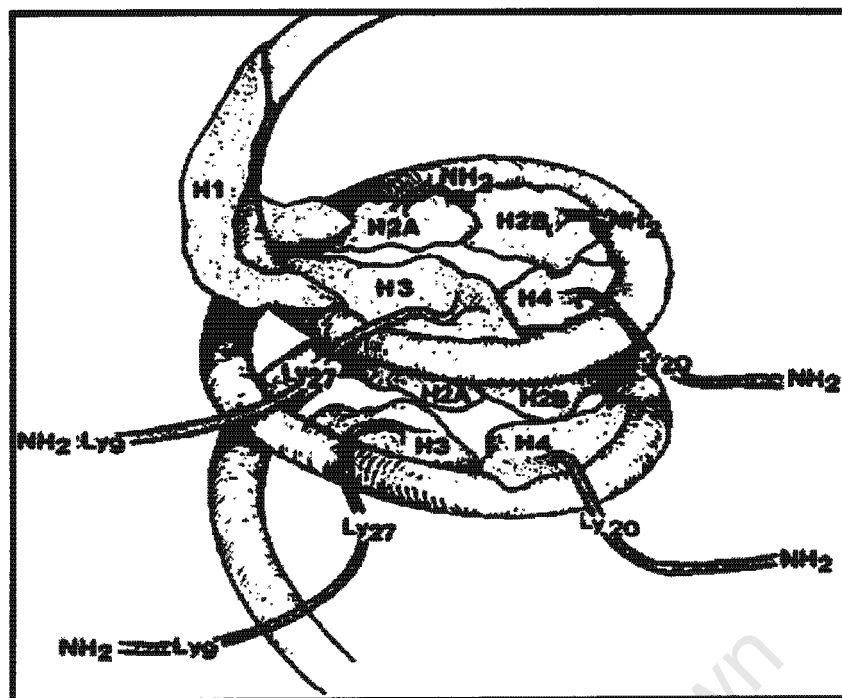


Fig 1 Possible arrangement of histones within the nucleosome (Paik and Kim, 1990, pg. 127).

Histone Acetylation

There are two types of histone acetylation. The first is the acetylation of N-terminal serine residues which occurs in the cytoplasm (Oliver, 1974). This usually happens on newly synthesised histone H4. This type of acetylation seems to be involved in deposition of histones onto the chromatin (see Grunstein, 1997, for a review). The second type of acetylation occurs in the nucleus on the ϵ -amino group of N-terminal lysines of the core histones. These acetylation sites are highly conserved. Acetylation is a dynamic process and in some cases turns over rapidly due to the presence of both acetylases and deacetylases (Twaits *et al*, 1976). Acetylation has been correlated with transcriptional activity while hypoacetylation has been linked with transcriptional repression (Turner, 1993). It has been postulated (Wolffe, 1997) that acetylation reduces the net positive charge and consequently weakens electrostatic interactions between histones and DNA,

thereby allowing transcription to occur. Others believe that hyperacetylation modifies the higher order structure of the chromatin (van Holde, 1996).

Histone methylation

K. Murray first observed the presence of methylated lysine residues in histones in 1964. Since then many researchers have attempted to investigate the functional significance of this chemical modification. Despite these efforts very little is known as to the structural and functional significance of histone methylation in chromatin.

Methylation is one of the postsynthetic modifications of histones, which occurs on the basic N-terminal tails of mainly histones H3 and H4. The sites of methylation have been highly conserved in a large number of uni- and multi-cellular organisms. Histone H4, with only two known exceptions, is methylated at lysine residue 20. H3 is always methylated at lysine residues 9 and 27, but there have also been reports of methylation of lysines at sites 4 and 36 (van Holde, 1989). See table 1 for a list of some of the known sites of lysine methylation in histones H3 and H4.

Table 1 Methylation sites in Histones H3 and H4

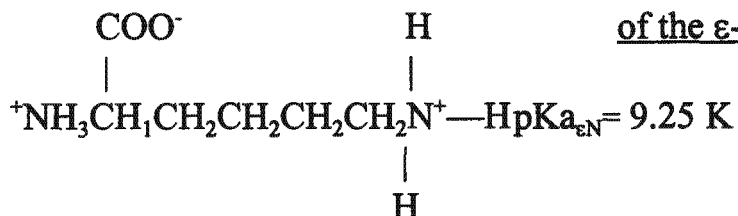
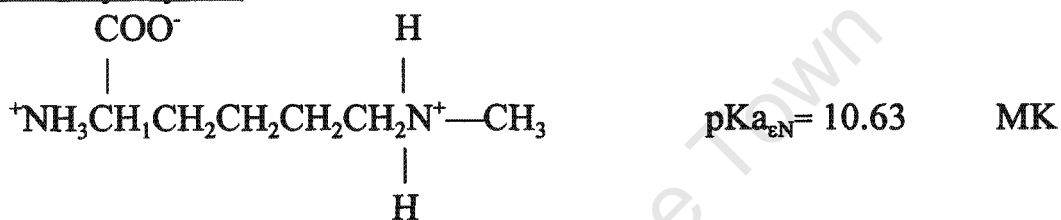
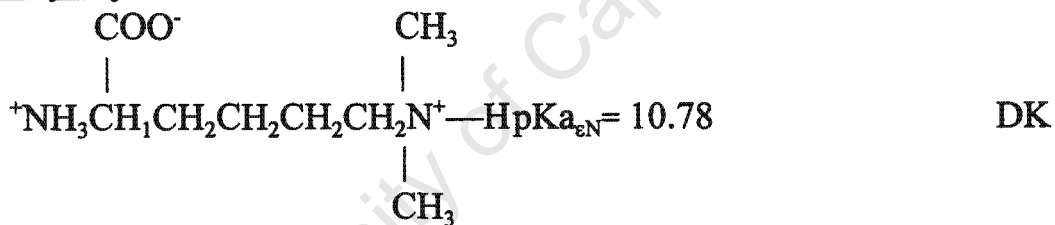
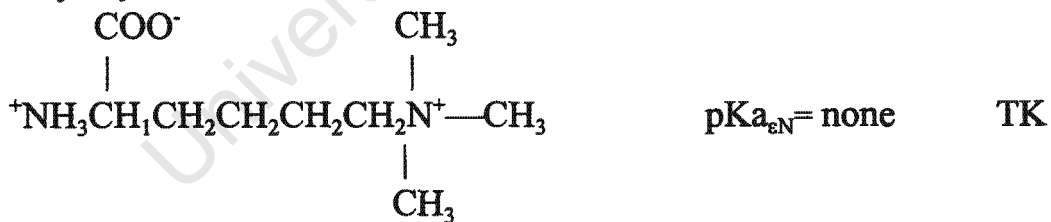
Organism (tissue)	H4	H3	Reference
Human (spleen)	20	9,27,36	Hayashi <i>et al</i> (1982) Ohe and Iwai (1981)
Calf (thymus)	20	9,27	DeLange <i>et al</i> 1969b,1973
Chicken (erythrocytes)	20	9,27,36	Urban <i>et al</i> (1979) Brandt and von Holt (1974)
Trout (testes)	20	4,9,27,36	Honda <i>et al</i> (1975b)
Sea urchin (sperm)	20	9,27,36	Wouters-Tyrou <i>et al</i> (1976) von Holt <i>et al</i> (1979)
Pea (embryos)	None	9,27	Patthy <i>et al</i> (1973)

Reference van Holde, 1989, pg. 120

Methylated Lysine amino acid derivatives

Lysine

Dissociation constant
of the ϵ -amino group Abrev.

Monomethyl LysineDimethyl LysineTrimethyl Lysine

Ref: WK Paik and S Kim, in Protein Methylation (ed. A Meister) John Wiley & Sons, New York, 1980, 69

Methylation is an enzymatic reaction performed by three classes of enzymes. Methylation of lysine residues in histones is the result of the activity of Type III methylases, also referred to as protein-lysine methyltransferases. This enzyme is found exclusively in the nucleus. The histone lysine methyltransferase is tightly bound to the chromatin, consequently complicating the characterisation of the enzyme. Attempts to purify the enzyme usually cause it to lose its activity. The lysine methylase preferentially methylates the ϵ -amino group of lysines. Histone methylation seems to be a progressive reaction. Wallwork *et al* (1977) showed that methylation of lysine progresses stepwise from mono- to di- to trimethyllysine, all performed by the same enzyme. Waterborg (1993) came to the same conclusions during his studies on the incorporation of labelled methyl groups into Alfalfa histone.

The isolated enzyme also methylates arginine residues (Aniello *et al*, 1989). This loss of specificity seems to indicate that the enzyme must be in a specific conformation and/or environment for methylating lysines in histones H3 and H4. This is also apparent in studies (Wallwork *et al*, 1977) in which free histones from both young and old rats could be methylated to similar degrees, whereas when they were complexed in nucleosomes significant differences were found. It has also been established that these methyl groups are fairly permanently incorporated into the histones as the half-lives of isotopically labelled histones and methyl groups are very similar. A histone lysine demethylase enzyme has been purified from rat kidney by Paik and Kim (1973) suggesting that there may be an occurrence of a limited and localised turnover of methyl groups in histones.

In addition to the type III methylase, a second methylase, type II, has been extensively characterised. This enzyme methylates the β and γ carboxyl groups of aspartic and glutamic acid respectively. At this stage there is little proof that this enzyme methylates histones. A complicating factor in studying the products of type II methylases is that they are acid-labile and are therefore lost during histone isolation procedures (Paik and Kim, 1971).

There are conflicting reports as to whether addition of a methyl group onto lysine and arginine residues in histones increases or decreases their affinity for DNA. Byvoet and Baxter (1975) have found that increasing methyl groups on the ϵ -amino group of lysine leads to a progressive decrease in charge density. The non-polar methyl group does not change the charge on the amino group but enhances the hydrophobicity of the lysine residue and decreases its ability to form hydrogen bonds. This increase in hydrophobicity may enhance the ability of the basic histone tails to condense chromatin into a more compact and inactive structure.

Histone methylation occurs in the N-terminal tails of mainly H3 and H4. These tails, particularly of the (H3-H4)₂ tetramer, stick out from the solenoid and appear to be involved in the stability of the solenoid and in generation of the DNA supercoil (see Allan *et al*, 1982). Methylation could alter the interaction of the tails with the DNA (nucleosome-DNA interaction) and modulate the nucleosome structure. Alternatively it could be involved in altering nucleosome-nucleosome interactions and thus be involved in altering the higher order structure of chromatin. The crystal structure of the nucleosome to 2.8Å resolution (Luger *et al*, 1997) has revealed that the histone tails extend from the nucleosome and may interact with neighbouring nucleosomes favouring the latter hypothesis. Methylation may also be involved in modulating the ability of histones in packaging DNA with different physical properties based on variation in the local composition. Large differences in the flexibility as a function of composition exists. There is conflicting experimental evidence in the literature indicating that histone methylation may be involved the regulation of transcription of DNA on the one hand or that it plays a role the general packaging of DNA on the other.

It has been postulated that histone methylation may be involved in nucleosome assembly and condensation of the chromatin during mitosis. Histone methylation has been shown in many cases to have little turnover (Shepherd *et al*, 1971, Byvoet *et al*, 1972, Honda *et al*, 1975) and it appears that this modification is a widespread rather than a localised event in chromatin. An increase in methylase activity appears to be correlated with mitosis in synchronous cell cultures. Many

researchers have investigated methylation patterns in relation to the cell cycle. Tidwell *et al* (1968) did studies on methylation of histones in regenerating rat liver and found that maximal histone methylation took place after the majority of DNA synthesis had occurred. Shepherd *et al* (1971) also found that histone methylation occurred mainly in the G2 phase of the Chinese Hamster ovary cell cycle. Similarly, in HeLa S-3 cells very little methylation occurs during the cell cycle until the end of the S-phase when methylation suddenly increases twofold (Borun *et al*, 1972). Methylation reaches a peak approximately 3 hours after DNA-synthesis with a parallel increase in methylase III activity. This increased methylation coincides with the condensation that starts in G2 and continues through to metaphase in mitosis, suggesting that there may be some link between the two. Honda *et al* (1975) has also proposed that methylation of histones plays a role in chromatin condensation or mitosis. Several reports argue against histone lysine methylation playing a more global role in chromatin structure. Instead it has been suggested that histone methylation may be more dynamic and therefore involved in transcription (Borun *et al*, 1972, Hempel *et al*, 1979).

Henzel and Davie (1989 and 1991) found a correlation between transcriptionally active chromatin and increased levels of histone methylation. They suggest that methylation may therefore be involved in helping to maintain a transcriptionally active and unfolded state. They also found that acetylated species of H3 and H4 that are complexed with active genes are selectively methylated. Thus lysine methylation might provide a way to modulate the potential for histone acetylation, which is associated with transcriptionally active chromatin. It is possible that acetylation may occur before histone methylation, thus increasing the accessibility to methylase enzymes. Therefore these two modifications may be linked whereby one influences the other. For example dimethylation of the ϵ -amino group (tertiary amino group) would block the acetylation of this lysine residue. Waterborg (1990) did studies on the acetylation and methylation of histones H3.1 and H3.2 from Alfalfa. He found that in general the lysines of the N-terminal region of H3 were either methylated or acetylated. The three major sites of

acetylation that were identified were lysines 14, 18 and 23 while the major sites of methylation were lysines 4, 9 and 27 (Brandt and von Holt (1974)).

Apart from the most common sites of methylation in H3 and H4, there have been reports on methylation of other sites and in other histones. However these cases seem to be the exception rather than the rule.

Natural occurrence of methylated lysine residues of H1 has only been reported in the eukaryote *Physarum* (Jerzmanowski and Maleszewski, 1985). However H1 from calf thymus has been shown to be a substrate for lysine methyl transferase III isolated from *Euglena gracilis*. In *Physarum Polycephalum*, which undergoes synchronous cell division, H1 is methylated between late S phase (when newly synthesised histones are deposited in an unmodified state onto the chromatin) and mid-G2 phase, which is just prior to super-phosphorylation that occurs just before the mitotic phase. This once again shows that post-synthetic modifications are linked to chromatin condensation, which occurs just prior to and during mitosis.

Heat shock (37°C) in *Drosophila* changes the pattern of histone methylation (Camato and Tanguay, 1982, Arrigo, 1983 and Desrosiers and Tanguay, 1988). There is a rapid increase in the level of H2B methylation; consistent with observations that histone methylation may not be such a static process as was originally thought. A proline residue at the N-terminal region of histone H2B is methylated during the heat shock. Furthermore heat shock induces a decrease in the level of lysine methylation and new methylation of arginine residues in H3. It is postulated that these changes in methylation may be involved in the restructuring of the chromatin in order to inactivate certain genes in response to the stress. Heat shock also blocks cell replication and a gradual decrease of DNA synthesis occurs. Inhibition of synthesis of heat shock proteins had no effect on the methylation changes, suggesting further that these changes could be involved in the extensive transcriptional regulation that occurs in these cells during heat shock. Heat shock also induces de-acetylation of the core histones, which resumes slowly when the cells return to 25°C (Camato and Tanguay, 1982, Arrigo, 1983 and Desrosiers and Tanguay, 1988).

Waterborg *et al* (1983) have reported that histone H4 in *Physarum polycephalum* is partially methylated at lysine in position 79. This residue is located in the loop (L2) that connects two α -helices. It occurs next to an arginine R78 and although it is located close to the DNA backbone, faces away from it and makes hydrogen bonds with aspartate D85 of H4 (Luger *et al*, 1997).

In summary, it would appear that we do not yet have a clear picture of the structural and functional significance of histone methylation. It seems unlikely that histone methylation is a random event with no functional significance as it occurs on specific lysine residues, which have been highly conserved through evolution. The fact that methylation occurs on the tail region of histones makes it all the more likely that it may be linked to the modulation of the chromatin structure. It is speculated that the terminal ends of histones are involved in the dynamic aspects of nuclear function as well as the more static aspects of overall chromatin structure (i.e. Luger *et al*, 1997).

The purpose of this research project was to investigate the distribution of histone lysine methylation in various tissues and cell types. This was done on cells at different stages of differentiation with different levels of cell replication, and on tissue of different ages, in order to determine any correlation. The rate of histone methylation was then measured at various times after mouse erythroleukemia (MEL) cells had been induced to differentiate. Finally, the effect of histone methylation on chromatin stability was studied using UV thermal denaturation and calorimetry.

2 AN INVESTIGATION INTO THE LEVELS OF METHYLATION OF H3 AND H4 FROM VARIOUS TISSUES USING AMINO ACID ANALYSIS

2.1 SUMMARY

1) In the first part of this research project the distribution of methylated lysines residues in histones H3 and H4 isolated from various mouse tissues was investigated.

The amount of ϵ -N mono-, di- and trimethyllysine present in histones H3 and H4 was determined in mouse brain, liver and spleen, and compared to that from an actively growing (mouse erythroleukemia, MEL) cell culture line as well as various other mouse tissues.

Total histones were acid extracted and then acetone precipitated from purified nuclei of each sample. The total histones were separated by high performance liquid chromatography (HPLC) and the fractions containing H3 and H4 were analysed for methyl lysine content¹ by amino acid analysis. It was found that the total methylation (i.e. ϵ -N mono-, di-, and trimethyllysine) content of the H3.1 histone variant from the rapidly dividing MEL cells was 7.2%. This was lower than the levels found in mouse tissues, which ranged from 15.0%, 17.9% and 19.2% for spleen, liver and brain respectively. A similar trend was found in H3.2/H3.3 variants (which were analysed together) where the methyl lysine content was found to be 12.2% in MEL cells and increased to 15.3% in spleen, 22.0% in liver and 23.9% in brain. Methyl lysine content of H4 increased from 7.3% in MEL cells to 8.8% in liver tissue and 12.5% in brain tissue.

¹ 'methyl lysine content' does refer to ϵ -N mono-, di- and trimethyllysine content from here on.

The rate of histone methylation at various stages of differentiation of MEL cells was also investigated. The addition of hemin induces these cells to differentiate into erythrocyte-like cells, which have the ability to synthesise hemoglobin. These cells then cease to divide and die within a week after the hemin addition. Methyl lysine content of H3 and H4 was examined in MEL cells that were rapidly dividing as well as from MEL cells that had been induced to differentiate for four days. Methyl lysine content of the H3.2/H3.3 variants was found to increase from 12.1% in uninduced MEL cells to 15.1% in induced cells. No significant increase was noted for the H3.1 variant.

3) Methyl lysine content of histone H3 was also examined in mouse olfactory (OP4) cells, another immortalised cell culture line. These cells grow optimally at 33 °C and cease to divide when the temperature is increased to 39 °C. Methyl lysine content was examined in H3 histone isolated from cells growing at 33°C and cells that had been growth arrested at 39°C for one month. The total methyl lysine content of H3 from actively growing cells was 11.6%, which is similar to that found for MEL cells. After cells had been arrested for one month the methyl lysine content of H3 histone increased to 16.4%. These cells retained their ability to divide after reducing the temperature back to 33 °C after a month of growth arrest.

