Functional characterisation of the histone H2A variant, H2A.F/Z.

by

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STATEMENT

The research described in this thesis was solely and entirely conducted by the author unless acknowledgment is made in the text. It has not been presented for any other degree.

Michael Clarkson
December 2000

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ABSTRACT

Since the DNA in eukaryotes is packaged into chromatin it is perhaps not surprising that cellular activities that involve DNA, such as replication, recombination, transcription and mitosis also intimately involve chromatin. In each of these cellular activities, particular chromatin structures have been identified that have characteristic biochemical properties. These different properties can be generated by altering the composition of chromatin and/or by the action of specialised enzymes on chromatin constituents.

The basic subunit of chromatin is the nucleosome which packages 147bp of DNA by wrapping it twice around an octameric protein complex consisting of two molecules each of histones H2A, H2B, H3 and H4. The functional and biochemical properties of chromatin can be altered at the nucleosome level by; post-translational modification of histones, ATP-dependent remodelling of histone-DNA contacts in the nucleosome or by incorporation of histone variants.

This thesis details the characterisation of a histone H2A variant, H2A.F/Z. A unique and important role has been ascribed to this protein on the basis of demonstrations that null mutations in the H2A.F/Z gene are lethal in mouse (Thonglairoam, 1996), *Tetrahymena thermophila* (Liu *et al.*, 1996a) and *Drosophila melanogaster* (van Daal and Elgin, 1992). Although the actual function of this histone is unknown, an enrichment of H2A.F/Z in transcriptionally competent chromatin has lead to the hypothesis that it is involved in the establishment or maintenance of transcription in the nucleus (Gabrielli *et al.*, 1981; Wenkert and Allis, 1984; Allis *et al.*, 1986; Huang *et al.*, 1986; Ridsdale and Davie, 1987; Stargell *et al.* 1993).

In chapter 3, experiments were conducted in *Drosophila* to identify domains of His2AvD that functionally distinguish it from the core H2A histone *in vivo*. Prior to the commencement of this project, it had been demonstrated that null mutant lethality in *Drosophila* could be rescued with a transgene derived from a 4.1kb genomic DNA fragment containing the *His2AvD* gene (van Daal and Elgin, 1992). Based on this result, a strategy was employed here where regions or "cassettes" encoding amino acids in the *His2AvD* rescue fragment were mutated, *in vitro*, to the equivalently positioned H2A.1 residues. Lines of flies containing stably integrated wild type and mutant *His2AvD* transgenes were generated by P-element mediated transformation of *Drosophila*. These transgenes were then tested for their ability to rescue *His2AvD* null mutant lethality. Interestingly, this experiment demonstrated that unique features of His2AvD reside in a C-terminal region of the protein not in the histone fold. This C-terminal region is part of a short _-helix that, in H2A, is buried deep inside the nucleosome core and appears to

be important for the stability of the histone octamer (Luger *et al.*, 1997). To characterise the extent of rescue afforded by the mutant transgenes, the null mutant phenotype was characterised using phenotypic and molecular developmental markers. Analysis of the phenotype of *His2AvD* null mutants found that these individuals undergo a protracted third instar and then die without entering pupation. Consistent with this observation, transcripts from developmental genes activated late in third instar are not detected in *His2AvD* null mutant individuals. Interestingly, heat shock genes can still be induced after this developmental block.

In chapter 4, the distribution of His2AvD was characterised in *Drosophila* using a transgene that encoded His2AvD with green fluorescent protein (GFP) fused to the C-terminus. It was demonstrated that the transgene could provide functional His2AvD protein by being able to rescue null mutant lethality. During early embryonic development, the appearance of His2AvD in nuclei coincided with the onset of transcription. Subsequently, GFP associated fluorescence was observed in all nuclei at all stages of embryonic and larval development and in adult somatic tissues. In nuclei, His2AvD was widely, but not homogeneously, distributed. His2AvD-GFP fusion protein remained associated with chromatin throughout the cell cycle, including during mitosis when transcription is shut down.

The tissue specific expression and protein distribution of mouse H2A.Z was also examined. Mice were used to examine H2A.Z expression and protein concentration in different tissues because tissue samples from mice are more convenient to obtain and are less subject to contamination by other tissues on dissection than they are from *Drosophila*. In adult mice, the amount of H2A.Z transcript varies by up to two orders of magnitude between tissues and is directly proportional to the rate of cell turnover. H2A.Z protein is present at the same concentration, relative to the core histones, in all tissues examined. The subcellular location of mouse H2A.Z was also investigated on tissue sections and cell culture monolayers using antibodies directed against the C-terminus of the protein. Histone H2A.Z containing chromatin is generally distributed throughout nuclei but is not associated with transcriptionally silent satellite DNA sequences.

In summary, experiments conducted in this thesis identified that His2AvD provides its unique function through a region at the C-terminus of the protein. In addition, results presented here support the temporal and spatial association of histone H2A.F/Z with transcriptional activity.

PUBLICATIONS

Clarkson, M. J.,V. Tonglairoam and J. R. E. Wells. (2000) Histone H2A.Z gene sequence, expression and protein distribution. In prep for *Biochemical Journal.*

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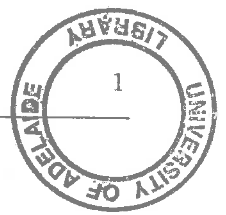


Chapter 1: Introduction

1.1 Introduction

Histones are the most abundant proteins in the eukaryotic nucleus. Two molecules of each histone; H2A, H2B, H3 and H4 form an octameric protein complex that packages 147bp of DNA at intervals of 200±40bp. Numerous molecular, biochemical and genetic studies have also demonstrated that histone proteins participate in the regulation of various cellular processes that involve DNA like replication, recombination, transcription and mitosis.

The central aim of the work conducted in this thesis was to investigate the function of histone H2A variants of the H2A.F/Z class. Gene knock out studies have shown that H2A.F/Z null mutations are lethal in mouse (Thonglairoam, 1996), *Tetrahymena thermophila* (Liu *et al.*, 1996a) and *Drosophila melanogaster* (van Daal and Elgin, 1992). While these results dramatically demonstrate that H2A.F/Z is a fundamentally important molecule in these organisms, its actual function is unknown. To date, the most compelling data in relation to the function of H2A.F/Z is a temporal and spatial colocalisation of the protein with transcriptional activity in *Tetrahymena* nuclei (Stargell *et al.*, 1993). In *Schizosaccharomyces pombe*, a null mutation in the H2A.F/Z gene is not lethal but the demonstration that these cells are defective in minichromosome maintenance has lead to the suggestion that this protein may have a role in chromosome dynamics during mitosis (Carr *et al.* 1994).

In this thesis, the histone *Drosophila* histone H2A.F/Z variant, *His2AvD*, was further characterised by determining the domain that distinguishes its function from the core H2A and by examining its distribution in chromatin. The functional domains of His2AvD were identified in an amino acid swap experiment where a *His2AvD* gene was mutagenised *in vitro* so that regions encoding amino acid residues unique to His2AvD were replaced with sequences that encoded the equivalently positioned H2A.1 residues. Mutated *His2AvD* genes were transformed into *Drosophila* and tested for their ability to overcome *His2AvD* null lethality. To determine the extent of rescue that the mutant *His2AvD* genes were able to provide, the stage where development was disrupted by the *His2AvD*^{810} null mutation was also characterised. The distribution of His2AvD in chromatin was examined during the activation of transcription in *Drosophila* embryogenesis and throughout the cell cycle to investigate the proposed roles of this variant in transcription and chromosome dynamics, respectively. In a complementary

analysis, the distribution and expression of histone H2A.Z in mouse tissues was also determined.

This introduction provides background information relevant to the functions that histone H2A.F/Z has been associated with. H2A.F/Z is classified as a variant histone because it has features which distinguish it from the major or core histones. Initially, this introduction will review the gene structure and regulation of core histones so the differences that classify a histone as a variant can be outlined. The contribution of histone variants to the generation of specific chromatin structures that have a particular function in the nucleus will then be presented. Next, the biochemical characteristics of H2A.F/Z and features that have linked its function with chromosome dynamics and transcription will be reviewed. Possible ways that H2A.F/Z could influence chromosome dynamics and transcription will then be considered in relation to information derived from the analysis of core histones in these processes.

1.2 Core histones

The core histone proteins are synthesised during the S-phase of the cell cycle to package the newly replicated DNA. In higher eukaryotes histone production is regulated at the level of transcription, pre mRNA processing and mRNA stability. During S-phase, the synthesis of histone mRNA synthesis increase 3 to 5 fold over the levels of basal transcription in G1 cells (reviewed in Osley, 1991; Heintz, 1991). Widely different strategies are employed to regulate this cell cycle specific transcription of histone genes both between different histone genes in the same organism and between the same histone genes in different organisms (reviewed in Osley, 1991; Heintz, 1991). In addition to the transcriptional activation of histone genes, the regulation of pre mRNA processing and mRNA stability increases the levels of histone message in the cytoplasm a further 5 to 6 fold during S-phase. Histone precursor RNA is processed into mature mRNA with a conserved 3' stem loop structure (Busslinger *et al.*, 1979; Hentschel and Birnstiel, 1981; Wells, 1986; Birchmeier *et al.*, 1983) and transported from the nucleus in S-phase but not in G1 or G0 (Luscher and Schumperli, 1987; Stauber and Schumerli, 1988). Once in the cytoplasm, mature histone mRNA has a longer half life during S-phase than when S-phase is inhibited. Histone messages are targeted for degredation outside the S-phase of the cell cycle by the position and location of the 3' stem loop structure (Alterman, *et al.*, 1985; Whitelaw *et al.*, 1986; Levine *et al.*, 1987; Pandey and Marzluff, 1987; Chodchoy *et al.*, 1987) and by translation (Capasso *et al.*, 1987; Graves *et al.*, 1987). In the lower eukaryotes, fungi and ciliates, histone mRNAs do not have the stem loop structure and are instead polyadenylated (Fahrner *et al.*, 1980; Bannon *et*

al., 1983; Choe *et al.*, 1985; Horowitz *et al.*, 1987; Butler *et al.*, 1990). Despite this, the yeast histone messages also appear to have an element in the 3' sequences that targets the mature transcripts for degradation during G1 and G2 phases of the cell cycle (Lycan *et al.*, 1987; Xu *et al.*, 1990).

In almost all organisms the genes encoding core histons do not contain introns (Smith, 1984; Choe *et al.*, 1985; Matsumoto and Yanagida, 1985; Wells, 1986). The only exceptions that have been identified so far are found in the lower eukaryotes *Neurospera* (Woudt *et al.*, 1983), *Aspergillus* (Ehinger *et al.*, 1990; May and Morris, 1987) and *Physarum* (Wilhelm and Wilhelm, 1987).

In all eukaryotes the genes encoding replication dependent (core) histones are part of a multigene family and in higher eukaryotes the linker histone (*H1*) genes are also present in multiple copies. The copy number of histone genes varies from as few as two copies in yeast (Hereford *et al.*, 1979) to as many as several hundred copies in invetebrates (Lifton *et al.*, 1977; Hentschel and Burnstiel, 1981; Maxson *et al.*, 1983). The organisation of histone genes in the genome varies widely among organisms. In invetebrates, the histone genes are part of a reiterated tandem repeat of the five histone genes (Lifton *et al.*, 1977; Kedes, 1979). This arrangement is not found in vetebrates or lower eukaryotes where the genomic organisation is largely dispersive, although some clustering does occur (Heintz *et al.*, 1981; Sittman *et al.*, 1981; Engel and Dodgson, 1981; Sierra *et al.*, 1982; Maxson *et al.*, 1983; Marzluff and Graves, 1984; Engel, 1984, Stein *et al.*, 1984).

Histone amino acid sequences are highly conserved both between the individual copies found in a particular eukaryote and across species. Some minor variations do occur in both of these situations, however, and these are seen as allelic variations that that can be accommodated with maintanance of function (Wu *et al.*, 1986; Wells and McBride, 1989).

Recently the three dimensional structure of the histone octamer core particle and the nucleosome core has been resolved by X-ray crystallography (Arents *et al.*, 1991; Luger *et al.*, 1997). These studies have revealed that all of the core histones share a common structural motif called the histone fold. The histone fold consists of an 11-residue helix (α1), followed by loop (L1), a long 27 residue helix (α2), another loop (L2) and a final 11-residue helix (α3). The X-ray crystal structure has allowed individual histone residues which contribute to histone-histone and histone-DNA interactions in the nucleosome to be identified. These specific interactions will be detailed further in the

consideration of how the incorporation of histone H2A.F/Z might alter the properties of the nucleosome (section 1.5.1).

1.3 Variant histones

Histone variants are distinguished from their core histone counterparts on the basis of differences in gene structure, regulation and/or amino acid sequence. Unlike the core histone genes, many of the variant histones exist as a single copy and contain introns. Some histone variants are expressed at a particular stage of development or differentiation, suggesting that they may produce particular chromatin structures in a particular cell type or at a specific stage of development. The most striking example of developmental regulation of histone variants occurs in sea urchins. During embryogenesis in these organisms an early set of histone variants (H2A, H2B and H1) are transcribed and then in later stages of embryogenesis the expression of these genes is downregulated while that of a second set coding for the late histone variants is upregulated. This transcriptional switch occurs primarily during the blastula stage, a time when cell division rates markedly decline and morphogenesis and cell differentiation commence (von Holt *et al.*, 1984). Some of these sea urchin histone variants are also expressed tissue specifically in mature animals (Angerer *et al.*, 1984). In vertebrates, tissue specific expression of variant histones has also been reported. In birds and fish the linker histone variant, H5, accumulates during the maturation of the transcriptionally silent erythrocyte nuclei (Doenecke and Tonjes, 1986). In mice there is a similar accumulation of a particular linker histone variant, $H1^{o}$, in terminally differentiating cells from many lineages at about the time when the cells cease dividing (Panyim and Chalkley, 1969; Pehrson and Cole, 1980). Knock out experiments have shown that this variant histone is not required for normal mouse development (Sirotkin *et al.*, 1995). Mice also contain at least six other non-allelic subtypes of histone H1 including the somatic variants, H1a through H1e, and a testis specific variant H1t (Lennox and Cohen, 1984). The somatic histone variants, H1a through H1e, have different tissue specific distributions as a consequence of being synthesised at different relative rates in dividing and non-dividing cells. The testis specific variant H1t is not found in chromatin until the pachytene stage of spermatogenesis (Doenecke *et al.*, 1997).

While the core histones are expressed during S-phase, expression of the histone variants H2A.X, H2A.F/Z, H2A.3, H3.3 and the linker variants $H1^{o}$ and H5 is cell cycle independent (Zweidler, 1980; Wu and Bonner, 1981; Zweidler, 1984; Grove and Zweidler, 1984; Doenecke *et al.*, 1997). Analyses of the H3.3 homologue, hv2, in

Tetrahymena have indicated that the critical function of this gene is its constitutive expression and not the production of a distinct protein with minor sequence changes compared to the core H3 histone (Yu and Gorovsky, 1997). This suggests that the function of some variant histone genes is to replace histones which are turned over in the normal course of metabolism. Since H3.3 is synthesised at basal levels throughout the cell cycle and its synthesis continues after cells have ceased dividing, it accumulates in non-dividing cells (Zwiedler, 1984). A similar accumulation of variant histone H2A.3 in non-dividing cells has also been reported (Zwiedler, 1984).

Analysis of the function of some histone variants has shown that they are associated with particular chromatin structures in the nucleus. A centromere specific histone variant was identified in a characterisation of proteins that cross-reacted with centromere specific autoantibodies from patients with limited systemic sclerosis (CREST) syndrome. This protein, CENP-A, contains extended regions of homology to the core H3 histone, is present in nucleosome like structures following micrococcal nuclease digestion of nuclei and copurifies with nucleosome core particles under stringent isolation conditions (Palmer *et al.*, 1987). CENP-A localises to the heterochromatic interior of the centromere, underlying the kinetochore (Palmer *et al.*, 1987). Mutagenesis studies have shown that the histone fold region of CENP-A directs this localisation (Sullivan *et al.*, 1994; Shelby *et al.*, 1997). A functional role for this protein in centromere function was suggested after it was demonstrated that a null mutation in the *S. cerevisiae* CENP-A homologue, *CSE4*, exhibits defects in chromosome disjunction and are unable to progress through mitosis (Stoler *et al.*, 1995).

Another histone variant, which has been isolated from, and characterised in mouse cells, localises specifically to the inactive X chromosome (Xi) of adult female animals. This variant is called macro H2A1.2 because the first third of the protein contains a region which is 64% identical to full length mouse core H2A (Costanzi and Pehrson, 1998). Motif analysis on the remaining two thirds of the protein have identified a region with homology to the leucine zipper motif which acts as a dimerisation domain in some transcription factors (Pehrson and Fried, 1992; Pehrson and Fuji, 1998). The function of macro H2A1.2 during X inactivation has been investigated in ES cells that are induced to differentiate by removal of LIF. In undifferentiated XX ES cells both X chromosomes are transcribed. Then, during differentiation, one X chromosome is randomly inactivated. During day 1-3 of differentiation the inactivated X chromosome becomes coated with X inactive specific transcript (Xist), then from days 7-9 macro H2A1.2 colocalises with Xist RNA in the nucleus (Mermoud *et al.*, 1999). The binding

of Xist RNA to the inactive X chromosome appears to be important for the specific localisation of macro H2A1.2 because deletion of the *Xist* gene in mouse embryonic fibroblasts disrupts the specific localisation of macro H2A1.2 to the inactive X chromosome (Csankovszki *et al.*, 1999). Interestingly, the disruption of macro H2A1.2 localisation does not affect the maintainance of transcriptional silencing (Csankovszki *et al.*, 1999). Since macro H2A does not bind to the X chromosome until after it is silenced and disruption of localisation does not affect silencing, macro H2A1.2 does not appear to be required for either establishment or maintainance of X inactivation.

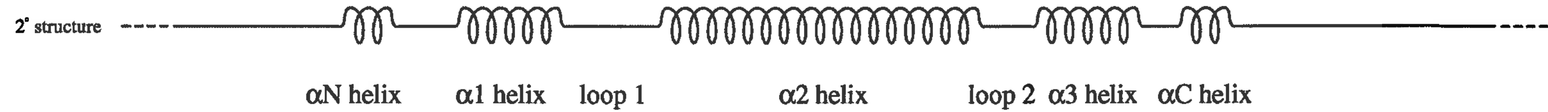
Clearly histone variants provide an opportunity to alter the basic structure of the nucleosome in order to carry out a specialised function. Another histone H2A variant, H2A.F/Z, has also been associated with particular processes in the nucleus. Since this variant was the focus of work conducted in this thesis its function will be considered in detail below.

1.4 Histone H2A.F/Z

Genes encoding H2A.F/Z histones have been characterised in a variety of eukaryotes including human (H2A.Z), mouse (H2A.Z), chicken (H2A.F), sea urchin (H2A.F/Z), *Drosophila melanogaster* (D2, H2A.2, *H2AvD, His2AvD*),*Tetrahymena thermophila* (hv1), *S.pombe* (*pht1*) and *S.cerevisiae* (*HTA3*) (Jackson *et al.* 1996). In these organisms the H2A.F/Z gene is expressed at almost constant levels throughout the cell cycle, is present as a single copy gene (except in sea urchin where there may be 4 copies) and contains introns (except in fission and fusion yeast) (Wu and Bonner, 1981; Ernst *et al.*, 1987; White and Gorovsky, 1988; Dalton *et al.*, 1989; van Daal *et al.*, 1990; Carr *et al.*, 1994). Within a species the amino acid sequences of H2A and H2A.F/Z differ by 40% (Fig 1.1). Across species, the H2A.F/Z amino acid sequence is more extensively conserved than the core histones (van Daal *et al.* 1990). This extraordinary degree of conservation and lethality of the null mutant state in mice (Thonglairoam, 1996), *Drosophila melanogaster* (van Daal and Elgin, 1992) and *Tetrahymena thermophila* (Liu *et al.*, 1996a) confirm that H2A.F/Z plays a fundamental role in eukaryote life. Lethality of the null mutant state also demonstrates that H2A.F/Z function cannot be provided by H2A. Conversely, the demonstration that viability of a yeast strain lacking an endogenous H2A gene could be restored with a *Tetrahymena thermophila* H2A gene but not with the *hv1* gene indicates that H2A function cannot be provided by H2A.F/Z proteins (Liu *et al.* 1996b).

Figure 1.1: Amino acid sequence comparison of *Drosophila* H2A.1 and His2AvD. The amino acid sequence of *Drosophila* H2A.1 (top) and the amino acids unique to His2AvD (bottom) are shown. Gaps represent identical residues, dashes are used to maximize homology between the two sequences and represent sites where there is not an equivalently located amino acid residue. Comprehensive comparison of H2A and H2A.F/Z variant sequences are given in Wells and McBride, 1989 and Jackson *et al.*, 1996.

```
H2A.1     -SGR-GK-FFKVKGKAKSRSNRAGLQFPVGRIHRLLRKGNYAE-RVGAGAPVYLAAVMEYLTAEVLALAGNAARDNKKTRIIPPRHLQLAIRNDEELNKLLSGVTIAQGGVLPNIQAVLLPKKTEKKA-------------
His2AvD   AS KA  DS  A A  V   A               H KSRTTSHG   T A  S  IL                SK L VK  T          G    DS I-KA   G   I H HKS IG  E TVQDPQRKGNVILSQAY
```



Although the precise function of histone H2A.F/Z is not known, studies conducted in the ciliate *Tetrahymena thermophila* have demonstrated that this protein temporally and spatially colocalises to regions of transcriptional activity in the nucleus (Stargell *et al.*, 1993). In this single cell organism there are two nuclei, a transcriptionally inert micronucleus which serves as the germ line of the cell and a transcriptionally active polyploid macronucleus which is destroyed during sexual conjugation (Gorovsky, 1973; 1980). Immunohistochemical studies with antibodies directed against the *Tetrahymena* H2A.F/Z homologue, hv1, found that the protein was specifically located in the transcriptionally active macronucleus and was only found in the micronucleus during a brief period in conjugation when transcription takes place (Wenkert and Allis, 1984; Allis *et al.*, 1986; Stargell *et al.* 1993). Antibodies directed against hv1 also stain the nucleolus of selected mammalian cell lines (Allis *et al.*, 1982) further supporting the association of H2A.Z with transcriptionally active chromatin. The caveat with this last result, however, is that although antibodies directed against hv1 cross react with a protein in western analysis of histone preparations from mouse, wheat and yeast they do not cross react with any protein in histone extracts from calf or *Drosophila* (Allis *et al.*, 1986).

Immunolocalisation studies conducted in *Drosophila* with antibodies directed against the H2A.F/Z homologue, *His2AvD*, indicate that there may not be a simple relationship between transcriptional activity and the presence of histone H2A.F/Z. In an analysis of the distribution of protein on salivary gland polytene chromosomes, His2AvD was found in the euchromatic interband regions and on developmentally activated puffs but is not present on puffs induced by heat shock (Donahue *et al.*, 1986).

Fractionation of nuclear chromatin using a number of different protocols has shown that H2A.F/Z is enriched in fractions which are also enriched in transcribed sequences (Gabrielli *et al.*, 1981; Ridsdale and Davie, 1987). In these, and other fractionation experiments, it has been demonstrated that transcriptionally active chromatin is also enriched in the high mobility group (HMG) -14/-17 proteins (Gabrielli *et al.*, 1981; Postnikov *et al.*, 1991; Bustin and Reeves, 1996), contains histones which are more highly acetylated than bulk chromatin (Sealy and Chalkley, 1978; Vidali *et al.*, 1978; Allegra *et al.*, 1987; Ridsdale and Davie, 1987; Walker *et al.*, 1990) and is depleted of linker histones (Gabrielli *et al.*, 1981; Dimitrov *et al.*, 1990; Kamakaka and Thomas, 1990; Bresnick *et al.*, 1992; Dedon *et al.*, 1991; Postnikov *et al.*, 1991).

Another different function has been ascribed to H2A.F/Z based on the phenotype of deletion mutants in *Schizosaccharomyces pombe*. Unlike *Tetrahymena*, *Drosophila* and

mouse, deletion of the H2A.F/Z homologue, *pht1*, in *S.pombe* is not lethal. Cells lacking a functional copy of *pht1* gene have an increased resistance to heat shock, grow slower than wild type cells and are defective in minichromosome maintenance (Carr *et al.* 1994). This last result led to the suggestion that H2A.F/Z may have a role in chromosome dynamics during mitosis (Carr *et al.* 1994).

In addition to these functional analyses, the stability of H2A.F/Z binding in chromatin has been characterised using chromatin fragments released from chicken erythrocyte nuclei following digestion with micrococcal nuclease (Li *et al.*, 1993). After immobilising chromatin on a hydroxyapatite column, histone proteins were eluted with a salt gradient and analysed by electrophoresis through acid urea triton gels. Core histone H2A is the first histone detected in the eluates from these columns at < 0.7M NaCl whereas histone H2A.F/Z does not elute from these columns until 0.9M NaCl. This study indicates that histone H2A.F/Z is more stably associated with chromatin than the core H2A. A more stable association of histone H2A.F/Z with chromatin could arise from an increased affinity of this histone for DNA in the nucleosome. Since H2A.F/Z elutes from these columns just before, or coincident with, the elution of histones H3 and H4 it is also possible that histone H2A.F/Z could exhibit a higher affinity for chromatin than H2A because it forms a more stable association with the $(H3\text{-}H4)_2$ tetramer than core H2A. Interestingly, one form of histone H2B, H2B.2, eluted at the same salt concentrations as histone H2A.F/Z from the immobilised chromatin. Thus, H2A.F/Z might preferentially associate with this form of H2B in the nucleosome.

Since a direct effect of H2A.F/Z on a particular aspect of transcription or chromosome segregation is to be demonstrated, the rest of this review will examine how this histone variant may be involved in these processes. The most obvious way that H2A.F/Z could participate in a particular function is to produce specific chromatin structures that are required for that function. Thus, the contribution of core histones to chromatin structure will be reviewed so the effect of incorporating H2A.F/Z in the production of alternate chromatin structures can be considered. The role of H2A.F/Z in chromosome segregation will then be addressed in relation to the effect of chromatin on DNA replication, metaphase chromosome condensation and sister chromatid disjunction. Mutations in the genes involved in these processes produce similar defects in chromosome segregation as those found in *S. pombe pht1* null mutants. The colocalisation of histone H2A.F/Z with transcriptionally active chromatin suggests that it could be involved in establishing or facilitating transcriptional processes. Alternatively, or in addition, H2A.F/Z might become enriched in these regions as a

consequence of transcription. In this case histone H2A.F/Z might function as an epigenetic marker for transcribed regions or as a regulator to promote or repress the expression of active genes. The role of H2A.F/Z in transcription will thus be considered as a requirement for, or as a consequence of, transcription on the basis of studies which have shown how the packaging of DNA into particular chromatin strucures influences gene expression. In this analysis of H2A.F/Z function, the possibility that histone H2A.F/Z might also directly mediate or abrogate the function of other proteins that interact with chromatin will also be examined.