4) The ϵ -N methyllysine content of H3 and H4 isolated from brain tissue of another species, viz. ox and foetal calf brain was also investigated and compared to the results obtained for mouse brain. The brain tissue was separated into crude preparations enriched in neuronal and glial cells. Methyl lysine content was found to be in the region of 20%-23% as in mouse brain tissue, and there was no significant difference in the preparations enriched in neuronal and glial cells.

2.2 RESULTS

2.2.1 An investigation of the levels of lysine methylation of histones H3 and H4 in various mouse tissues

2.2.1.1 Isolation and purification of histones H3 and H4 from mouse liver and brain and MEL cells

Balb C mice of no specific age were used in this study. The mice were starved overnight and then killed by cervical dislocation. Liver, brain and spleen were removed immediately. These tissues were homogenised in cold, buffered sucrose and nuclei were harvested by centrifugation, as described in materials and methods. A similar procedure was used to isolate nuclei from mouse erythroleukemia (MEL) cells that were grown to logarithmic phase in suspension cultures.

Total histones were acid extracted from the purified nuclei of each sample with H_2SO_4 . The histones were then precipitated in acetone, collected by centrifugation and dried. These precipitates were analysed by denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). The gels confirmed that purified total histones were obtained from each sample (see figure 2).

Individual histones were purified by high performance liquid chromatography (HPLC) on a reverse phase C18 column using an acetonitrile gradient in trifluoroacetic acid (TFA) (figure 3). The histone H4 fraction was still slightly contaminated with H2A and was therefore further chromatographed as before but with a C4 column and substituting TFA with heptafluorobutyric acid (HFBA).

Purity of separated histones was assessed and confirmed by means of gel electrophoresis. A SDS polyacrylamide gel of purified histones H3 and H4 from MEL cell, mouse liver and mouse brain is shown in figure 4. Results showed that each fraction contained a single band, running in line with H3 or H4 of the total histones standard.

It has been postulated that the changes in levels of histone variants with age are related to the reduction in the number of mitotic cycles in a given cell population (Zweidler, Histone Genes, 1984). In order to establish the histone variant distribution in the various mouse tissues investigated, purified histone fractions were electrophoresed on polyacrylamide gels in the presence of a non-ionic detergent, triton-X 100 and 6 M urea (figure 5), which is sensitive to small conformational differences. Variants cannot be resolved via SDS gel electrophoresis due to the fact that they have nearly identical molecular masses.

The triton gel in figure 5 shows that the H3.3 variant is not present in MEL cells, but is present in increasing amounts in mouse liver and brain tissue respectively (compare for example lanes 4,8 and 14). See also table 7.

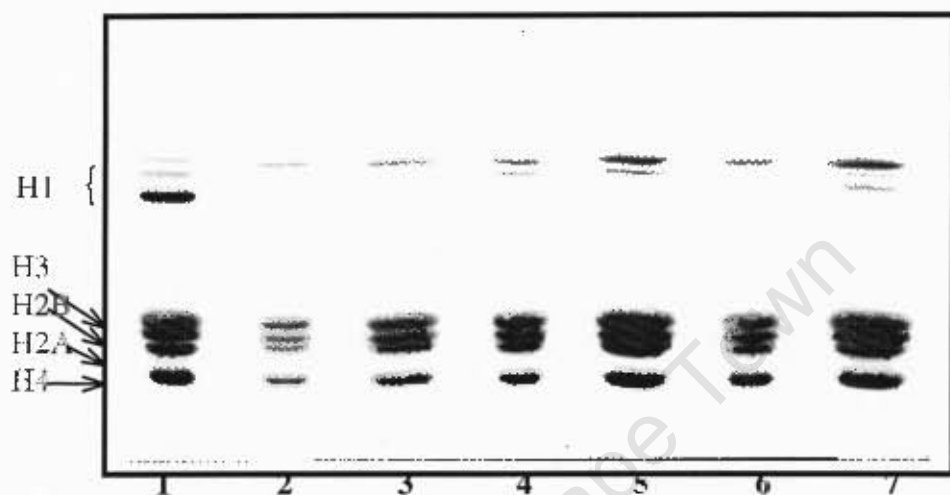


Figure 2: SDS PAGE gel to show purified total histones isolated from MEL cells, mouse liver and mouse brain. Total histones were acid extracted with H_2SO_4 from the nuclei of each sample. The histones were then precipitated with acetone, collected by centrifugation and dried. Lane 1-chicken std, lanes 2 and 3-brain histones, lanes 4&5-MEL histones, lanes 6&7- liver histones.

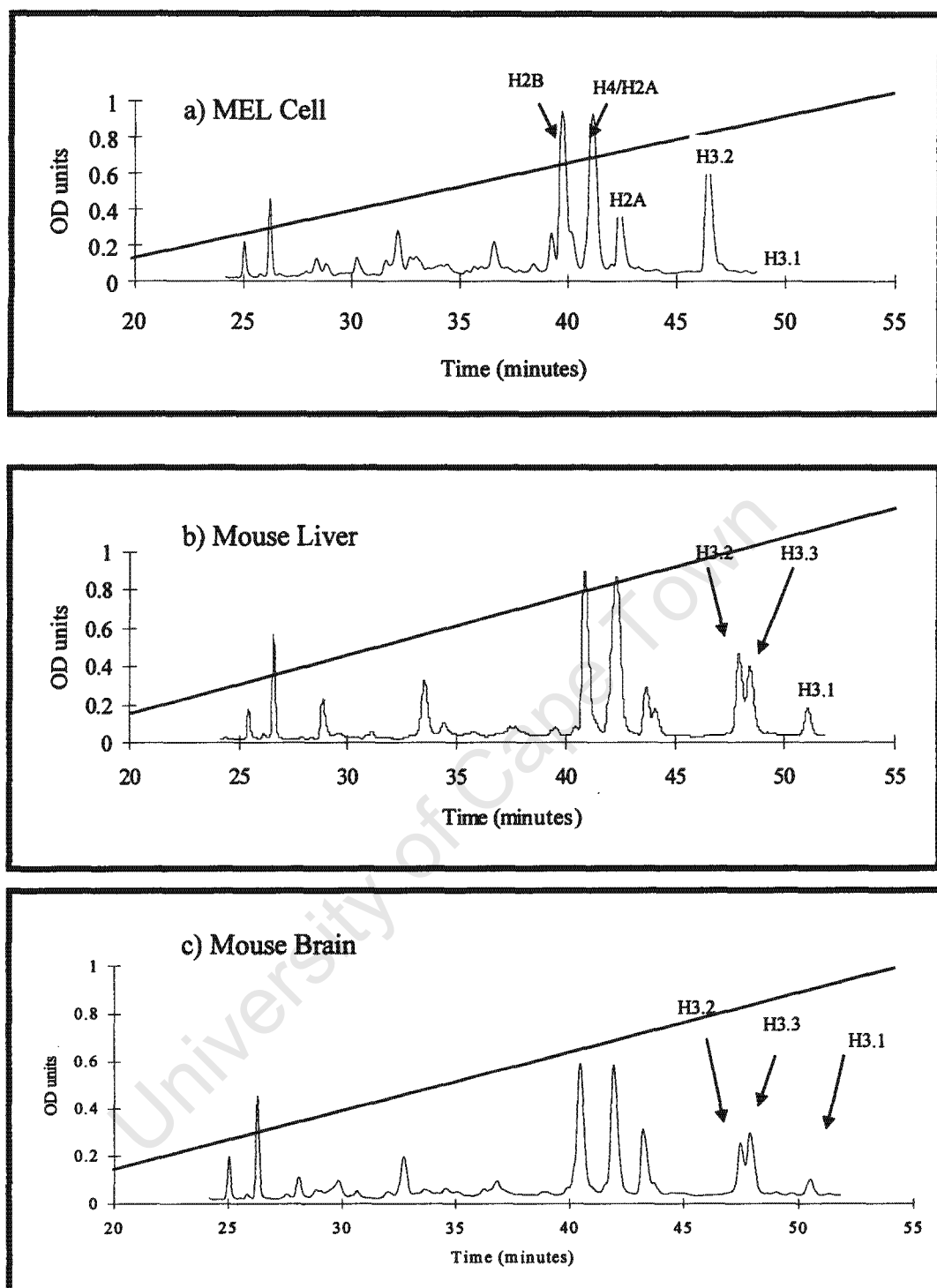


Figure 3 HPLC profiles to show the separation of purified total histones from MEL cell, mouse liver and mouse brain in parts a, b and c of the figure respectively. The histones were separated by reverse-phase HPLC on a C18 column, using an increasing acetonitrile gradient. The H4/H2A fractions were

purified further with a similar method, using a C4 column. The acetonitrile gradient (—) is given as the fraction as of the total eluent (0 to 1).

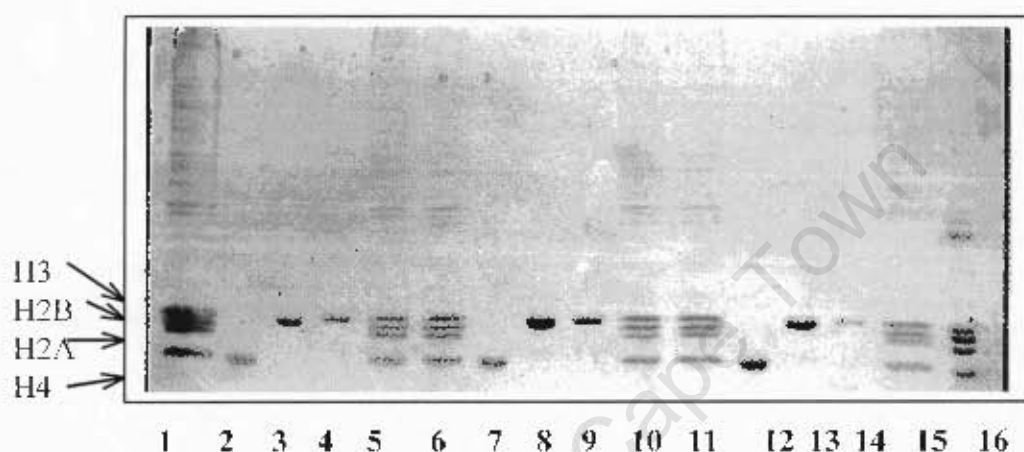


Figure 4 SDS PAGE gel of histones H3 and H4 from MEL cells, mouse liver and mouse brain, which were purified by HPLC. Lanes 1&5, MEL total histones; lane 2, MEL H4; lane 3, MEL H3.2 and H3.3; lane 4, MEL H3.1; lanes 6 & 10, liver total histones; lane 7, liver H4; Lane 8, liver H3.2 and H3.3; lane 9, liver H3.1; lanes 11 and 15, brain total histones; lane 12, brain H4; lane 13, brain histones H3.2 and H3.3; lane 14, brain histone H3.1; lane 16, chicken total histone std.

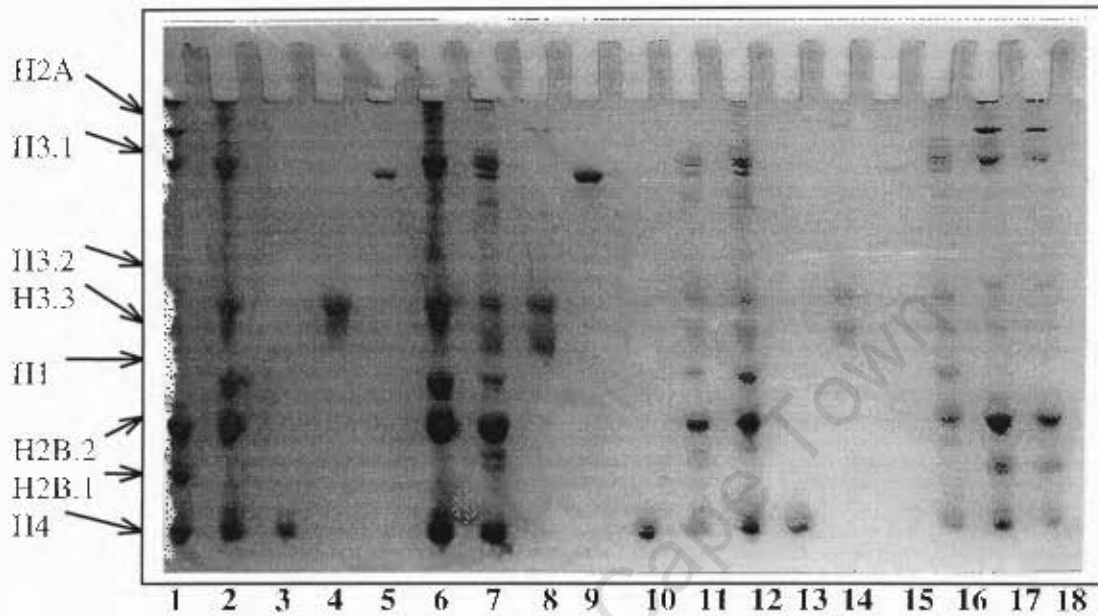


Figure 5 A triton-acid urea gel showing the histone variants of H3.1, H3.2 and H4, separated by HPLC, from MEL cell, mouse liver and mouse brain. The variants of these histones do not separate on SDS PAGE gels. Lanes 1, 17 and 18-chicken std, lanes 2 and 6 - MEL total histone std, lane 3 - MEL H4, lane 4- MEL H3.2, lane 5-MEL H3.1. Lanes 7 and 11 - Liver total histone std, lane 8 liver H3.2 and H3.3, lane 9- liver H3.1, lane 10- liver H4. Lane 12 and 16- brain total histone std, lane 13-brain H4, lane 14- brain H3.2 and H3.3, lane 15 - brain H3.1.

2.2.1.2 Methyl lysine content determination of histones H3 and H4 by amino acid analysis

Amino acid analysis was performed on hydrolysed samples of histones H3 and H4. Earlier studies have shown that methylation occurs mainly in these two histones. Reports on the presence of methyl groups in other histone types could not be confirmed by sequence analysis (van Holde, 1989).

Once purified, the histone fractions H3 and H4 were subjected to acid hydrolysis. The individual amino acids including those modified by methylation (i.e. ϵ -N mono-, di- and trimethyllysine) were separated by ion exchange chromatography using o-phthalaldehyde (OPA) post-column derivatisation methodology. The methyl lysine content of the histones was then calculated by determining the number of residues of the methylated lysines present relative to the histidine (see figure 6 for a typical HPLC profile of an amino acid analysis run). All variants of H3 as well as H4 possess two histidine residues. There are 13 lysine residues in H3 and 11 in H4 and these values were used to calculate the percentage methylation¹. The experimental values for non-methylated lysine were unreliable due to the fact that their concentration is always much higher relative to the methylated lysines and thus fell into the non-linear range. Furthermore, lysine reacts with OPA at both the α and ϵ amino groups resulting in quenching of the fluorescence due to proximity of the two aromatic rings. No quenching has been observed in the methylated lysines (Oates and Jorgenson, 1990).

Note 1: The %methylation is defined as the % of the total lysines residues per histone carrying one, two or three methyl groups that is monomethyl-, dimethyl and trimethyl-lysine.

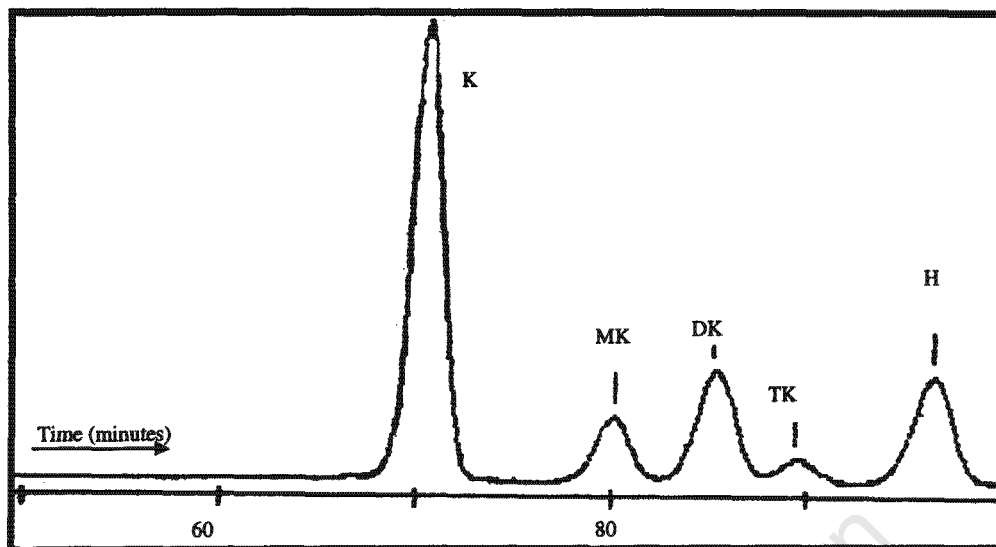


Figure 6a A typical HPLC profile of an amino acid analysis run showing the separation of purified H3 into mon- di- and trimethyl lysine (MK, DK and TK respectively, K is lysine and H is Histidine). This example is of mouse brain H3.1

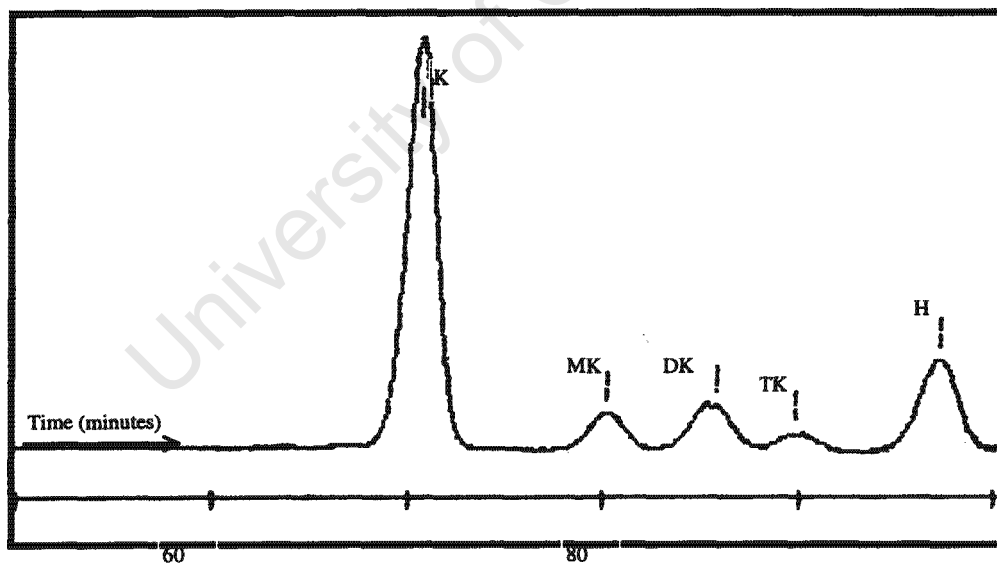


Figure 6b A typical profile of an amino acid analysis run of mouse histone H3 in MEL cells

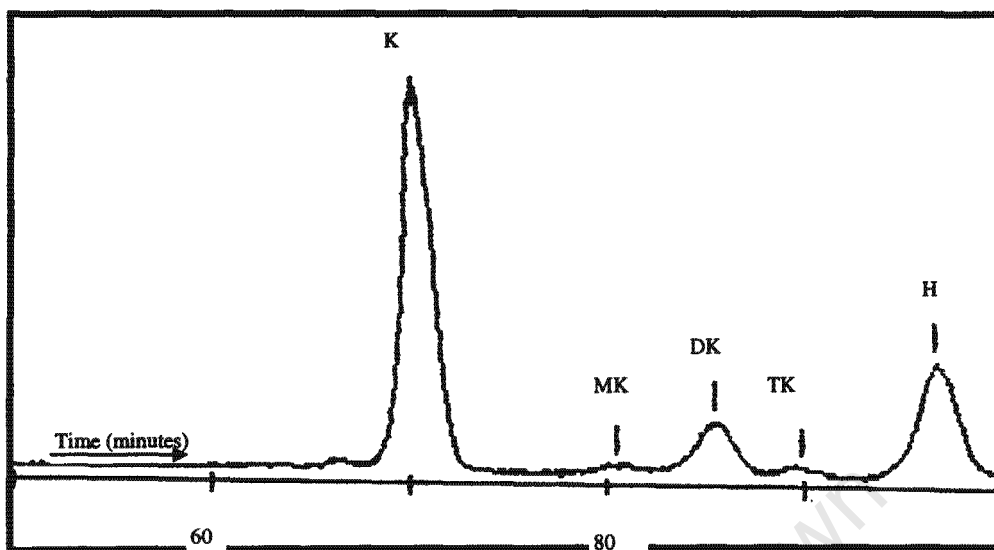


Fig 6c A typical profile of amino acid analysis of H4. (This example is mouse brain H4). Note the high relative amount of DK, characteristic of the H4 histones analysed, as compared to parts a and b of this figure.