1.5 Histones and chromatin structure

In the nucleus of all eukaryotic cells, DNA is complexed with a similar mass of histones and non-histone proteins to form chromatin. Histones contribute to the organisation of DNA into chromatin at a number of different levels. Initially, 146bp of DNA is wound 1.75 times around an octameric protein complex consisting of two molecules of the histones H2A, H2B, H3 and H4 to form the basic repeating subunit of chromatin, the nucleosome core. In most eukaryotes the nucleosome core subunits are spaced at approximately 200bp intervals. In this arrangement, nucleosomes can self-associate to form higher order structures like the 30nm chromatin filament which is widely observed in sections of interphase nuclei. Specific histone residues are also required for the condensation of chromosomes during mitosis. Thus, the substitution of a core histone with a variant histone has the potential to influence DNA packaging from the level of the individual subunit to the highly condensed metaphase chromosome. In the following sections the role of core histones in the packaging of DNA will be examined.

1.5.1 Nucleosome structure

After the nucleosome core was identified as the basic subunit of chromatin and its composition was determined its physical properties were characterised. The histone octamer complex is unstable in the absence of DNA at physiological salt concentrations. Under these conditions the octamer dissociates into two H2A-H2B dimers and a $(H3\text{-}H4)_2$ tetramer (Eickbush and Moudrianakis, 1978; Godfrey *et al.*, 1980). This tripartite nature of the octamer core was confirmed when its three dimensional structure was resolved using X-ray crystallography (Arents *et al.*, 1991). In this analysis the histone octamer was found to be comprised of a centrally located $(H3\text{-}H4)_2$ tetramer which was flanked by two H2A-H2B dimers. The octamer structure derived from X-ray crystallography was also of sufficiently high resolution to reveal the tertiary folding of the individual core histones. All four core histones share a common

structural motif, the histone fold. The histone fold consists of an 11-residue helix (α1), followed by a loop (L1), a long 27 residue helix (α2), another loop (L2) and a final 11-residue helix (α3). Within each heterodimer, the histone partners, ie; H3 and H4 or H2A and H2B, are arranged head to tail in what Arents *et al.* (1991) have called a "handshake" motif. Juxtapositioned molecular surfaces of each dimer partner interact through a series of hydrophobic and hydrogen bonds. Dimer pairing in this way aligns L1 of one histone with L2 of its partner to create two L1L2 DNA binding sites (Arents *et al.*, 1991; Arents and Moudrianakis, 1993; Luger *et al.*, 1997). The α1 helices also pair and form a third DNA binding site, the α1α1 site. DNA interacts with amino acid residues at these binding sites through hydrogen bonds with phosphate atoms, ionically with the DNA phosphate oxygen atoms and through nonpolar contacts with the deoxyribose groups. Phosphate atoms in the DNA also interact with the helix dipole positive charge at the amino terminus of α1 helices in H2B, H3 and H4 and the α2 helices of all four histones.

Heterodimers in the octamer interact primarily through contacts made between the α2 and α3 helices of one histone and another. In the $(H3\text{-}H4)_2$ tetramer, α2 and α3 helices of the H3 molecule in one H3-H4 heterodimer interact with the α2 and α3 helices of H3 in the other H3-H4 heterodimer. Similarly, the α2 and α3 helices of histone H4 interact with the α2 and α3 helices of H2B in a H2A-H2B dimer. The H2B-H4 interface has less hydrogen bonds and a more extensive hydrophobic component than the H3-H3 interface in the $(H3\text{-}H4)_2$ tetramer which may explain why the dimer-tetramer interaction is less stable than interactions between dimers in the tetramer.

Amino acid residues outside the histone fold, at the N- and C-termini of each histone, also contribute significantly to DNA binding and octamer stability (Luger *et al.*, 1997). In addition to the helices in the histone fold, H3, H2B and H2A also have helical regions at the amino (αN), carboxy (αC) or both termini respectively. Extrafold helices in H3, H2B and at the N-terminus of H2A associate with DNA at the surface of the octamer. The H2A αC helix interacts with the H3 αN helix and the short H4 C-terminal tail forming a docking domain to stabilise a dimer/tetramer alignment which provides a continuous helical ramp for DNA to follow around the octamer.

In the X-ray crystal structure of the nucleosome, imaged amino acid residues in the H2A, H2B and H3 amino-terminal tails were resolved as random-coils that extend away from the surface of the octamer and lie within the minor grove of the nucleosomal DNA (Luger *et al.*, 1997). Interestingly, part of the H4 N-terminal tail rich in basic amino acids (K16 to N25) interacts with an acidic patch on a H2A-H2B dimer in the adjacent

nucleosome. This interparticle connection may contribute to the assembly of higher order chromatin structures (see section 1.5.3).

Unfortunately, the complete three dimensional structure of N-terminal sequences of histones H2B, H3 and H4 and the C-terminus of H2A were not resolved in the X-ray crystal structure of the nucleosome. These unimaged regions also extend away from the surface of the octamer and have a high proportion of basic amino acids. While the N-terminal tails of all four histones and the carboxy terminus of H2A have been shown to bind DNA *in vitro* (reviewed in Bohm and Crane-Robinson, 1984; van Holde, 1988; Hansen, 1997; Wolffe, 1998), recent studies indicate that these particular interactions occur as a consequence of chromatin fragmentation to produce the mononucleosomes which were used for characterising histone tail-DNA interactions (Fletcher and Hansen, 1996; Hansen, 1997). Although the structure of histone N-termini remains the subject of investigation these regions have been shown to play important structural and functional roles in many processes including higher order compaction of chromatin, regulation of transcription, nucleosome assembly during S-phase and maintenance of genome integrity (see below).

Comparison of the amino acid sequences of the core H2A and variant H2A.F/Z histones illustrates that there are amino acid differences across the entire length of the proteins (Fig 1.1). Since amino acid differences are found in regions of H2A which are involved in dimerisation, octamer formation, and DNA binding it is possible that histone H2A.F/Z could influence the structure and/or function of nucleosomes through altered histone-histone and/or histone-DNA interactions.

1.5.2 The chromatosome

DNA in the eukaryote nucleus is packaged by histone octamer complexes with a periodic interval of 200±40bp. In nuclear chromatin, a molecule of linker histone interacts with each nucleosome and with the linker DNA between adjacent nucleosomes. This interaction transiently protects 20bp of linker DNA immediately contiguous to the nucleosome core from micrococcal nuclease digestion (Simpson, 1978) and reduces the mobility of nucleosomes on chromatin templates (Pennings *et al.*, 1994; Ura *et al.*, 1995). The metastable complex containing a nucleosome core, linker DNA and the associated linker histone is called a chromatosome (Simpson, 1978).

The precise location of linker histone in the chromatosome is controversial (reviewed in Travers, 1999a; Thomas, 1999; see also An *et al.*, 1998a). Two different models of

linker histone binding to chromatosomes have been proposed. These different models have arisen from the analysis of chromatosomes produced by different means. In one model a DNA binding domain in the linker histone that is structurally similar to the DNA binding region of transcription factor HNF-3γ contacts chromatosomal DNA at one end of the nucleosome and a nearby patch of basic amino acids contacts DNA at, or near, the dyad (Muyldermans and Travers, 1994; Travers and Muyldermans, 1996; Goytisolo *et al.*, 1996; Zhou *et al.*, 1998). In the other model the linker histone is bound under one of the gyres of the nucleosome and contacts DNA through its HNF-3γ DNA binding domain only (Pruss *et al.*, 1996; Hayes, 1996).

Another unresolved aspect of chromatosome structure is the location of the 20bp of DNA that is protected from endonuclease digestion relative to the nucleosome core. Studies of chromatosomes isolated from bulk, cellular chromatin are consistent with the protection of 10bp of linker DNA either side of the nucleosome (Stanov and Crane-Robinson, 1988; Zhou *et al.*, 1998). In contrast, chromatosomes reconstituted on DNA fragments with nucleosome positioning sequences suggest that there is an asymmetrical protection of 20bp on one side of the nucleosome and 0bp on the other (Wong *et al.*, 1997; An *et al.*, 1998b).

The interaction of core histones with DNA changes as a consequence of linker histone binding. In the absence of linker histone, H3 and H4 crosslink to the DNA in the nucleosome core within 30bp of the dyad axis, histones H2A and H2B crosslink 40-80bp away from the dyad axis and H2A also crosslinks to the dyad. In the presence of linker histone, H4, H2B and H2A contacts are reduced on the side of the nucleosome where linker histone confers protection against micrococcal nuclease digestion while on the other side of the nucleosome the contacts are similar to those observed in the absence of linker histone (Guschin *et al.*, 1998). A reorganisation of the contacts that the carboxy-terminus of H2A makes with DNA in the presence of linker histone has also been characterised (Usachenko *et al.*, 1994; Lee and Hayes, 1998).

All of the data and proposals presented above are supported by experiments which show that linker histone interacts with a H2A/H2B dimer. An intimate interaction between the globular domain of histone H1 and H2A was characterised by Boulikas *et al.* (1980) using a water soluble carbodiimide to catalyse the formation of covalent bonds between residues of proteins in electrostatic contact. In another study, it was found that histones H2A and H2B were necessary for the stable association of H5 with a nucleosome (Hayes *et al.*, 1994).

Although the available data makes it difficult to discern where the linker histone binds and how it protects linker DNA from nuclease digestion it has been found that linker histone incorporation into chromatin influences aspects of transcription (section 1.6) and chromosome condensation (below). Thus, histone H2A.F/Z might influence processes that involve linker histones by interacting with them in a different manner to the core H2A.

1.5.3 Higher order chromatin structures

Arrays of chromatosomes released from chicken erythrocyte nuclei after mild nuclease treatment form 30nm chromatin fibres in physiological salt (Thoma *et al.*, 1979). 30nm fibres have also been produced in a defined *in vitro* system using chromatin assembled onto a DNA fragment containing 12 tandem repeats of a 208bp 5S rDNA sequence from the sea urchin *Lytechinus variegatus* (Simpson *et al.*, 1985). The 5SrDNA sequence directs the positioning of nucleosomes on the repeat fragment so that they are separated by a defined length of linker DNA to produce an array which artificially recreates the spacing which is observed *in vivo*. Analytical ultracentrifugation studies conducted on native and reconstituted chromatosome arrays have demonstrated that the compact fibre, which has a sedimentation co-efficient of 55S, decondenses in two steps to form structures with sedimentation co-efficients of 40S and 29S respectively as the as the ionic strength is decreased from physiological levels (Thoma *et al.*, 1979; Garcia-Ramirez, *et al.*, 1992; Schwarz and Hansen, 1994; Schwarz *et al.*, 1996). Nucleosome arrays, like chromatosome arrays, can compact to form moderately (40S) and highly (55S) condensed chromatin fibres, but at slightly higher salt concentrations than chromatosome arrays indicating the structures formed are less stable (Hansen *et al.*, 1989; Garcia-Ramirez *et al.*, 1992; Hansen and Wolffe, 1992; Schwarz and Hansen, 1994; Fletcher and Hansen, 1995; Tse and Hansen, 1997). Nucleosome arrays assembled using histones which have been digested with trypsin to remove the N-terminal tails do not form highly condensed structures at physiological salt concentrations (Garcia-Ramirez *et al.*, 1992; Fletcher and Hansen, 1995; Tse and Hansen, 1997). Thus, the ability of arrays to form compact chromatin fibres is mediated by both the degree of DNA charge neutralisation and the inherent properties of nucleosome tails in a process which is stabilised by H1. Small perturbations in the composition of the chromatin fibre can cause marked differences in the higher order compaction of chromatin. When the 12 octamer binding arrays used in *in vitro* studies are assembled into 10 or 11 nucleosomes the ability of these arrays to form compact fibres is compromised and folding is abolished altogether when less than 7 octamers are present (Schwarz and Hansen, 1994; Fletcher *et al.*, 1994)

Nucleosome arrays also self-associate in a cooperative and reversible manner to form large soluble oligomers at physiological salt concentrations (Schwarz and Hansen, 1994; Schwarz *et al.*, 1996). This oligomerisation is thought simulate, *in vitro*, the extensive interfibre interactions which have been observed in intact nuclei (McDowall *et al.*, 1986; Giannasca *et al.*, 1993). Arrays assembled with fully trypsinised histones do not oligomerise (Schwarz *et al.*, 1996; see also, Moore and Ausio, 1997; Tse and Hansen, 1997). Oligomer formation is also disrupted in arrays that are not fully assembled into nucleosomes (Schwarz *et al.*, 1996).

One way in which core histone tails may function in chromatin compaction or oligomerisation was unexpectedly revealed when the X-ray crystal structure of the nucleosome was solved (Luger *et al.*, 1997). In the nucleosome crystals, basic residues in the H4 amino terminal tail (K16 to N25) make multiple ionic and hydrogen bonds with a patch of acidic residues on the surface of the H2A-H2B dimer (derived from H2A residues E56, E61, E64, D90, E91, E92 and H2B E110) in the adjacent nucleosome core particle. In H2A.F/Z containing nucleosomes the nature of this interaction may vary because the acid patch is extended slightly by replacement of the H2A residues N93 and K95 with D and S respectively. The demonstration that higher order chromatin structures are less stable when they are assembled with trypsinised H2A/H2B dimers than they are with intact H2A/H2B dimers indicates that the N-terminal tail regions of these proteins are important for compaction (Tse and Hansen, 1997). In the amino terminal 15 amino acids of H2A that are removed by trypsinisation there are sequence differences between it and H2A.F/Z at 8 sites (Fig 1.1).

1.5.4 Metaphase chromosome condensation

During cell division chromatin dispersed throughout the interphase nucleus condenses to produce physically separate compact structures observed as mitotic chromosomes by various forms of microscopy. A number of models for the arrangement of chromatin in the mitotic chromatid have been proposed, but difficulties in tracing the folding of individual chromatin fibres throughout large chromosomal regions has hampered the direct verification or contradiction of these models. One structure which has been observed in the mitotic chromosomes of human, CHO and *Drosophila* cells is a fibre of 100-130nm diameter (Belmont *et al.*, 1989; Belmont and Bruce, 1994). Analysis of thin serial sections of nuclei during chromosome condensation and decondensation suggest that these fibres are produced by twisting a 60nm chromatin fibre which is in turn derived from folding of the 30nm chromatin filament (Belmont and Bruce, 1994).

Metaphase chromatin, like interphase chromatin, is not homogeneous. Different regions of metaphase chromosomes absorb DNA stains to different extents to produce characteristic banding patterns which indicates that the concentration of DNA, or its accessibility, varies across the chromatid. In support of the latter possibility it has been demonstrated that regions of metaphase chromosomes that contain sequences which are transcribed during interphase are more sensitive to DNAse1 and hydroxy radicals than regions which are transcriptionally silent (Gazit *et al.*, 1982; Kerem *et al.*, 1984; Michelotti *et al.*, 1997). The molecular determinants of these different chromatin structures in metaphase chromosomes are not known.

Although the arrangement of chromatin in metaphase chromosomes is yet to be resolved, two molecular requirements for chromosome condensation have been identified; phosphorylation of serine residue 10 of histone H3 (Gurley *et al.*, 1978; Th'ng *et al.*, 1994; Guo *et al.*, 1995; Ajiro *et al.*, 1996a; Ajiro *et al.*, 1996b; Hendzel *et al.*, 1997) and the presence of a functional condensin complex (Hirano and Mitchinson, 1994; Strunnikov *et al.*, 1993, 1995; Saka *et al.*, 1994; Kimura and Hirano, 1997; Kimura *et al.*, 1998; see also Hirano, 1998; Hirano, 1999). An essential role for the phosphorylation of Serine 10 of histone H3 in chromosome condensation was conclusively established recently in *Tetrahymena thermophila* when it was shown that replacement of the endogenous histone H3 with a mutant form of H3 which could not be phosphorylated at position 10 (S10A) disrupted chromosome condensation and segregation (Wei *et al.*, 1999). Chromatin condensation during mitosis also requires a function provided by the condensin protein complex (Hirano and Mitchinson, 1994; Hirano *et al.*, 1997; Strunnikov *et al.*, 1993, 1995; Saka *et al.*, 1994; Bhat *et al.*, 1996; see also Hirano, 1998; Hirano, 1999). Functional analysis of a purified *Xenopus* condensin complex *in vitro* has lead to the proposal that it might contribute to chromosome condensation by generating superhelical tension after a condensin subunit, XCAP-D2, is phosphorylated by the mitosis specific cdc-2 kinase (Kimura and Hirano, 1997; Kimura *et al.*, 1998).

Two models have been proposed that link H3 phosphorylation and the activity of condensins (Wei *et al.*, 1999). Phosphorylation of histone H3 at serine 10 may create a binding site for chromatin condensation components. Alternatively, by reducing the net positive charge of the H3 amino terminal region, the phosphorylation of histone H3 serine 10 may disrupt histone-DNA interactions in a way that facilitates condensation factor access to DNA in chromatin. Considering that yeast strains homozygous for a histone H2A.F/Z null mutation have chromosome segregation defects (Carr *et al.*, 1994)

it is possible that this histone could function in conjunction with phosphorylated histone H3 in either of these processes.

1.5.5 Centromeric chromatin and chromosome segregation

In addition to chromosome condensation, defects in chromosome segregation have been reported for mutation in genes that encode proteins involved in centromere formation, DNA replication, chromosome segregation, transcription of cell cycle genes, histone deacetylation, and chromatin remodelling complexes.

Centromeres have been divided into three subdomains which reflect their participation in different elements of centromere function; the pairing domain, the kinetochore domain and the central domain. The pairing domain is the region of the inner centromere that faces the same region on the sister chromatid and is involved in sister chromatid cohesion. The central domain is comprised of centromeric DNA sequences packaged into heterochromatin to produce a region which is transcriptionally silent and recombination deficient. The kinetochore domain resembles a trilaminar button like structure on the outer centromere and is responsible for the association with microtubules and motor proteins that segregate the divided sister chromatids towards the spindle poles in mitosis.

As discussed in section 1.3, inactivation of the gene encoding the *S. cerevisiae* centromere specific, histone H3 variant *CSE4* in yeast disrupts chromosome segregation (Stoler *et al.*, 1995). Mutations in some of the genes that produce proteins associated with heterochromatin have also been shown to disrupt chromosome segregation (Ekwall *et al.*, 1995; Allshire *et al.*, 1995; Kellum and Alberts, 1995; Karpen and Allshire, 1997). Thus, H2A.F/Z could be involved in chromosome segregation by influencing the formation of heterochromatin or centromere specific structures. These possibilities seem unlikely however because histone H2A.F/Z localises to euchromatic regions on *Drosophila* polytene chromosomes (Donahue *et al.*, 1986) and does not appear to be enriched in the centromeric chromatin of human cells (see Fig 3 in Pashev *et al.*, 1983).

The minichromosome maintainance (*MCM*) genes were identified in yeast in a screen for mutations which caused defective segregation of a minichromosome. Some of these genes have subsequently been found to be involved in the initiation and processivity of DNA replication (reviewed in Kearsey and Labib, 1998; Pasero and Gasser, 1998). Since origins in heterochromatin fire later than origins in euchromatin it appears that chromatin structure can influence the efficiency of replication (Maillet *et al.*, 1996;

Raghuraman *et al.*, 1997; Ferreira *et al.*, 1997). H2A.F/Z could therefore influence origin firing either directly through interaction with replication proteins and/or indirectly through its effects on chromatin structure.

Characterisation of the *MCM1* gene has shown that a protein involved in the regulation of transcription can be involved in chromosome segregation. Mcm1p is a transcription factor which regulates the expression of a diverse range of genes (Passmore *et al.*, 1988; Messenguy and Dubois, 1993; Kuo and Grayhack, 1994; Althoefer *et al.*, 1995). The recent demonstration that Mcm1 is required for the G2 to M phase specific expression of *CLB1*, *CLB2*, *CDC5*, *SWI5* and *ACE2* has lead Althoefer *et al.* (1995) to propose that Mcm1 deficient cells lack the factors essential for the initiation and execution of mitosis. These results provide an example of how disrupted expression of the appropriate genes can indirectly influence the correct segregation of chromosomes.

Histones in mitotic chromosomes are underacetylated relative to those in interphase chromatin (Chahal *et al.*, 1980; D'Anna *et al.*, 1983; Turner, *et al.*, 1989; Turner and Fellows, 1989; Jeppesen *et al.*, 1992). In *S. pombe*, treatment of wild type cells with the histone deacetylase inhibitor trichostatin A (Grewal *et al.*, 1998; see also Yoshida *et al.*, 1995) or null mutations in the putative histone deacetylase, *clr6* (cryptic loci regulator), reduce fidelity of chromosome segregation leading to chromosome loss (Grewal *et al.*, 1998). While it has been shown that *S. pombe* cells treated with TSA or containing a mutation in *clr6* increase the expression of centromere proximal genes the effect that these experimental systems have on the function of the centromere, chromosome condensation, DNA replication or in the regulation of genes involved in chromosome segregation have not been directly addressed.

Chromatin remodelling complexes have also been implicated in chromosome segregation in recent studies which showed that a temperature sensitive mutation of the ATPase subunit of the remodels the structure of chromatin (RSC) complex causes missegregation of minichromosomes at the nonpermissive temperature (Tsuchiya *et al.*, 1998). In this study it was further shown that DNA in the vicinity of the centromere becomes more accessible to nucleases at the elevated temperatures. While this study suggests that RSC is involved in the production of an active centromere through chromatin remodelling it did not rule out the possibility that this effect might also occur as a consequence of disrupted transcription.

Thus, the chromosome segregation defects seen in H2A.F/Z knock out yeast could reflect a direct role for this histone in chromosome condensation, centromere formation

or DNA replication. Alternatively, this phenotype could also arise from disrupted expression of genes which encode proteins involved in these processes or in the expression of genes involved in chromosome segregation. The possible role of H2A.F/Z in gene expression will be considered in the following section.

1.6 Chromatin and gene expression

Gene expression is a multistep process (reviewed in Hernandez, 1993; Zawel and Reinberg, 1995; Lee and Young, 1998). Activation of gene transcription is thought to be initiated by the binding of sequence specific transcription factors to enhancer and/or promoter regions. This action influences recruitment of the general transcription factors which direct promoter recognition by RNA polymerase. *In vitro* studies have demonstrated that the packaging of DNA into chromatin can inhibit the binding of both sequence specific and general transcription factors. After the initiation of RNA synthesis, chromatin can further disrupt transcription by inhibiting RNA polymerase elongation. In addition to these direct influences on transcriptional processes chromatin also provides a protein scaffold to which other positive or negative regulators can bind.

Recently it has become apparent that many genes employ similar strategies for overcoming histone-mediated repression of transcription. These strategies utilise processes which make DNA in chromatin more accessible to the transcription apparatus. In some instances chromatin structure is disrupted by transcription factors which can bind directly to sites in chromatin. Direct binding of transcription factors can be influenced by cooperative interactions with other transcription factors, histone binding proteins or nucleosome positioning. Two activities have also been identified which alter chromatin structure and facilitate transcription apparatus binding to the promoters of a number of genes. One activity is derived from the histone acetyltransferase (HAT) family of conserved proteins which enzymatically catalyses the addition of an acetyl group to lysine residues in the amino terminal regions of histones. The other activity uses the energy of ATP hydrolysis to remodel histone/DNA interactions in the nucleosome and is also derived from a family of highly related proteins.

Histone H2A.F/Z colocalises with regions of chromatin that are enriched in transcribed sequences (see section 1.4). This co-localisation suggests that H2A.F/Z could either be involved in establishing/facilitating transcriptional processes or might be incorporated into chromatin as a consequence of transcription. Incorporation of H2A.F/Z into chromatin as a consequence of transcription could also influence further transcription in

a regulatory capacity or as an epigenetic marker for potentially active regions. This section will therefore review how the incorporation of histone H2A.F/Z could influence transcription based on studies which have shown how the transcription apparatus overcomes core histone mediated repression. In addition, the possible consequences of H2A.F/Z incorporation into chromatin will also be considered.

1.6.1 Chromatin structure of active genes

Chromatin structure has been investigated in isolated nuclei since the late 1970s using probes which cut or modify DNA (reviewed in Gross and Gerrard, 1988). The endonuclease DNAse I has been widely used in this kind of analysis because it can enter nuclei and cut accessible DNA with minimal sequence specificity. Different regions of the genome differ in their sensitivity to DNAse I. Potentially active and actively transcribed sequences are enriched in regions of chromatin which are an order of magnitude more sensitive to DNAse I than bulk chromatin. In addition, the genome is punctuated by discrete sites which are two to three orders of magnitude more sensitive to DNAse I than the rest of the genome and are consequently referred to as hypersensitive sites. Hypersensitive sites map to sequences that function in replication, recombination and chromosome segregation. Their presence is usually associated with the binding of a particular protein at, or in the vicinity of, the hypersensitive site. All expressed and some inducible genes contain hypersensitive sites in their control regions. The promoters of constitutively active genes have a characteristic pattern of hypersensitive sites which are as ubiquitous as their expression. The promoters of inducible genes have been categorised according to their DNAse I digestion profile before and after induction. Inducible promoters with similar patterns of DNAse I hypersensitivity before and after induction are said to be "preset". At "remodelling" promoters, hypersensitive sites appear following gene induction and prior to, or concomitant with, transcription (reviewed in Gross and Garrard, 1988). These features of hypersensitive sites suggest that particular chromatin structures are either required for, or produced as a consequence of, transcription *in vivo*.

Treatment of isolated nuclei with endonucleases has shown that DNA in the transcribed region, as well as at the promoter, becomes more accessible to nucleases during activation. Within the transcribed region of the human hsp70 gene, disruption of chromatin over the first 400bp is resistant to the RNA polymerase II transcription inhibitor α amanitin. At sites further downstream, the disruption of chromatin was sensitive to α amanitin indicating that RNA polymerase movement caused, and was required to maintain, the disrupted structure in this region (Brown and Kingston, 1997).

In a crude *in vitro* system, transcription factor activation domains were found to direct promoter proximal chromatin disruption. When templates containing a stalled RNA polymerase were assembled into chromatin, a BamH1 site at position +154 (relative to the start of transcription, +1) was much more accessible when a transcription factor with the heat shock factor (HSF) activation domain was present (Brown and Kingston, 1997).

As discussed in section 1.4, biochemical fractionation of chromatin has shown that transcriptionally active regions are enriched in variant histone H2A.F/Z, high mobility group (HMG) -14/-17 proteins (Gabrielli *et al.*, 1981; Postnikov *et al.*, 1991; Bustin and Reeves, 1996), contains histones which are more highly acetylated than bulk chromatin (Sealy and Chalkley, 1978; Vidali *et al.*, 1978; Allegra *et al.*, 1987; Ridsdale and Davie, 1987) and is depleted of linker histones (Gabrielli *et al.*, 1981; Dimitrov *et al.*, 1990; Kamakaka and Thomas, 1990; Bresnick *et al.*, 1992; Dedon *et al.*, 1991; Postnikov *et al.*, 1991).

1.6.2 The influence of chromatin structure on gene expression

A number of *in vivo* and *in vitro* experiments have demonstrated that the assembly of DNA into chromatin inhibits transcription. These demonstrations prompted complementary *in vitro* biochemical and *in vivo* genetic experiments to identify the nature of chromatin mediated repression and have shown that chromatin can inhibit sequence specific and general transcription factor binding and RNA polymerase elongation.

Inhibition of sequence specific and general transcription factor binding by nucleosomes *in vitro* has been reported by many investigators. These studies have demonstrated that the affinity of different transcription factors for their particular binding site is reduced to different extents by the packaging of their recognition sequences into nucleosomes (reviewed in Owen-Hughes and Workman, 1994; Workman and Kingston 1998).

Transcription factor binding affinity is also influenced by the position of its site in the nucleosome. The glucocorticoid receptor (GR) has a lower affinity for its consensus binding site when it is oriented towards the histones in the octamer than an outward facing site (rotational position) (Li and Wrange, 1995). The affinity of GR and Gal4p for their consensus binding sites (GRE and UAS respectively) is also lower for sites close to the nucleosome dyad than sites at the periphery of the nucleosome (translational position) (Vettesse-Dadey *et al.*, 1994; Li and Wrange, 1993).

The binding of one transcription factor to a nucleosome can facilitate binding of other transcription factors to their site in the same nucleosome through cooperativity (Giniger and Ptashne, 1988; Vettesse-Dadey, *et al.*, 1994; Adams and Workman, 1995; Ng *et al.*, 1997; Tanaka, 1996). Analysis of the mechanics of cooperative binding has found that sites at the nucleosome periphery become occupied first (Vettesse-Dadey, *et al.*, 1994).

Binding of a transcription factor to nucleosomes disrupts histone-DNA contacts and produces a metastable ternary complex consisting of the transcription factor, histones and DNA (Workman and Kingston, 1992; Bresnik *et al.*, 1992; McPherson *et al.*, 1993; Truss *et al.*, 1995; Owen-Hughes and Workman, 1996; Steger and Workman, 1997). Removal of the transcription factor from the ternary complex using double stranded DNA oligonucleotides containing the transcription factor consensus binding site results in the reformation of a canonical nucleosome. However, in the presence of nonspecific naked DNA or the histone binding proteins nucleoplasmin or NAP-1, the canonical nucleosome does not reform after Gal4p is removed by competitor DNA indicating that the nucleosome has been displaced (Workman and Kingston, 1992; Chen *et al.*, 1994; Walter *et al.*, 1995; this is not universal, see, Steger and Workman, 1997). Two different mechanisms for histone octamer displacement have been identified. Nucleosplasmin and NAP-1 requires disruption of the dimer-tetramer interface but octamer transfer to naked DNA does not (Walter *et al.*, 1995).

As well as having an inhibitory influence on transcription factor binding, the assembly of templates into nucleosomes or $(H3\text{-}H4)_2$ tetramers has been found to almost completely block the elongation of RNA polymerase II *in vitro* (Izban and Luse, 1991; 1992; Chang and Luse, 1997). A protein complex comprised of a 140KDa and an 80kDa peptide which can facilitate the elongation of RNA polymerase II on chromatin templates has been purified from HeLa extracts by Orphanadies *et al.* (1998). Additional biochemical characterisation of this complex, called FACT (for facilitates chromatin transcription), has shown that it does not require ATP or a transcriptional activator to function and it does not stimulate transcription initiation (Orphanadies *et al.*, 1998; LeRoy *et al.*, 1998).

Chromatin can also cause RNA polymerase II to pause at specific sites during elongation (reviewed in Reines *et al.*, 1996). Addition of a human SWI/SNF chromatin remodelling preparation (see section 1.6.5) augmented elongation through a pause site at position +45 of the human hsp70 gene, but on activator bound templates only (Brown *et al.*, 1996).

The effects of histone H1 on transcription are gene specific with repressive, minimal and activating functions being detected in different systems (Shimamura *et al.*, 1989; Laybourn and Kadonaga, 1991; 1992; Bouvet *et al.*, 1994; Sandaltzopoulos *et al.*, 1994; Shen and Gorovsky, 1996; Wong *et al.,* 1997; Panetta *et al.*, 1998; Lee and Archer, 1998). A similar phenotype to histone H1 knock out in *Tetrahymena thermophila* was produced when the endogenous H1 was replaced with a version that simulated a phoshorylated form of H1 suggesting that phosphorylation mimics removal (Dou *et al.*, 1999; see also Lee and Archer, 1998). Repression of transcription by histone H1 is thought to occur as a result of its stabilisation of higher order chromatin structures (Hansen *et al.*, 1989; Schwarz and Hansen, 1994; Carruthers *et al.*, 1998; section 1.5.3) and/or restriction of nucleosome mobility (Pennings *et al.*, 1994; Ura *et al.*, 1995). Recently it has been demonstrated that HMG-14 (Ding *et al.*, 1997) and HMG-I/-R (Nagpal *et al.*, 1999) can overcome repressive effects of histone H1 on transcription. Since HMG-I interacts with ligand bound RAR, c-Jun, CBP, PPARγ, retinoid X receptor (RXRα) and is capable of displacing histone H1 from A+T rich DNA (Zhao *et al.*, 1993) it has been proposed that this activation of transcription involves displacement of histone H1 (Nagpal *et al.*, 1999).

Histone H2A.F/Z could therefore be involved in transcription by promoting the access of sequence specific and/or general transcription factors to their binding sites in chromatin. The demonstration that histone H2A.F/Z is more tightly associated with chromatin does not necessarily preclude this possibility. For example, if H2A.F/Z binds more tightly to chromatin because it has a higher affinity for the $(H3\text{-}H4)_2$ tetramer it could promote transcription factor binding by having a lower affinity for DNA which would make regions at the nucleosome periphery more accessible. If H2A.F/Z has a higher affinity for DNA than the core H2A histone it could promote transcription factor access by destabilising the dimer-tetramer interaction to make binding sites near the dyad more accessible. Alternatively, or in addition, H2A.F/Z could facilitate the elongation of transcripts by RNA polymerase, either directly through producing chromatin structures which are more amenable to the elongation of RNA polymerase, or indirectly, for example, by promoting the binding of proteins like HMG-14 which increase the processivity of RNA polymerase (Ding *et al.*, 1994).

1.6.3 Chromatin remodellers

The convergence of numerous biochemical and genetic studies has resulted in the identification of protein complexes that are able to overcome the inhibitory effects of chromatin on the binding of sequence specific and general transcription factors to DNA.

These complexes are referred to as nucleosome remodellers because they function by changing the accessibility of nucleosomal DNA to solvent or by promoting nucleosome sliding. Characterisation of the composition of these complexes has revealed that they all contain a subunit with a nucleic acid dependent ATPase domain (Carlson and Laurent, 1994). To date, three families of proteins, with this domain, that have chromatin remodelling activity have been identified; SWI2/SNF2, ISWI and Mi2 (recently reviewed in Felsenfeld *et al.*, 1996; Hartzog and Winston, 1997; Tsukiyama and Wu, 1997; Gregory and Horz, 1998; Varga-Weisz and Becker, 1998; Cairns, 1998; Workman and Kingston, 1998; Kingston and Narlikar, 1999; Travers, 1999b; Bjorklund *et al.*, 1999).

The effect that these complexes have on chromatin structure has been analysed using mononucleosomes, nucleosome arrays and plasmids assembled into nucleosomes. Yeast and human complexes containing the SWI2/SNF2 subunit (or its homologues) remodel histone-DNA interactions in the nucleosome so that the DNA is more accessible to transcription factors and nucleases (Cote *et al.*, 1994; Kwon *et al.*, 1994; Imbalzano *et al.*, 1994; Cairns *et al.*, 1996; Owen-Hughes *et al.*, 1996; Cote *et al.*, 1998; Lorch *et al.*, 1998). This effect is not due to histone displacement since all of these structures contain the same histone concentration and stochiometry (Cote *et al.*, 1998; Lorch *et al.*, 1998; Schnitzler *et al.*, 1998). The Swi2/Snf2 containing chromatin remodeling complex (SWI/SNF complex), however, does evict histones from mononucleosomes that contain Gal4p binding sites in the presence of Gal4p and ATP (Owen-Hughes *et al.*, 1996). The conformation that a nucleosome adopts as a consequence of remodelling is not known, however, the remodelling of mononucleosomes by SWI/SNF produces a reaction product which appears to be a dimer of two complete mononucleosomes in gel filtration, gradient sedimentation and EMSA analysis (Schnitzler *et al.*, 1998).

Human, *Drosophila* and *S. cerevisiae* complexes which contain ISWI family members have also been shown to facilitate transcription factor binding (Tsukiyama and Wu, 1995; Varga-Weisz *et al.*, 1997; Mizuguchi *et al.*, 1997; Ito *et al.*, 1997; Tsukiyama *et al.*, 1999; Corona *et al.*, 1999). Complexes containing ISWI appear to function in one of two ways either by promoting nucleosome sliding (Varga-Weisz *et al.*, 1997; Ito *et al.*, 1997; LeRoy *et al.*, 1998; Tsukiyama *et al.*, 1999) or by disrupting the structure of the nucleosome (Tsukiyama and Wu, 1995; LeRoy *et al.*, 1998; Tsukiyama *et al.*, 1999).

Recent results have indicated that, in yeast, chromatin remodelling complexes are recruited to the promoter regions of genes by transcription factors with acidic activation domains (Neely *et al.*, 1999; Yudkovsky *et al.*, 1999). In mammalian systems, interactions between the SWI/SNF chromatin remodelling complex and transcription factors, including steroid hormone receptors (Fryer and Archer, 1998; Ostlund *et al.*, 1997) and ELKF (Armstrong *et al.*, 1998) have also been demonstrated.

At the *HO* promoter, a time course analysis of factor binding showed that the transcription factor, Swi5p, recruits SWI/SNF and then the Gcn5p containing histone acetylase complex, SAGA, associates with the promoter (Cosma *et al.*, 1999; see also section 1.6.5). The combined activity of these factors then facilitates binding of the transcription factor SBF (Cosma *et al.*, 1999). This demonstration that transcription is regulated by a coordinated interplay between transcription factors, chromatin remodellers, histone acetylases and the transcription apparatus has also been demonstrated in other systems (Pollard and Peterson, 1997; Drysdale *et al.*, 1998; Natarajan *et al.*, 1998; 1999; Krebs *et al.*, 1999; Biggar and Crabtree, 1999; Sudarsanam *et al.*, 1999; Kingston and Narlikar, 1999).

In addition to having a stimulatory role in transcription, nucleosome remodellers have also been associated with the repression of transcription. In microarray experiments it was found that SWI/SNF appears to act more frequently in repression events than in activation events (Holstege *et al.*, 1998; see also, Moreira and Holmberg, 1999; Murphy *et al.*, 1999). It has recently also become apparent that repression of transcription might be directed by a coordinated interplay between chromatin remodellers and histone deacetylases (Trouche *et al.*, 1997; LeGouy *et al.*, 1998; Muchardt *et al.*, 1998; Brehm *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). Indeed, a protein complex has been identified in human and *Xenopus* cells which has both nucleosome remodelling and histone deacetylase activities (NuRD) (Tong *et al.*, 1998; Wade *et al.*, 1998; Xue *et al.*, 1998; Zhang *et al.*, 1998a). The conserved Mi2 subunit of this complex contains a DNA dependent ATPase domain (Tong *et al.*, 1998; Wade *et al.*, 1998; Zhang *et al.*, 1998a). Functional studies have shown that NuRD complexes might function in the establishment and maintenance of repression by interactions with methylated DNA (Jones *et al.*, 1998; Wade *et al.*, 1998; Zhang *et al.*, 1999), transcription factors like Ikaros (Kim *et al.*, 1999), hunchback (Kehle *et al.*, 1998), TR (Xue *et al.*, 1998) or possibly co-repressors like TR (Xue *et al.*, 1998).

In *S. cerevisiae*, a number of mutations in histone genes have been found to alter the requirement for the SWI/SNF complex in the expression of genes dependent on SWI/SNF function. SWI/SNF independent (Sin) mutations in histones H3 and H4 are located in the histone fold domains, predominantly in DNA binding loop motifs, which are located close to the dyad axis of the nucleosome (Kruger *et al.*, 1995). Analysis of the ability of some Sin versions of H3 and H4 to introduce superhelical turns into plasmid DNA has demonstrated that while the H4 Sin mutant R45H has an impaired ability to constrain supercoils, the H3 Sin mutants E105K, R116H and T118I package DNA in a similar manner to wild type core histones (Wechser *et al.*, 1997; Kurumizaka and Wolffe 1997). Further *in vitro* and *in vivo* characterisation of chromatin containing H3 and H4 Sin mutations has shown that, in all but one case (H3 E105K), the DNA packaged by these histones is more susceptible to endonuclease digestion and Dam methyltransferase activity than DNA packaged by wild type histones (Wechser *et al.*, 1997; Kurumizaka and Wolffe 1997).

Particular H4 mutants were constructed to investigate the role of histone H4 tyrosine residues Y72, Y88 and Y98 which, in the X-ray crystal structure of the octamer, appeared to be involved in interactions between the $(H3\text{-}H4)_2$ tetramer and the H2A-H2B dimer (Arents *et al.*, 1991; Arents and Moudrianakis, 1993). H4 tyrosines 72 and 88 contact one H2A-H2B dimer subunit and tyrosine 98 contacts the other dimer subunit (Arents *et al.*, 1991; Arents and Moudrianakis, 1993). The H4 mutants Y88-G and Y98-H grow slowly and the H4 mutant Y72-G causes cells to arrest in the G1 phase of the cell cycle (Santisteban *et al.*, 1997). The demonstration that cell cycle arrest by Y72-G coincides with decreased expression of genes involved in progression through the G1 phase indicates that mutation of these residues disrupts an event in gene activation. Interestingly, these mutant versions of histone H4 only have a partial Sin phenotype in that they are able to restore transcription of the SWI/SNF dependent gene *INO1*, but not *SUC2*, in cells lacking a functional SWI/SNF complex (Santisteban *et al.*, 1997). These results have lead to the proposal that while SWI/SNF function is sufficient for the activation of *INO1* and Ty elements, an additional step is required to overcome chromatin mediated repression at *SUC2* (see also, Hirschhorn *et al.*, 1995).

In considering the role of H2A.F/Z in transcription it is therefore possible that this variant histone could be involved in transcription by facilitating the role of chromatin remodellers. It could promote the action of chromatin remodellers by increasing the binding of transcription factors (see section 1.6.3) that recruit chromatin remodelling complexes, catalysing the transfer of remodelling complexes from one nucleosome to the next (see Logie *et al.*, 1999), stabilising the action of remodellers or stabilising the

remodelled structure. In relation to the results obtained with dimer interface mutants of H4 (Santisteban *et al.*, 1997) indicate that gene activation requires an event downstream or independent from the action of chromatin remodellers. Since histone acetylases are recruited after SWI/SNF at the *HO* promoter (Cosma *et al.*, 1999), and the histone acetylase Gcn5p is required for full expression of *SUC2* (Pollard and Peterson, 1997) this additional step in gene activation might involve histone acetylation. The possible function of H2A.F/Z in relation to histone acetylation is considered in the following section.

1.6.4 Histone acetylation, gene expression and chromatin structure

Since the original demonstration by Allfrey *et al.* in 1964 that the ε-amino groups of lysine residues in the core histones are posttranslationally modified by acetylation a large body of evidence has accumulated which indicates that this modification plays an important role in regulating gene expression and chromatin assembly (reviewed in Kaufmann, 1996; Imhof and Wolffe, 1998; Kuo and Allis, 1998; Mizzen and Allis, 1998; Pollard and Peterson, 1998; Struhl, 1998; Workman and Kingston; 1998; Kingston and Narlikar; 1999). *In vivo*, only the lysine residues on the N-terminal tails of histones are modified by acetylation. Histones H2B, H3 and H4 have 4 lysine residues in their N-terminal tails which can be modified by acetylation whereas histone H2A only has one (Hansen *et al.*, 1998).

Fractionation of nuclear chromatin using a number of different protocols has shown that acetylated histones are enriched in fractions that are also enriched in transcribed sequences (Gorovsky, 1973; Sealy and Chalkley, 1978; Vidali *et al.*, 1978; Vavra *et al.*, 1982; Allegra *et al.*, 1987; Ridsdale and Davie, 1987; Ip *et al.*, 1988; Lin *et al.*, 1989; Boffa *et al.*, 1990; Tazi and Bird, 1990; Walker *et al.*, 1990; O'Neil and Turner, 1995). Immunolocalisation studies on polytene chromosomes in *Drosophila* has shown that H4 isoforms acetylated at lysines 5, and 8 are distributed in overlapping but non-identical islands throughout euchromatin and, interestingly, H4 acetylated at lysine 16 is found only on the hyperactivated (dosage compensated) X-chromosome in males (Turner *et al.*, 1992; Bone *et al.*, 1994). Conversely, heterochromatin in *Drosophila* and *S. cerevisiae* contains histone H4 which is acetylated less frequently at positions 5, 8 and 16 than bulk chromatin, however, histone H4 is acetylated more frequently in this chromatin on lysine residue 12 relative to bulk chromatin (Turner *et al.*, 1992; Braunstein *et al.*, 1993). In human cells, histones on the inactive X chromosome and in centromeric and telomeric heterochromatin are also hypoacetylated, however they do not appear to be selectively acetylated on lysine 12 (Jeppsen and Turner, 1993).

A number of studies have also demonstrated that histone acetylation changes the properties of nucleosomes. At the β-globin locus, the presence of acetylated histones coincides with increased nuclease sensitivity (Hebbes *et al.*, 1994). Plasmids assembled with acetylated histones restrain fewer superhelical turns of DNA than non acetylated histones (Norton *et al.*, 1989; Norton *et al.*, 1990; Thomsen *et al.*, 1991) Nucleosome arrays assembled with acetylated histones are less able to form higher order chromatin structures than arrays assembled with acetylated histones (Tse *et al.*, 1998). Electron microcroscopy and gel electrophoresis studies have also shown that nucleosomes assembled with acetylated histones are less compact than nucleosomes assembled with non acetylated histones (Oliva *et al.*, 1990; Pennisi, 1997; Tse *et al.*, 1998). Despite this less compact structure, nucleosomes assembled with acetylated histones still protect 146bp of DNA from micrococcal nuclease digestion and do not exhhibit increased nucleosome mobility (Ura *et al.*, 1997). Linker histones also bind acetylated and unacetylated nucleosomes with equal efficiency (Dimitrov *et al.*, 1993; Ura *et al.*, 1994; Ura *et al.*, 1997). Investigations into the consequences of this modification on the binding of transcriptional regulatory proteins have shown that histone acetylation can increase the binding affinity of TFIIIA (Lee *et al.*, 1993) and sequence specific transcription factors including derivatives of Gal4p and Usfp (Vettesse-Dadey *et al.*, 1996). Histone acetylation has also been shown to increase *in vitro* transcription from chromatin templates containing genes for 5SrRNA (Ura *et al.*, 1997) and *Drosophila hsp26* (Nightingale *et al.*, 1998). The stimulatory effect of acetylation on the transcription of 5SrRNA genes by RNA polymerase III is repressed by the binding of linker histone (Ura *et al.*, 1997).

1.6.5 Histone acetylases

Recently multiprotein complexes with histone acetylase and deacetylase activities have been identified. Biochemical and genetic analysis have demonstrated that these complexes have an integral role in the regulation of gene expression (reviewed in Imhof and Wolffe, 1998; Kuo and Allis, 1998; Mizzen and Allis, 1998; Pollard and Peterson, 1998; Struhl, 1998; Workman and Kingston; 1998; Kingston and Narlikar; 1999)

The histone acetyltransferases (HATs) have been grouped into two categories according to their function within a cell. Type A HATs acetylate chromosomal histones in the nucleus and type B HATs acetylate free cytoplasmic histones prior to chromatin assembly.

The first direct link between histone acetylation and gene activation *in vivo* was established when the first type A HAT to be isolated was found to be encoded by *GCN5* (for general control nonrepressed) (Brownell *et al.*, 1996). Previously, this gene had been identified in genetic studies as a transcriptional coactivator which is required for the full level transcription of a number of genes (Georgakopoulos and Thireos, 1992; Marcus *et al.*, 1994; Brandl *et al.*, 1996; see also Pollard and Peterson, 1997). Subsequent fractionation of yeast nuclear extracts has shown that there are six multisubunit type A HATs in this organism (Grant *et al.*, 1997; Saleh *et al.*, 1997). Characterisation of these complexes found that two (ADA and SAGA) preferentially acetylate histone H2B and H3 and contained Gcn5p, one (NuA3) acetylates histone H3 and one (NuA4) acetylates histone H4 (Saleh *et al.*, 1997). The catalytic subunit of NuA4, Esa1p (Allard *et al.*, 1999), is a member of the MYST family - so called after its founding members MOZ, YBF2/SAS3, SAS2 and TiP60 (Borrow *et al.*, 1996; Reifsnyder *et al.*, 1996; Yamamoto and Horikoshi, 1997). Esa1p is also a direct homologue of MOF, a putative histone acetylase associated with dosage compensation in *Drosophila* (Hilfiker *et al.*, 1997). Analysis of the function of yeast acetylase complexes on preassembled nucleosome arrays has shown that ADA, SAGA, NuA3 and NUA4 increase *in vitro* transcription and nuclease access to DNA (Steger *et al.*, 1998). SAGA and NuA4 specifically stimulate transcription in the presence of Gal4p derivatives with the acidic VP16 activation domain but not with glutamine or proline rich activation domains (Ikeda *et al.*, 1999). In addition, preincubation of nucleosomal templates with NuA4 enhanced transcription from all activators tested which indicates that H4/H2B acetylation by NuA4 leads to a general activation of transcription that is independent of activator-NuA4 interactions (Ikeda *et al.*, 1999).

In Gcn5p deficient *S. cerevisiae* strains it has been shown that acetylation can have a specific effect on chromatin structure *in vivo*. These strains have a different arrangement of nucleosomes at the *HIS3* promoter (Filetici *et al.*, 1998) and at a synthetically activated *PHO5* promoter (Gregory *et al.*, 1998) compared to wild type cells.

A screen for human homologues of *S. cerevisiae GCN5* has resulted in the identification of two homologous proteins, hGcn5 and PCAF (Candau *et al.*, 1996; Yang *et al.*, 1996). PCAF interacts with other transcriptional coactivators including p300/CBP (Yang *et al.*, 1996) and ACTR/SRC (Chen *et al.*, 1997; Spencer *et al.*, 1997). These coactivator proteins also posses acetyltransferase activity (Yang *et al.*, 1996; Ogryzko *et al.*, 1996; Bannister and Kouzarides, 1996; Chen *et al.*, 1997; Spencer *et al.*, 1997) and in the case of CBP it has been shown that its HAT domain is capable of stimulating transcription *in*

vivo (Martinez-Balbas, *et al.*, 1998a). These results indicate that large acetylation complexes may assemble at promoters to regulate transcription.

In addition to histones it has also been shown that sequence specific (Gu and Roder, 1997; Boyes *et al.*, 1998; Zhang and Bieker, 1998) and general (Imhof *et al.*, 1997) transcription factors can be modified by acetylation. Interestingly, the acetylation of the sequence specific transcription factors p53 (Gu and Roder, 1997) and GATA1 (Boyes *et al.*, 1998) stimulates their DNA binding ability whereas acetylation of HMG-I/-Y in the IFNβ enhanceosome destabilises the complex so that it dissasembles, attenuating IFNβ expression (Munshi *et al.*, 1998). It is therefore likely that HATs may modify histones and/or other factors at promoters to positively or negatively modulate their activity.

Recent evidence has shown that histone acetylases are required for the full expression of all SWI/SNF dependent genes tested (Pollard and Peterson, 1997) which indicates that they perform complementary functions in gene activation. Although they cooperate at some promoters, chromatin remodelling and acetylation activities perform overlapping or redundant functions at other promoters. For example, inactivation of both the SWI/SNF and Gcn5p complexes causes transcriptional defects in genes which were previously thought to be SWI/SNF and GCN5 independent (Biggar and Crabtree, 1999). Other genetic studies have also demonstrated interaction (Roberts and Winston, 1997) and partial functional redundancy (Sudarsanam *et al.*, 1999) of the SWI/SNF and SAGA complexes.

In relation to the proposed role of H2A.F/Z in transcription it is therefore possible that this variant histone may promote the function of acetylases. It could do this directly by altering the conformation of a nucleosome so that it was a more suitable substrate for acetylation or indirectly by increasing the binding of transcription factors (see section 1.6.2) that recruit histone acetyltransferases. Considering that at least some histone acetylation complexes require chromatin remodelling, H2A.F/Z may also augment the action of acetylases by ameliorating the function of chromatin remodellers (see section 1.6.3). In addition to promoting the action of acetyltransferases, histone H2A.F/Z could complement the effect of acetylation by forming chromatin which is refractory to processes that repress the stimulatory effect of acetylation on transcription, like the binding of histone H1 (Ura *et al.*, 1997) or histone deacetylation (considered following).