Key to tables 2a-c on pg. 22

N	=	number of experimental determinations
n/d	=	not determined.
Std. Deviation	=	$(\sum X_i - X) / n$, where X_i = individual sample values; X = the mean value of n samples
% Deviation	=	$(\text{deviation} / X) \times 100$

Table 2a Methyl lysine content of histone H3.1 from various mouse tissues

Tissue Type	%Methylation	Std. Dev. (\pm)	% Deviation	N
MEL	7.3	0.7	9.6	3
Spleen	15.0	n/d	n/d	1
Liver	19.2	1.2	6.3	4
Brain	17.9	3	16.8	3

Table 2b Methyl lysine content of histone H3.2/H3.3 from various mouse tissues

Tissue Type	%Methylation	Std. Dev. (\pm)	% Deviation	N
MEL	12.2	1.3	10.6	4
Spleen	15.3	n/d	n/d	1
Liver	22.0	0.7	3.2	3
Brain	23.9	0.7	2.9	3

Table 2c Methyl lysine content of histone H4 from various mouse tissues

Tissue type	%Methylation	Std. Dev. (\pm)	% Deviation	N
MEL	7.2	0.5	6.9	4
Spleen	7.4	n/d	n/d	1
Liver	8.8	0.4	4.5	4
Brain	12.5	0.02	0.2	2

Each amino acid analysis result was taken from an average of at least two separate experiments except for the spleen, which was performed once. Results for each experiment are tabulated below. H3.2 and H3.3 was analysed together as they were difficult to separate.

The error in the determination of methylated lysines by amino acid analysis was found to be in the region of 5% to 10% (standard deviation). The larger error in histone variant H3.1 (table 2) is due to the fact that the quantities of this variant were very small (see figure 3) compared to the other histone variants (see materials and methods, section 6.1.10 for details on linearity).

Table 3 Summary of tables 2a-c to show the percentage methyl lysine content in histone H3 and H4 from different tissues of the mouse as determined by amino acid analysis

TISSUE TYPE	H3.1	H3.2/H3.3	H4
MEL	7.2	12.2	7.3
Spleen	15.0	15.3	7.4
Liver	19.2	22.0	8.8
Brain	17.9	23.9	12.5

The percentage methyl lysine content of H3.1 and H3.2/H3.3 was found to be lowest in MEL cells, with values of 7.2% and 12.2% respectively. The highest methyl lysine content was found in brain tissue where the percentage methyl lysine content was 23.9% for the H3.2/H3.3 variants.

2.2.2 An investigation into the levels of histone lysine content of H3 in cell culture cells

The observed correlation between the levels of histone methylation of H3 and H4 and the rate of replication was investigated in more detail using a cell line that can be induced to differentiate into mortal cells.

MEL cells are derived from the spleen of mice after transformation with the Friend's leukaemia virus. A photograph of the cells is shown in figure 7. These cells are precursor erythroid cells that have been blocked in their development to mature red blood cells. They are cancer cells and will continue to divide until they are induced to differentiate into mature cells resembling mature red blood cells, which then cease to divide. Examples of differentiating reagents are hexamethylenebisacetamide (HMBA), dimethylsulphoxide (DMSO), hemin, erythropoietin, sodium butyrate and many others. When cells become terminally differentiated there is an increased expression of some genes e.g. the genes involved in the production of haemoglobin. There is also a repression of certain other genes e.g. the genes involved in mitosis. Furthermore, the chromatin becomes condensed, cells become smaller, iron uptake is increased and membrane changes occur. Haemoglobin is produced, which causes the cells to stain blue on addition of the benzidine reagent (Ching Lo *et al*, 1981).

Growth patterns of MEL cells were studied in conjunction with differentiation of MEL cells with various inducing agents. Amino acid analysis was then performed on histone H3 isolated from induced and uninduced MEL cells in order to determine the methyl lysine content.

Cells that are actively dividing grow logarithmically, with a doubling time of approximately 14 hours. When cells are treated with hemin, they cease to divide after approximately one to two days (see figure 8). After cells have become

differentiated they can divide a maximum of four times (Neumann *et al*, 1978). There have been reports that there is maximal differentiation after four days in cells that have been treated with dimethylsulphoxide. (Richon *et al*, 1991, Peterson and McConkey, 1976).

One day after the addition of hemin the cells stained positive with benzidine. This together with the observation that the cells ceased to divide led to the conclusion that the cells had indeed become differentiated in the presence of hemin. Ching Lo *et al* (1991) investigated the effect of hemin on MEL cells and they found that excess heme that is not bound to the globin plays an important role in shutting down the expression of those genes that are involved in cell division. It is also important to note that although heme may be necessary, it is not sufficient to generate genuine red blood cells (Ching Lo *et al*, 1991).

During the experiment the medium of the hemin treated cells was not changed as the cell density remained between 4 to 20 X 10⁴ cells/ml and the nutrients in the medium were estimated to be enough to sustain the cells for the duration this study.

Other agents that were tested were sodium butyrate and DMSO. DMSO did not cause the cells to stain benzidine positive and the cell number decreased. The DMSO therefore did not cause the cells to differentiate and further experiments were abandoned. Sodium Butyrate at a concentration of 5mM (Reeves and Cserjesi, 1979) killed the cells almost immediately, possibly due to the fact that the concentration used was too high for these MEL cells. The only reagent that caused the cells to differentiate was hemin, which yielded cells that stained benzidine positive after one day. Figure 8 shows the graphical representation of the growth of the MEL cells before and after induction with hemin. From this graph it is apparent that the number of cells do not increase when cells differentiate into erythrocyte-like cells.

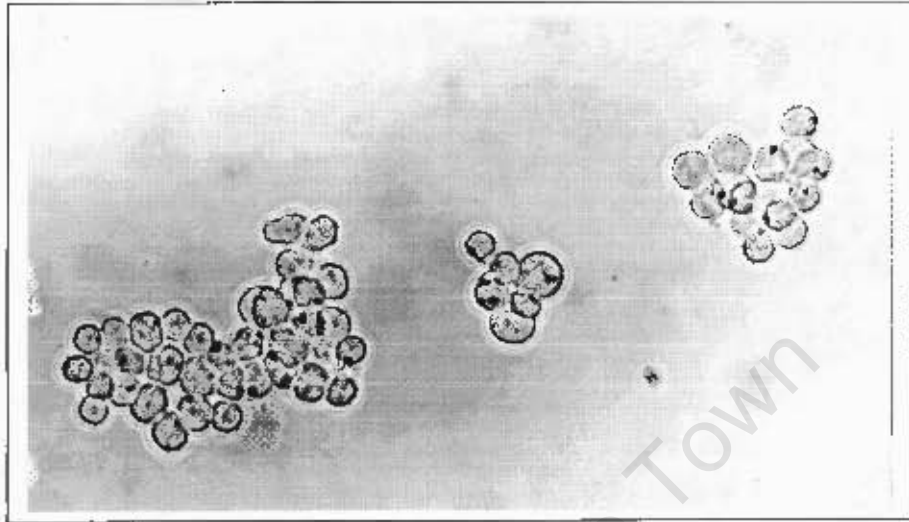


Figure 7 MEL cells taken at 400x magnification

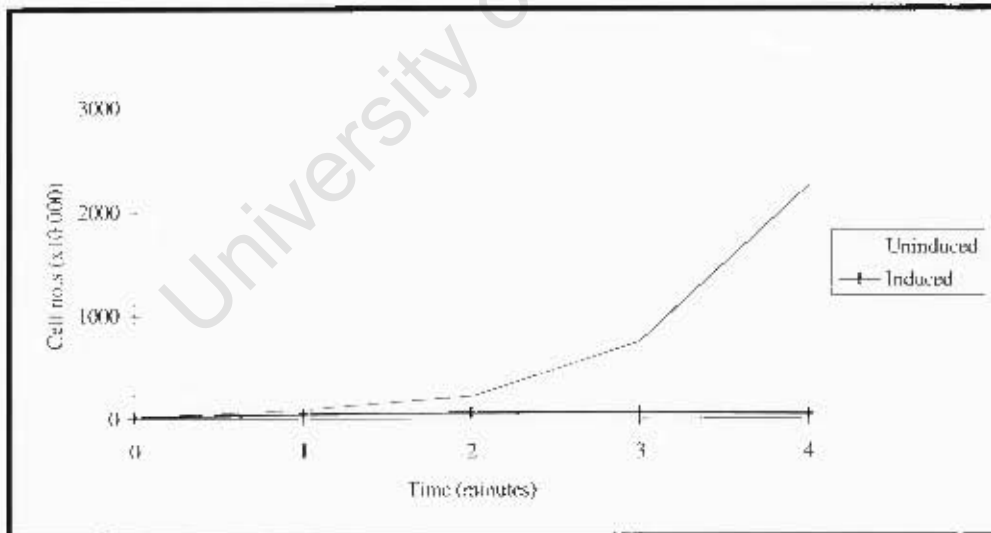


Figure 8 Growth curve of MEL cells in log phase (uninduced) and after induction with hemin (induced)

Table 4 The percentage lysine methylation of histone H3 from MEL cells

Cell type	H3.1	H3.2/H3.3
MEL uninduced	7.2	12.2
MEL induced	6.7	15.1

2.2.3 An investigation of the levels of lysine methylation of histones H3 & H4 from bovine brain tissue of different ages

Brain tissue was obtained from freshly slaughtered animals of ox and foetal calf. The tissue was then washed with cold saline and gently homogenised in buffered sucrose. Nuclei were obtained as described in materials and methods. These nuclei were then separated into crude preparations of neuronal and glial nuclei by differential sucrose centrifugation. From this point onwards the procedure for isolation of histones and analysis of histone methyl lysine content was the same as that used for all other samples. Amino acid analysis was performed once per sample.

Table 5 Amino acid analysis results showing percentage methylation of H3 and H4 from ox and foetal brain, neuronal and glial cells.

TISSUE TYPE	H3.1	H3.2/H3.3	H4
Neuronal Ox Brain	19.9	22.8	4.7
Glial Ox Brain	19.5	21.2	5.0
Neuronal Foetal Brain	21.1	19.4	5.4
Glial Foetal Brain	21.3	20.0	7.2

2.3 DISCUSSION

2.3.1 Methyl lysine content determination of histone H3 from various mouse tissues

The histones were extracted as described, and the levels of lysine methylation determined by amino acid analysis. Histone H3 was separated by HPLC into two fractions. The first fraction contained variants H3.2 and H3.3, while the last eluting fraction contained variant H3.1. Amino acid analysis was performed on the two fractions and the results are shown in Tables 2 and 3 (pp. 21, 22 and 23). From the tables it is evident that the percentage of histone lysine methylation of H3.1 variant is 7.2% in rapidly dividing MEL cells and increases to 15.0%, 19.2% and 17.9% in spleen, liver and brain tissue respectively. The difference in the percentage methylation between MEL cells and other tissues is more pronounced in the fraction containing variants H3.2/H3.3. In this case it increases progressively from 12.2% to 15.3%, 22.0% and 23.9% in MEL, spleen, liver and brain tissue respectively. In both cases the percentage methylation in MEL H3 histone variants is less than half of that found in brain. MEL tissue differs from all other tissues investigated in its rapid growth rate.

The distribution of mouse histone variants in various tissues has been investigated in great detail by Zweidler. The somatic variants are divided into four main groups. The two categories that are of interest here are the replication independent variants and the strictly replication dependent variants. The replication independent variants e.g. H3.3, are continually expressed at a low rate even in non-dividing cells. They tend to accumulate in differentiated cells as a function of time. The strictly replication dependent variants, e.g. H3.1 and H3.2, are induced at the beginning and repressed at the end of DNA synthesis, which occurs in S-phase (Zweidler, 1984).

The H3 histone variants H3.1 and H3.2 have identical amino acid sequences except that in H3.2 there is a serine at position 96 instead of a cysteine. Variant H3.3 has the same sequence as H3.2 with a substitution of Ile-Gly at positions 89-90 by Val-Met (Franklin and Zweidler, 1977), changing it to the more hydrophobic variant (see table 6, which gives a summary of the sequence differences of H3 variants in the mouse).

Table 6 The sequence differences of H3 variants in the mouse. The rest of the sequences are the same.

VARIANT	Positions 89, 90, 96
H3.1	V ₈₉ M ₉₀ C ₉₆
H3.2	V ₈₉ M ₉₀ S ₉₆
H3.3	I ₈₉ G ₉₀ S ₉₆

The triton gel in figure 5 shows the variants of H3 that were chromatographically separated by HPLC. Interestingly the H3.3 variant is not present in the MEL cells (lane 3). It has been well documented by Zweidler and colleagues (Histone Genes, 1984) that there is a change in expression of histone variants with age in the mouse liver. Specifically, with H3 there is an increase in the amount of H3.3 and a decrease in the amount of H3.2 that is present in the liver as a function of age. This change in levels of variants is thought to be related to the levels of mitosis, with the increase in the H3.3 variant being correlated with increasing age and a decreasing rate of cell division; i.e. H3.3 variant is independent of replication. MEL cells are immature cells that have a high rate of replication, which explains the absence of the H3.3 variant. The results show that the replication independent variants accumulate as tissues mature, for example in the brain.

Table 7 gives the relative amounts of each of the H3 variants present in each tissue type, as determined from the peak heights of HPLC profiles (figure 3). In MEL cells the predominant variant is H3.2, which represents 82% of the total. There is no H3.3 variant present, consistent with the high rate of cell growth. There is also a progressive decrease in the amount of H3.1 and H3.2 variants that are present in MEL, liver and brain tissues respectively and a parallel increase in the H3.3 variant. In liver, H3.2 comprises only 45% of the total with a concomitant increase in the H3.3 variant to 39%. There is a further increase in the amount of H3.3 to 48% in the brain. This indicates that the brain tissue should be the oldest with low levels of mitotic activity. In all three tissues studied here the methyl lysine content of the H3.2/H3.3 variants was found to be higher than that of the H3.1 variant. The increase of the H3.3 variant in liver and brain tissue may suggest that increasing methyl lysine content of H3.2/H3.3 could play a role in inhibiting the mitotic cycle of these cells. This may be a general event that proceeds as a function of time consolidating inactive chromatin into a permanent repressed state.

Table 7 Relative amounts of H3 variants present in different tissues as determined by heights of HPLC peaks.

	H3.1	H3.2	H3.3
MEL	18	82	0
Liver	16	45	39
Brain	12	40	48

From figure 3 it is apparent that H3.3 elutes later than H3.2 even though, unmodified, it is the less hydrophobic of the two (see table 6). This difference in the elution could be due to increased levels of methylation, as the addition of methyl groups increases hydrophobicity. Both H3.2 and H3.3 elute before H3.1, which is therefore the most hydrophobic. The difference in the levels of histone

lysine methylation that is observed in the H3.3/H3.2 variant suggest that these variant/s may be located in different environments to that of the H3.1 variant. It is possible the H3.3 variant is in a region that is more accessible to histone lysine methyl transferases.

The heights of the HPLC peaks of H3 were used as ratios for the determination of the total percentage methyl lysine that is present in each tissue (i.e. that of H3.1, H3.2 and H3.3 together). This is shown in the table below.

Table 8 Total methyl lysine content in all H3 variants and the percentage variation of lysine methylation between H3.1 and H3.2/H3.3 (Refer to tables 2a and 2b).

Tissue Type	% Total H3 Methylation	Diff. in % Methylation H3.1 – H3.2+H3.3
MEL	11.3	4.9
Spleen	15.2	0.3
Liver	21.6	2.8
Brain	23.4	6.0

Note 1: The %methylation is defined as the % of the total lysines residues per histone carrying one, two or three methyl groups that is monomethyl-, dimethyl and trimethyl-lysine.

An interesting observation in figure 6a and 6b is that the levels of mono- and dimethyllysine are more or less equal in MEL cell H3 histone whereas in liver and brain histones the amount of dimethyllysine is much higher than that of the monomethyllysine.

There are a total of thirteen lysine residues in H3. Amino acid sequence analysis of H3 histones revealed that 4 out of the 13 lysine residues are recipients of the

methyl moiety. It is thus tempting to speculate that complete methylation of four lysine residues would yield a maximum total methyl lysine content of approximately 30%. The observed 23.4% methyl lysine content, determined in the mouse brain (see table 8) therefore represents an 80% occupation of methylation sites (i.e. of all the possible lysine residues that can be methylated). The level of methylation of histone H3 in brain tissue is thus relatively high.

It has been shown (Lee and Duerre, 1974) that methylase activity is much greater in liver and brain extracts from young animals than that from old animals. This is consistent with the observation that methylation increases as cells mature. Histones H3 and H4 isolated from brain tissue, which is fully differentiated, have the highest levels of methylation and therefore should be poor substrates for the methylase enzyme as most of the accessible methyl sites on the lysines are already occupied. Histones H3 and H4 do not turn over in adult brain and this would imply that the existing histones have to become progressively more methylated.

Duerre and Chakrabarty (1975) investigated the levels of methylation of histones from various organs of the rat. They found that in all organs tested only H3 and H4 were methylated. H4 was predominantly dimethylated and H3 was mono- di- and trimethylated. They found that the ratio of ϵ -N mono-, ϵ -N di-, and ϵ -N trimethyl-lysine was in the approximate molar ratio of 0.55:1.0:0.35 and did not differ significantly from organ to organ. Shown below are the ratios that were found in the mouse liver and brain and MEL cell H3 variants, which can be compared to those found by Duerre and Chakrabarty.

Table 9 Distribution of the methylated lysine residues in histone H3 variants isolated from various mouse cells

Tissue Type	H3.1			H3.2/H3.3		
	MK	DK	TK	MK	DK	TK
MEL	0.98	1.00	0.24	0.89	1.00	0.37
Liver	0.45	1.00	0.36	0.55	1.00	0.28
Brain	0.42	1.00	0.20	0.53	1.00	0.14

Values given are relative to dimethylated lysine (DK). MK and TK correspond to the mono and trimethylated lysine derivative.

Table 10 Distribution of the various methylated lysine derivatives in histone H3 isolated from various mouse tissues.

Tissue Type	MK	DK	TK
MEL	0.41	0.44	0.13
Liver	0.27	0.55	0.18
Brain	0.29	0.61	0.10

Values given for the methylated lysine derivatives are expressed as fraction of total methylated lysines present for all the H3 variants.

Average ratios of mono- di- and tri- methylated lysines in liver are essentially the same as those found by Duerre and Chakrabarty (1975). In the brain however, the ratio of TK is much lower (0.10) and in the MEL the MK ratio is much higher than that found by Duerre and Chakrabarty, even though the total methylation content of MEL is much lower than that of liver and brain.

2.3.2 Methyl lysine content determination of histone H4 from various mouse tissues

H4 is almost always methylated at residue 20 except in the pea and *Tetrahymena*, where it is totally absent (van Holde, 1989, pg. 120). It has been suggested that the methylation of H4 involves a different mechanism to that of H3 (Thomas *et al* (1975), Sarnow *et al* (1981), Duerre *et al* (1982)). It is also possible therefore that methylation of H4 could have a different role to that of H3 methylation.

The methylation content of H4 was determined as described for H3 (table 3, pg. 23). Although only one methylation site has been reported in histone H4, results shown here suggest that more than one site may exist. With only one of the eleven lysines methylated, the maximum methylation that should be observed is 9.1%. Since 12.5% total methyl lysine content was found for the brain, it follows that there are approximately 1.4 sites that are methylated out of a possible 11. Although K20 is the only lysine on H4 that has been reported to be methylated, it is possible that K16, which also occurs in the N-terminal region, could also be methylated, as it is potentially accessible to methylases. However, this could not be confirmed by protein sequencing since H4 is blocked at the N-terminal.

In every case studied the H4 was mostly dimethylated. A table of the moles per mole of protein of ϵ -N mono-, di- and trimethyllysine is shown in table 11.

Table 11 Moles of ϵ -N mono-: di-: tri-methyllysine per mole histone H4

Tissue Type	MK	DK	TK
MEL	0.1	0.7	0.0
Liver	0.1	0.7	0.1
Brain	0.2	1.0	0.2

The K16 to N25 region of the positively charged N-terminal tail of H4 makes extensive contacts with the negatively charged dimer surface of H2A-H2B of a neighbouring nucleosome (Luger *et al*, 1997). Salt bridges and hydrogen bonds exist between the side chains at K16, R19, K20 and R23 of H4 that make six contacts with H2A and one with H2B. In nucleosomes, methylation at K20 in H4 could disrupt or modify these contacts, thereby altering the interaction between neighbouring nucleosomes.

2.3.3 Methyl lysine content of H3 from tissue culture cells

Histones were isolated from cells that were actively dividing as well as from cells that had been induced to differentiate for four days with hemin. The methylation content was determined as previously described.

The amino acid analysis results presented in table 4, show that in the H3.2/H3.3 variant the methyl lysine content increases when differentiation is induced. This is not the case with the H3.1 variant where the levels of methylation remain constant. As differentiation of the cells progresses, mitosis ceases rapidly and a concomitant change in the proportions of the H3 variants occurs. H2A and H2B variant changes occur much more rapidly (Grove and Zweidler, 1982).

When MEL cells are induced to differentiate several changes occur. The chromatin becomes condensed and the cells become smaller, which also suggests a link between condensation and histone methylation. However there is also an increase in transcription of certain genes in response to differentiation. For example, transcription of the β^{maj} -globin gene increases 5 to 100 fold (Reddy and Shen, 1993). There is also an increased synthesis of heme and various enzymes required for erythropoiesis. This could be interpreted to mean that histone methylation plays a role in transcriptional activation. The results of this experiment do not allow a conclusion as to whether histone methylation is involved in gene regulation or general packing of chromatin or both. During the differentiation of the MEL cells the bulk of the chromatin is remodelled into inactive heterochromatin and the more likely role of methylation is that it plays a role in this condensation. Studies by Reboulleau and Shapiro (1983) on the differentiation of MEL cells using circular dichroism (CD) spectra and thermal denaturation have also indicated that the process of cell differentiation involves several post synthetic modifications such as DNA and histone methylation.

The results presented here show that there is a correlation between the methyl lysine content of the histones, the stage of differentiation and age of differentiated tissue. Upon differentiation of rapidly dividing MEL cells the methyl lysine content of histone H3 increases from $\pm 12\%$ to 15%. However the level of methyl lysine content in histones of differentiated MEL cells remains lower than that of brain cells (mouse, ox and foetal brain). The MEL cells are rapidly dividing as opposed to the low cell turnover in mouse liver and brain. If MEL cells were able to last for a longer period of time after differentiation then it is postulated that the levels of histone lysine methylation would increase even further over time.

Experiments on the MEL cells led to the interesting observation that the methyl lysine content of histones is low during rapid growth. The methyl lysine content increases substantially in other mouse tissues. This observation led to the question of whether the low level of methylation is in fact typical for actively dividing cells. Cells used for these experiments were a gift from Prof. N. Illing and at the

time of the experiments had not been fully characterised. They are mouse olfactory neuronal cells that grow at 33°C and cease to divide when transferred to 39°C due to the presence of a heat sensitive T antigen. Methyl lysine content of histone H3 was determined from cells that were actively dividing at 33°C and cells that had been growth inhibited at 39°C. Cells that were cultured at 33°C were found to contain similar amounts of methyl-lysine when compared to MEL cells (11.6% total methylation). In order to establish the progression of methylation as a function of age the cells were kept at 39°C for one month. The methyl lysine content of H3 histone had increased to 16.4%, as compared to 11.6% of rapidly dividing and recently growth arrested cells. Furthermore, when the cells were transferred to 33°C, cell division and growth resumed. These cells therefore retained their potential to divide although the histone H3 methylation had increased to 16 %. It is speculated that the overall level of methylation in the growing cells would decrease again to 11 %. Due to time constraints, and the incomplete characterisation of these cells, it was not possible to pursue these experiments any further. However, it would indeed be interesting to establish the levels of methyl lysine in histones from cells that had been growth arrested for longer periods and its effect on cells growth upon re-incubation at 33°C. One might speculate that the levels of histone lysine methylation would increase as the time span of growth arrest increases and that at a critical level of histone methylation, cell division would be retarded or permanently inhibited.

2.3.4 Methyl lysine content of histones H3 and H4 from ox and foetal calf brain

Studies on the methylase activity of rat brain liver with ageing (Lee and Duerre, 1974) have shown that the levels of histone methylases and the ability of brain histones to accept methyl groups decreases during development. This is consistent with increased levels of histone methylation observed in more differentiated tissues. Amino acid analysis of methyl lysine in mouse brain histones, presented in section 2.2.1.2. (Pg. 19) was performed on animals of no

specific age. Methylation of histones noted to be higher in brains of adult animals than of juvenile ones prompted further analysis of lysine methylation content in histones from brains of foetal calf and ox brain.

Brain cells can be separated into neuronal and glial enriched fractions by differential sucrose gradients (Thompson, 1973). Crude preparations enriched in neuronal and glial nuclei were prepared from both ox and foetal calf brain. Methyl lysine content of histones H3 and H4, extracted from each of these preparations, was determined by amino acid analysis.

Neuronal cells are responsible for transmitting signals between different regions of the brain. There are many different types of neurones but the basic structural feature of most neurones is similar. There is a large cell body, which receives signals from fibre-like extensions called dendrites. The axon extends from the cell body and transmits the signal to synaptic receptors on the next cell. Neuronal nuclei are much larger than glial nuclei and are rich in dispersed chromatin. RNA synthesis in the neuronal nuclei is three times higher than in glial nuclei (Thomas and Thompson, 1977).

The glial cells are the supporting cells of the nervous system. They do not transmit impulses. There are 10 to 50 times more glial cells than neuronal cells. These cells are responsible for the structural integrity of the nervous system. Glial cells are much smaller and denser and consist mainly of heterochromatin. One type of glial cells called oligodendrocytes forms myelin sheaths that are responsible for coating the neuronal cell axons (Campbell, 1990).

It has been shown that the *in vitro* acetylase activity in neuronal cells of the rat brain is greater than that of glial nuclei (Sarkander *et al*, 1975). It has also been shown that there are more extensive modifications (acetylation and phosphorylation) in euchromatin (more dispersed) than in heterochromatin (Allfrey, 1971).

Once neuronal cells are differentiated, they do not divide. As cells stop dividing the replication independent variants begin to accumulate. The methylation of these variants (H3.2/H3.3 fractions) was found to be higher than that of the replication dependent variants (H3.1) (see table 5).

Amino acid analysis results presented in table 5, pg. 27, show no significant difference between the levels of histone lysine methylation of neuronal and glial cells. Therefore no correlation seems to exist between the levels of histone lysine methylation and the levels of RNA synthesis and chromatin condensation in these two cell types. There is also no difference between the levels of lysine methylation of histone H3 isolated from foetal and ox brain and between H3.1 and H3.2 /H3.3 fractions. It might be interesting to conduct more careful studies on the levels of histone lysine methylation and the exact developmental stages of the brain cell during gestation and during development.

Although there are no significance differences between the levels of histone lysine methylation of the different cell types of the brain, the percentage of methylation in brain cells in general is higher than that of rapidly dividing cells (e.g. MEL cells).

These results concur with those of Duerre and Chakrabarty (1975) who found no difference between the mole ratios of mono-, di- and trimethyllysine in H3 from adult organs and those of young rats. However the percentage methylation of lysines is generally the lowest in rapidly dividing tissue culture cells. On average the percentage methyl-lysine in histone H3 from both the ox and the foetal calf brain is 20%, which is slightly lower than that of the mouse brain and liver cells.

When neurones differentiate there is a pre-programmed sequence of gene activations and repressions (Kuenzle *et al*, 1983). Non-histone chromosomal proteins as well as histones are thought to be involved in this process. At some stage during the development of neurones there is a point where cells can no

longer divide. There are two events that occur during the development of neurones. The first is the commitment of continuously proliferating, multipotent stem cells to potential neuronal cells. The second is the conversion of committed precursor cells into non-dividing terminally differentiated neuroblasts (Kuenzle *et al*, 1983).

In rat, for example, stem cells of cortex neurones that are committed to differentiation start to lose their capacity to divide and migrate to the periphery of the hemispheres of the brain. The ability to divide is only effectively stopped after birth. Differentiation however, begins as soon as the cells have migrated to their relevant locations. Could there be a correlation between either of these two events i.e. differentiation or cessation of cell division and histone methylation? The development of rat cerebellar neurones occurs from rapidly dividing precursor cells. This proliferation occurs from late foetal stages to 20 days postnatal (Kuenzle *et al*, 1983). Migration to the cortex occurs three days after birth, with a maximum at day 7 after birth. Two to three days after the cells have reached their destination they begin to differentiate. The rate of methylation of histones of rat brain increases for the first few days after birth (Lee and Duerre, 1974). Approximately 11 days after birth this rate decreases progressively throughout the life of the animal. This change in the rates of histone methylation corresponds with the start of terminal differentiation of the cerebellar neurones of the rat brain. Differentiation of the cortex neurones begins to occur a few days after birth. There is therefore a correlation between cessation of mitosis at birth and histone methylation. The rate of methylation of histones seems to depend on age. After birth the rate of histone methylation is high. This occurs at the same time as the brain cells are ceasing to divide (Kuenzle *et al*, 1983).

It is possible that histone methylation may be involved in both cessation of mitosis and neuronal differentiation. Once cells have been earmarked and are committed to differentiate methylation increases and the cells stop dividing. This coincides with the arrival of the pre-neuronal cells at their destination. The rate of histone

methylation then begins to decrease with differentiation but the levels of histone methylation continue to increase. It is postulated therefore that as histone methylation progresses there comes a critical stage, when the histones are fully methylated, and at this point the cells can no longer divide. The methylation of the histone tails induces a progressive compaction of chromatin.

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2.4 SUMMARY OF RESULTS

The most interesting observations of amino acid analysis are summarised as follows:

- There is a general increase in the total histone lysine methylation in histones H3 and H4 from MEL to liver to brain that correlates with a decrease in the replicative ability of these cells.
- Dimethyl-lysine is the predominated lysine derivative in histone H4 isolated from MEL cells (induced and uninduced), mouse spleen, liver and brain, ox and foetal calf brain. The mono- and trimethylated derivative occurs in relative small amounts (see figure 6c).
- Superimposed on a progressive increase in the levels of lysine methylation in MEL, liver and brain histones, there is a progressive increase in relative amounts of dimethyl-lysine- (see figure 6 and table 9 and 10) and small increase in the levels of trimethyl lysine.
- MEL and OP4 tissue culture cells (cancer cells) were found to have the lowest level of lysine methylation for both histone H3 and H4. The levels of mono- and dimethyllysine were roughly the same in histones from these cells (see figure 6b and table 2 and 3).

3 AN INVESTIGATION INTO THE INCORPORATION OF RADIOACTIVE METHYL GROUPS INTO HISTONES OF NUCLEI FROM ACTIVELY DIVIDING AND DIFFERENTIATED MEL CELLS

3.1 SUMMARY

Logarithmically growing MEL cells were induced to differentiate with hemin for 0, 1 and 4 days. Nuclei were isolated from the cells and pulse labelled with S-adenosyl-L-methionine-methyl-³H. Incorporation of radioactivity into each histone was measured. It was found that H2B, H2A and H1 were not significantly methylated while H3 and H4 were. H3 had the highest incorporation of radioactivity. The rate of methylation of H3 and H4 was found to be constant between day 0 and 1 and then decreased to approximately half the original rate between day 1 and day 4. Histone H3 remained the predominant acceptor of methyl groups at all times.

3.2 RESULTS

In order to measure the rate of incorporation of methylated groups into lysine residues, nuclei were isolated from MEL cells which had been induced to differentiate with hemin for 0, 1 and 4 days. Histones were labelled by incubating the isolated nuclei with S-adenosyl-L-methionine-methyl-³H for one hour at 37°C. The reaction was terminated by addition of SDS sample application buffer and boiling for one minute. The nuclear proteins were then separated on a SDS polyacrylamide gel and stained with Coomassie Brilliant Blue. The protein bands were quantitated by densitometric scanning using a Hoefer gel-scanner and the supplied software. Each band was then excised from the gel, cut into pieces and soaked in water. SDS was added and the mixture was then incubated at 55°C for 12 hours. Scintillation fluid was added to the preparation, which was then counted for one minute on a Beckman Liquid Scintillation Counter as counts were deemed sufficiently large and the error acceptably low for this length of time. Results are tabulated in tables 11 and 12. Specific activity was determined as disintegrations per minute (dpm) per unit area as determined by densitometric quantitation.

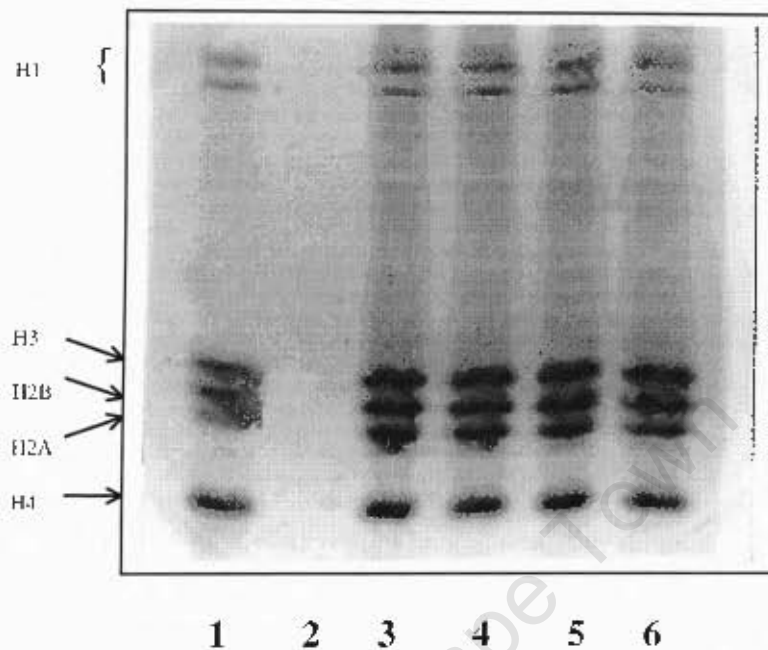


Figure 9 A typical Coomassie stained SDS PAGE gel showing histones of radiolabelled MEL cell nuclei. Nuclei isolated from MEL cells induced with hemin for 0,1 and 4 days were incubated with S-adenosyl-L-methionine-methyl- ^3H . After one hour the reaction was terminated and the nuclei run on a SDS PAGE gel. Lane 1 Calf Thymus Standard, lane 2-nothing, lanes 3-6 - labelled MEL cell nuclei (induced with hemin for 4 days).