1.6.6 Histone deacetylases

As well as histone acetylation, complexes have been identified which have histone deacetylation activities. Like the acetylase complexes, deacetylation contain different subunits, are targeted differently and have different functions within the cell. These deacetylation complexes have been classified according to their subunit composition as either Sin3A/HDAC complexes or NuRD complexes (reviewed in Roth and Allis, 1996; Grunstein, 1997; Pazin and Kadonaga, 1997; Wade *et al.*, 1997; Workman and Kingston, 1998; Wu, 1997; Struhl, 1998; Kingston and Narlikar, 1999).

Analysis of histone deacetylase complexes has shown that they can be recruited in three ways to negatively regulate transcription. The mammalian histone deacetylase HDAC and yeast homologue Rpd3p are recruited directly by DNA binding transcription regulators YY1 in mammals (Yang *et al.*, 1996) and Ume6p in yeast (Kadosh and Struhl, 1997; see also Kadosh and Struhl, 1998; Rundlett *et al.*, 1998). Histone deacetylases can also be recruited indirectly by interactions with the corepressors Rb (Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998), NCoR (Alland *et al.*, 1997; Heinzel *et al.*, 1997; see also, Laherty *et al.*, 1998), SMRT (Nagy *et al.*, 1997) and CIR (Hsieh *et al.*, 1999). The recruitment of another histone deacetylase, HDAC4, is regulated by nuclear transport. HDAC4 shuttles between the nucleus and cytoplasm in a process that involves active nuclear export (Miska *et al.*, 1999). When in the nucleus it associates with the myocyte enhancer factor, MEF2A, and represses transcriptional activation by this factor (Miska *et al.*, 1999).

Characterisiation of *RPD3* deletion mutations in *Drosophila* and *S. cerevisiae* has found that in addition to being involved in repression of transcription this histone deacetylase is also involved in derepression. Deletion of either *RPD3* (or the homologous gene *HDA1*) in *S. cerevisiae* leads to increased levels of histone H3 and H4 acetylation *in vivo* and also results in the 2-5 fold derepression of uninduced genes (Vidal and Gaber 1991; Rundlett *et al.*, 1996; De Rubertis *et al.*, 1996). In these strains however, there is also a 2-5 fold reduction in the expression of activated genes (Vidal and Gaber 1991; Rundlett *et al.*, 1996; De Rubertis *et al.*, 1996). Similarly in *Drosophila*, deletion of the *RPD3* homologue enhances the silencing effect experienced by a reporter gene located adjacent to heterochromatin in a position effect variegation (PEV) assay (De Rubertis *et al.*, 1996).

Treatment of cells with histone deacetylase inhibitors sodium butyrate and/or trichostatin A (TSA) also has stimulatory and inhibitory effects on transcription. While deacetylase inhibitors increases expression from the HIV-1 promoter (Bohan *et al.*, 1989; Golub *et al.*, 1991; Shahabuddin *et al.*, 1992; Laughlin *et al.*, 1993; Sadaie and Hager, 1994; Van Lint *et al.*, 1996a) and the $H1^{o}$ gene (Almouzni *et al.*, 1994) they also decrease glucocorticoid receptor dependent transcription of the MMTV promoter (Bresnick *et al.*, 1990; Bresnick *et al.*, 1991), transcriptional activation by MyoD and myogenin (Johnston *et al.*, 1992) and expression from the c-*myc* gene (Van Lint *et al.*, 1996b). These results indicate that transcriptional regulation by histone deacetylases might involve more than just the deacetylation of histones to form repressive chromatin structures. To this effect it will be interesting to see if histone deacetylases, like the histone acetylases, are able to modify transcription factors.

The NuRD deacetylase complex isolated from human and *Xenopus* possesses both histone deacetylation and chromatin remodelling activities. The function and targeting of this complex has been considered in section 1.6.3.

It is also worth noting that not all transcriptional repressors function via deacetylases. Acr1p, ERF2 and Tup1p do not require Rpd3 or Sin3 to mediate transcriptional repression (Heinzel *et al.*, 1997; Kadosh and Struhl 1996; see also Edmonson *et al.*, 1996).

H2A.F/Z could therefore function in transcription by inhibiting the action of histone deacetylases. It could do this by restricting the access of histone deacetylases to acetylated residues on the core histones or by disrupting the association of deacetylases with chromatin. Alternatively, histone H2A.F/Z could promote transcription by disrupting the binding of repressors like Acr1p, ERF2 or Tup1p that do not rely on histone deacetylation to function.

1.6.7 Gene expression during mitosis

Transcription of the endogenous cellular complement of genes is shut down during chromatin condensation in mitosis and resumes after decondensation of the metaphase chromosomes (Taylor, 1960; Prescott and Bender, 1962; Johnson and Holland, 1965). This cessation of transcription appears to occur through a combination of inhibition and displacement of the proteins involved in transcription. Inhibition of RNA polymerase II and III transcription by a mitosis-specific phosphorylation has been reproduced *in vitro* (White *et al.*, 1995; Segil *et al.*, 1996). Immunocytochemical analysis of protein

localisation has shown that a number of transcription factors are displaced from chromatin during mitosis, incuding; Oct-1 (Fletcher *et al.*, 1987), Oct-2 (Scheidereit *et al.*, 1988), Ets-1 (Ghysdeal *et al.*, 1986), B-Myb (Gonda *et al.*, 1985), c-fos (Bohmann *et al.*, 1987), E2F-1 (Kovesdi *et al.*, 1986) Bcl-6 (Ye *et al.*, 1993), HSF1 (Martinez-Balbas *et al.*, 1995), Sp1 (Martinez-Balbas *et al.*, 1995), GBF (Martinez-Balbas *et al.*, 1995) C/EBP (Martinez-Balbas *et al.*, 1995). Transcriptional coactivators which are displaced from chromatin during mitosis include; the human SWI/SNF chromatin remodelling subunits hBRM and BRG-1 (Muchardt *et al.*, 1996), HMG-1/-2 (Falciola *et al.*, 1997) and HMG-14/-17 (Hock *et al.*, 1998). Although the displacement of factors involved in transcription from mitotic chromosomes appears to be a common phenomena, the detection of transcription factors AP-2 (Williams *et al.*, 1988), serum response factor (Gauthier-Rouviere *et al.*, 1991) and GAGA factor (Raff *et al.*, 1994) on mitotic chromosomes demonstrates that it is not a universal event. In addition, at the end of prophase 80-90% of the nuclear TBP is released into the mitotic cytoplasm. However, 10-20% remains associated with chromatin in mitotic chromosomes (Segil *et al.*, 1996).

After chromosomes decondense, and the refractory influences on transcription are abrogated, the mass action of displaced or inhibited elements may then restore transcription. Alternatively, or in addition, transcriptionally active regions might be marked through mitosis for reactivation in G1. In support of the latter possibility it has been known for some time that actively transcribing chromatin is an order of magnitude more sensitive to the endonuclease activity of DNAse I than bulk chromatin both during interphase and metaphase (Gazit *et al.*, 1982; Kerem, *et al.*, 1984). More recently, Michelotti *et al.* (1997) have characterised mitosis specific, $KMnO_4$ hypersensitive sites in the TATA boxes of expressed genes. A protein appears to be required for this "bookmarking" of active genes because inhibition of protein synthesis with cyclohexamide in mitotic HeLa cells reduces $KMnO_4$ hypersensitivity in transcribed genes. Considering that most of the observed mitotic DNA pertubations occur at the TATA box it may also be possible that TBP (or TFIID) functions as the bookmark. The proteins, Dr1 and Mot1, are interesting candidates for bookmarking because they bind TBP and inhibit RNA polymerase II transcription (Dr1 also inhibits RNA polymerase III transcription) (Inostroza *et al.*, 1992; Auble *et al.*, 1994 White *et al.*, 1994). This combination of functions, activated during mitosis, would therefore make it possible for expressed genes to be silenced and bookmarked simultaneously.

The punctate nature of the recently characterised mitosis specific bookmarks means it is unlikely that histone H2A.F/Z is involved in this process. However it could be involved

in the formation of domains which are rapidly reactivated following mitosis through formation of chromatin structures that facilitate transcription. As discussed above H2A.F/Z could affect transcription by increasing the affinity of transcription components such as sequence specific or general transcription factors, facilitating RNA polymerase elongation, promoting the activity of chromatin remodelling and/or histone acetylation complexes or by decreasing the binding of inhibitory elements to chromatin.

1.7 Thesis aims

The lethality of H2A.F/Z null mutations in mice (Thonglairoam, 1996), *Drosophila melanogaster* (van Daal and Elgin, 1992) and *Tetrahymena thermophila* (Liu *et al.*, 1996b) clearly establish that this variant histone plays a fundamental role in eukaryotic life. From the observations that H2A.F/Z is enriched in transcriptionally active chromatin it has been hypothesised that it may be involved in establishment and/or maintainance of transcription in the nucleus. Although H2A.F/Z must clearly function by modifying the biochemical properties of chromatin very little is known about the effect that histone H2A.F/Z has on chromatin structure and function. In addition, no studies have addressed which regions of the H2A.F/Z histones distinguish their function from the core H2A histones.

The overall aim of experiments conducted in this thesis were to further characterise the role of H2A.F/Z histones. The specific aims of this investigation were to;
- identify which regions of H2A.F/Z protein functionally distinguish it from core H2A,
- determine the distribution of H2A.F/Z protein during development and at different stages of the cell cycle and
- characterise H2A.F/Z expression and protein concentration in different tissues.

Prior to the commencement of this project, a null mutation had been generated in the *Drosophila His2AvD* gene (van Daal and Elgin, 1992). It had also been demonstrated that lack of a functional *His2AvD* gene was lethal and that viability could be restored with a transgene derived from a 4.1 kb genomic DNA fragment containing the *His2AvD* gene (van Daal and Elgin, 1992). These features made *Drosophila* the most suitable organism to perform a mutagenesis study of the H2A.F/Z histone gene to identify the domains of the protein that functionally distinguish it from the core H2A histone *in vivo*. To this end, sequences which encode amino acids in the *His2AvD* genomic fragment were mutated, *in vitro*, to encode the equivalently positioned H2A.1 residues. An additional mutant was also produced (Q127->stop) to examine the functional contribution of the C-terminal 14 amino acids which are present only in the *Drosophila*

H2A.F/Z homologue. Mutated genes encoding modified versions of this histone were then transformed into *Drosophila* and tested for their ability to rescue null mutant lethality. To characterise the extent of rescue provided by mutant versions of *His2AvD* it was first necessary to determine the stage of development that *His2AvD* null mutant individuals die. This characterisation was conducted using a combination of molecular and phenotypic developmental markers.

The distribution of His2AvD protein during stages of development and the cell cycle was examined in lines of *Drosophila* that contained a transgene encoding *His2AvD* with green fluorescent protein (GFP) fused to the C-terminus. To demonstrate that the transgene produced functional His2AvD protein, it was initially tested for its ability to rescue null mutant individuals from lethality. The distribution of His2AvD-GFP fusion protein was analysed in early *Drosophila* embryos to investigate whether the temporal association of H2A.F/Z with actively transcribing chromatin occurred in species other than *Tetrahymena*. In the initial stages of *Drosophila* development there are 10 rapid, synchronous nuclear divisions in a syncitial blastoderm (Foe and Alberts, 1983). No zygotic transcripts have been detected in *Drosophila* embryos prior to nuclear cycle eight. During this stage, some of the nuclei in some of the embryos begin to produce specific transcripts. The number of nuclei with zygotic transcripts then gradually increases during cycles 9 and 10 until it is observed in all nuclei of all embryos by the end of cycle 10 (Pritchard and Schubiger, 1996). The distribution of the His2AvD-GFP fusion protein was also analysed throughout the cell cycle to determine whether His2AvD remained associated with chromatin during mitosis, when transcription is silenced.

In addition to the analysis of *Drosophila* His2AvD some characteristion of the mouse homologue, H2A.Z, was conducted. H2A.Z expression and protein concentration in different tissues was examined using Northern and Western analysis respectively. Mice were chosen for this investigation because the isolation of tissue samples from this organism is more convenient and less subject to contamination than *Drosophila.* The subcellular location of mouse H2A.Z was also investigated on tissue sections and cell culture monolayers using antibodies directed against the carboxy terminus of the protein.

Results obtained from these studies were then used to derive models for the function of histone H2A.F/Z.

Chapter 2: Materials and general methods

2.1 Abbreviations

A_{xxx}	absorbance at xxx nm
APS	ammonium persulphate
ATP	adenosine triphosphate
bp	base pair
bisacrylamide	N,N'-methylene-bisacrylamide
βme	βmercaptoethanol
BSA	bovine serum albumin (Fraction V)
cDNA	complementary deoxyribonucleic acid
CIP	calf intestinal phosphatase
cm	centimeter
DAB	diaminobenzidine
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DTE	dithioerythritol
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid, disodium salt
EGTA	ethylene glycol bis(aminoethyl ether)tetraacetic acid
ELISA	enzyme linked immunological surface assay
EMS	ethylmethanesulphonate
FBS	foetal bovine serum
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FPLC	fast protein liquid chromatography
GuHCl	guanadine hydrochloride
h	hour
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
IPTG	isopropylthiogalactopyranoside
kb	kilobasepairs
kDa	kilodalton molecular weight
Krpm	thousands of revolutions per minute
kV	kilovolts
l	litre

LB	Lauria broth
M	molar
mA	milliampes
MOPS	[3-(N-morpholino)-propanesulfonic acid]
mg	milligram
ml	millilitre
mm	millimeter
mM	millimolar
mRNA	messenger ribonucleic acid
msec	millisecond
ng	nanogram
nm	nanometer
OD_{xxx}	optical density at xxx nm
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PBS	phosphate buffered saline
pBS	pBluescript
PEG	polyethylene glycol
pM	picomolar
PMSF	phenylmethylsulfonylflouride
PNK	T4 polynucleotide kinase
psi	pounds per square inch
rATP	ribo-adenosine triphosphate
RSP	reverse sequencing primer
RNA	ribonucleic acid
RNAse A	ribonuclease A
SD	"super duper" buffer
SDS	sodium dodecyl sulphate
sec	seconds
SSC	sodium citrate buffer
TAE	Tris, acetate, EDTA buffer
TBE	Tris, borate, EDTA buffer
TE	Tris, EDTA buffer
TEMED	N,N,N',N-tetramethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
U	units of enzyme activity
μCi	microCurie
μg	microgram

µl	microlitre
USP	universal sequencing primer
UV	ultraviolet light
V	volts

2.2 Materials

2.2.1 Chemicals and reagents

All chemicals were obtained from departmental stocks and were of analytical grade or the highest purity available. Most chemicals and reagents were obtained from a wide range of suppliers, the major sources of the more important chemicals and reagents are listed below:

Acrylamide, ampicillin, agarose, chloramphenicol, DAB, dNTPs, DTT, EDTA, EGTA, ethidium bromide, IPTG, PMSF, rATP, salmon sperm DNA, SDS, spermidine, spermine, tetracyclin, thiodiglycol, Tris: Sigma
bisacrylamide, TEMED: Bio-rad
APS, boric acid, sodium acetate, urea: BDH (Merck)
glycogen, CsCl: Boehringer Mannheim
sepharose CL-6B: Pharmacia
PEG 6000: Stansen surgical
Phenol : Wako pure chemical industries, Ltd.
Sodium hypochlorite: (White King): Kiwi Australia
DMEM, FBS: Gibco BRL

2.2.2 Enzymes

Enzymes were obtained from the following sources:

AMV Reverse Transcriptase: Pharmacia
Calf intestinal phosphatase: Boehringer Mannheim
E.Coli DNA polymerase 1 (Klenow fragment): Bresatec
Lysozyme : Sigma
Polynucleotide kinase: Boehringer Mannheim
Restriction endonucleases: Bresatec
Pharmacia
New England Biolabs
Boehringer Mannheim
Ribonuclease A: Sigma

T4 DNA ligase: Boehringer Mannheim
T7 DNA polymerase: Boehringer Mannheim
Taq DNA polymerase: Perkin Elmer/Cetus Corporation

2.2.3 Kits

Kits were obtained from:

Amersham:	ECL western blotting detection system
	Megaprime DNA labelling system kit
Bresatec:	Bresa-Clean DNA purification kit
	Cyclone Cycle Sequencing System
	T7 Super-base Sequencing kit
PROMEGA	Altered Sites *In Vitro* Mutagenesis System

2.2.4 Radionucleotides

α^{32}PdATP (specific activity 3000 Ci/mM, concentration 10mCi/ml), α^{35}SdATP (specific activity 1500 Ci/mM, concentration 12.5mCi/ml and γ^{32}PdATP (specific activity 4000 Ci/mM, concentration 10mCi/ml) were obtained from Bresatec.

2.2.5 Nucleic acid molecular weight standards

All markers were obtained from Bresatec.
SPP-1 phage restricted with EcoRI
λ phage restricted with HindIII
pUC restricted with HpaII

2.2.6 Buffers and solutions

Agarose gel loading buffer (10X)	50mM Tris pH 7.4
	50% (v/v) glycerol
	5mM EDTA
	0.1% (w/v) bromophenol blue
	0.1% (w/v) xylene cyanol
AH (100X)	10mM hypoxanthine
	0.58mM azaserine
Denhardt's solution (100X)	2% (w/v) Ficol 400
	2% (w/v) polyvinylpyrrolidone

	2% (w/v) BSA
Drosophila embryo injection buffer (10X)	1mM PO_4 pH 6.8
	50mM KCl
Formamide load buffer	72% (v/v) formamide
	20mM EDTA
	0.05% (w/v) bromophenol blue
	0.05% (w/v) xylene cyanol
Hewish and Burgoine buffer A (10X)	100mM HEPES pH 7.4
	600mM KCl
	150mM NaCl
	5mM spermidine
	1.5 mM spermine
	1mM EDTA
Ligation buffer (5X)	25mM Tris pH 7.5
	5mM $MgCl_2$
	0.5mM rATP
	0.5mM DTT
	25% (v/v) PEG
Lysis buffer (10X)	100mM HEPES pH 7.5
	100mM $MgCl_2$
	50mM KCl
MOPS buffer (10X)	400mM MOPS pH 7.0
	100mM sodium acetate
	10mM EDTA
OPI (100X)	115mM oxaloacetate
	45mM sodium pyruvate
	20U/ml bovine insulin
PBS	10mM Na_2HPO_4
	1.8mM KH_2PO_4 pH 7.3
	140mM NaCl
	2.7mM KCl
PNK buffer (10X)	600mM Tris pH 7.5
	90mM $MgCl_2$
Primer extension mix	10mM Tris pH 8.3
	14mM $MgCl_2$
	14mM DTT
	700μM dNTPs
SD buffer (10X)	330mM Tris pH 7.8

	625mM KAc
	100mM MgAc
	40mM spermidine
	5mM DTE
SDS PAGE gel buffer (4X)	1.5M Tris pH 8.8
	0.4% (w/v) SDS
SDS PAGE gel loading buffer (2X)	60mM Tris pH 7.0
	10% (v/v) glycerol
	2% (w/v) SDS
	0.25% (w/v) bromophenol blue
	5.7 % (v/v) βme, added just prior to use
SDS PAGE running buffer (5X)	125mM Tris
	1mM glycine
	0.5% (w/v) SDS
SSC (20X)	300mM sodium citrate pH 7.0
	3M NaCl
TAE	40mM Tris pH 8.2
	20mM sodium acetate
	1mM EDTA
Taq reaction buffer (10X)	670mM Tris pH 8.8
	166mM $(NH_4)_2SO_4$
	2mg/ml gelatin
	4.5% (v/v) Triton X-100
TBE	89mM Tris pH 8.3
	89mM boric acid
	2.5mM EDTA
TE	10mM Tris pH 7.5
	1mM EDTA
Western transfer buffer	50mM Tris Base
	40mM Glycine
	0.0384 % (w/v) SDS
	20% (v/v) methanol

2.2.7 Bacterial Strains

Escherichia coli :

BL21(DE3): F^-, *ompT hsdS*$_B$ (r_B^-, m_B^-) pLysS.

DH5α: *supE44 ΔlacU169* (ø80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*.

CJ236: F' *cat* (=pCJ105; M13^{s}Cm^r)/*dut ung1 thi-1 relA1 spoT1 mcrA* .

JM101: *supE thi Δ* (*lac-pro AB*) [F' *lacI^{q}ZΔM15 raD36 proAB*]

MC1061: F$^-$ *araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac)X74 rpsL*(Str^r) *hsdR2*(r_K^-, m_K^+) *mcrA mcrB1*.

XL1-Blue: *recA1 endA1 gyrA96 thi hsdR17*(r_K^-, m_K^+) *supE44 relA1 lac* [F' *proAB$^+$ lacI^{q}ZΔ M15* ::Tn10(Tet^r)].

BMH 71-18 mut S *thi supE Δ*(*lac proAB*) [*mutS*::Tn10] {F' *proAB lacI^{q}ZΔ M15*]

2.2.8 Bacterial Media

(i) Liquid media

Antibiotics were added from sterile stock solutions after the media had been autoclaved.

L-broth	1% (w/v) NaCl
	1% (w/v) bactotryptone
	0.5% (w/v) yeast extract
	pH 7.0.
SOC broth	2% (w/v) bactotryptone
	0.5% (w/v) yeast extract
	0.05% (w/v) NaCl
	pH 7.0
	After autoclaving glucose was added, from a sterile stock solution, to a final concentration of 2% (w/v).
2YT broth	1.6% (w/v) bactotryptone
	1% (w/v) yeast extract
	0.5% NaCl
	pH 7.0

(ii) Solid media

L-agar plates: 1.5% (w/v) bactoagar was added to L-broth.

Antibiotics were added from sterile stock solutions after the media had been autoclaved and had cooled to 55°C.

2.2.9 Oligonucleotides

Oligonucleotides used were obtained from Bresatec and Gibco BRL.

The sequences of the oligonucleotides are as follows;

(i) Chapter 3 oligonucleotides

5C actin primer 5' ACTTCTTCGTCACACATTTTGTAAG 3'

Sgs4 primer 5' AGCCAGCCCCACCAATAACACAACC 3'

hsp23 primer 5' TACTTTCGCTTTAGCTGTTATCGC 3'

hsp70 primer 5' TTTGCTTAGCTTTCGCTTAGCGACG 3'

FRT top

5'AATTCGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCAG3'

FRT bottom

5'GCTTCAAGGATAAGGCTTCAAGGATAAGAGATCTTTCATATCCTTGAAGTCTTAA3'

pCaSpeR *white* 5' 5' ACATTCTTCAAAAAAGGG 3'

USP 5' GTAAAACGACGGCCAGT 3'

RSP 5' AACAGCTATGACCATG 3'

amp^{r} 5' GTTGCCATTGCTGCAGGCATCGTGGTG 3'

M1 5' CTATTCGCAGTCTGGCCGCGGTAAGGGTGGCAAGGTCAAGGCGAAGGCGG 3'

M2 5' GGGCAAGGCCAAGGGCAAGGCGAAGAGCCGTTCCAATCGCGCGGGTCTTC 3'

M3 5' CCGACGCGTTCAGCGTAGTTACCCTTACGTAACAGACGATGGATGCG 3'

810 5' GTTATTGTGCTGCTGCGG 3'

M4 5' TGTACGCGTCGGAGCCGGAGCACCCGTGTACTTGGCTGCCGTAATGGAATACC 3'

M4a 5' TAAACAACTGATAATGTGCA 3'

M5 5' CGAGGAATGATACGTGTCTTTTTGTTGTCCCTCGCTGCGTT 3'

M6 5' GCGATGGTGACTCCCGAGAGCAGCTTGTTCAGCTCCTCGTCGTTGCGAAT 3'

M7 5' TTCTTGGGGAGCAGCACCGCCTGTATGTTCGGAAGACACCGCCTTGAGCG 3'

CT 5' GAAGGAGGAAACGGTGTAGGATCCGCAGCGGAAG 3'

2374 5' CTCGTCGTGTTAAGCTTG 3'

(ii) Chapter 5 oligonucleotides

H2A.1 5' 5' CGCTGAGCACCATGGCTGGACGTGG 3'

H2A.1 3' 5' CTTTGAAAAGCTAGGCCTTCTTC 3'

His2AvD 5' 5' CGATTGAAGACATATGGCTGGCGG 3'

His2AvD 3' 5' CGAAGATCTTAGTAGGCCTGCGACAG 3'

2.2.10 Synthetic peptides

Synthetic peptides used were obtained from Chiron Mimotopes.
The sequences of the synthetic peptides are as follows;

H2A.1 CNIQAVLLPKKTESHHKAKG

H2A.Z	CHIHKSLIGKKGQQKTV
His2AvD	CVQDPQRKGVNVILSQAY

The peptides were also provided conjugated biotin and Diphtheria toxoid via the cysteine residue.

2.2.11 Clones and cloning vehicles

pC4B was a gift from Angela van Daal.
pCasper 1-4 and pCasper hs were gifts from Vincenzo Pirrotta.
pBluescript SK(-) was a gift from Blair Hopwood.
pDM420-FLP was a gift from Kent Golic.
pET.AZ was a gift from Varaporn Thonglairoam.
pET3b was a gift from Barry Egan.
pUAST was a gift from Andrea Brand.

2.2.12 Fly Media

Grape juice agar plates:	3% (w/v) agar
	3% (w/v) sucrose
	25% (v/v) unsweetened grape juice
	3% (v/v) tegosept mix.
Fortified (F1) *Drosophila* medium:	1% (w/v) agar
	18.75% (w/v) compressed yeast
	10% (v/v) treacle
	10% (w/v) cornmeal (polenta)
	2.5% (v/v) tegosept mix.
Tegosept mix:	10% (w/v) methylparahydroxybenzoate in ethanol.