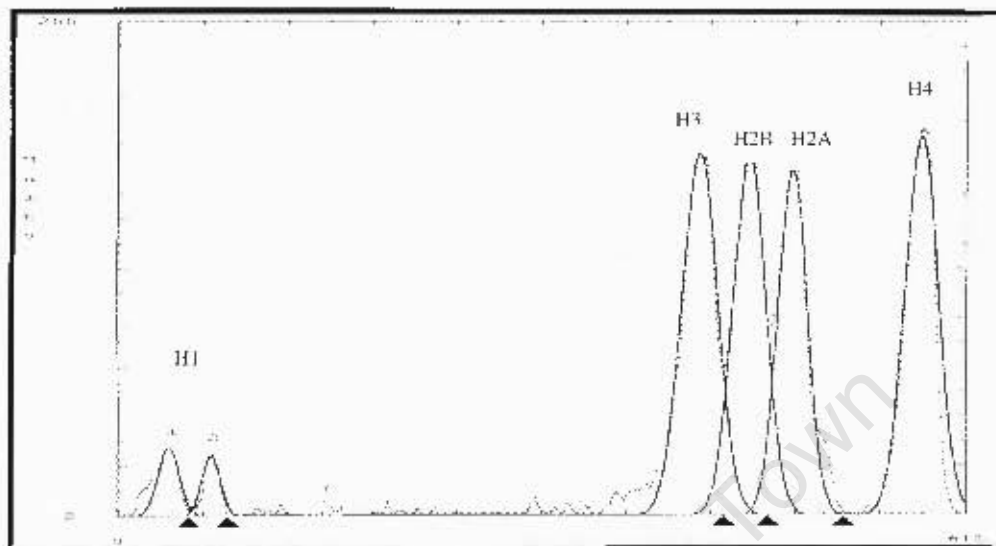


Figure 10 A typical densitometric scan of SDS PAGE gel of histones isolated from labelled MEL nuclei. The areas of the peaks were used to determine the relative amounts of histones in each sample. This together with the radioactive counts was used to determine the specific activity of the histones from each of the samples.

Densitometric analysis of gels, stained with Coomassie Blue, was performed on every lane of each gel. A typical profile is shown in figure 10. Densitometric quantification showed that the relative amounts of histones remained constant.

A source of error for this experiment could occur during the process of excising the bands out of the gel. H2B and H3 run very close together making it difficult to cut them out separately. This problem is however eliminated by running the gels for a longer period and by the fact that H2B and H2A are very little methylated. Each determination was performed in duplicate. The reproducibility was in the region between 2% and 12% and was deemed satisfactory for the

purposes of this experiment. Other possible sources of error in the experiment are due to the staining and destaining of the gel and the densitometric scanning. Reproducibility of the densitometric scanning and gaussian analysis was determined by scanning each lane twice. Error in reproducibility was found to be between 5% and 8%.

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Table 12 Incorporation of radioactive methyl groups into histones H1, H2A and H2B from nuclei isolated from cells growing logarithmically (Log cells), and cells induced with hemin for 1 and 4 days (Hemin 1 day and Hemin 4 days respectively). Specific activity is calculated as radioactive counts (dpm) per unit area.

	H1	H2B	H2A
Log cells			
Area (rau)	1956	3645	2744
Dpm	3436	5725	1337
Specific Activity	1.8	1.6	0.5
Hemin 1 day			
Area (rau)	1831	5842	4513
Dpm	5445	3264	717
Specific Activity	2.9	0.6	0.2
Hemin 4 days			
Area (rau)	2036	2043	1734
Dpm	1484	794	686
Specific Activity	0.7	0.4	0.4

Note: The areas are relative area units (rau) as determined by the densitometric software (Hoeffer). Specific activity is the ratio between the radioactive count (dpm) and the area (rau).

Table 13 Incorporation of radioactive methyl groups into histones H3 and H4 from nuclei isolated from cells growing logarithmically (Log cells), and cells induced with hemin for 1 and 4 days (Hemin 1 day and Hemin 4 days respectively). Specific activity is calculated as radioactive counts (dpm) per unit area.

	H3	H4
Log cells		
Peak Area (rau)	2440	3270
Dpm	118 560	24 815
Specific Activity	48.6	7.6
Hemin 1 day		
Peak Area (rau)	3956	4996
Dpm	189959	53330
Specific Activity	48.0	10.7
Hemin 4 days		
Peak Area (rau)	959	1853
Dpm	24123	5654
Specific Activity	25.2	3.1

Note: The areas are relative area units (rau) as determined by the densitometric software (Hoeffer). Specific activity is the ratio between the radioactive count (dpm) and the area (rau).

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3.3 Discussion

Nuclei, isolated from MEL cells that had been induced with hemin for 0, 1 and 4 days, were pulsed labelled with S-adenosyl-L-methionine methyl-³H for 1 hour. Specific activity of the radioactive incorporation into each histone was calculated as described in materials and methods and is summarised in table 14.

Table 14 Specific Activity of the incorporated radioactive methyl lysine into MEL histones. (a summary of tables 12 and 13, showing specific activity only).

Days of induction	H1	H2A	H2B	H3	H4
0	1.8	0.5	1.6	48.6	7.6
1	2.9	0.2	0.6	48.0	10.7
4	0.7	0.4	0.4	25.2	3.1

As nuclei and not whole cells were used for the experiment, it can be assumed that no new synthesis of histones occurred during labelling, as histones are synthesised in the cytoplasm and then transported to the nucleus where they become deposited onto the chromatin. The incorporation measured therefore represents the modification of existing histones that are complexed into nucleosomes.

The enzyme responsible for methylation of histones (methylase III (Paik and Kim, 1970 and 1971) is located in the nucleus and is tightly bound to the chromatin (Aniello *et al.*, 1989). The enzyme specifically methylates lysine residues. The growth of the MEL cells was monitored after induction with hemin and is shown in figure 1f. From the growth curve it is apparent that cells doubled twice in the first day, giving a doubling time of 12 to 14 hours. After induction the growth begins to decrease and reach a stationary phase on the third day, followed by a decrease in cell numbers due to cell death (figs 11a&b).

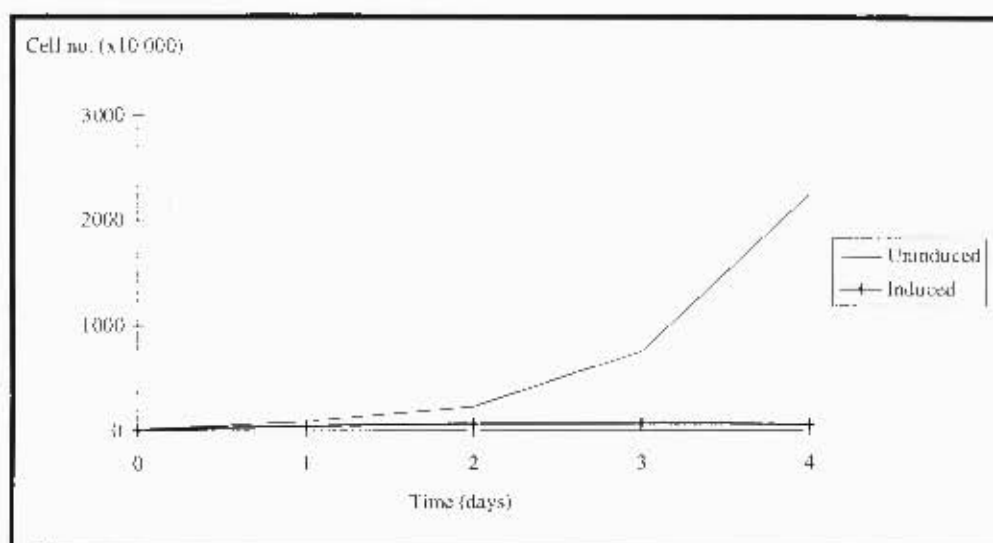


Figure 11a Growth of MEL cells in log phase and after addition of hemin. Cell density was monitored each day by counting on a hemocytometer.

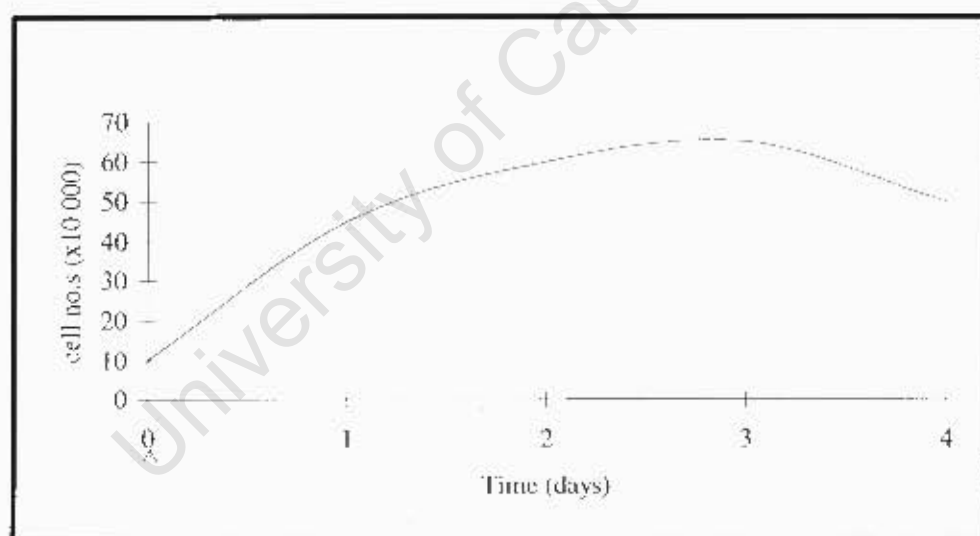


Figure 11b Growth of MEL cells after hemin addition. The arrow indicates the time of hemin addition. This figure is a reproduction of figure 11a in which the Y-axis as been magnified.

The incorporation of radioactive methyl groups (i.e. S-adenosyl-L-methionine-methyl- ^3H) into histones H2B, H2A and H1 was found to be very low compared to that of H3 and H4. The specific activities of H2B, H2A and H1 prior to

addition of hemin, for example, were 0.5, 1.6 and 1.8 respectively, while those of H3 and H4 were 48.6 and 7.6 respectively (see table 14). H1 shows an increased incorporation after one day of induction, with an increase in specific activity from 1.8 to 2.9. This indicates a low level of methylation of this histone. There have been reports of H1 from lower organisms being methylated on lysine residues (Jerzmanowski and Maleszewski, 1985 and Frost *et al.*, 1989).

The incorporation of radioactive methyl groups into histone H3 was much higher than that of the other histones. On day 0 and day 1 after hemin induction, the specific activity of H3 was 48.6 and 48.0 respectively while the next highest activity recorded was for histones H4 with a value of 10.7.

The specific activity of H3 remains at a high level between day 0 and 1 after induction. This may partly be attributed to cell growth and methylation of newly synthesised histones. The benzidine-staining test revealed the presence of hemoglobin after day 1, indicating cell differentiation into hemoglobin producing cells. Four days after hemin addition, the rate of histone H3 methylation decreased to approximately half the initial values. A graphic representation of the specific activity of all histones, monitored over the four days, is shown in figure 12.

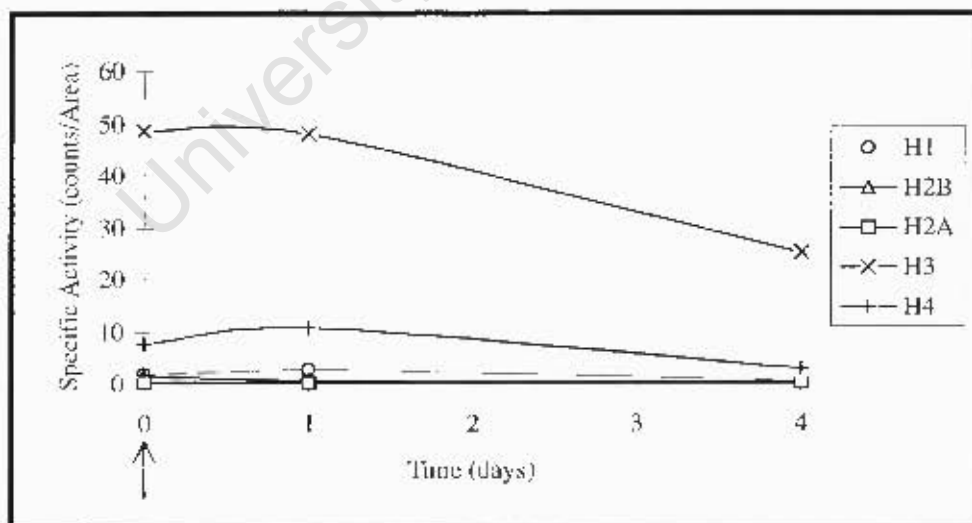


Figure 12 Graphic representation of the incorporation of radioactive methyl groups into histones from MEL cells growing

in log phase, and induced with hemin for one and four days. The arrow indicates the time of hemin addition (refer to table 14).

The incorporation of radioactive methyl groups into histone H4 was found to be approximately 6 times lower than that of H3 on day 0, and 4.5 times lower on day 1. There is a slight increase in the incorporation of radioactivity into H4 from 7.6 to 10.7, one after day of hemin addition, whereas that of H3 remains approximately the same (± 48). The rate decreases to 3.1 for H4 and 25.2 for H3 on the fourth day after hemin addition. Since there is only one known site of methylation on H4 and two to four possible sites on H3, difference in the specific activity were expected.

The observed decrease in the rate of methylation could be either due to the decrease in the availability of methylation sites and/or a decrease in enzyme activity. The latter possibility could be investigated by repeating the experiment with addition of exogenous histones. It is also possible that the chromatin matures in some way after replication, reducing accessibility of the histone to the methylating enzyme. A third possibility is that the decrease in the incorporation of methyl groups is due to a removal of incorporated methyl residues. This is however unlikely as the half-life of the histone H3 and that of methyl groups are very similar ().

Lee and Duerre (1974) performed a similar experiment to the one presented here, on old and young rat brains. They monitored incorporation of activity into histones from old and young rat brain and found that methylase activity increased during the first few days after birth and then decreased progressively throughout the life of the rat. Activity of methylase from the brains of old rats was still lower than that of the methylase from younger brains, even after using histones from the young brain as a substrate. The results of this experiment indicate that it is likely that the decreased incorporation of radioactive methyl groups observed here could be due to decreased levels or activity of the enzyme, which is unaffected by substrate concentration.

Analysis of the total methyl lysine content of histones H3 and H4, presented in chapter 2, has shown that there is an increase in the levels of histone lysine methylation when MEL cells are induced to differentiate. One can thus conclude that the reduction in the rate of methylation as a function of time could be due a decrease the availability of methylation sites as well as a reduction enzymatic activity. The rate of histone H3 methylation is maintained after one day of induction and then decreases but continues at a higher rate than that of the other histones after four days of induction. Methylation therefore continues after DNA replication has stopped. This is in agreement with investigations by others, including Tidwell *et al* (1968) and Borun *et al* (1972).

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4 AN INVESTIGATION OF THE EFFECT OF HISTONE METHYLATION ON THERMAL DENATURATION OF NUCLEOSOMES AND CHROMATIN

4.1 SUMMARY

Amino acid analysis results have shown that MEL, liver and brain cells have different levels of histone methylation. Thermal stability studies were used to investigate the functional significance of the different levels of histone methylation found in these tissues. This was done by melting core particles, isolated from mouse brain and liver and MEL cell chromatin. The core particles were obtained by digesting the chromatin with Mnase. The core particles were purified by sucrose gradient fractionation. The purified core particles were used for UV thermal denaturation studies. Additional thermal denaturation studies were performed on soluble chromatin isolated from the same samples. Differential scanning calorimetric experiments were performed on nuclei from the same tissues in an attempt to reveal chromatin stability due to the methylation of histones.

4.2 RESULTS

4.2.1 UV thermal denaturation of core particles isolated from mouse liver and MEL cell chromatin

Core particles were prepared from nuclei, which were isolated as previously. The nuclei were briefly M_nase digested to obtain soluble, long chromatin, which was extracted in 0.6 M NaCl to remove H1 and purified on a 5-20% sucrose gradient. After extensive dialysis in 5 mM NaCl, 0.1 mM EDTA, 10 mM TRIS-HCl, pH 7.5, a trial M_nase digest was performed to determine the optimum digestion time to obtain the cores. The bulk digestion was then performed and the reaction terminated by addition of EDTA. The core particles were purified on a second 5-20% sucrose gradient. The purified core particles were then dialysed extensively against 5 mM NaCl, 0.1 mM EDTA, 10 mM TRIS-HCl, pH 7.5. A 6% acrylamide DNA gel of the purified core particles confirmed DNA length at 146 base pairs (see figure 13).

Purified core particles were heated from 25°C-100°C in a Pye Unicam spectrophotometer equipped for heating the sample cell.

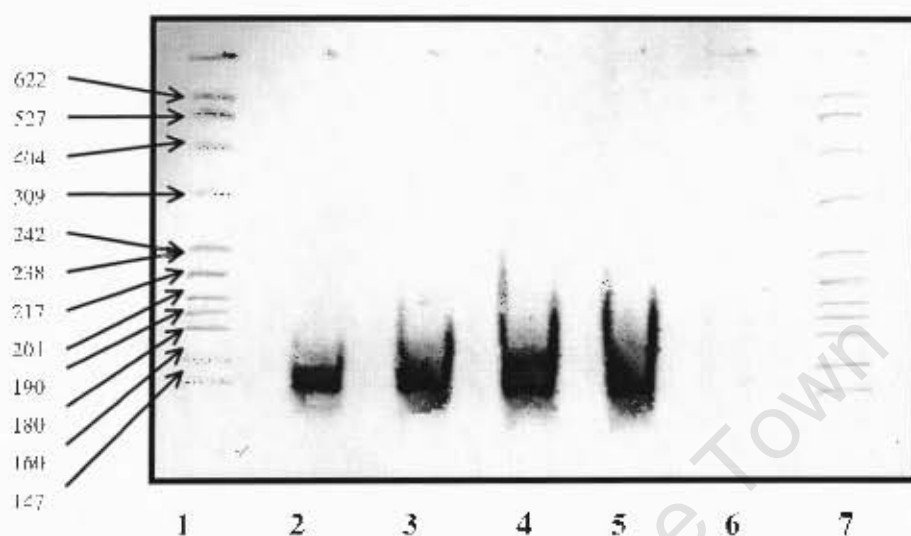


Figure 13 6% acrylamide gel showing purified core particles at 146 bp of DNA. Core particles were prepared from purified nuclei of mouse liver and MEL cells by digestion with Mnase. Lanes 1, 6 and 7; PBR 322 std (Hpa II digest); lanes 2 - 5, core particles isolated from MEL cells. The nos. refer to base pairs of DNA.