2.2.13 Fly Strains

Fly strains were provided as gifts from:

Richard Tearle;

$$\frac{w^+}{w^+ \text{ or } y};\frac{+}{+};\frac{st\ sbd\ e^s\ ro\ ca}{st\ sbd\ e^s\ ro\ ca}$$

$$\frac{w}{w \text{ or } y};\frac{+}{+};\frac{ru\ st\ e\ His2AvD^{810}\ ca}{\text{TM6b}\ Hu\ e\ Tb}$$

$$\frac{w}{w \text{ or } y};\frac{+}{+};\frac{Df(3R)ro^{XB3}}{\text{TM6b}\ Hu\ e\ Tb}$$

$\frac{w}{w \text{ or } y}$; $\frac{CyO}{Sp}$; $\frac{+}{+}$

white ^{1118}

Robert Saint; w^{m4}

$\frac{w}{w \text{ or } y}$; $\frac{+}{+}$; $\frac{ru\ st\ e\ ca}{ru\ st\ e\ ca}$

2.3 General Methods

2.3.1 Large-scale isolation of plasmid DNA using CsCl gradients

100ml of 2YT broth containing the appropriate antibiotic(s) was inoculated with 1ml of overnight culture and grown up overnight at 37°C with shaking. Cells were pelleted by centrifugation for 5min at 6Krpm (SS-34 rotor in a Sorvall RB-5C centrifuge) then resuspended in 2ml of 50mM Tris pH 8.0, 5mM EDTA, 15% (w/v) sucrose, 4mg/ml lysozyme and left at room temperature for 20min. To the resuspended cells, 4ml of 0.2M NaOH, 1% (w/v) SDS was added. This was mixed by gentle inversion and left on ice for 20min. Next, 2.5ml of 3M NaAc pH 4.6 was added and the material was again mixed gently and left on ice for 20min. Denatured cellular material was pelleted by centrifugation at 16Krpm for 30 min (SS-34 rotor in a Sorvall RB-5C centrifuge) and the supernatant containing plasmid DNA was decanted into a fresh tube. Plasmid DNA was pellted from the supernatant by addition of 0.6 volumes of isopropanol and centrifugation at 15Krpm for 15min (SS-34 rotor in a Sorvall RB-5C centrifuge). The pellet was air dried, resuspended in 720µl of water and added to 1.26g CsCl. After the CsCl had completely dissolved (this sometimes required the addition of a further 50-100µl of water) 120µl of 10mg/ml ethidium bromide was added to it. This solution was then used as an underlay for two heat sealable 2ml Beckman TL100 centrifuge tubes that each contained 1.4ml of 65% (w/v) CsCl. Tubes were balanced with 65% (w/v) CsCl, sealed and spun at 100Krpm for 3h (TL100.2 rotor in Beckman TL100 centrifuge). After centrifugation, tubes were removed from the rotor and visualised under long wavelength UV light (302nm). The lower band in the gradient containing supercoiled plasmid DNA was extracted using an 18 gauge needle attached to a 1ml syringe. Samples from both gradients were pooled and treated to remove the ethidium bromide by three extractions with an equal volume of water saturated butanol. From this material, plasmid DNA was pelleted by addition of one volume of water and two

volumes of nuclease free ethanol followed by centrifugation for 10min in a microfuge (12Krpm). The DNA pellet was washed with 70% (v/v) nuclease free ethanol and, once this was aspirated, allowed to air dry. Purified DNA was resuspended in 100µl of water and stored at -20°C.

2.3.2 Plasmid minipreps

A single colony was used to inoculate 1.5ml of LB containing the appropriate antibiotic(s) and grown up overnight at 37°C on a rotating wheel. Culture was transferred to an eppendorf tube and cells were pelleted by 1min centrifugation in a microfuge (11Krpm). Culture media was aspirated and the pellet resuspended in 200µl of STET buffer (50mM Tris pH 8.0, 8% (w/v) sucrose, 5% (v/v) Triton X100, 50mM EDTA). 20µl of lysozyme (5mg/ml) was added to the resuspended cells immediately prior to heating at 100°C for 45sec. Lysed cells were then spun for 15min in a microfuge (12Krpm) at 4°C and cell debris removed with a sterile toothpick. To pellet the plasmid DNA 200µl of isopropanol was added to the remaining solution and the sample was spun for 10min in a microfuge (12Krpm). Pellet was washed twice to remove excess salt by the addition of 500µl of 70% (v/v) ethanol followed by a 5min centrifugation in a microfuge (12Krpm). After aspiration of the second 70% (v/v) ethanol wash the pellet was air dried and resuspended in 20µl of water.

2.3.3 Calculation of the amount of plasmid DNA in solution

1µl of DNA sample was added to 999µl of water and the absorbance was measured at 260nm against a blank of water in a Shimadzu UV spectrophotometer. Concentration of the DNA was calculated where concentration (µg/ml) = dilution factor x 50 x A_{260}x 1000.

2.3.4 Phenol/chloroform extractions

An equal volume of 1:1 phenol/chloroform saturated with buffer (100mM Tris pH 8.0, 5mM EDTA) was added to the nucleic acid solution (minimum 100µl made up with water), vortexed briefly and spun 2min in a microfuge (12Krpm). The aspirated aqueous layer was added to an equal volume of chloroform, vortexed, spun in a microfuge as before and the aqueous layer saved. This was then ethanol precipitated, vacuum dried and resuspended in an appropriate volume of water.

2.3.5 Ethanol precipitation of DNA in solution

For a given DNA sample in solution, one tenth volume 3M NaAc pH 4.6, 3 volumes ethanol and 1µl 20mg/ml glycogen was added. This material was mixed, stored at -20°C for a minimum of 30min and centrifuged at 12Krpm for 10min. Liquid was aspirated and the pellet washed by addition of 500µl of 70% (v/v) ethanol followed by centrifugation at 12Krpm for 5min. After removal of the 70% (v/v) ethanol wash the DNA pellet was dried under vacuum before resuspension in water.

2.3.6 Agarose Gel Electrophoresis of DNA

Agarose was dissolved in TAE to the required concentration (0.7-3% w/v) and poured into the appropriate gel mould. DNA sample plus one tenth volume of 10X agarose loading buffer was loaded into each well. Electrophoresis was carried out in TAE buffer at 10V/cm (between electrodes) until the bromophenol blue dye had migrated aproximately three quaters of the length of the gel. Gels were stained for a few minutes in 0.5 µg/ml ethidium bromide, visualised under short wave UV light (254nm) and photographed.

2.3.7 Polyacrylamide gel electrophoresis of DNA

DNA fragments less than 500bps were resolved by electrophoresis through 10% (w/v) acrylamide/bisacylamide (30:1), 1XTBE, 1% (w/v) APS, 0.1% (v/v) TEMED vertical slab gels (14cm x 14cm x 0.5mm) in 1XTBE buffer. After 30min of pre-electrophoresis at a constant current of 30mA samples were loaded onto the gel. Electrophoresis was continued until the bromophenol blue dye had migrated approximately three quarters of the length of the gel. Gels were stained for a few minutes in 0.5µg/ml ethidium bromide, visualised under short wave UV light (254nm) and photographed.

2.3.8 Generation, modification and subcloning of restriction fragments into prepared plasmid vectors

(i) Restriction endonuclease digestion of DNA

Restriction endonuclease digests were carried out in 1XSD buffer. To ensure complete digestion 2-5U of enzyme was added per µg of DNA and the reaction was incubated at 37°C for at least 1 hour.

(ii) End modification of vectors and DNA fragments

(a) 3' overhang end blunting

3' overhanging ends were blunted by incubating the DNA with 10U of T4 DNA polymerase per μg of DNA in 1XSD buffer at 14°C, initially for 10min, and then for a further 30min after the addition of dNTPs to a final concentration of 0.1mM.

(b) 5' overhang end filling

5' overhang end filling was conducted in 1XSD buffer containing 5U of Klenow fragment per μg of DNA and 0.1mM dNTPs. This reaction was incubated at 37°C for 30min.

(c) Calf intestinal phosphatase treatment of vector ends

Plasmid DNA that had been subject to restriction enzyme digestion and end blunting or filling (if required) was treated with 1U calf intestinal phosphatase per μg of DNA in 1XSD buffer. This reaction was incubated at 37°C for 15min and then at 55°C for 15min. Since this was the final DNA modification performed before cloning, load buffer was added to the reaction and the sample was then subject to agarose gel electrophoresis.

(iii) Isolation of DNA fragments from agarose gels

DNA vectors and fragments larger than 500bp that had been digested with the appropriate restriction enzyme(s) and end modified (if required) were subject to agarose gel electrophoresis. The required fragment or vector was located on the agarose gel by staining with ethidium bromide staining and illumination with long wavelength UV light (302nm). This material was then excised from the gel and purified using either of the following methods:

(a) Bresa-Clean DNA purification kit

This technique uses a silica matrix suspension and is based on the method developed by Vogelstein and Gillespie (1979) and was performed according to the manufacturers instructions.

(b) DNA isolation from agarose gels using siliconised glass wool

Gel slices were placed on a bed of siliconised glass wool in the bottom of a PCR tube with a hole in the bottom. This assembly was then placed in a 1.5ml eppendorf and spun in a microfuge for 10min (6.5Krpm). Eluent liquid containing DNA from the gel

slice was then made up to 100μl with water, phenol chloroform extracted and ethanol precipitated. After drying down under vacuum in a "Spedivac" pellet was resuspended in 20μl water.

(iv) Isolation of DNA fragments from polyacrylamide gels

The DNA fragment of interest was located by illumination of the ethidium bromide stained polyacrylamide gel with long wavelength UV light (302nm). Once identified, the fragment was excised and eluted into 200μl of TE, 0.1% (w/v) SDS by overnight incubation at 37°C. DNA containing eluent was then phenol chloroform extracted, ethanol precipitated and resuspended in 20μl of water.

(v) Ligation of restriction fragments to vector DNA

Gel purified DNA fragments were resuspended in 20μl of water. 2μl of this material was electrophoresed on a 0.7-2% (w/v) agarose gel alongside SPP1 markers to facilitate approximate quantitation of the remaining DNA. Ligation reactions were performed in 20μl of 1X ligation buffer containing 1U of ligase, 50ng of vector and a three molar excess of fragment. After incubation for half an hour at room temperature the reactions were phenol chloroform extracted, ethanol precipitated and resuspended in 20μl of water ready for transformation into *E.coli.*

2.3.9 Transformation of plasmid DNA into bacteria by electroporation

(i) Preparation of cells

Cells were prepared and stored in 10% (v/v) glycerol at -70°C as described in the instructions for the preparation of cells for use with the Bio-Rad Gene-Pulser.

(ii) Electrotransformation of cells

Cells were electroporated at 2.0kV for 4.7msec with plasmid DNA, as described in the users manual for the Bio-Rad Gene-Pulser. If the plasmid DNA was from a ligation reaction, the ligation mix was phenol/chloroform extracted, ethanol precipitated and resuspended in 20μl of water before electroporation into cells. Routinely, transformation of 2μl of this material gave 150-200 colonies per plate.

2.3.10 DNA Sequencing

(i) Sequencing reactions

Dideoxy chain termination sequencing (Sanger *et al.* 1977) was performed on double stranded DNA templates using the T7 Super-base sequencing kit (Bresatec) according to the manufacturers supplied protocol. α^{35}SdATP was used as the source of radio label. 2μg of plasmid DNA disolved in 8μl of water was prepared for sequencing by treatment with 2μl of RNase A (10mg/ml) at 37°C for 15min followed by denaturation with 2.5μl of 1M NaOH, 1mM EDTA at 37°C for 15min and then purified by centrifugation (2min at 1.8Krpm in a Jouan C312 benchtop centrifuge) through a Sepharose CL6B column.

Template derived sequencing compressions were resolved using the Cyclone Cycle Sequencing System from Bresatec according to the manufacturers instructions. This protocol is based on a method described by Murray (1989) and Craxton (1991).

(ii) Electrophoresis of sequencing reactions

Dideoxy chain termination sequencing reaction products were heated at 100°C for three miutes and electrophoresed on 6% (w/v) acrylamide:bisacrylamide (20:1), 8M urea, 1XTBE, 1% (w/v) APS, 0.1% (v/v) TEMED gels in 1XTBE buffer. The gels were 40cm long, 30cm wide, 0.2mm thick and were run at a constant current of 40mA. Prior to sample loading gels were pre-electrophoresed for 30min. After electrophoresis was complete the gel was transferred to a sheet of Whatman 3MM chromatography paper, covered in plastic wrap and dried on a flat-bed heated vacuum drier for 30min. The dried gels were autoradiographed overnight at room temperature.

2.3.11 Kinasing of oligonucleotides

(i) Radiolabeling of oligonucleotides

500ng of oligonucleotide was labelled in 10μl of 1XPNK buffer containing 3U of PNK and 40μCi γ^{32}PdATP. After incubating this reaction at 37°C for 45min the oligonucleotide was purified as described in 2.3.12.

(ii) Non radioactive kinasing of oligonucleotides

500ng of oligonucleotide was labelled in 10µl of 1XPNK buffer containing 3U of PNK, 10mM DTT and 10mM rATP. This reaction was incubated at 37°C for 45min and the oligonucleotide was then purified as described in 2.3.12.

2.3.12 Purification of oligonucleotides

A vertical 14cm x 14cm x 0.5mm slab gel containing 20% (w/v) acrylamide/bisacylamide (30:1), 1XTBE, 1% (w/v) APS, 0.1% (v/v) TEMED was prepared and pre-electrophoresed at a constant current of 30mA for 30min in 1XTBE buffer. Oligonucleotide sample(s) were heated at 65°C for 5min after the addition of one tenth sample volume of 10X agarose load buffer. Imeadiately after heating, samples were loaded onto the purification gel and electrophoresed continued until the bromophenol blue dye had migrated approximately three quaters of the length of the gel. The oligonucleotide was located either by UV shadowing (non-radioactive samples) or exposure of X-ray film (radioactively labelled samples). Once located, the oligonucleotide was excised from the gel and placed in an eppendorf tube containing 400µl of water. The oligonucleotide was eluted from the polyacrylamide by overnight incubation at 37°C after which time the liquid was collected and stored at -20°C.

2.3.13 Radiolabelling of DNA probes

Oligo-labelling of DNA fragments was performed using an Amersham Megaprime DNA labelling kit according to the manufacturer' s instructions. This protocol is based on a method described by Feinberg and Vogelstein (1983).

2.3.14 Polymerase chain reaction (PCR)

PCR amplification of DNA was conducted in 50µl of 1X Taq reaction buffer containing 500ng of each primer, 2mM $MgCl_2$, dNTPs at 0.2mM each, 5U Taq/Pfu (80U:1U) DNA polymerase mix and 100ng of template DNA. Once the reactions were set up they were overlayed with mineral oil and placed in an M.J. Research PTC-100-60 (BRESATEC) thermal cycler. Cycling conditions were optimised for each reaction from a starting protocol of; 1min at 95°C followed by 35 cycles of (95°C for 30sec, 50°C for 30sec and 72°C for 1min).

2.4 Containment Facilities

All manipulations involving recombinant DNA were carried out in accordance with the regulations and approval of the Genetic manipulation advisory committee and the University Council of the University of Adelaide.

Chapter 3: Histone His2AvD differs from the core H2A in a function provided by its C-terminal region

3.1 Introduction

The demonstration that *His2AvD* homologues are essential for survival in mouse (Thonglairoam, 1996), *Drosophila melanogaster* (van Daal and Elgin, 1992) and *Tetrahymena thermophila* (Liu *et al.*, 1996a) affirms that His2AvD function is not provided by H2A. Interestingly, it also appears that H2A function cannot be provided by His2AvD because the viability of a *S.cerivisiae* strain lacking endogenous major H2A can be restored with *Tetrahymena* major H2A genes but not with the *hv1* gene (Liu *et al.*, 1996b).

In this chapter, domains of the His2AvD protein that functionally distinguish it from the core H2A histone, *in vivo*, were identified. The *His2AvD* coding sequence in a 4.1kb genomic DNA fragment which could rescue *His2AvD* null lethality were mutated, *in vitro*, so they encoded amino acids located at the same position in H2A. Mutant *His2AvD* genes were then transformed into *Drosophila* and tested for their ability to rescue null mutant lethality. To determine the extent of rescue it was first necessary to identify the stage of *Drosophila* development when *His2AvD* null mutants died. This was done using a combination of phenotypic and molecular developmental markers. Phenotypic markers indicated that *His2AvD* null mutant individuals reached the third larval instar stage but did not pupate. Analysis of developmentally activated transcripts demonstrated that *His2AvD* null mutants arrest during the first half of the third larval instar stage. Based on these results, mutant *His2AvD* transgenes were tested for their ability to restore the development of null mutant individuals into both pupation and adulthood. Interestingly, the only *His2AvD* mutants that did not rescue null mutant lethality encoded proteins with amino acid changes in the C-terminal region, not in the histone fold domain. In H2A.1, the X-ray crystal structure of the nucleosome suggests that this region might be required to stabilise the dimer-tetramer interface and may align this interface such that the periodicity of DNA docking sites on the octamer is continuous (Luger *et al.* 1997).

3.2 Methods

3.2.1 Developmental staging of *Drosophila* larvae

Larvae were selected at the transition from second to third instar larvae on the basis of different anterior spiracle morphology between these two stages. In second larval instar the anterior spiracles end in a node whereas in third larval instar this structure is branched (see Ashburner, 1989a Fig 8.19).

Embryos were collected over a 30min period on 3% agar plates supplemented with grape juice and transferred to a flyfood vial. After 70h at 25°C second instar larvae were individually selected and transferred to a yeasted agar dish. Larvae were allowed to develop for a further 2h at 25°C after which time third instar larvae were selected and transferred to a flyfood vial. The time of third instar larvae selection was taken as 0±2h.

3.2.2 RNA isolation from *Drosophila* larvae

Up to 5 *Drosophila* third instar larvae were homogenised in 200μl of 0.2M sodium acetate pH 5.0 saturated phenol. To the homogenate, 360μl of 0.2M sodium acetate pH 5.0 and 40μl 10% (w/v) SDS was added. Samples were then incubated at 65°C for 5min, vortexed and allowed to cool to room temperature before the addition of 200μl of chloroform. After centrifugation of this material for 2min in a microfuge (12Krpm) the aqueous phase was transferred to a fresh tube and extracted with 400μl of chloroform. The aqueous phase was again removed to a fresh tube and 2.5-3 volumes of ethanol and 1μl of 20mg/ml glycogen was added to it. Since RNA prepared in this manner was to be used in primer extension analysis, 1ng of radioactively labelled oligonucleotide was also added at this stage. RNA and oligonucleotide were pelleted by centrifugation for 10min in a microfuge (12Krpm) after incubating samples for 16h at -20°C or 30min at -70°C. 500μl of 80% (v/v) ethanol was used to wash the pellet and was then aspirated. The RNA/oligonucleotide pellet was air dried and resuspended in 10μl of primer extension hybridisation buffer (10mM Tris pH 8.3, 200mM NaCl).

3.2.3 Primer extension analysis of RNA

RNA and radioactively labelled oligonucleotide in primer extension hybridisation buffer (10mM Tris pH 8.3, 200mM NaCl) was prepared as outlined in section 3.2.2 above. Oligonucleotide was hybridised to RNA by heating samples at 80°C for 3min then cooling slowly to 42°C. To the hybridised samples, 24μl of primer extension

buffer mix and 3U (1μl) of AMV reverse transcriptase was added. Reactions were incubated for 1h at 42°C after which time they were extracted with phenol/chloroform (2.3.4), ethanol precipitated (2.3.5) and resuspended in 10μl formamide load buffer. Samples were electrophoresed in 1XTBE buffer as per sequencing reactions (2.3.10(ii)) on 10% (w/v) polyacrylamide/bisacrylamide (20:1), 8M urea, 1XTBE, 1% (w/v) APS, 0.1% (v/v) TEMED gels.

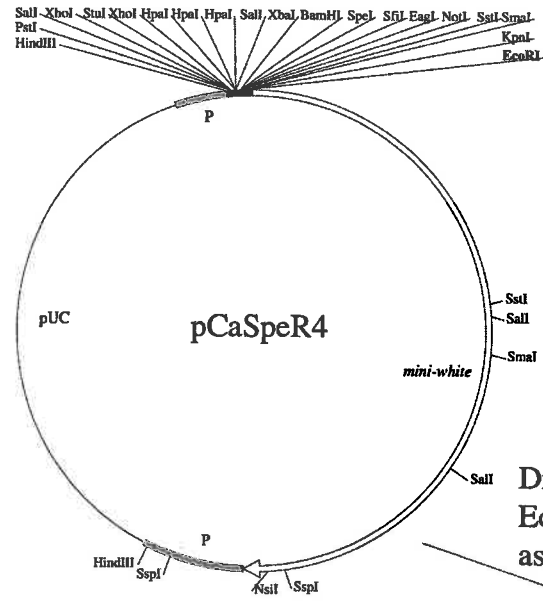
3.2.4 Construction of the transformation vector, pONIX

The *Drosophila* P-element transformation vector used in this study, pONIX, was created by cloning two FRT sites into the pCaSpeR4 vector produced by Vincenzo Pirrotta (1988) so that they flanked the *mini-white* transgene marker as direct repeats (Fig 3.1).

DNA fragments containing FRT sites with *EcoR*I restriction ends were generated by annealing two complementary, kinased, 55mer synthetic oligonucleotides (FRT top; FRT bottom, 2.2.9(i)). The resultant FRT fragment contained two inverted 13bp repeats and an 8bp spacer to provide the minimal FLP recognition sequence (Gronostajski and Sadowski, 1985; Senecoff *et al.*, 1985; Jayaram, 1985) plus an additional 13bp repeat to augment the reactivity of the minimal site (Andrews *et al.*, 1985; Jayaram, 1985). The 8bp spacer region contains an off-center *Xba*I site that specifies the orientation of the FRT (Senecoff and Cox, 1986).

To produce pONIX, an FRT was first cloned into the pCaSpeR4 *EcoR*I restriction enzyme site at the junction between the polylinker and the upstream control region of the *white* gene. The FRT copy number of individual clones were characterised by agarose gel electrophoresis after an *Xho*I/*EcoR*Vrestriction enzyme digest. This digest produced a 254bp fragment in clones that had incorporated a single FRT at the *EcoR*I site. Single copy clones were then sequenced to determine orientation of the FRT using a primer complementary to sequences in the upstream control region of the *white* gene (pCaSpeR *white* 5', 2.2.9(i)). The clone pCaSpeRFRT 8 was selected and another FRT was cloned into the *Nsi*I restriction enzyme site 551bps downstream from the *white* gene polyadenylation site. For this cloning the 3' *Nsi*I overhang of the vector and the 5' *EcoR*I overhang of the FRT fragment were blunted before cloning (2.3.8(ii)a). Individual clones that had integrated a single FRT at this site were identified by the production of a 744bp fragment in a *Ssp*I digest. To determine FRT orientation in these clones the *Ssp*I fragment was subcloned into a *EcoR*V digested pBluescript and partially

Figure 3.1: Construction of the *Drosophila* P-element transformation vector pONIX. Two synthetic FRT sites were cloned into the *Eco*RI and *Nsi*I sites of pCaSpeR4 so that they flanked the *mini-white* gene as direct repeats (section 3.2.4 of text for details).

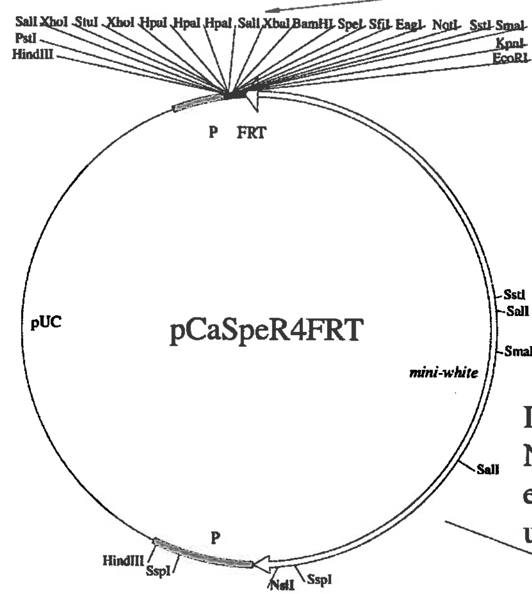


Digested with EcoRI for use as vector

FRT

FRT with EcoRI ends

Ligation

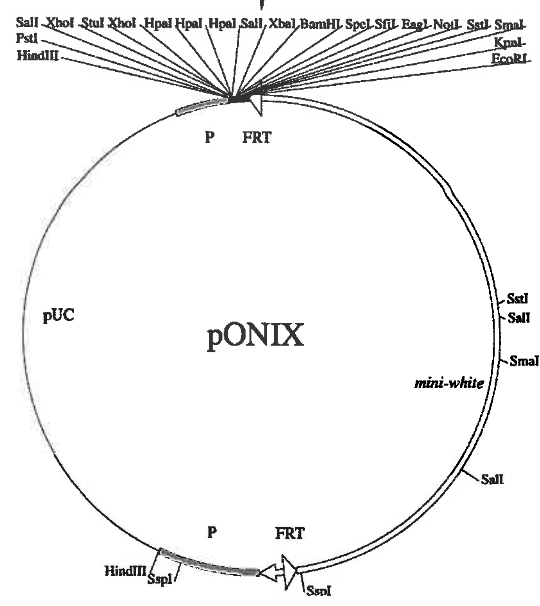


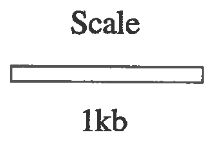
Digested with NsiI and 3' end filled for use as vector

FRT

FRT with EcoRI ends 5' end filled

Ligation





1kb

sequenced (2.3.10) with the primers USP or RSP (2.2.9(i)). A clone that contained the two FRT sites as direct repeats was selected and renamed pONIX.

3.2.5 Construction of pONIX.AvD

A 4.1kb genomic fragment containing the *His2AvD* gene was cloned into the vector pONIX to produce pONIX.AvD (Fig 3.2). The *His2AvD* genomic fragment was derived from the *Drosophila* transformation construct, pC4B, which had previously been used by van Daal and Elgin (1992) to rescue null mutant lethality. The entire 4.1kb genomic insert from pC4B was excised with the restriction endonucleases *Sal*I and *Sma*I and cloned into *Xho*I/*Hpa*I sites in pONIX. Mutated *His2AvD* sequences were cloned into this plasmid for transformation into *Drosophila.*

3.2.6 Construction of phsFLP

phsFLP was created by cloning the FLP recombinase coding region into the vector pCaSpeR-hs (Fig 3.3). pCaSpeR-hs is a P-element transformation vector which was designed for heat shock induced expression of open reading frames cloned into a polylinker and was a gift from Vincenso Pirrotta. The plasmid used as a source of the FLP recombinase coding region, pDM420-FLP, was a gift from Kent Golic. pDM420-FLP was digested with *Sal*I, subjected to 5' overhang end filling (2.3.8(ii)b) and then digested with *Xba*I to obtain a fragment containing the FLP recombinase gene that was suitable for cloning into an *Xba*I/*Hpa*I digested pCaSpeR-hs.

3.2.7 Mutagenesis of the histone *His2AvD* gene in pONIX.AvD

Several different strategies were employed to mutate coding seqences on the *His2AvD* genomic fragment to encode the equivalently positioned H2A.1 amino acids. In each mutagenesis several codons in a particular region were exchanged simultaneously to produce *His2AvD* mutants that encoded "cassettes" of H2A.1 amino acids. The location and specific amino acid sequence alterations of each His2AvD mutant are illustrated in Fig 3.12.

(i) Creation of *His2AvD* mutants M1 and M2

Two *His2AvD* N-terminal mutants, M1 and M2 were created using the Altered Sites *In Vitro* Mutagenesis Kit™ from PROMEGA (an overview of pONIX.M1 and pONIX.M2 production is given in Fig 3.4). The plasmid, pSelM1,2, was constructed for the

Figure 3.2: Production of the plasmid, pONIXAvD, for creation of *Drosophila* lines with a stably integrated *His2AvD* gene and FRT-flanked *mini-white* transgene marker. The *His2AvD* sequence was isolated from a *SalI/Sma*I digest of pC4B and cloned into a *Xho*I/*Hpa*I digested pONIX (see section 3.2.5 of text for details).

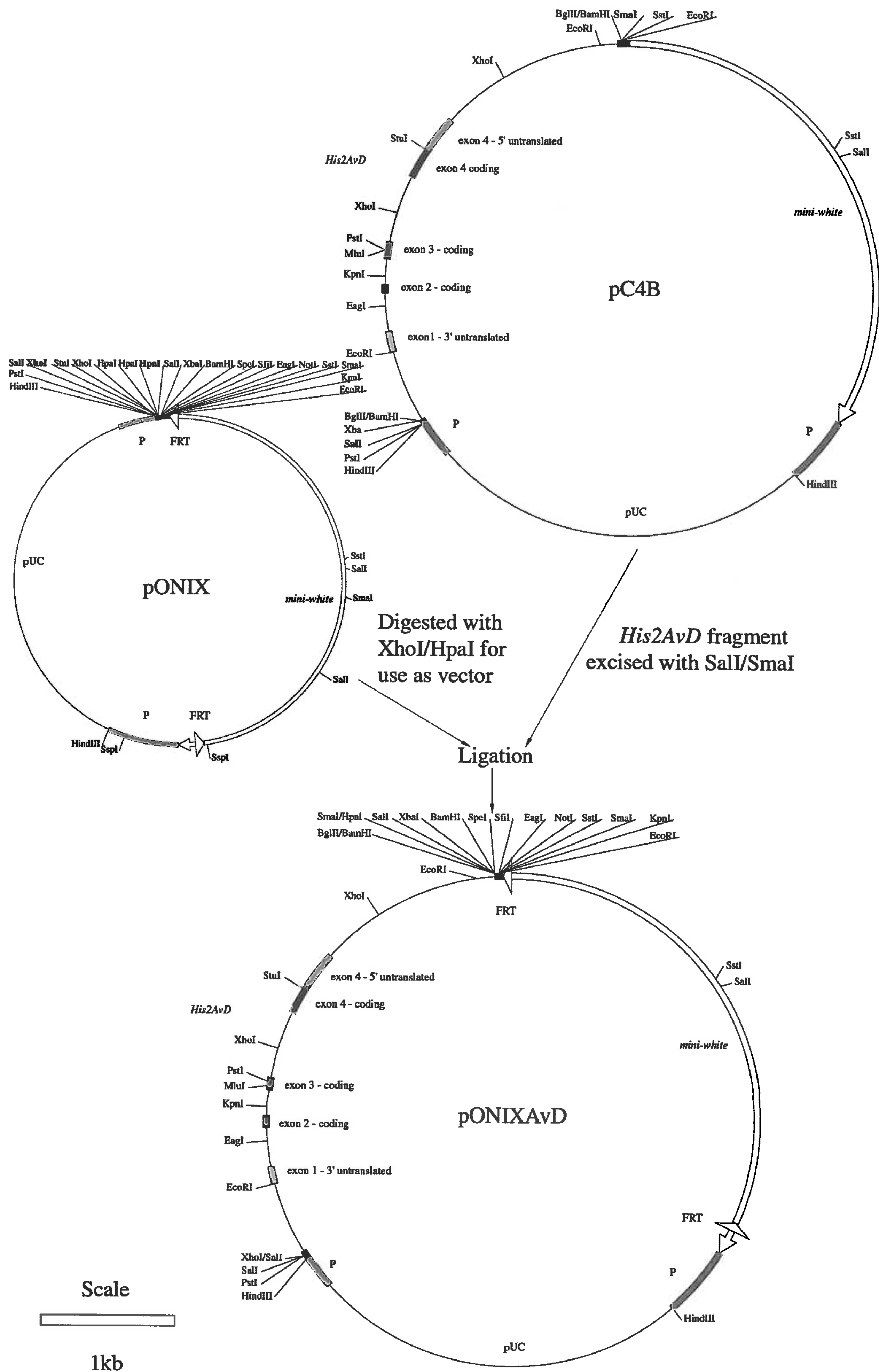

pC4B
His2AvD
exon 4 - 5' untranslated
exon 4 coding
exon 3 - coding
exon 2 - coding
exon1 - 3' untranslated
mini-white
pUC
pONIX
FRT
Digested with XhoI/HpaI for use as vector
His2AvD fragment excised with SalI/SmaI
Ligation
pONIXAvD
Scale
1kb

Figure 3.3: Construction of the phsFLP vector for heat shock induced production of FLP recombinase in transformed *Drosophila* lines. The FLP recombinase coding sequence was isolated as a *Sal*I/*Xba*I fragment and cloned into hsCaSpeR digested partially with *Xba*I and to completion with *Hpa*I (see section 3.2.6 of text for details).

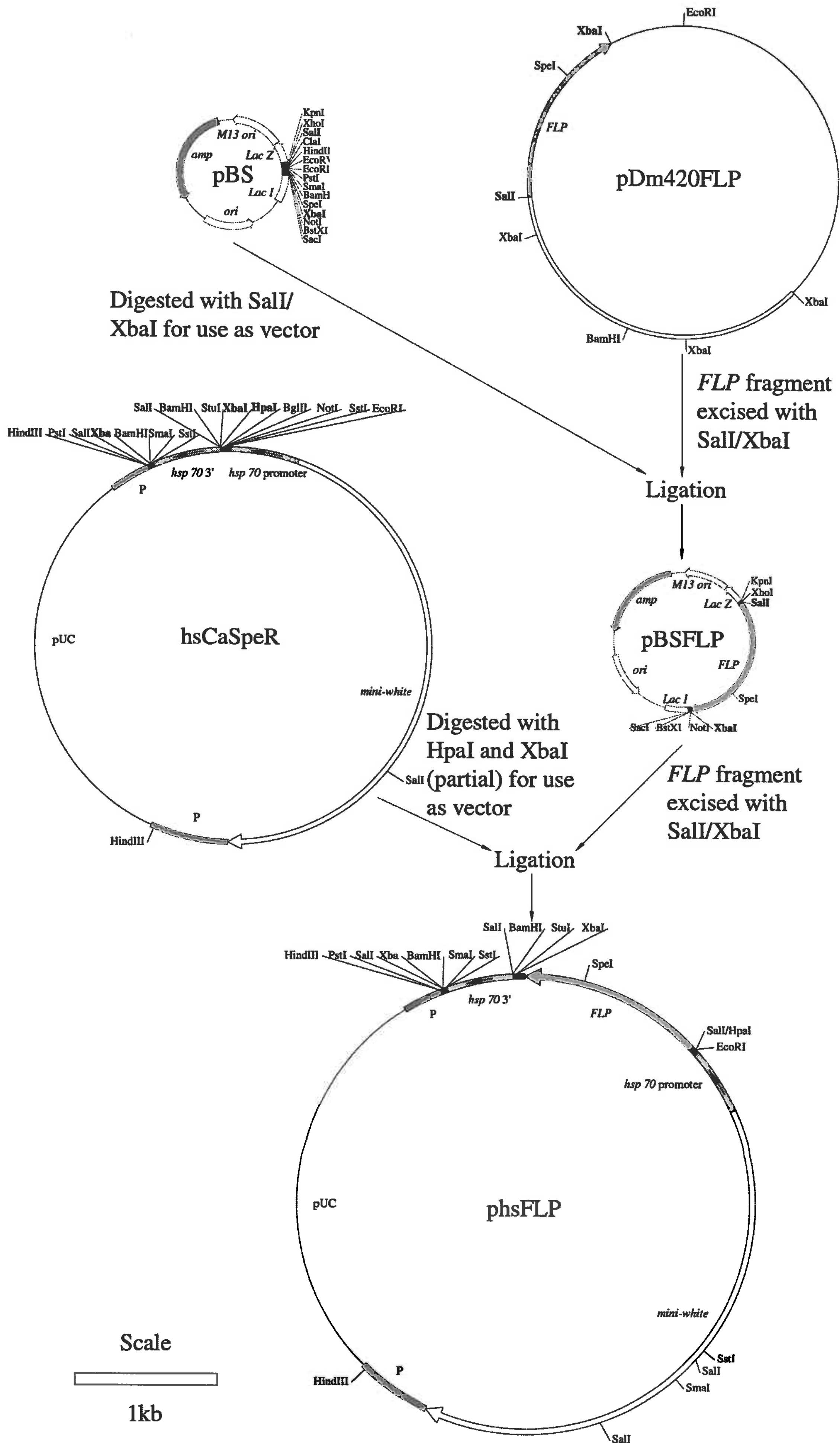
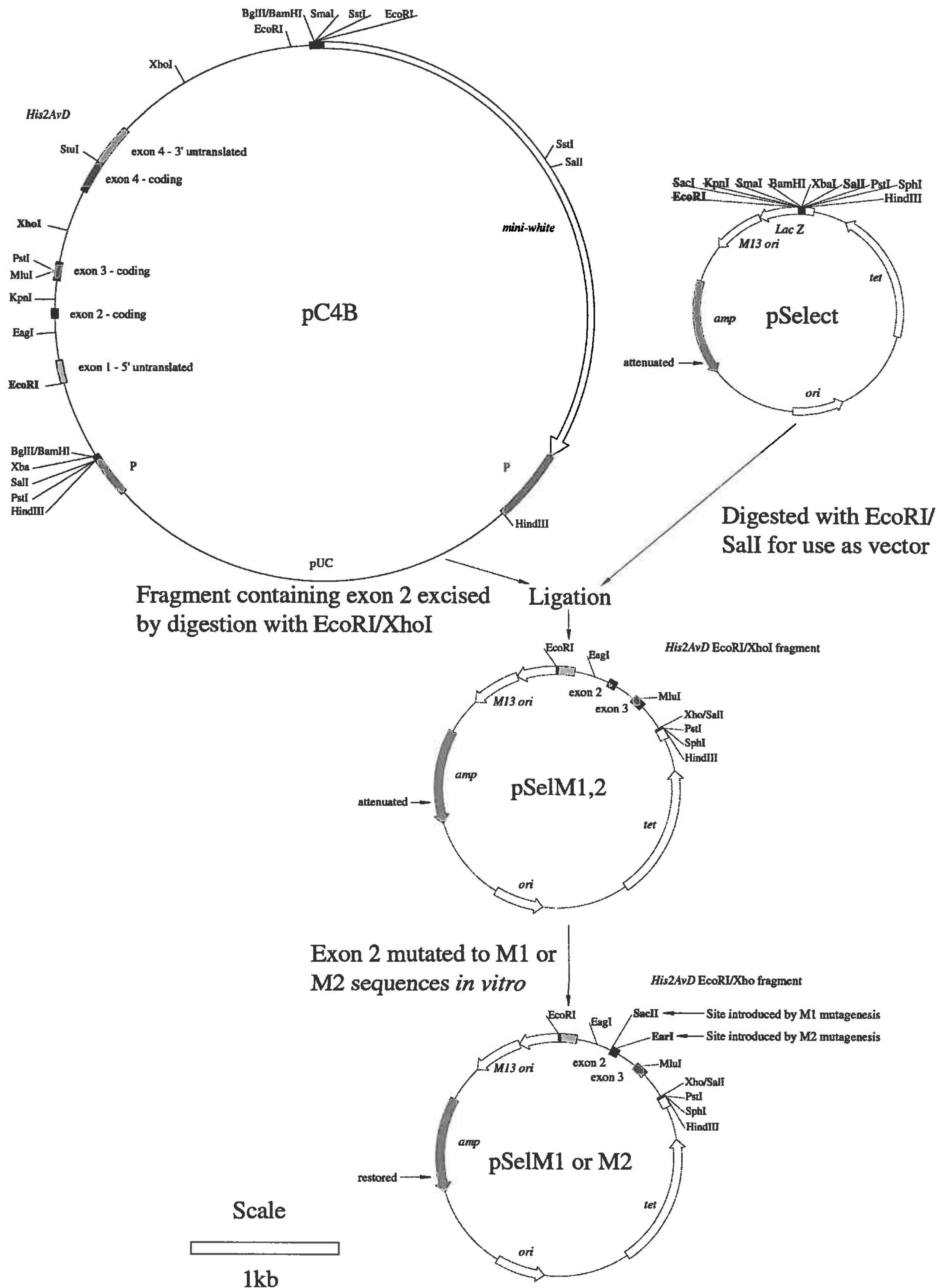
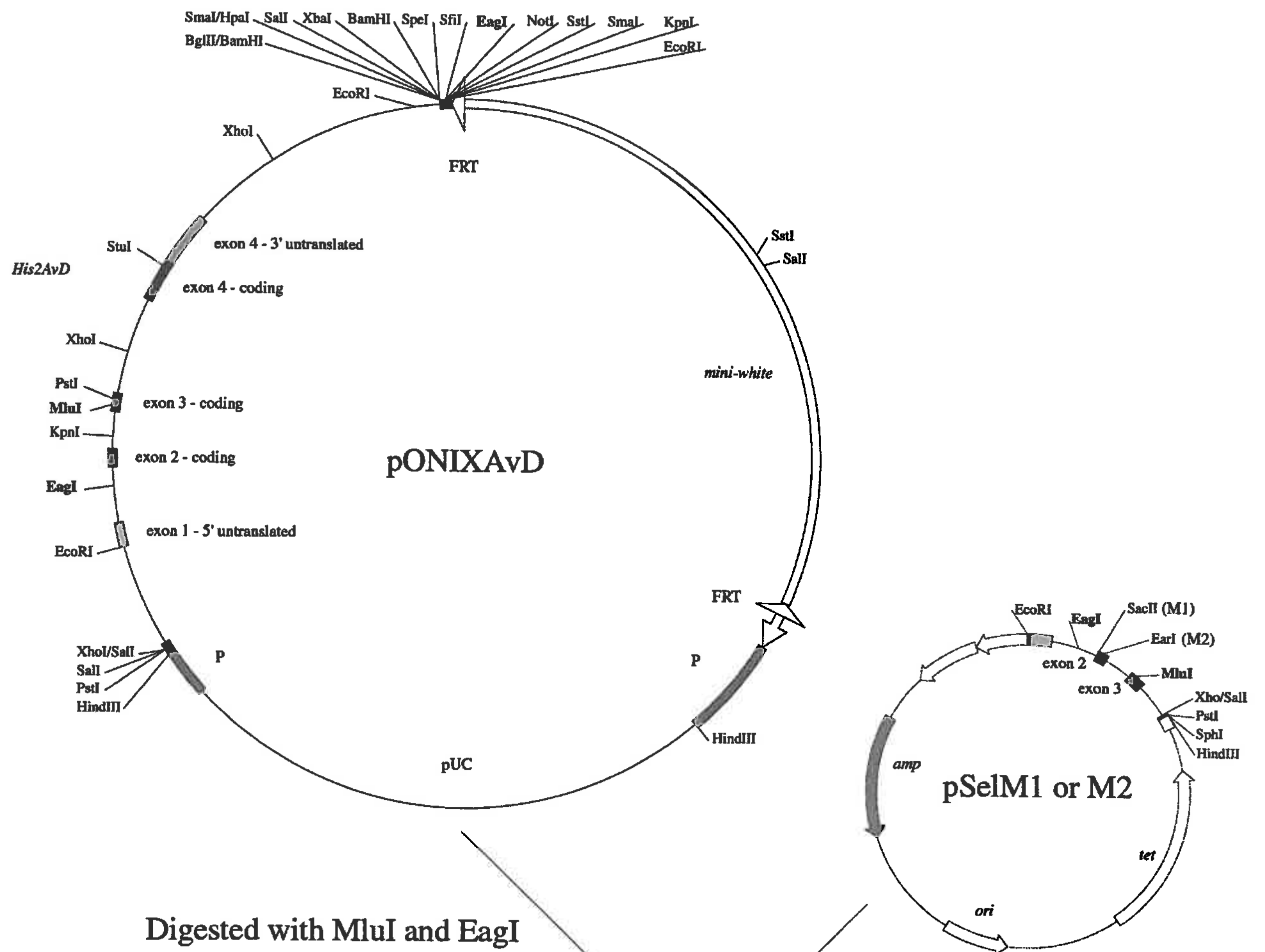

pBS
M13 ori
amp
Lac Z
Lac I
ori
KpnI
XhoI
SalI
ClaI
HindIII
EcoRV
EcoRI
PstI
SmaI
BamHI
SpeI
XbaI
NotI
BstXI
SacI
pDm420FLP
EcoRI
XbaI
SpeI
FLP
SalI
XbaI
XbaI
BamHI
XbaI
Digested with SalI/
XbaI for use as vector
FLP fragment
excised with
SalI/XbaI
Ligation
hsCaSpeR
SalI BamHI StuI XbaI HpaI BglII NotI SstI EcoRI
HindIII PstI SalI Xba BamHI SmaI SstI
hsp 70 3'
hsp 70 promoter
P
pUC
mini-white
SalI
P
HindIII
pBSFLP
M13 ori
amp
Lac Z
KpnI
XhoI
SalI
ori
FLP
Lac I
SpeI
SacI BstXI NotI XbaI
Digested with
HpaI and XbaI
(partial) for use
as vector
FLP fragment
excised with
SalI/XbaI
Ligation
phsFLP
SalI BamHI StuI XbaI
HindIII PstI SalI Xba BamHI SmaI SstI
SpeI
P
hsp 70 3'
FLP
SalI/HpaI
EcoRI
hsp 70 promoter
pUC
mini-white
SstI
SalI
SmaI
SalI
HindIII
P
Scale
1kb

Figure 3.4: Creation of *His2AvD* mutants M1 and M2. An *EcoRI/Xho*I fragment of the *His2AvD* gene was cloned into the vector pSelect and mutagenised with either the M1 or M2 primers. The mutagenised regions were excised by digestion with endonucleases *Eag*I and *Mlu*I and cloned into a similarly digested pONIXAvD (see section 3.2.7(i) of text for details).

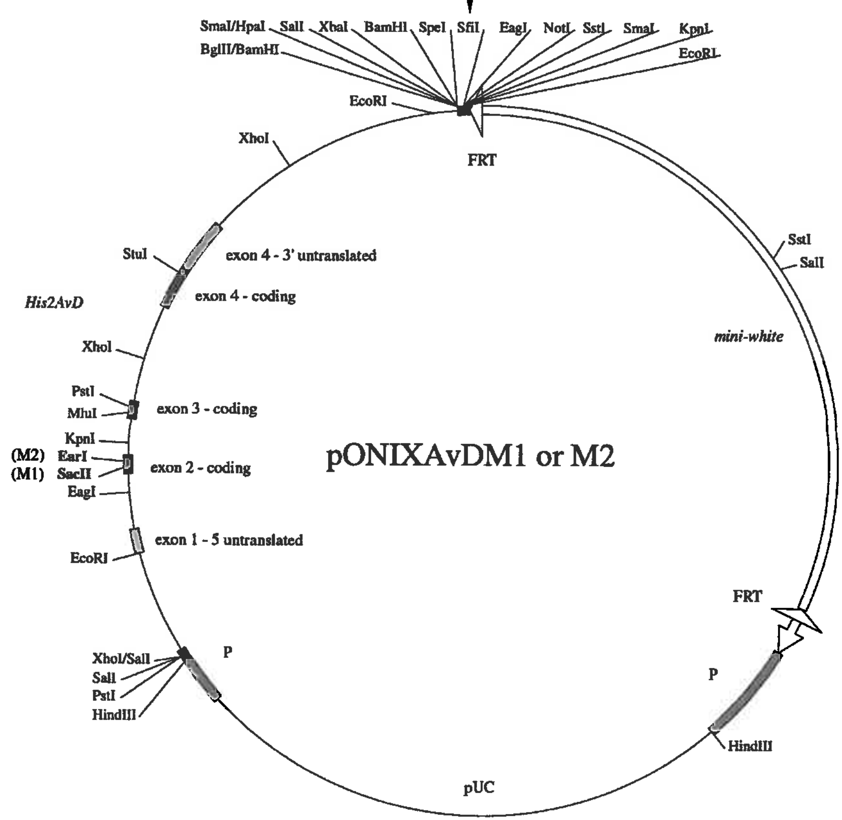




Digested with MluI and EagI (partial) for use as vector

Fragment containing M1 or M2 mutation excised with EagI/MluI

Ligation



production of mutants M1 and M2 by cloning a 1080bp *EcoRI/XhoI* fragment containing the N-terminal coding sequence of His2AvD from pC4B into an *EcoRI/SalI* digested pSelect. The vector pSelect contains a functional tetracycline resistance gene and a non-functional ampicillin resistance gene. An oligonucleotide provided in the kit (*amp*^{r}) restores the function of the ampicillin resistance gene on one strand during the mutagenesis reaction. Oligonucleotides for introducing the M1 and M2 mutations were designed to anneal to the same strand as the *amp*^{r} oligonucleotide and were present at a five molar excess in the mutagenesis reaction. This ratio created a bias favouring the presence of the desired mutation on plasmids where the function of the ampicillin resistance gene had been restored.

To prepare single stranded DNA template for mutagenesis, 0.05pM of pSelM1,2 plasmid DNA in 10µl of water was denatured by addition of 2.5µl of 1M NaOH, 1mM EDTA and incubated at 37°C for 15min. After this material was centrifuged through a Sepharose CL6B column (1.8Krpm for 2min in a Jouan C312 benchtop centrifuge), *amp*^{r} and M1 or M2 oligonucleotides were annealed to the single stranded templates. Closed circular plasmid DNA was produced from oligonucleotide bound, single stranded pSelM1,2 DNA templates using T4 DNA polymerase and DNA ligase in accordance with the Altered Sites™ mutagenesis protocol. These plasmids were transformed into a repair deficient strain of *E.coli* (BMH 71-18 mut S) and plated on media containing ampicillin and tetracycline. Plasmid DNA prepared from individual BMH 71-18 mut S colonies was then retransformed into DH5α and plated on on media containing ampicillin and tetracycline. Plasmid DNA prepared from individual colonies that grew under this selection regimen were then screened for incorporation of the M1 or M2 mutations by digestion with the restriction endonucleases *Sac*II or *Ear*I respectively (the mutagenic oligonucleotides had been designed to incorporate these restriction endonuclease recognition sites to simplify the screening process).

Plasmid clones derived from the M1 and M2 mutagenesis reactions containing either the *Sac*II or *Ear*I restriction endonuclease sites respectively were digested with *EagI/MluI* to excise a 429bp fragment containing the specific mutation. These fragments were cloned separately into pONIX.AvD digested partially with *Eag*I and to completion with *Mlu*I to produce the clones pONIX.M1 and pONIX.M2. The correct incorporation of mutations in each of these plasmid constructs was confirmed by sequencing (2.3.10) both strands of the *EagI/MluI* fragments with USP and RSP (2.2.9(i)) after they had been subcloned as *EagI/PstI* fragments into a similarly digested pBluescript.

(ii) Creation of *His2AvD* mutants M3 and M4

The *His2AvD* mutants M3 and M4 were created by PCR amplifying a *His2AvD* DNA fragment with primers containing the specific mutations and a restriction endonuclease site that was also present in the *His2AvD* sequence. This DNA fragment was then cut with suitable restriction endonucleases and cloned into pONIX.AvD. The specific details of *His2AvD* mutant M3 and M4 production is outlined following and in Figs 3.5 and 3.6 respectively.