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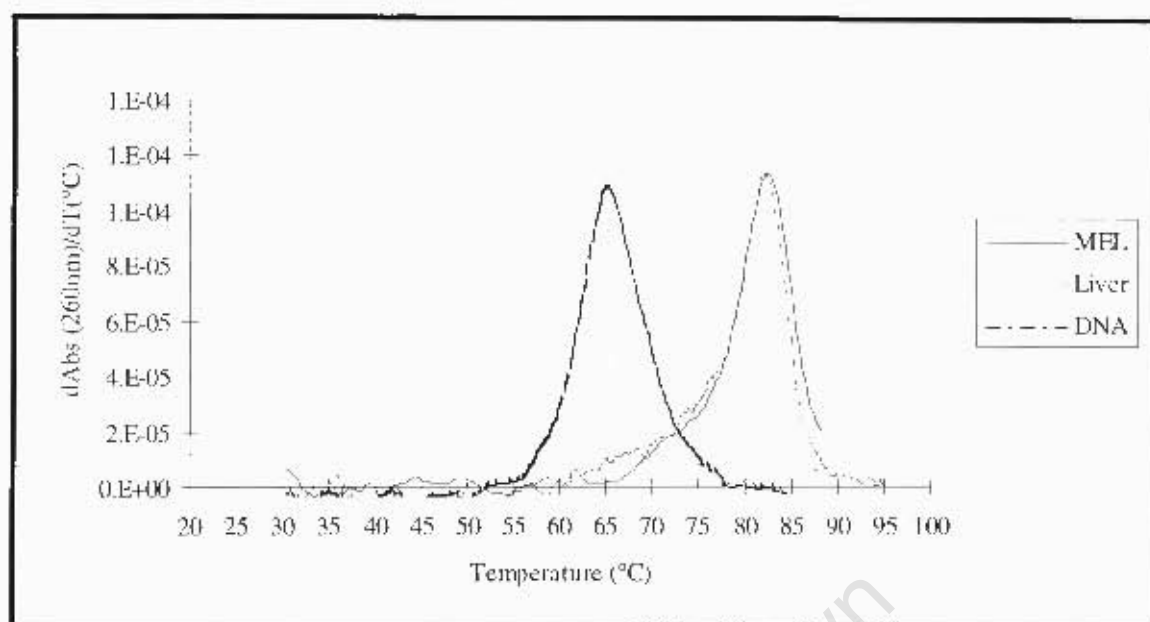


Figure 14 Thermal melting profiles of liver and MEL core particles. H1 depleted chromatin isolated from mouse from liver and MEL cells was digested with M_nase and purified on a sucrose gradient to produce the core particles. These particles were melted from 25°C to 100°C and the absorbance measured at 260nm in 5mM NaCl, 0.1mM EDTA 1mM cacodylate. The first derivative of each sample is shown as a function of temperature.

4.2.2 Thermal denaturation of MEL cell, mouse liver and mouse brain chromatin

Soluble chromatin was isolated from mouse liver and brain as well as from MEL cells as described in materials and methods. Purified nuclei were briefly digested with M_nase and chromatin extracted in 0.6 M NaCl. Soluble chromatin was purified on a sucrose gradient and then extensively dialysed against 5 mM NaCl, 0.1 mM EDTA, 1 mM cacodylate. Chromatin was melted, as previously, in a Pye Unicam spectrophotometer with an attached heating cell.

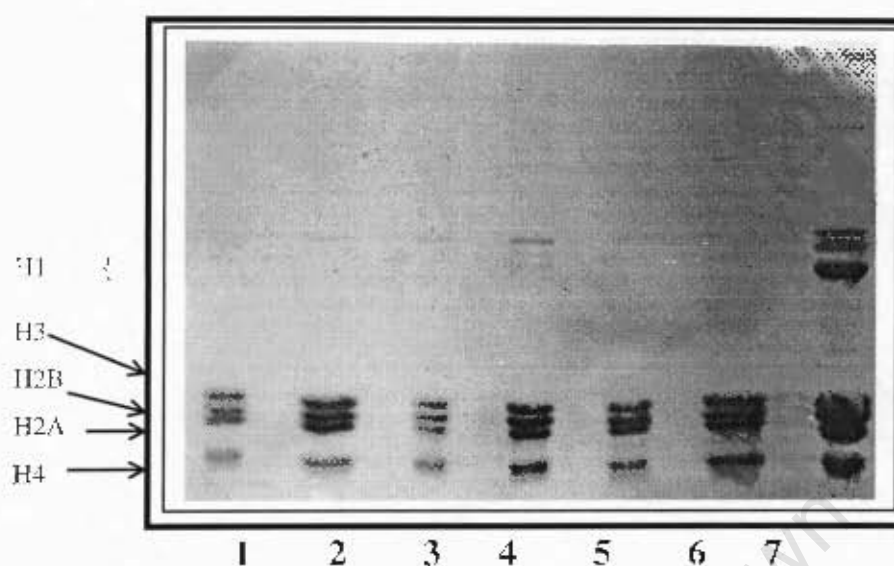


Figure 15 20% SDS gel to show intact histones from MEL cell, mouse liver and mouse brain chromatin samples used for melting. Lanes 1-2, MEL; lanes 3-4, liver; lanes 5-6 brain; lane 7, chicken total histone standard

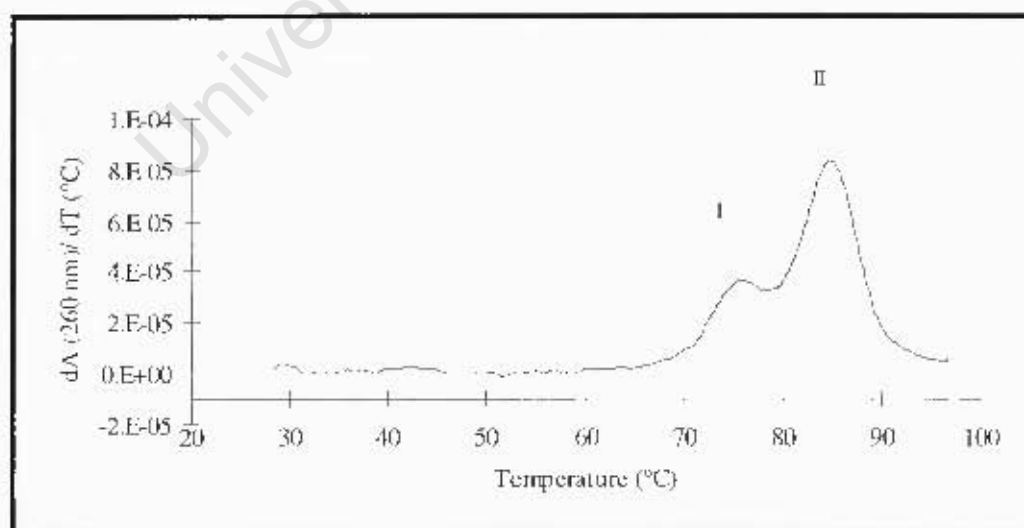


Figure 16 The first derivative melting profile of H1-depleted MEL cell soluble chromatin in 5mM NaCl, 0.1mM

EDTA 1mm cacodylate (I and II are the transitions as discussed in the text).

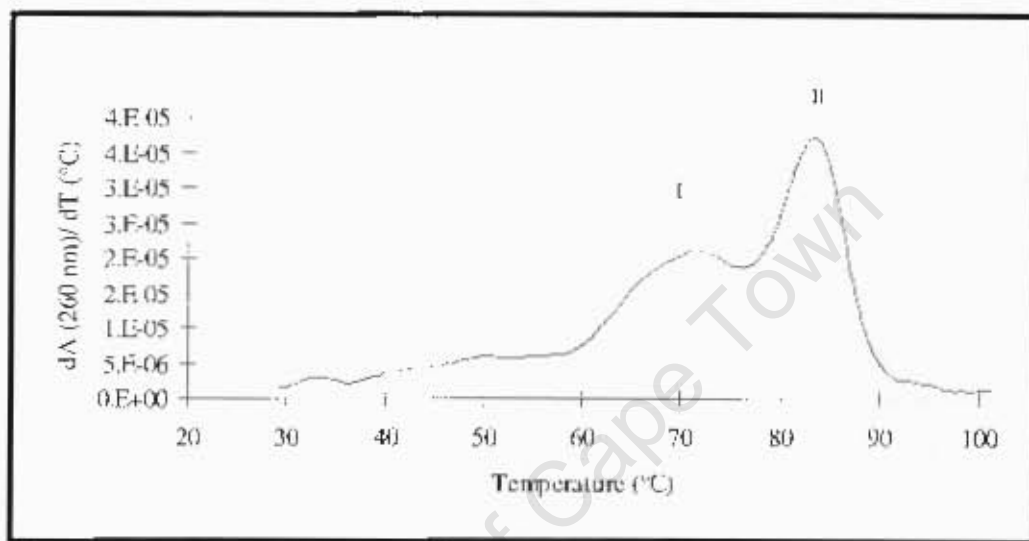


Figure 17 The first derivative melting profile of HI-depleted soluble chromatin from mouse liver in 5mM NaCl, 0.1mM EDTA 1mm cacodylate (I and II are the transitions as discussed in the text).

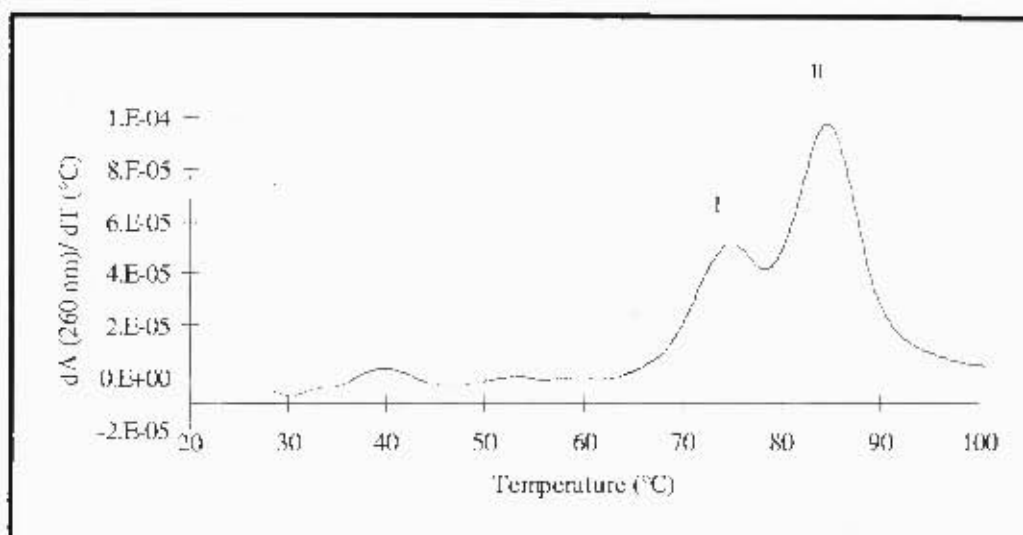


Figure 18 The first derivative melting profile of H1-depleted soluble chromatin from mouse brain in 5mM NaCl, 0.1mM EDTA 1mM cacodylate (I and II are the transitions as discussed in the text).

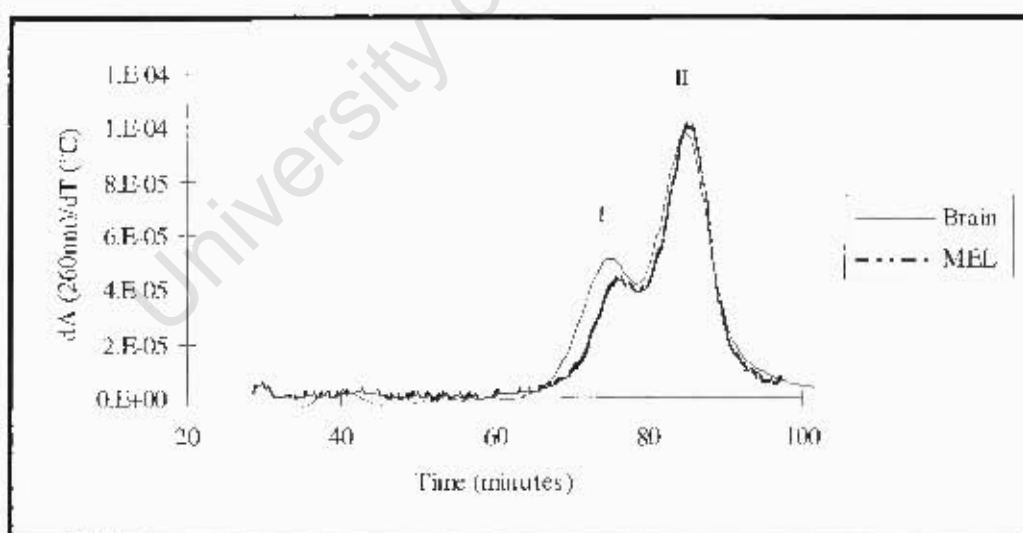


Figure 19 Overlay of the first derivative thermal denaturation of mouse brain and MEL cell chromatin.

4.2.3 Calorimetry of MEL cell and mouse brain nuclei

Thermal denaturation experiments were performed on soluble chromatin from MEL cell and mouse brain nuclei. It is possible that isolation of soluble chromatin could select for a certain population of the chromatin and thus give a biased result. Also, isolation procedures for this sub-section of the population could possibly have influenced the high order structure and selected against modifications. While these experiments are useful at a lower level of chromatin organisation, it would be informative to investigate thermal denaturation of chromatin in its native state in intact nuclei. Differential scanning calorimetry (calorimetry) can be performed on opaque samples, and was therefore performed on whole, purified nuclei. Calorimetry of whole nuclei can be used to show the thermodynamic transitions of chromatin structures (Touchette and Cole, 1985).

Table 15 Transition temperatures in °C from calorimetry of MEL and brain nuclei (see figures 20 and 21)

Tissue	I	II	III	IV	V
MEL	54°C	68°C	82°C	86°C	97°C
Brain	55°C	77°C	82°C	85°C	95°C

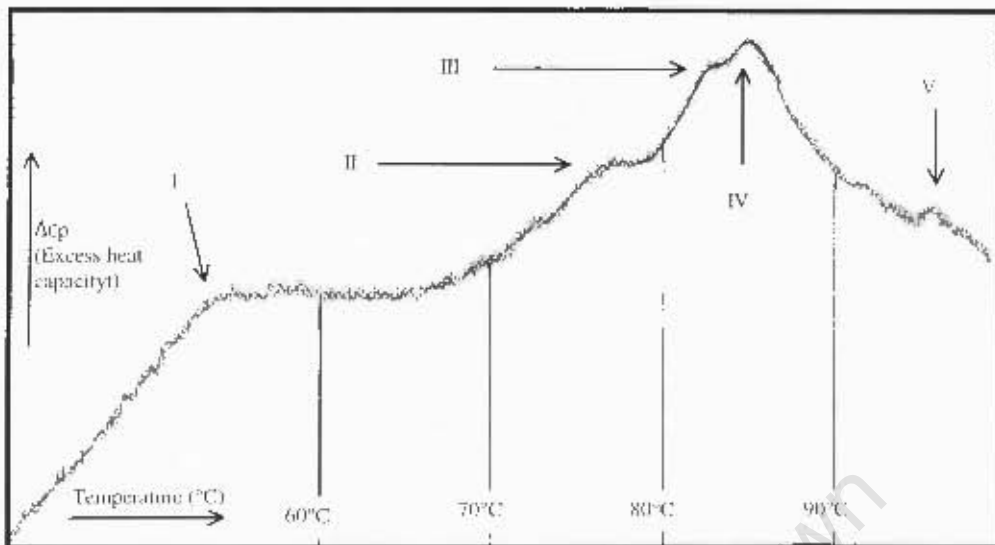


Figure 20 Differential Scanning Calorimetry of purified MEL cell nuclei. Heat absorption is shown as a function of temperature in $^{\circ}\text{C}$. I-V indicates the transitions as discussed in the text.

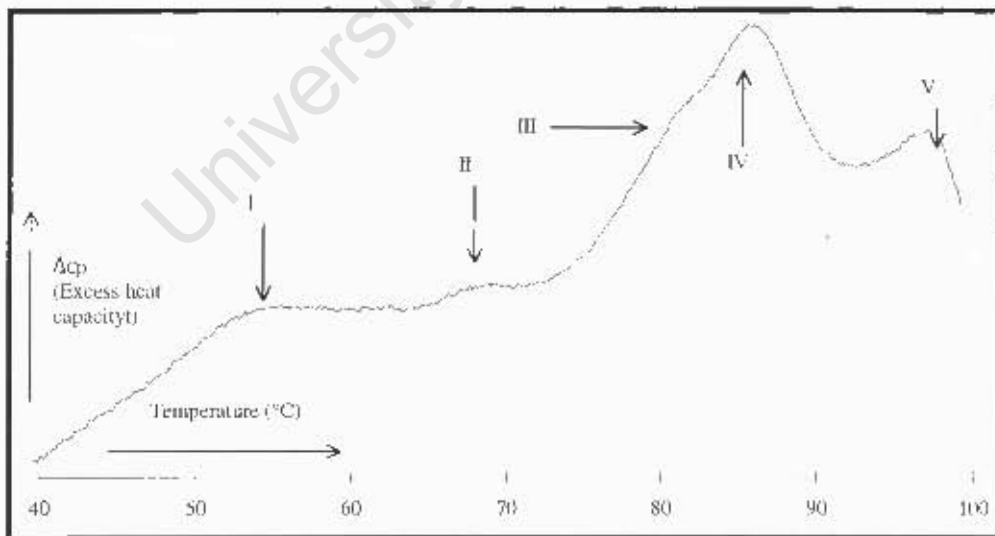


Figure 21 **Differential Scanning Calorimetry of purified mouse brain nuclei. Heat absorption is shown as a function of temperature in °C. I-V indicates the transitions as discussed in the text.**

4.3 DISCUSSION

4.3.1 **Melting of core particles isolated from mouse liver and MEL cells**

Amino acid analysis, which was used to determine the ϵ -N-mono-, di- and trimethyllysine content of histones H3 and H4 (chapter 2), showed that the levels of methyl lysine in histones from mouse erythroleukemia (MEL) cells differs from that of mouse liver and mouse brain histones. MEL cells have low levels of histone methylation ($\pm 12\%$) whereas liver and brain cells have much higher levels (± 20 - 25%), (table 3, pg. 23).

The thermal denaturation of the DNA complexed with histones into core particles was studied to determine whether histone lysine methylation contributes to the stability of the DNA of the core particle. Thermal denaturation of DNA in solution measures the shift in absorbance of ultraviolet light at 260 nm in response to heating. The purine and pyrimidine rings of the DNA bases absorb light strongly around 260 nm. In native DNA these bases are in close association (stacking) and the absorbance of light is less than the when the DNA strands are dissociated. Increasing the temperature results in a co-operative unstacking of these bases from helix to random coil and causes a hyperchromic shift in the absorbance of ultraviolet light. The double helix is held together until it is on the verge of instability and then dissociates (melts) over a very narrow temperature range (Matthews and van Holde, 1990). This transition is referred to as melting. There

are various factors that may affect this transition, for instance the association of the DNA with proteins or the base composition of the DNA. This technique is suitable to analyse conformational transitions of DNA, as well as any influence methyl lysine may have on the stability of the DNA.

Core particles, containing no H1, were isolated from mouse liver and MEL tissues, which contain histones that are methylated to different levels. Thermal denaturation of these core particles (first derivative) is shown in figure 14. Naked DNA isolated from the core particles served as a control. The T_m for naked DNA is 69°C and yields a symmetrical transition. The T_m of core particles lies at a higher temperature than that of the naked DNA. This shows that the histones of the core particle stabilise the DNA double helix. The derivative curves of both core particle preparations are asymmetric and show a low melting component. There is no difference in the melting of the core particles isolated from MEL cells or mouse liver. The different levels of histone lysine methylation of these core particles apparently do not contribute to the stability of the DNA of the core particle (figure 14).

Studies of the thermal denaturation of chicken core particles by Weischet *et al* (1978) showed that the melting of core particles is biphasic, with two distinct transitions. In the denaturations presented here, the first transition appears to have merged with that of the second transition. Weischet *et al* showed that the difference in T_m of the two transitions of the core particle denaturation decreases as the ionic strength of the melting buffer is increased. These observations were confirmed for the denaturation of the MEL and mouse liver core particles. TRIS, even in low concentrations (10 mM), was found to increase the melting temperature of naked DNA. Hence TRIS buffers are unsuitable and all further experiments were done in the absence of TRIS. However, due to no observable difference in denaturation of core particles isolated from MEL and mouse liver, as well as the tails not contributing to the stability of the core particle (discussed later) these thermal denaturation experiments were not repeated.

Interestingly another thermal denaturation was performed on samples of core particles isolated from both MEL and liver cells in which the histones had been partially degraded by proteolysis. The melting profiles of the core particles containing undegraded histones was found to be similar to that of degraded core particles (data not shown). As the histone tails are the first to undergo proteolysis, it is possible that in these samples the basic conformation of the core particle was still intact even after mild proteolysis had occurred. This is further evidence that the tails are not involved in the stability of the core particle and are therefore not involved in protein-DNA interactions within the core particle (protein-protein interactions between neighbouring nucleosomes have been demonstrated (Luger *et al.*, 1997)).

A model that is derived from electron microscopic studies by Poon and Seligy (1980) suggests that as the temperature increases the DNA core ends melt first giving rise to the first hyperchromic shift. The final transition, occurring at $\pm 80^{\circ}\text{C}$, is due to the complete melting of DNA after protein denaturation.

Thermal denaturation of core particles with different levels of histone tail methylation are not expected to influence core particle stability and hence hyperchromicity. This is because the histone tails extend from the core particle, and are not attached to the DNA of the particle (as shown by the melting of proteolysed core particles).

In summary, comparisons of the melting curves of mouse liver and MEL core particles demonstrate that the methylation of H3 and H4 histone tails has no noticeable effect on the stability of the DNA of the core particle and therefore on the interaction of the tails with the core particle. As the melting was unaffected by mild degradation of the histones, one would not expect methylation of these tails to be of significance in the stability of the DNA core particles.

4.3.2 UV thermal denaturation of soluble chromatin isolated from MEL cells, mouse liver and mouse brain

Soluble chromatin, with H1 removed, was isolated from mouse liver and brain tissue and MEL cells. This chromatin was 'melted' in a buffer of 5 mM NaCl, 0.1 mM EDTA, 1 mM cacodylate. At this ionic strength chromatin depleted of H1 adopts a 'beaded-string' conformation and shows the typical nucleosome characteristic of a regular zigzag pattern (Van Holde, 1980). At higher ionic strengths (i.e. 20 mM -100 mM), chromatin that is depleted of H1 aggregates whereas at much lower ionic strengths (<5 mM) the stripped chromatin loses most of its structure and forms irregular thick fibres (Thoma *et al.*, 1979).

H1 makes contacts with the core histones, including H2A and H3, and is known to be involved in the condensation of chromatin. At the ionic conditions that are used here chromatin containing H1 is more tightly condensed, and the linker DNA is associated with H1. Melting done on H1-depleted (stripped) chromatin would eliminate any heterogeneity between samples and was pursued. The purpose of this experiment was to determine whether histone lysine methylation would influence interactions between neighbouring nucleosomes in the nucleosome chain.

Condensation of chromatin involves the tails of the histones (Allan *et al.*, 1982). If methylation played a role in chromatin condensation or higher order structure, it would most probably be a subtle one, involving weak interactions. Other molecules that are not present in the purified chromatin fractions used in the thermal stability experiments could mediate such interactions. It is well known that acetylation has a more pronounced effect on the charged state of lysine residues in the histones and their involvement in transcription is well documented. It is conceivable that the irreversible methylation of lysine residue will prevent acetylation of the relevant histones and in this indirect way influence transcription. Methylation may thus not translate into a pronounced physical difference in the

purified chromatin. Although highly acetylated chromatin has been found to be associated with transcriptionally active chromatin (Hebbes *et al.*, 1988), it is not a pre-requisite for transcription.

The first derivative of the melting of the chromatin from MEL and mouse brain chromatin is shown in figures 16 and 18 respectively. An overlay of the two is shown in figure 19. Both samples show two distinct transitions, in agreement with results of Weischet *et al.* for chicken erythrocyte core particles (1978). In both cases the sharp first transition (I) is clearly distinguishable from that of the second (II). The peak of the derivative of the first transition occurs at 74°C for the brain and 75°C for the MEL. The peak of the derivative of the second transition occurs at approximately the same temperature for both MEL and brain chromatin (85°C). Essentially there is no difference in the melting of the MEL and brain chromatin. The melting profile for liver chromatin shows a similar pattern to that of the MEL and mouse brain chromatin except that the first transition is broader.

The first transition of core particle denaturation can be attributed to the melting of the ends of the DNA of the core particle (Weischet *et al.*, 1978 and Poon and Seligy, 1980). The unprotected regions of the DNA melt first, therefore it is possible that the first transition of the soluble chromatin shown here is due to the melting of the linker DNA between nucleosomes and is not anymore stabilised by HU. Removal of HU from chromatin results in an increase in the early melting sub compounds and hence shows peak broadening. This was shown by Richin and Harrington (1987) in thermal denaturation experiments of chromatin from HeLa nuclei.

The second transition noted is most probably due to the denaturation of the DNA and protein of the nucleosome as was shown to be the case when core particles were melted (Weischet *et al.*, 1978).

If methylation influences higher order structure of chromatin then one might expect that soluble chromatin could have a different level of methylation to that of bulk chromatin because it represents a sub-population enriched in less tightly packed and enzymatically accessible chromatin. However preliminary experiments indicated very little difference exist in histone methylation levels of bulk and soluble chromatin (Brandt, W. F., personal communication). These experiments are to be pursued at a later stage. In this thesis no attempt was made to differentiate between the soluble and bulk chromatin during thermal denaturation experiments.

If the interaction of the tails with the neighbouring nucleosomes is weak then it will not be seen as a transition in these experiments. The different levels of histone methylation in the MEL and mouse brain showed no effect on thermal stability of the DNA of the soluble chromatin preparations (figure 19). This situation could arise if the chromatin is not representative and a certain subsection was selected during isolation. It could also mean that histone methylation plays no role in chromatin structure or that the interactions of the methylated tails are too weak to be detected before the first transition. In order to investigate these possibilities calorimetry was done on whole nuclei so that a selection for one subsection of the chromatin is avoided.

4.3.3 Calorimetry of MEL cell and mouse brain nuclei

Differential scanning calorimetry is a useful technique to study structural features of chromatin since it does not require solubilisation. Whole nuclei can be tested where chromatin is native. This technique was used to study the structural features of chromatin from MEL cell and mouse brain nuclei. Isolation of chromatin by M_nase digestion induces a risk of structural damage, whereas nuclei represent the native structure of chromatin (Touchette and Cole, 1985). Unlike

thermal denaturation calorimetry allows transitions of all structures to be monitored and not only that of DNA.

In whole nuclei H1 is present in the chromatin. Calorimetry of MEL cell and mouse brain nuclei is shown in figures 20 and 21 respectively. In both cases there are five transitions (numbered I-V in the figures). Transitions I, III, IV and V occur at the same temperatures in both cases. Transition II occurs at a higher temperature in the MEL cells. It occurs at $\pm 77^{\circ}\text{C}$ in the MEL nuclei and at 68-69 $^{\circ}\text{C}$ in the brain nuclei.

Comparison of the calorimetry data to that of the UV thermal denaturation data reveal that the first, second and fifth transitions that occur in the calorimetric scans are characteristic of whole nuclei. In soluble chromatin depleted of H1 these transitions (I, II and IV) are not present.

The calorimetric profiles of MEL and mouse brain nuclei are similar. There is however an increase in magnitude of the fifth transition in mouse brain nuclei. Whether this transition is linked to the higher levels of histone lysine methylation present in brain nuclei cannot be said for certain at this point. Many researchers have postulated that the upper transition that occurs when nuclei are melted is due to a higher order structure of chromatin, which is not present in soluble chromatin. Touchette and Cole (1985) found four distinct transitions were present in HeLa nuclei, occurring at approximately 55-60 $^{\circ}\text{C}$, 76 $^{\circ}\text{C}$, 88 $^{\circ}\text{C}$ and 105 $^{\circ}\text{C}$. In isolated chromatin, however, only two major transitions occurred, at 72 and 85 $^{\circ}\text{C}$ with transitions I and IV being absent. They therefore suggest that transitions I and IV are due to non chromosomal proteins or to some kind of higher order structure. They further propose that their fourth transition (i.e. the higher one) is a measure of the cell's replication potential. Results on both cell types show the type V transition which is much more pronounced in the brain cells and may reflect the lower (or absent) cell replication rate of these cells. (Note: Giartosio *et al* (1992) hypothesise that since this transition is disrupted with either mechanical shearing

or nucleases that it is more likely to be an indication of the integrity of the chromatin, than of the replication potential of a cell).

Further evidence that the transition V is due to a higher order structure of chromatin is provided by Giartosio *et al* (1992) who showed that the first three transitions of chicken erythrocyte nuclei are fairly reversible, but that the fourth transition is not. This is consistent with the proposal that the fourth transition is due to a super structured form of DNA, which is lost after protein and/or DNA denaturation and cannot be reconstituted to its original form. Cavazza *et al* (1991) also show that the higher order structure of chromatin is destroyed at high temperatures and that any shearing or digestion of the chromatin with Mnase results in a loss of this proposed higher order structure and the higher transitions are transformed in to the lower ones.

Russo *et al* (1995) have showed that transition V is converted to transition IV upon removal of H1 from the chromatin. Although their experiments were at quite different conditions to those used here, they show that H1 can change the thermal denaturation of the chromatin by removing the upper transition. It is possible that the upper transition that is observed may be due to the presence of H1 that causes a condensation of the chromatin, even though this can still occur in the absence of H1 (Yao *et al*, 1991). Selective trypsinisation of N-terminal tails of the core histones has shown that these tails are also involved to a large extent in this process (Russo *et al*, 1995. Garcia-Ramirez *et al*, 1992).

Note that although other researchers have found differences in the number of transitions and/or T_m 's that are observed, this could be attributed to differences in instrumentation, in sample concentration and to the slower scanning rate, which is used here ($\pm 1^\circ\text{C}/\text{min}$), and gives a lower T_m (Giarosio *et al*, 1992, found similar results using a slower scanning rate). In all the examples mentioned above it is the upper transition that is referred to, which in the case of the MEL and mouse brain nuclei is transition V.

Transition IV is most probably the same transition that occurs during denaturation of chromatin and is due to melting of the nucleosomes, as postulated for core particles and chromatin of the same samples (sections 4.3.1 and 4.3.2). The first transition could be due to denaturation of the nuclear scaffold structure (Balbi *et al.*, 1989) or lamins (Giartosio *et al.*, 1992, who did calorimetry on whole nuclei from chicken erythrocytes). It is possible that transitions II and/or III are due to linker DNA, which is seen as transition I in the chromatin samples (figure 19). This was proposed to be the case by Balbi *et al.* (1989) and Cavazza *et al.* (1991).

Amalgamor and Cole (1989) found that transitions II and III in HeLa chromatin at 150 mM salt do not depend on the higher order structure of the chromatin because they are unaffected by nuclease digestion to mononucleosomes. They propose that transition II, which depends on the integrity of both DNA and histones, represents the denaturation of core proteins of the nucleosome. Transition III does not depend on the presence of protein and represents the unstacking of bases of the DNA.

In summary, results obtained from calorimetric studies of mouse brain and MEL cell nuclei are overall very similar. There is a difference in the upper transition (V), which is larger in brain than in MEL nuclei. This upper transition is postulated to be due the melting of a higher order structure of the chromatin. It is therefore possible that in the brain nuclei there is more chromatin in a condensed state than there is in the MEL nuclei. At this stage it is uncertain whether this could be due to the increased levels of histone lysine methylation that is observed in the brain tissues.

5 CONCLUSION

Eukaryotic cells contain large amounts of DNA, which holds all the genetic information required during the life cycle of an organism. This DNA is stored in a compact form, nevertheless allowing complete access during cell division on the one hand and restricting access to a small fraction of the genome after cell differentiation on the other. During transcription only those genes involved in specific functions are accessed, involving only between 5% and 10% of a cell's DNA (Mathews and van Holde, 1990). Histones are very closely associated with the DNA and may thus be crucial role players in regulating access to the DNA during replication and transcription.

A considerable amount of structural detail of the chromatin has been elucidated, in which histones play a very important role. From the structural details it is apparent that the basic histones tails protrude from the nucleosomal structure and may thus be involved in the higher order packaging of DNA. Postsynthetic modifications of these "arms" could modulate the structure of the chromatin. The N-terminal ends would be readily accessible to the various enzymes, which fits the observation that acetylation and methylation occurs in these regions. Experiments performed in this investigation attempt to answer the question as to what the significance of histone methylation could be.

It was established that the level of methylation of the bulk of histones was the lowest in rapidly dividing cells. This level increased in liver cells and was maximal in brain cells. Results revealed that the methylation levels increases after cells differentiate and stop dividing. Quantitation of the methylated lysines in fetal and adult bovine brain showed that the levels remained the same. The methyl content in histones seems to correlate with the time that has elapsed since the last mitotic cycle. This maturation of chromatin structure may reflect permanent packing of chromatin, preventing replication of DNA and thus mitosis. In addition this maturation may also give rise to a permanent transcriptional inactivation of genes not required by a specific cell. One might speculate that

spurious gene expression of cell replication might be detrimental, especially for brain cells that have to survive for many years. Experiments on the mouse olfactory cancer cells, containing a heat sensitive antigen showed that preventing cell division (at 39°C) for a month increased the methyl content from 10 to 16 %. At this stage the cell had not lost the ability to divide if returned to normal growth conditions at 33°C. A longer period of cell growth inhibition and consequently higher methylation of histones may prevent resumption of growth. Due to time restrictions this experiment was not performed.

It is possible then that methylation of lysine residues on histones H3 and H4 reaches a threshold, above which no replication or access to inactive genes can be gained. Above this threshold transcription and replication may be initiated only in exceptional circumstances by the intervention of specific de-repressors. This may resemble the methylation and inactivation of specific genes in DNA. Pulse labelling experiments on transcriptionally active enriched genes from erythrocytes (Hendzel and Davie, 1989) seemed to show that methyl groups were preferentially incorporated into the accompanying histones. Moreover it was shown that the acetylated histones H3 and H4 were also preferentially methylated. Acetylation of histones has been shown to be associated with active genes (Hebbes *et al*, 1988). It is therefore postulated that acetylation and methylation of histones at their N-terminal tails may alter the higher order chromatin structures and facilitate transcription (Hendzel and Davie, 1989). These results seem to contradict the hypothesis that methylation is involved in formation of permanently inactivated chromatin. However this conclusion is based on pulse labelling experiments and may reflect only localised and short-term events. The estimation of the methyl content of histones by amino acid analysis represents the composition of the bulk of chromatin.

In conclusion, results obtained seem to indicate that histone lysine methylation is involved in the stability of chromatin and state of differentiation of cells. As cells become older the levels of histone lysine methylation increase, until a critical stage is reached after which the bulk of chromatin is highly compacted and fairly

permanently inhibited. This may also contribute to the inhibition of the mitotic cycle of the cell. The labelling experiments showed that even when cell growth and DNA synthesis ceases, methylation continues at a high rate for some time and then finally decreases, possibly due the fact that all accessible sites become methylated or are folded into more compact structures. Methylation may aid the compaction of chromatin and the levels required may be sensitive to the environment and the local composition of the DNA. The experiments on the influence of methylation on the nucleosomal structure revealed no difference in DNA stability and seem to confirm that the histone tails are not involved in nucleosome stability. Alternatively methylation may cause only minor changes in the physical properties of chromatin, which are not revealed by experimental measurements performed. The calorimetric measurements on brain and MEL (cancer) cells reveal differences, but the results are difficult to interpret due to the complex nature of a nucleus. However, the results do not contradict the postulated role of histone methylation. The elucidation of the structural significance of histone methylation remains a major challenge in the understanding of the structure and function of chromatin.

6 MATERIALS AND METHODS

6.1 AMINO ACID ANALYSIS

6.1.1 Cell culture

6.1.1.1 Growth conditions of MEL cells

Mouse Erythroleukemia (MEL) cells, obtained from Highveld Biologicals, were grown in suspension in RPMI-1640 medium (Highveld Biologicals) supplemented with 20% foetal calf serum. Cells were grown in a humidified incubator containing 5% CO₂ in air at a temperature of 37°C. Cells were maintained at a density of 4-20 x 10⁴ cells/ml.

6.1.1.2 Sterile conditions and procedures

All operations were carried out in a laminar flow hood and all glassware used was rinsed thrice with sterile water and then autoclaved. All equipment used, including pipettes and flasks, was sterile.

The medium, which was made up in pyrogen-free, sterile water, was initially filtered through a 0.45µm Whatmann filter and then through a 0.22µm filter under nitrogen gas pressure. Each time medium was made up a small aliquot was placed in the incubator to check for contamination and the rest was stored at 4°C

6.1.1.3 Trypan blue test for viable cells

Periodically a trypan blue test was executed to test for viable cells. Viable cells have a clearly defined membrane and thus cannot take up the trypan blue stain.

Membranes of non-viable or dead cells become permeable to the dye and therefore stain blue.

6.1.1.4 Procedure for freezing MEL cells

Cells were pelleted by spinning at 1,000 rpm in a SS34 rotor for five minutes, washed with phosphate buffered saline (PBS, see appendix), and then resuspended in RPMI-1640 at a cell density of $2-6 \times 10^6$ cells/ml. Dimethyl sulfoxide (DMSO) was then added to a final concentration of 15 %. Cells were kept on ice for ten minutes and then aliquotted into cryotubes. Cryotubes were then placed on ice for a further ten minutes, at -20°C for 40 minutes, on dry ice for 10 minutes and finally stored in liquid nitrogen (Cells may be stored at -70°C for approximately one month, but for longer storage liquid nitrogen is required).

6.1.1.5 Procedure for thawing MEL cells

Cryotubes were removed from liquid nitrogen and immediately placed at 37°C in a circulating water bath to thaw as quickly as possible. The cells were then transferred into a 25 cm^2 tissue culture flask which contained 10 - 15 ml of RPMI-1640 medium supplemented with 20% FCS.