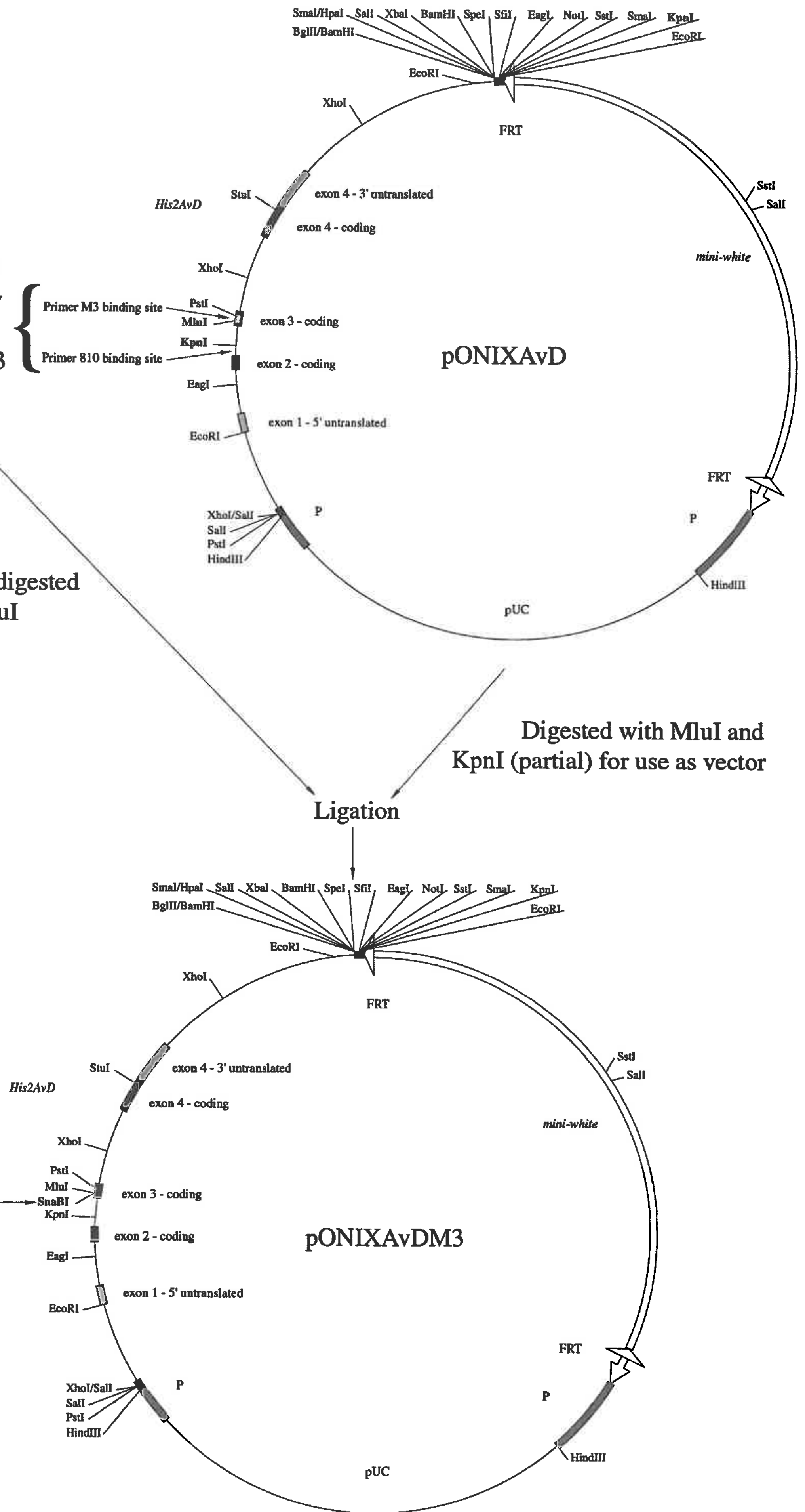
pONIX.AvD was used as the template for PCR amplification (2.3.14) of a fragment containing the M3 mutations using the primers M3 and 810 (2.2.9(i)). This PCR product was cut internally and within the primer sequence with the restriction endonucleases *Kpn*I and *Mlu*I respectively. The resulting 189bp fragment was cloned into pONIX.AvD that had been digested partially with *Kpn*I and to completion with *Mlu*I to create pONIX.M3. Clones were identified by digestion with the restriction endonuclease *SnaB*I which cut at a site introduced by the mutagenesis. pONIX.M3 was digested with *Mlu*I, subject to 5' overhang end filling (2.3.8(ii)b) and then digested with *Kpn*I to produce a fragment containing the M3 mutation which was cloned into an *EcoR*V/*Kpn*I restricted pBluescript. Sequencing (2.3.10) both strands of the subcloned insert with the primers USP and RSP (2.2.9(i)) revealed mutations in this sequence existed only where they had been specifically introduced.

The primers M4 and M4a (2.2.9(i)) were used to incorporate the M4 mutation into a fragment that was PCR amplified (2.3.14) from a pONIX-AVD template. This fragment was restricted with the enzymes *Mlu*I and *Xho*I which cut the fragment internally and within the M4 primer sequence respectively. pONIX.M4 was created by cloning this fragment into pONIX-AvD that had been digested partially with *Xho*I and to completion with *Mlu*I. Clones were identified by restriction with *Pst*I because this site is present in the *His2AvD* sequence but is abolished in M4 mutants. As with pONIX.M3 the specific incorporation of M4 mutations was confirmed by sequencing (2.3.10) both strands of the insert with the primers USP and RSP (2.2.9(i)) after it had been subcloned into pBluescript. For this analysis, pONIX.M4 was digested with *Mlu*I, subject to 5' overhang end filling (2.3.8(ii)b) and then digested with *Xho*I to produce the M4 mutation containing fragment which was cloned into an *EcoR*V/*Xho*I restricted pBluescript.

Figure 3.5: Production of the plasmid, pONIXAvDM3, for creation of *Drosophila* lines with a stably integrated *His2AvD* gene incorporating the M3 mutation. A PCR product incorporating the M3 mutation was generated with the M3 and 810 primers, digested with *KpnI*/*MluI* and cloned into pONIXAvD digested partially with *Kpn*I and to completion with *Mlu*I (see section 3.2.7(ii) of text for details).

Primers M3 and 810 used to PCR amplify a *His2AvD* fragment incorporating the M3 mutation

PCR product digested with KpnI/MluI

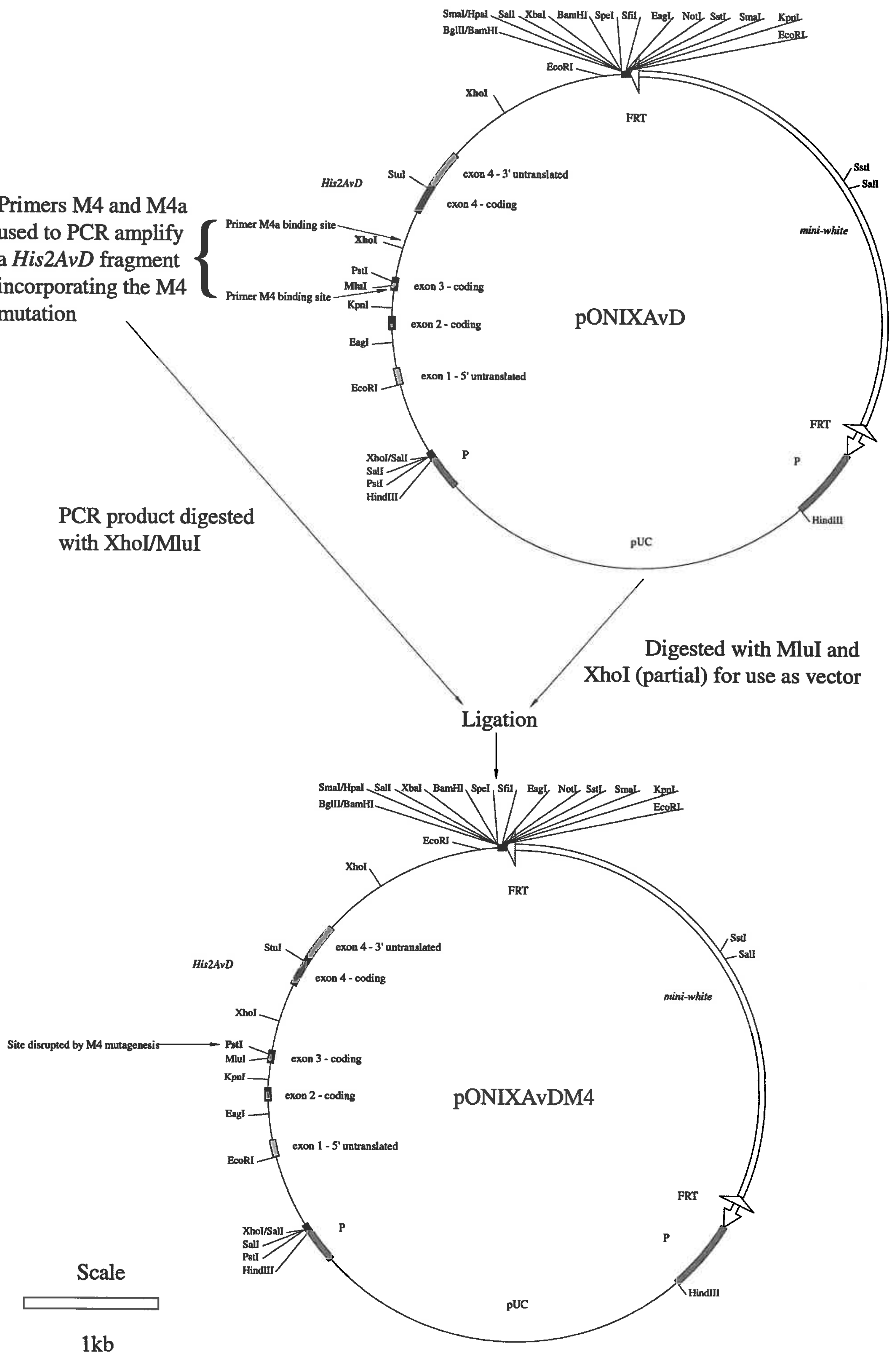


Site introduced by M3 mutagenesis

Scale

1kb

Figure 3.6: Production of the plasmid, pONIXAvDM4, for creation of *Drosophila* lines with a stably integrated *His2AvD* gene incorporating the M4 mutation. A PCR product incorporating the M4 mutation was generated with the M4 and M4a primers, digested with *Xho*I/*Mlu*I and cloned into pONIXAvD digested partially with *Xho*I and to completion with *Mlu*I (see section 3.2.7(ii) for details).



SmaI/HpaI
SalI
XbaI
BamHI
SpeI
SfiI
EagI
NotI
SstI
SmaI
KpnI
BglII/BamHI
EcoRI
EcoRI
XhoI
FRT
StuI
exon 4 - 3' untranslated
His2AvD
exon 4 - coding
SstI
SalI
Primers M4 and M4a used to PCR amplify a *His2AvD* fragment incorporating the M4 mutation
Primer M4a binding site
XhoI
mini-white
PstI
MluI
exon 3 - coding
Primer M4 binding site
KpnI
exon 2 - coding
pONIXAvD
EagI
exon 1 - 5' untranslated
EcoRI
FRT
XhoI/SalI
SalI
PstI
HindIII
P
P
HindIII
PCR product digested with XhoI/MluI
pUC
Digested with MluI and XhoI (partial) for use as vector
Ligation
SmaI/HpaI
SalI
XbaI
BamHI
SpeI
SfiI
EagI
NotI
SstI
SmaI
KpnI
BglII/BamHI
EcoRI
EcoRI
XhoI
FRT
SstI
SalI
StuI
exon 4 - 3' untranslated
His2AvD
exon 4 - coding
mini-white
XhoI
Site disrupted by M4 mutagenesis
PstI
MluI
exon 3 - coding
KpnI
exon 2 - coding
pONIXAvDM4
EagI
exon 1 - 5' untranslated
EcoRI
FRT
XhoI/SalI
SalI
PstI
HindIII
P
P
HindIII
Scale
pUC
1kb

(iii) Creation of His2AvD mutants M5, M6, M7 and CT

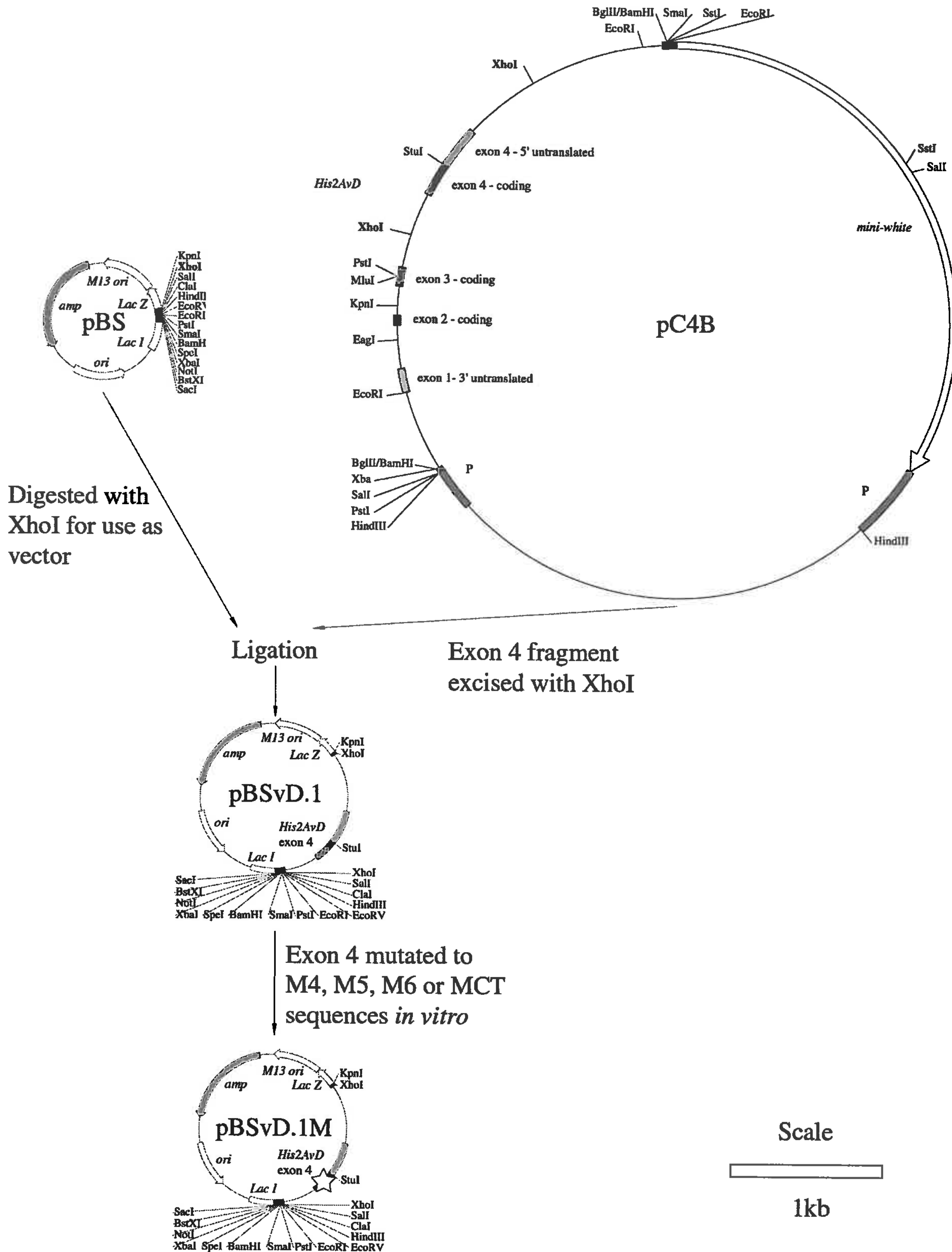
Mutations in the carboxy-terminal coding region of the *His2AvD* gene were introduced by the method developed by Kunkel (1985) and Kunkel *et al.* (1987). Templates for the mutagenesis reaction were grown in the *dut*$^-$ *ung*$^-$ F' bacterial strain CJ236 (2.2.7). This bacterial strain incorporates uracil nucleotides in place of thymine during DNA replication. The DNA sequence to be mutated was cloned into a phagemid, in this case pBluescript, and transformed into CJ236 cells. Single stranded phagemid DNA was isolated from a culture of these cells after they were co-infected with the "helper phage" M13K07. Mutagenic oligonucleotides were then annealed to this single stranded template and used to prime the *in vitro* synthesis of a complementary DNA strand. The resultant closed circular plasmid DNA was transformed into the *dut*$^+$ *ung*$^+$ strain DH5α which degrades the uracil containing template causing preferential replication of plasmids from the *in vitro* synthesised mutagenic strand. Plasmids in DH5α cells transformed with the mutagenesis reaction were separated by isolating and re-transforming the DNA from these cells into DH5α. The *His2AvD* mutants M5, M6, M7 and CT were produced using the mutagenesis strategy detailed below and in Fig 3.7.

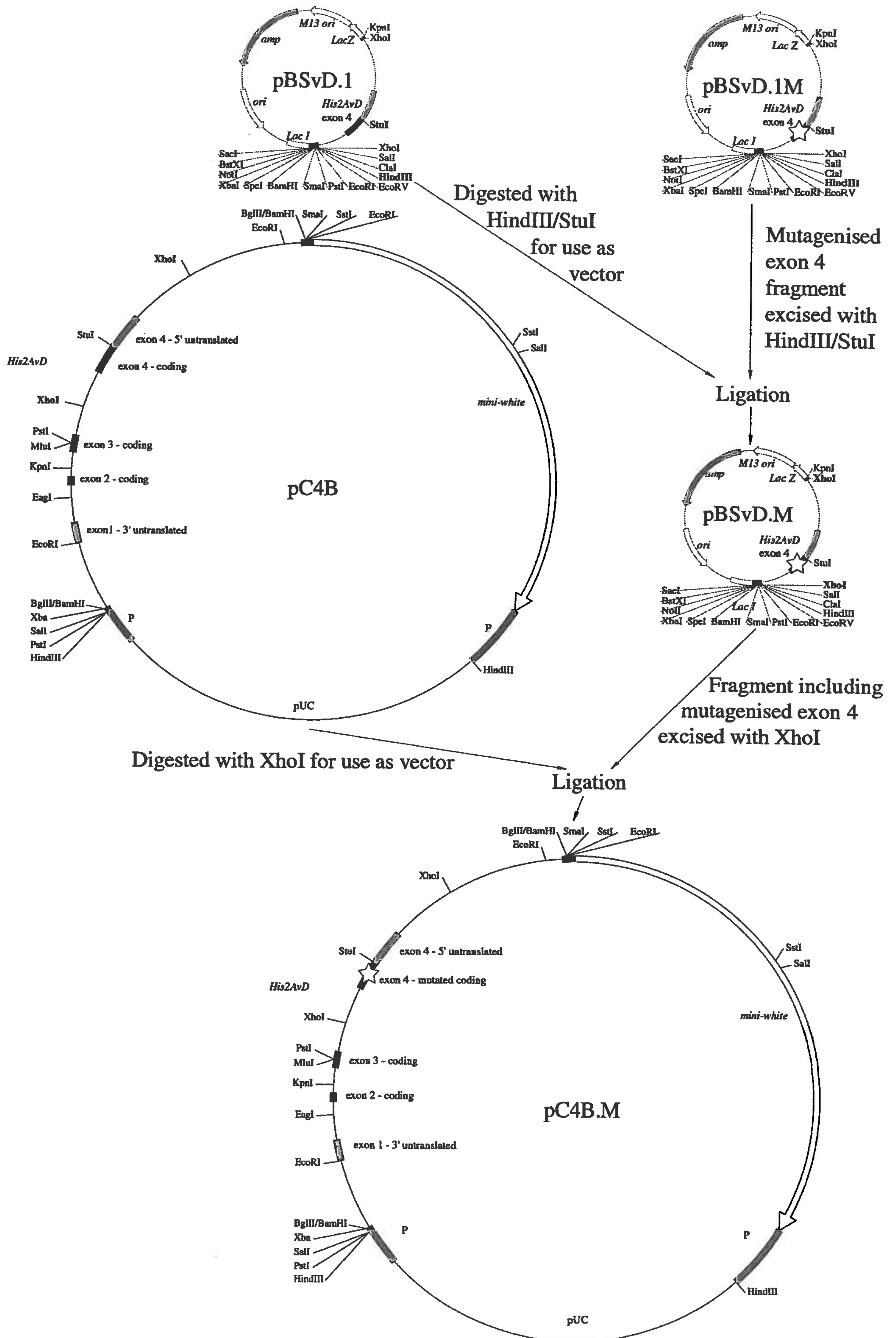
A plasmid template for mutagenesis, pBSvD.1, was created by cloning a 1.4kb *Xho*I fragment of the *His2AvD* gene from pC4B into a similarly digested pBluescript. The *His2AvD* sequence in pBSvD.1 was mutagenised as described above using one of the primers; M6, M7, M8 or CT (2.2.9(i)). Mutant clones were selected by sequencing (2.3.10) across the last exon of *His2AvD* with the primer 2374 (2.2.9(i)) which binds to the 3' untranslated region 84bp downstream from the stop codon. The mutated region, contained on a 541bp *XhoI/Stu*I fragment, was cloned back into the parent vector, pBSvD.1, to produce pBSvD.1M. This step was performed to reduce the amount of sequencing required to check for the misincorporation of bases during mutagenesis. Mutated *His2AvD* sequences were excised from pBSvD.1M as 1.4kb *Xho*I fragments and cloned into a similarly digested pC4B. Mutant *His2AvD* genes were then cloned into pONIX as outlined in 3.2.5. 541bp *XhoI/Stu*I fragments from pONIX.AvD mutants M6, M7, M8 and CT were cloned into the *XhoI/Sma*I sites of pBluescript and sequenced (2.3.10) from both directions with the primers USP and RSP (2.2.9(i)).

3.2.8 Transformation of *Drosophila melanogaster* with pONIX.AvD wild type, pONIX.AvD mutants and phsFLP

DNA was integrated into the germline chromosomes of *Drosophila* using the transposition mechanism of P-elements as per Rubin and Spradling (1982) and

Figure 3.7: Creation of *His2AvD* mutants M5, M6, M7 and CT. A *Xho*I fragment of the *His2AvD* gene was cloned into the vector pBS to create pBSvD.1. *His2AvD* seqences were then mutagenised with the M5, M6, M7 or CT primers. The mutagenised regons were excised by digestion with *Hind*III and *Stu*I and cloned back into the original pBSvD.1 vector to produce pBSvD.1M. Mutagenised *His2AvD* sequences were excised from pBSvD.1 and cloned into pC4B. Mutant *His2AvD* genes were cloned into the *Drosophila* P-element transformation vector pONIX as per wild type *His2AvD* in Fig 3.2. Specific details of the cloning and mutagenesis strategy used to create *His2AvD* mutants M5, M6, M7 and CT are given in section 3.2.7(i) of the text.





pBSvD.1
pBSvD.1M
pBSvD.M
pC4B
pC4B.M
M13 ori
amp
LacZ
ori
Lac I
His2AvD
exon 4
KpnI
XhoI
StuI
SacI
BstXI
NotI
XbaI
SpeI
BamHI
SmaI
PstI
EcoRI
EcoRV
HindIII
ClaI
SalI
Digested with
HindIII/StuI
for use as
vector
Mutagenised
exon 4
fragment
excised with
HindIII/StuI
Ligation
Fragment including
mutagenised exon 4
excised with XhoI
Digested with XhoI for use as vector
Ligation
BglII/BamHI
EcoRI
SstI
exon 4 - 5' untranslated
exon 4 - coding
exon 4 - mutated coding
exon 3 - coding
exon 2 - coding
exon1 - 3' untranslated
MluI
EagI
Xba
mini-white
P
pUC

Spradling and Rubin (1982). P-elements are a family of *Drosophila* transposases that are characterised by short inverted repeat sequences at their termini (reviewed in Engels, 1989). Transposition of a P-element requires these terminal inverted repeats and transposase enzyme, Δ2-3. The transposition mechanism of P-elements has been exploited for transformation of DNA into the germ line chromosomes of *Drosophila* (Rubin and Spradling, 1982; Spradling and Rubin, 1982).

For the production of transformant lines of *Drosophila* the DNA fragment of interest is first cloned into a vector between inverted P-element terminal repeats and then injected into the posterior end of embryos with a source of transposase. Embryos are injected within 90min of being laid so that DNA can access the germ cell nuclei. After this time the germ nuclei pinch off from the syncitium to form pole (germline precursor) cells. Adult flies that develop from the injected embryos are then crossed to obtain progeny derived from transformed germ cells.

The w^{1118} fly line was used to generate embryos for injection and in the cross to select transformants. This line lacks a functional *white* gene which is required for transport of pigment precursors into omatidia and consequently this line has unpigmented (white) eyes. The transformation vectors used here contain the *mini-white* transgene marker which restores eye pigmentation in these lines. Thus, the progeny of flies that developed from injected embryos with pigmented eyes were selected as transformants.

(i) Preparation of DNA

Plasmid DNA was purified using a CsCl gradient and then diluted in 1X injection buffer to a final concentration of 300-700ng/μl (construct DNA) and 300ng/μl (Δ2-3 DNA). 2μl of this material at a time was loaded into a needle for microinjection.

(ii) Egg collection, dechorionation and desiccation

Flies were allowed to lay for 30min on 3% agar plates supplemented with grape juice. Embryos were then transferred from the lay plates onto a microscope slide with a wet paint brush. Chorions were removed manually and the embryos were then lined up, facing the same way, on a strip of glue (Earth brand non-toxic rubber vulcanising cement, Muruni Co.) on a microscope slide. Prepared embryos were left on the bench for 8-10min to desiccate slightly before being covered in paraffin oil.

(iii) Microinjection

Embryos covered in paraffin oil were viewed under 20X magnification on an inverted microscope. The DNA filled needle, attached to a micromanipulator, was brought into the same field of view as the embryos. Injections were performed by moving the microscope stage such that the posterior end of the embryos were pierced by the stationary needle.

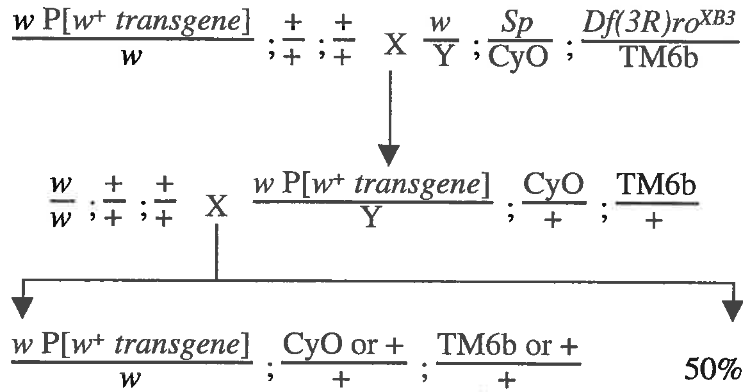
(iv) Post-injection care

After injection, the embryos on a slide were surrounded by a small amount of fresh yeast that had been made into a paste with water and placed on damp tissue in a covered petri dish at room temperature (18-22°C). Once the larvae had hatched, 24-48h later, they were removed with a small strip of Whatmann paper, transferred to a flyfood vial, and allowed to develop at 22°C.

3.2.9 Chromosome mapping of the integrated transgene

To genetically investigate the function of mutant *His2AvD* transgenes it was first necessary to determine which chromosome P-element constructs had integrated onto. *Drosophila melanogaster* has four chromosomes; X, two, three and four. Chromosomes containing an integrated P-element construct were identified in flies by production of eye pigment from the *mini-white* transgene marker. All strains used during chromosome mapping were null mutant in their endogenous *white* gene so that eye pigmentation produced from the *mini-white* transgene marker could be detected. Individual transformant lines were crossed with *w/w;Sp/*Cyo*;Df(3R)ro*^{XB3}/TM6b lines to obtain male flies that contained; the transgene; the 2nd marked balancer chromosome, CyO and; the 3rd marked balancer chromosome, TM6b. Male flies containing an integrated transgene on the X chromosome only produce daughters with pigmented eyes when crossed to w^{1118} females (Fig 3.8a). During gamete production in double balanced male flies, a transgene which had integrated onto either chromosome 2 or 3 segregates away from the CyO or TM6b balancer chromosomes respectively in meiosis. This segregation was observed, phenotypically, in the progeny of a cross with w^{1118} females (Fig 3.8b and c). When the transgene did not segregate away from the marked balancer chromosomes or maleness it was mapped, by default, to chromosome 4.

a.