6.1.1.6 Cell growth curves

Starter cultures were placed at a density of $4-20 \times 10^4$ cells/ml. Cell density was monitored each day by counting on a hemocytometer. When cell density exceeded 20×10^4 cells/ml, flasks were split and fresh medium was added.

6.1.1.7 Induction of MEL cells with hemin

Hemin stock solution was made by dissolving 13 mg of the hemin in 0.2 ml of 0.5 M NaOH. This was then buffered with 0.25 ml 1 M TRIS-HCl (pH 8.0) and made up to a final volume of 5.0 ml to give a 4 mM stock solution (Zahrie *et al.*, 1991).

For experimental purposes two flasks of cells were incubated with 10^{-4} M hemin in 20% FCS and the cell density was maintained at $4-20 \times 10^4$ cells/ml in accordance with Ching Lo *et al.* (1981).

Cell density was monitored each day by counting on a hemocytometer and the benzidine test (6.1.1.10) was performed to monitor induction and production of haemoglobin.

6.1.1.8 Induction of MEL cells with DMSO

Two flasks of cells were incubated with 2% DMSO (Sato *et al.*, 1971) in 20% FCS. As before the cells were maintained at a density of $4-20 \times 10^4$ cells/ml. Cell counts and benzidine tests were performed each day.

6.1.1.9 Induction of cells with Sodium Butyrate

Two flasks of cells were incubated with 5 mM filter sterilised sodium Butyrate (Reeves and Cserjesi, 1979). Cell densities were kept at $4-20 \times 10^4$ cells/ml.

6.1.1.10 Benzidine test

When MEL cells become differentiated they start to produce haemoglobin. This is generally used as a test for differentiation, because haemoglobin will stain blue on addition of the benzidine reagent.

A stock solution of benzidine was made up with 0.4% w/v benzidine hydrochloride in 0.5M acetic acid. This was stored in an amber bottle and at 4°C. When needed 2% H₂O₂ was added to the stock and 2µl of this solution was added to 200µl of cells which had been centrifuged and resuspended in PBS (Orkin *et al*, 1975). Fresh blood was used as a control for haemoglobin.

6.1.2 Isolation of MEL cell nuclei

Cells were grown to logarithmic phase in 75 cm² flasks. One day before isolation of nuclei these cells were transferred to 150 cm² flasks. Cells were spun at 1,000 rpm in a SS34 rotor and supernatant fluid discarded. The cells were then resuspended in 30 volumes of 0.32M sucrose, 1 mM MgCl₂, 0.25 mM PMSF, 0.5 % Nonidet P-40 in TKM buffer. The cells were spun at 2,500 rpm in a SS34 rotor for 20 minutes after which the supernatant was discarded. The pellet was resuspended in 2.3 M sucrose-TKM (see appendix) 9x the volume of the cell pellet. This was then centrifuged at 20,000 rpm (SS34 rotor) for 40 minutes and resuspended in 2.3 M sucrose-TKM 6x the volume of the cell pellet and spun for 20 minutes. The pellet contained fairly pure nuclei.

6.1.3 Isolation of mouse liver and spleen nuclei

Balb C mice were starved overnight and then killed by a cervical blow. All further procedures were carried out at 4 °C. The livers and spleen were excised and all connective tissue removed. The livers and spleen were chopped up with scissors and 0.32 M sucrose-TKM (pH 7.5) three times the weight of the livers or spleen was added. They were then homogenised in a Potter-Elvehjem homogeniser and filtered through two layers of cheesecloth. After spinning at 2,500 rpm for 20 minutes in a SS34 rotor the pellet was resuspended in 2.3 M sucrose-TKM (pH 7.5) at a final volume of nine times the volume of the cell pellet. This was then spun at 20,000 rpm for 40 minutes after which the

supernatant fluid was carefully poured off. The scum of cell debris was removed from the top of the tube with a tissue. The pellet was then resuspended in 5-6 volumes of 2.3 M sucrose-TKM and centrifuged once more at 20,000 rpm in a SS34 rotor for 20 minutes. The pellet contained fairly pure nuclei, which were either stored at -20°C in 50% glycerol: 50% buffer A or used immediately.

6.1.4 Isolation of ox, foetal calf and mouse brain nuclei

Ox and foetal calf brain were used from freshly slaughtered animals. All procedures were performed at 4°C . Brains were washed in 150 mM NaCl solution and then homogenised in 0.32 M sucrose, 1 mM MgCl_2 , 0.25 mM phenyl methyl sulfonyl fluoride (PMSF), 0.5 % Nonidet P-40 (buffer 1). The homogenate was filtered through one layer of cheesecloth and centrifuged at 10,000 rpm in an SS34 rotor for 20-25 minutes. The pellet was then resuspended in 2.0 M sucrose, 1 mM MgCl_2 , 0.25 mM PMSF (buffer 2), re-homogenised and centrifuged at 20,000 rpm for 40 minutes in a Beckman ultracentrifuge with SW28 rotor. This was then repeated for further purification to obtain a fairly pure nuclei pellet.

The same procedure was followed for mouse brain nuclei except that more care was taken with homogenising, as the cells from mouse brain seemed quite fragile and broke fairly easily. Cells were monitored under the microscope throughout homogenisation. Very low yields were obtained if homogenisation was too vigorous.

6.1.5 Separation of ox and foetal calf brain neuronal and glial nuclei

Nuclei isolated in 6.1.4 from ox and foetal calf brain were further purified into neuronal and glial nuclei. The pellet from 6.1.4 was resuspended in buffer 2 and

placed in SW28 centrifuge tubes on top of an equal layer of 2.4 M sucrose, 1 mM MgCl₂, 0.25 mM PMSF (buffer 3). This was centrifuged at 23,000 rpm for 40 minutes. The interface (neuronal nuclei) and pellet (glial nuclei) were resuspended separately in buffer A (see appendix) and further centrifuged at 10,000 rpm for 10 minutes in a SS34 rotor. At this point cells were checked under the microscope.

6.1.6 Extraction of total histones from nuclei

Nuclei that had been stored at -20°C in 2.3 M sucrose-TKM were allowed to thaw and then topped up with TKM buffer and centrifuged at 20,000 rpm for 10 minutes in a SS34 rotor. The pellet was washed twice with 0.15 M NaCl and then resuspended in water and thoroughly mixed, whereafter H₂SO₄ was added dropwise to a final concentration of 0.4 M. After spinning, the supernatants were pooled and eight volumes of ice-cold acetone was added. The histones were allowed to precipitate overnight at -20°C. They were then centrifuged at 10,000 rpm in a SS34 rotor for 10 minutes and washed with ice-cold acetone-0.02 % HCl and then again with pure acetone. The sample was then dried in the desiccator and the product weighed.

6.1.7 Purification of histones by High Performance Liquid Chromatography (HPLC)

Total histones were purified into separate histone fractions by reverse phase chromatography on a C18 column connected to a Shimadzu HPLC pump and software. Each sample was loaded in 6 M urea, 1 % 2-mercaptoethanol, 0.2 % TFA. Buffer A was 0.1 % TFA and Buffer B was 70 % acetonitrile, 0.1 % TFA. The gradient used was 0 % buffer B from 0 to 10 minutes, 0 to 70% buffer B from:

10 to 20 minutes, 70 to 100 % buffer B from 20 to 60 minutes. Fractions were freeze-dried and then resuspended in appropriate volumes of water.

H4 fractions were contaminated with H2A and were thus further purified by HPLC using a C4 column with buffers as previously except that TFA was substituted with HFBA. A linear gradient was used from 0% to 100% B over 60 minutes. The H4 and H2A peaks were well resolved.

6.1.8 SDS gel electrophoresis

The histone fractions collected from the HPLC were run on a SDS gel, with a 20% separating gel and a 6.7% stacking gel (*Laemmli* 1970), at 200 volts for approximately four hours. Chicken total histones were used as a standard. Gels were stained with Coomassie Brilliant Blue.

6.1.9 Triton acid urea gel electrophoresis

Histone fractions that were run on triton-acid urea gels were run with 6M urea, 6 mM Triton and 15% acrylamide. Gels were pre-electrophoresed until the voltage stabilised (100 volts for 4h). Samples were run at 70 volts overnight. Gels were then stained with Coomassie Brilliant Blue.

6.1.10 Amino Acid Analysis

Purified histone fractions were hydrolysed in constant boiling HCl at 110°C for ± 20 hours. The excess acid was evaporated under vacuum and the sample dissolved in running buffer. The samples were run on a cross-linked sulphonated cation exchanger column with post column derivitisation of amino acids by o-phthalaldehyde (OPA). The amino acids were eluted isocratically with 0.35M

Sodium Citrate pH 5.84. The column was kept at 29°C and the flow rate was 0.4 ml/min.

The reliable quantitation of the methylated lysine derivatives (MK, DK, TK) using o-phthalaldehyde (OPA) was verified by subjecting various concentrations, ranging from 0.5 pmoles to 4 pmoles, to amino acid analysis. Results revealed that the quantitative response was linear over the range tested and that their extinction coefficients were closely related. The percentage error for the amino acid analysis was generally in the region of 5 to 10%. Similar results have been observed using ninhydrin (Ref: WX Paik and S Kim, in *Protein Methylation* (ed. A Meister) John Wiley & Sons, New York, 1980, 72).

6.1.11 Quantitation of MK, DK and TK

The methyl lysine content of the histones was calculated by determining the number of residues of the methylated lysines present relative to the histidine (see figure 6 for a typical HPLC profile of an amino acid analysis run). In all variants of H3 as well as in H4 the number of histidine residues remains constant (two residues). There are 13 lysine residues in H3 and 11 in H4 and these values were used to calculate the percentage methylation. The experimental values for non-methylated lysine were unreliable due to the fact that their concentration is always much higher relative to the methylated lysines and thus fell into the non-linear range. Furthermore, lysine reacts with OPA at both the α and ϵ amino groups resulting in quenching of the fluorescence due to proximity of the two aromatic rings. No quenching has been observed in the methylated lysines (Oates and Jorgenson, 1990).

6.2 RADIOLABELLING EXPERIMENTS

Pulse-labelling experiment was performed on MEL cells nuclei at different stages of differentiation (after adding hemin) in order to determine the rate of histone methylation at various time intervals. The three cell stages that were analysed corresponded to the logarithmically growing cells, cells that had been induced by hemin after one and four days.

6.2.1 Incubation and induction of cells with hemin for radiolabelling

Three different sets of cells were needed for this experiment. The logarithmically growing cells were prepared by incubating two flasks each with 10 million cells in 20% FCS the day prior to the experiment. This would yield ± 30 million logarithmically growing cells per flask for the experiment. The cells induced with 10^{-4} M hemin for one and four days were prepared by incubating 15 and 10 million cells per flask respectively. This would allow for the growth of one to two generations of cells. Each experiment was designed to give as close to equal amounts of cells per sample as possible.

6.2.2 Isolation of MEL cell nuclei

On the day of harvesting the cells were centrifuged at 1.5K for 10 minutes washed once with 0.25M sucrose-TKM (pH 7.5) and then resuspended in 10 ml 0.25M sucrose-TKM and homogenised in a glass homogeniser. Cells, mixed with acridine orange, were checked under the microscope to see if breakage was sufficient. If the breakage was not sufficient further homogenising was done in a Teflon rotary potter homogeniser. The sufficiently broken cells were pelleted and resuspended in 2 ml of 0.25M sucrose-TKM to which was added 11 volumes of 2.3M sucrose-TKM. The solution was mixed well by inversion. The tubes were

centrifuged at 20,000 rpm in an SS34 rotor for 40 minutes. The nuclear pellet was used for the pulse labelling experiments.

6.2.3 Incubation of nuclei with radioactivity

The purified nuclei were transferred to a 1 ml plastic tube and washed twice in TKM buffer (pH 7.5). To the pellet 50 μ l of TKM (pH 7.5) and 40 μ Ci of S-adenosyl-L-methionine-methyl- 3 H was added. This was then incubated for exactly one hour at 37°C. The nuclei were then pelleted and washed twice with TKM (pH 7.5). 60 μ l of SDS sample application buffer was added to the nuclei, which were then boiled for one minute.

6.2.4 SDS gel electrophoresis

Each of the samples was run on a 20 cm SDS PAGE gel with calf thymus total histones used as a standard (20 μ g/lane). 10 μ l of each sample was loaded onto the gel and run at 200 volts for 5 hours.

6.2.5 Gel scanning

The histones were quantitated by using standardised staining and destaining of the gels and the histone bands were then quantitated on a Hoefer GS 300 scanning densitometer and GS 365 software. The staining intensity of the 4 core histones was approximately the same (see fig.2 & 10) assuming that the molar ratios equivalent. Non-equivalence should not affect the results of specific activity of any one histone isolated from different tissues.

6.2.6 Radioactive counting

Incorporation of the labelled methyl into the histones was quantified as follows: The stained bands were excised from the gel and soaked in water to expand the gel as much as possible. They were then cut up into small pieces and soaked in approximately 50 μ l of 10% SDS per band and incubated overnight at 55°C in a counting vial. Scintillation fluid (4 ml) containing triton-X-100 (to facilitate suspension of the sample) was then added to each fraction. Radioactivity was measured for one minute on the Beckman LS-5000 Liquid Scintillation Counter.

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6.3 UV THERMAL DENATURATION AND CALORIMETRY

6.3.1 Isolation of H1 depleted, long chromatin from nuclei

Stored nuclei were washed twice in buffer A to remove glycerol and then suspended in buffer A (see appendix) at a concentration of 2 mg/ml (Absorbance at 260 nm of 20 OD units is equivalent to 1 mg/ml). Nuclei were then briefly digested with micrococcal nuclease (Mnase) at a ratio of 50 ml nuclei at 2 mg/ml: 0.7 ml 100 mM CaCl₂: 20.8 µl Mnase at 20U/µl. The reaction was terminated by the addition of 5 ml of 100 mM EDTA. This was then centrifuged at 4,000 rpm in a SS34 rotor. 6 ml of 0.6M NaCl, 0.1 mM EDTA, 10 mM TRIS-HCl/1 mM Cacodylate (see discussion) pH 7 was added to the pellet and allowed to extract overnight by dialysing extensively in the same buffer. The dialysate was then centrifuged at 15,000 rpm in a SS34 rotor for 10 minutes. The absorbance of the supernatant, which contained the soluble chromatin, was measured at 260 nm and then loaded on the top of a 5%- 25% sucrose gradient at a maximum concentration of 6 mg/ml. This was spun in an ultracentrifuge at 28,000 rpm in a SW28 rotor for 16 hours after which the gradient was fractionated in an ISCO sucrose gradient fractionator. The top part of the gradient, which contained H1, was discarded and the rest kept. This was then dialysed extensively in 5 mM NaCl, 0.1 mM EDTA, 10 mM TRIS-HCl/1 mM Cacodylate (see discussion), pH 7.4.

6.3.2 Isolation of core particles

Long chromatin was concentrated to an absorbance at 260 nm of 15-25 AU (if necessary) using an Amicon ultrafiltration apparatus. A pilot Mnase digest was performed on the long chromatin in order to determine the exact digestion time needed to obtain core particles with DNA of 146 base pairs. The Mnase digested fractions were phenol extracted and ethanol precipitated and then run on a 6% acrylamide gel using PBR322 digested with Hpa II as a standard. A bulk digest

was then performed. the reaction stopped with excess EDTA, and the core particles purified by a further on a linear sucrose gradient 5-20% run for 16 hours in SW40 rotor at 35,000 rpm for 16 hours. The gradients were fractionated on an ISCO gradient fractionator. The fractions containing the core particles were dialysed extensively in 5 mM NaCl, 0.1 mM EDTA, 10 mM TRIS-HCl pH 7.5. An aliquot of core particles was added to an equal volume of sarcosyl buffer and electrophoresed for \pm 4 hours at 150 V on a 6% acrylamide gel to check the length of the DNA. PBR322 plasmid digested with Hpa II was used as a standard. A fraction was then run on a SDS gel to check the integrity of the histones.

6.3.3 Thermal denaturation

Hyperchromic shifts of chromatin samples were measured on a Pye Unicam spectrophotometer fitted with a heating cell and a temperature probe at a wavelength setting of 260 nm. Samples were heated from 25°C to 100°C at approximately 1°C/minute in a sealed chamber. The samples were degassed and kept under constant helium pressure throughout the run. The analogue data were converted using an A-D converter. The data was recorded on a computer with software written by Dr. Paul Büstler. The same software was used to determine the first derivative of the melting curves.

6.3.4 Calorimetry

Calorimetric measurements were made in a differential scanning micro-calorimeter, type DASM4. The sample cuvette was filled with a 1 ml suspension of nuclei in 5 mM NaCl, 0.1 mM EDTA, 10 mM Cacodylate buffer pH 7. The nuclear pellet was once washed in the same buffer. The reference cuvette was filled with buffer only. The chambers were degassed and sealed. Melting was performed using a heating rate of 1°C/minute.

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APPENDIX**Abbreviations**

DK	ϵ -N-dimethyl lysine
DMSO	dimethyl sulfoxide
Gly	glycine
H	histidine
HMBA	hexamethylenebisacetamide
HPLC	high performance liquid chromatography
Ile	isoleucine
K	lysine
Met	methionine
MEL	mouse erythroleukemia cells
MK	ϵ -n-monomethyl lysine
OPA	o-phthalaldehyde
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered Saline
PMSF	phenyl methyl sulphonyl fluoride
rpm	revolutions per minute
SDS	sodium dodecyl sulphide
TFA	trifluoroacetic acid

TK	ϵ -N-trimethyl lysine
TKM	TRIS, potassium, magnesium
Val	valine

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Solutions

- 1) Buffer A (10x) 150 mM TRIS-HCl, 650 mM NaCl, 600 mM KCl, 1.5 mM Spermine, 5 mM Spermidine, 2 mM EGTA, 2 mM EDTA, 50 mM 2-mercaptoethanol, 0.1 mM PMSF, pH 7.5
- 2) TKM 50 mM TRIS-HCl, 25 mM KCl, 1.5 mM MgCl₂, pH 7.5
- 3) Hanks buffered salt solution CaCl₂-0.18 g/l, KCl-0.4 g/l, KH₂PO₄ 0.06 g/l, MgSO₄ -0.2 g/l, NaCl-8.0 g/l, NaH₂PO₄ 0.06 g/l
- 4) Sarcosyl Buffer 1 ml 10 % sarcosyl, 2 ml 30 % glycerol, 1 ml 100 mM EDTA. Make to 10 ml with water. Add bromophenol blue to taste
- 5) PBS NaCl, 4g/l; KCl, 0.2g/l; Na₂HPO₄, 1.15g/l; KH₂PO₄, 0.2g/l

Note: Growth of OP4 cells and preparation of their nuclei was performed by Mrs F. Davids, therefore these methods are not presented in chapter 6.