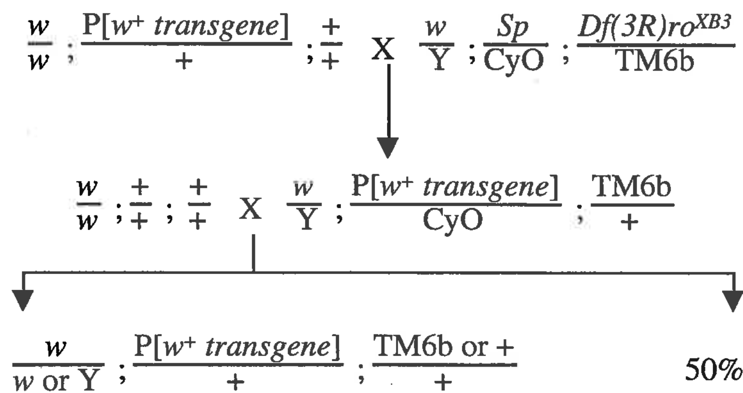


females with pigmented eyes; curly or straight wings; humeral or normal bristles.

$$\frac{w}{\mathrm{Y}} ; \frac{\mathrm{CyO\ or\ +}}{+} ; \frac{\mathrm{TM6b\ or\ +}}{+} \qquad 50\%$$

males with unpigmented eyes; curly or straight wings; humeral or normal bristles.

b.

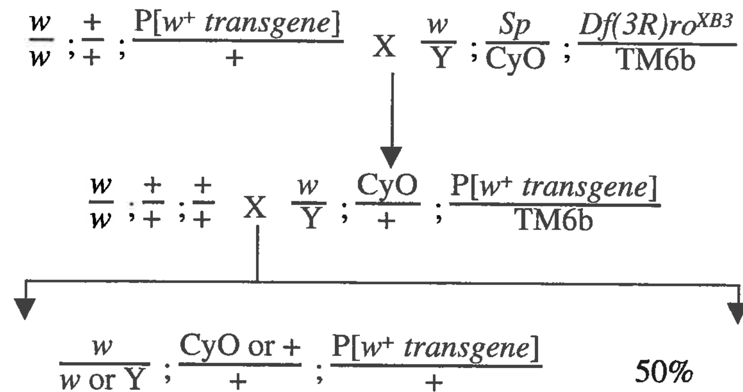


males or females; pigmented eyes; humeral or normal bristles.

$$\frac{w}{w\ \mathrm{or\ Y}} ; \frac{\mathrm{CyO}}{+} ; \frac{\mathrm{TM6b\ or\ +}}{+} \qquad 50\%$$

males or females with unpigmented eyes; curly wings; humeral or normal bristles.

c.



$\frac{w}{w \text{ or } Y}$; $\frac{\text{CyO or } +}{+}$; $\frac{P[w^+ \textit{transgene}]}{+}$ 50%

males or females; curly or straight wings; pigmented eyes and normal bristles.

$\frac{w}{w \text{ or } Y}$; $\frac{\text{CyO or } +}{+}$; $\frac{\text{TM6b}}{+}$ 50%

males or females; curly or straight wings; unpigmented eyes and humeral bristles.

Figure 3.8: Crosses performed to map a *mini-white* marked transgene to the chromosome into which it had integrated. All stocks used for chromosome mapping did not have a functional endogenous *white* gene (w^{1118} allele) and were, therefore, only able to produce eye pigment if they also contained the *mini-white* transgene marker. Transformant flies were crossed to male *w/Y;Sp/*CyO*;Df(3R)ro*^{XB3}/TM6b to obtain males which contained: the transgene; the 2nd marked balancer chromosome, CyO and; the 3rd marked balancer chromosome, TM6b. Male flies containing the integrated transgene on the X chromosome only produce daughters with pigmented eyes when crossed to w^{1118} females (**a**). When double balanced w^+ flies were crossed to w^{1118} females, a transgene which had integrated onto either chromosome 2 (**b**) or 3 (**c**) segregated away from the CyO or TM6b balancer chromosome markers, respectively, in the progeny. When the transgene did not segregate away from the marked balancer chromosomes or maleness it was mapped, by default, to chromosome four. Curly wings were used as the phenotypic marker for the CyO balancer chromosome and the presence of humeral bristles was used to identify flies containing the TM6b balancer chromosome.

3.3 Characterisation of His2AvD null mutants

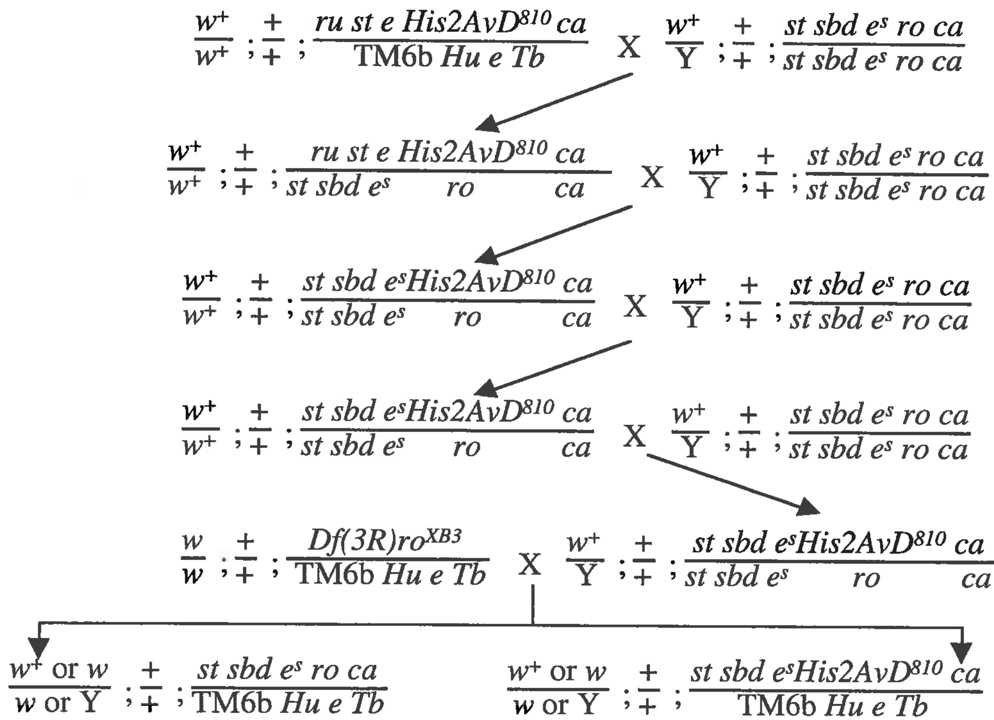
van Daal *et al.* (1988) mapped the *Drosophila His2AvD* gene to the 97CD region of the right arm of chromosome three by *in situ* hybridisation to third larval instar polytene chromosomes. Characterisation of ethylmethanesulfonate (ems) generated, lethal complementation groups in this region led to the identification of a 311bp deletion in the *His2AvD* gene (*His2AvD^{810}*) that removed the entire second exon. Although the mutant gene is transcribed to the same level as the wild type gene, no truncated protein product has been detected (van Daal and Elgin, 1992). The *His2AvD^{810}* mutation was shown to be directly responsible for the lethality of this complementation group when it was demonstrated that a *His2AvD* transgene could restore viability to lines carrying the *His2AvD^{810}* chromosome in combination with, a deficiency encompassing this region, *Df(3R)ro^{XB3}* (van Daal and Elgin, 1992).

Here the *His2AvD^{810}* null mutant lethal phase was further characterised so that the extent of rescue by modified versions of the gene could be assayed. For this experiment, regions of the original chromosome that may have been affected by the ems mutagenesis were removed by recombination with a homozygous viable third chromosome that contained the recessive markers *scarlet* (*st*), *stubbloid* (*stb*), *ebony sooty* (*e^S*), *rough* (*ro*) and *claret* (*ca*) (Fig 3.9a). Recombinant chromosomes containing *His2AvD^{810}* were selected for loss of the *ro* marker, located at 91.1, on the right arm of the third chromosome, in the vicinity of *His2AvD*. These lines were then tested for the presence of the *His2AvD^{810}* mutation, and lack of other lethal mutations, by examining whether the chromosome was lethal in combination with *Df(3R)ro^{XB3}* and whether *st, stb, e^S, His2AvD^{810}, ca* homozygotes could be generated in the presence of a *His2AvD* transgene (Fig 3.9c). Chromosomes that conformed to these criteria were maintained against the third chromosome balancer TM6b. The TM6b balancer chromosome contains the dominant markers *Tubby* (*Tb*) and *Humeral* (*Hu*). The *Tb* mutation causes larvae and pupae to be shorter and thicker than wild type larvae and the *Hu* mutation increases the number of bristles on the humerus in adult flies.

3.3.1 Phenotypic characterisation of *His2AvD^{810}* null mutants

Lines of flies containing the *st, stb, e^S, His2AvD^{810}; ca* chromosome balanced against TM6b were self crossed to generate progeny that were homozygous for *His2AvD^{810}*. Non-*Tb*, *His2AvD^{810}* null, larvae were produced from this cross but non-*Tb* pupae were not observed. To determine the larval instar stage that *His2AvD^{810}* null mutants progressed to, the anterior spiracles on non-*Tb* larvae were examined. Anterior

a.

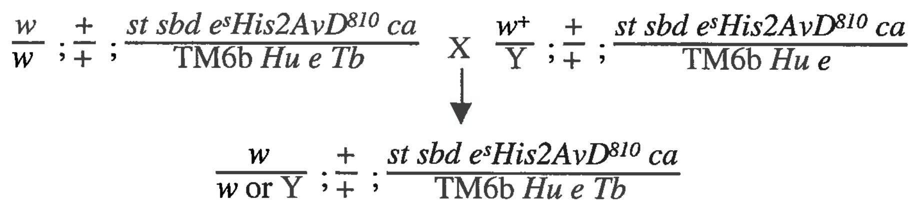


Stocks established from these progeny (Fig 3.9b)

$\frac{w^+ \text{ or } w}{w \text{ or } Y} ; \frac{+}{+} ; \frac{st\ sbd\ e^s\ ro\ ca}{Df(3R)ro^{XB3}}$ $\qquad$ $\frac{w^+ \text{ or } w}{w \text{ or } Y} ; \frac{+}{+} ; \frac{st\ sbd\ e^s His2AvD^{810}\ ca}{Df(3R)ro^{XB3}}$

Lethal if *His2AvD*^{810} retained

b.



c.

$$\frac{w}{w};\frac{P[w^+\ His2AvD]}{P[w^+\ His2AvD]};\frac{Df(3R)ro^{XB3}}{TM6b\ Hu\ e\ Tb}\ \mathrm{X}\ \frac{w^+}{Y};\frac{+}{+};\frac{st\ sbd\ e^s His2AvD^{810}\ ca}{TM6b\ Hu\ e\ Tb}$$

$$\downarrow$$

$$\frac{w}{w\ \text{or}\ Y};\frac{P[w^+\ His2AvD]}{+};\frac{st\ sbd\ e^s His2AvD^{810}\ ca}{TM6b\ Hu\ e\ Tb}\quad \mathrm{X}\ inter\ se$$

$$\frac{w}{w\ \text{or}\ Y};\frac{P[w^+\ His2AvD]}{P[w^+\ His2AvD]\ \text{or}\ +};\frac{st\ sbd\ e^s His2AvD^{810}\ ca}{TM6b\ Hu\ e\ Tb}\quad 55\%$$

$$\frac{w}{w\ \text{or}\ Y};\frac{P[w^+\ His2AvD]}{P[w^+\ His2AvD]\ \text{or}\ +};\frac{TM6b\ Hu\ e\ Tb}{TM6b\ Hu\ e\ Tb}\quad \text{lethal}$$

$$\frac{w}{w\ \text{or}\ Y};\frac{P[w^+\ His2AvD]}{P[w^+\ His2AvD]\ \text{or}\ +};\frac{st\ sbd\ e^s His2AvD^{810}\ ca}{st\ sbd\ e^s His2AvD^{810}\ ca}\quad 27\%$$

st, *sbd*, e^s, *ca* flies present if $His2AvD^{810}$ is the only lethal allele on the *st*, *sbd*, e^s, $His2AvD^{810}$, *ca* chromosome

$$\frac{w}{w\ \text{or}\ Y};\frac{+}{+};\frac{st\ sbd\ e^s His2AvD^{810}\ ca}{TM6b\ Hu\ e\ Tb}\quad 18\%$$

$$\frac{w}{w\ \text{or}\ Y};\frac{+}{+};\frac{TM6b\ Hu\ e\ Tb}{TM6b\ Hu\ e\ Tb}\quad \text{lethal}$$

$$\frac{w}{w\ \text{or}\ Y};\frac{+}{+};\frac{st\ sbd\ e^s His2AvD^{810}\ ca}{st\ sbd\ e^s His2AvD^{810}\ ca}\quad \text{lethal}$$

Figure 3.9: Removal of detrimental mutations on the ems mutagenised chromosome containing a null mutation in the *His2AvD* gene ($His2AvD^{810}$). **a.** Recombination of the $His2AvD^{810}$ mutation onto the homozygous viable *st*, *sbd*, e^s, *ro*, *ca* third chomosome. Genes are illustrated according to their relative location on the chromosome. Genotype abbreviations used here are as per Lindsley and Zimm (1992); *Humeral* (*Hu*), *Tubby* (*Tb*), *white* (*w*), *roughoid* (*ru*), *scarlet* (*st*), *stubbloid* (*sbd*), *ebony* (*e*), *ebony sooty* (e^s), *rough* (*ro*), *claret* (*ca*). TM6b is a balancer chromosome that is homozygous lethal and carries the markers *Hu*, *e* and *Tb* as indicated. **b.** Establishment of stable lines after the recombination protocol. **c.** Test for homozygous viability of recombined $His2AvD^{810}$ chromosomes in the presence of a wild type *His2AvD* transgene.

spiracles change morphology from a node to a branched structure in the transition from second to third instar, coincident with the second larval moult (Ashburner, 1989a see Fig 8.19). Non-*Tb* larvae were observed with branched anterior spiracles. Thus, *His2AvD*^{810} null mutants reach third larval instar but do not pupate.

The *His2AvD*^{810} null mutant third instar larvae survive for up to 10 days after their heterozygous sibs have pupated. During this protracted third instar they all accumulate melanotic tumors. Tumor formation occurs in nearly all *Drosophila* stocks but rarely occurs with a frequency greater than 1%. Although little is known about the triggers for tumor formation, it is known that the incidence of tumors in *Drosophila* is influenced by environmental and genetic factors. Environmental affects which have been shown to increase the incidence of tumors include X-irradiation or low temperatures while a reduction in the frequency of tumors in crowded culture conditions has been noted (reviewed in Sparrow, 1978). Some genetic factors which contribute to tumor formation have been identified. In *Tumorous lethal-1* (*Tum-1*)/*hopscotch* $^{Tum\text{-}1}$ (*hop*$^{Tum\text{-}1}$) (Harrison *et al.*, 1995) and *tumor suppressor gene lethal -1*/*aberent immune response-8* (*air-8*) (Watson *et al.*, 1992) mutants, melanotic tumor formation is associated with overproliferation of the larval lymph glands and blood cell neoplasia. In other strains that accumulate larval tumors including; *discs-large* (*dlg-1*), *lethal(2)giant larvae* (*l(2)gl*) and *fat* (*ft*) larval life is protracted and the imaginal discs overgrow, become disorganised and lose the ability to differentiate (see Lindsley and Zimm, 1992 and references therein). *His2AvD*^{810} mutant larvae, like the latter group of tumor forming mutants, have an extended larval life and bloated imaginal discs, however, the bloating of imaginal discs in *His2AvD*^{810} mutant larvae is not as pronounced and does not appear to be associated with over proliferation.

3.3.2 Gene expression in *His2AvD* null mutants

To determine whether His2AvD mutants completed third larval instar normally but then failed to enter pupation, levels of transcript produced from a gene activated during mid third instar, *Sgs4* (Mogneau *et al.*, 1993; Andres *et al.*, 1993), was compared in *w*^{1118} and *His2AvD*^{810} null mutant larvae. *Sgs4* transcript levels were investigated by primer extension analysis of RNA (3.2.3) extracted from larvae across the third instar stage at 14, 24, 40 and 50h after the second moult (3.2.2). As nearly constant levels of *5C actin* transcripts are present across this period (Andres *et al.*, 1993) it was used as an internal loading control. Since the amount of RNA in female larvae nearly triples during third instar (Ashburner, 1989a see Fig 8.23) primer extension analysis was conducted on

RNA extracted from three female larvae at 14h after the second moult, 2 larvae at 24h and 1 larvae at 40h and 50h.

In addition to examining the levels of a developmentally activated gene, the induction of *hsp23* and *hsp70* genes was also analysed in null mutants. This investigation was prompted by results obtained from characterisation of the His2AvD protein distribution on the polytene chromosomes of larval salivary glands. His2AvD was detected in interband regions but not in bands and on puffs that were developmentally activated but not on puffs that were induced by heat shock (Donahue *et al.*, 1986).

(i) *5C actin* transcript levels in w^{1118} and *His2AvD*^{810} larvae

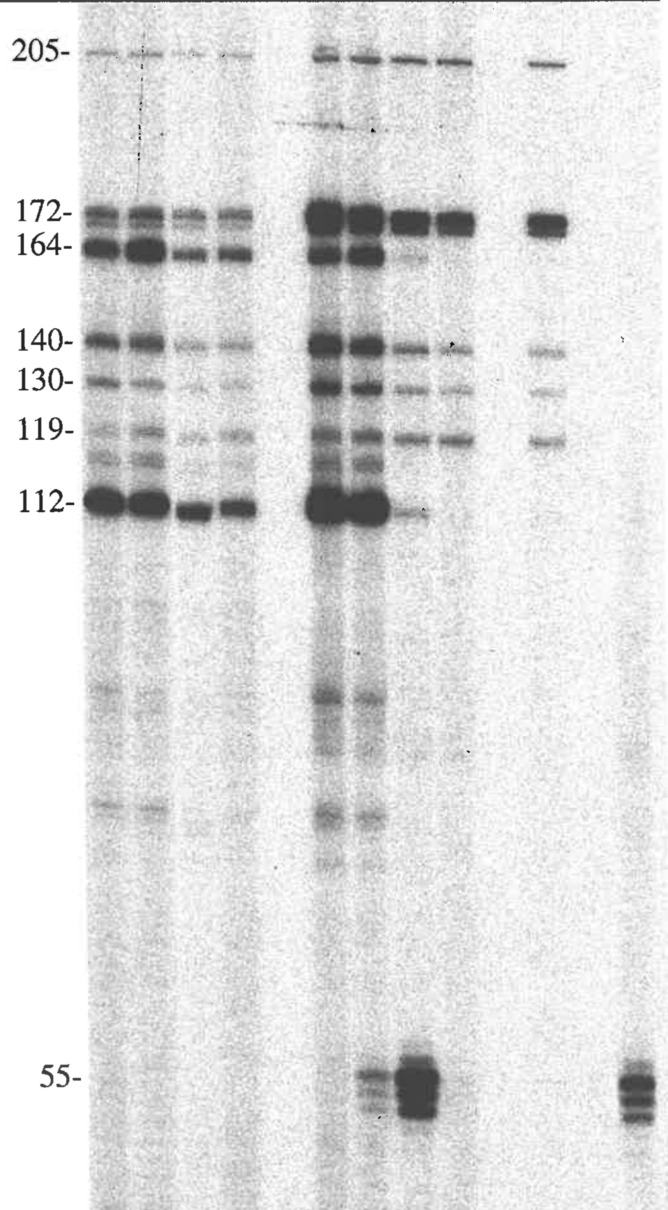
The *Drosophila 5C actin* gene encodes a cytoskeletal actin protein (Fyrberg *et al.*, 1983). Multiple mRNA species are produced from the *5C actin* gene by the use of alternative transcription start and polyadenylation sites (Tobin *et al.*, 1990; Burn *et al.*, 1989; Vigoreaux and Tobin, 1987). Alignment of the *5C actin* cDNA and genomic sequences has shown that different 5' untranslated regions are produced by the alternate splicing of either exon one or two onto exon three (Vigoreaux and Tobin, 1987). In addition to the transcription start sites identified at the start of exon 1 and 2, a third start site has been located within exon 2 by primer extension analysis (Vigoreaux and Tobin, 1987). The primer used in this investigation to detect *5C actin* transcripts (3.2.9(i)) was designed to hybridise to sequences in the third exon so that the relative usage of the three different initiation sites could be compared during the third larval instar.

When RNA samples from w^{1118} and *His2AvD*^{810} larvae were analysed by primer extension using the *5C actin* primer, three products were produced that corresponded to the expected lengths of *5C actin* transcripts initiated at exon 1 (172nt), exon 2 (140nt) and within exon 2 (130nt). During the time course there is a slight decrease in the amount of *5C actin* transcripts detected by primer extension (Fig 3.10). This result is in agreement with previous Northern analysis that also shows a slight decrease in *5C actin* transcript levels during third instar (Fyrberg *et al.*, 1983).

Additional extension products were also produced using this primer. In an effort to identify candidates for these additional products, the *5C actin* primer sequence was used in a BLASTN search of the Berkeley *Drosophila* genome project database. This search identified a high level of identity between the *5C actin* primer sequence and sequences in the 5 other *Drosophila* actin genes. The size of potential extension products from these other actin transcripts was calculated using actin cDNA sequences and the

Figure 3.10: Primer extension analysis of message levels in w^{1118} and *His2AvD*^{810} mutants during third larval instar using primers complementary to *5C actin* and *Sgs 4* message. The three extension products of 172nt, 140nt and 130nt match the expected size of *5C actin* extension products derived from 5C actin transcripts initiated at exon 1, exon 2 and within exon 2 respectively. The 205nt product is the same as the calculated length of product derived from the *88F actin* transcript and has a similar expression profile to that reported for *88F actin* across this period (Fyrberg *et al.*, 1983; Sanchez *et al.*, 1983). Similarly, the 119nt product is the same length as that which would be produced by extension with this primer from the *42A actin* message. The expression profile of the 119nt products also matches the expression profile that has been reported for *42A actin* (Fyrberg *et al.*, 1983; Andres *et al.*, 1993). Primer extension products of 164nt and 112nt may reflect levels of 57B actin transcript (see section 3.3.2 (i) for details). The 55nt product matches the expected size of material produced by primer extension from the *Sgs 4* message with the complementary *Sgs 4* primer used here. The 55nt *Sgs 4* product is induced to high levels 40h after the second moult in w^{1118} flies but not in *His2AvD*^{810} nulls. Primer extension analysis was conducted on RNA extracted from three female larvae at 14h after the second moult, 2 larvae at 24h and 1 larva at 40h and 50h to compensate for the three fold increase in RNA that occurs in female larvae during this period (Ashburner, 1989 see Fig 8.23).

Strain	*His2AvD* null				Wild type					
Primer *5C Actin*	+	+	+	+	+	+	+	+	+	–
Sgs 4	+	+	+	+	+	+	+	+	–	+
Hours after 2nd moult	14	24	40	50	14	24	40	50	40	40



location of the *5C actin* primer identified in the database screen (Table 3.1). Calculated sizes and reported expression profiles of the different actin transcripts throughout the third larval instar were then compared to the size and abundance of transcripts detected using larval RNA samples and the *5C actin* primer. A primer extension product of 206nt produced here is the same as the calculated length of product derived from the *88F actin* transcript. Constant levels of this 205nt extension product were detected throughout the third larval instar, resembling the reported expression profile of the *88F actin* gene during this time (Fyrberg *et al.*, 1983; Sanchez *et al.*, 1983). Similarly, an extension product of 119nt corresponds to the expected size that would be produced from the *42A actin* transcript (Couderc *et al.*, 1987). This product, like the *42A actin* transcript , is detected at a low, constant level throughout the third larval instar (Fyrberg *et al.*, 1983; Andres *et al.*, 1993). Primer extension products of 112nt and 164nt are dramatically down regulated during the period of this analysis in RNA samples derived from w^{1118} larvae. This expression profile matches that of *57B actin* (Fyrberg *et al.*, 1983; Tobin *et al.*, 1990). Although the 5' end of *57B actin* mRNA has not been determined, two coordinately regulated *57B actin* transcripts that differ in length by aproximately 50 nts have been detected in Northern analysis of third larval instar RNA (Tobin *et al.*, 1990). Products corresponding to the calculated size of *79B actin* (161nt) and *87E actin* (99nts) transcripts were not seen. Previous studies have demonstrated that these transcripts are either absent or present at very low levels during the period of this analysis (Fyrberg *et al.*, 1983; Tobin *et al.*, 1990). The *79B actin* and *87E actin* transcripts might be poor templates for extension with the *5C actin* primer because they are not complementary to the 3' extended end of the primer for the terminal 5 and 4nts respectively (Table 3.1).

Most of the primer extension products produced with the *5C actin* primer have similar relative levels of expression throughout third larval instar in w^{1118} and $His2AvD^{810}$ lines. The two exceptions are the 112nt and 164nt primer extension products. These products, which may reflect *57B actin* transcript levels, decrease dramatically mid way through third larval instar in w^{1118} lines but not in $His2AvD^{810}$ lines. The different expression profiles of these products in w^{1118} and $His2AvD^{810}$ lines suggest that the developmental regulation of genes in $His2AvD^{810}$ lines is disrupted by a stage corresponding to 40h into the third instar of w^{1118} larvae.

(ii) *Sgs-4* transcript levels in w^{1118} and $His2AvD^{810}$ larvae

In *Drosophila*, there is a group of seven genes which are induced to a high level in larval salivary glands during the second half of third instar (Korge, 1975, 1977;

<table>
<tr><th rowspan="2">Actin</th><th rowspan="2">Hybridisation and homology of 5C actin primer (top) to binding site on actin mRNA (bottom) from database search</th><th rowspan="2">Calculated extension product</th><th colspan="3">3rd instar expression</th><th rowspan="2">Refs.</th></tr>
<tr><th>E</th><th>M</th><th>L</th></tr>
<tr><td>5C</td><td></td><td></td><td></td><td></td><td></td><td rowspan="4">1, 2, 3, 4</td></tr>
<tr><td>Start at exon 1</td><td>3' GAATGTTTTACACACTGCTTCTTCA 5'
|||||||||||||||||||||||||
5' CUUACAAAAUGUGUGACGAAGAAGU 3'
MetCysAspGluGlu--></td><td>172nt</td><td>++</td><td>++</td><td>++</td></tr>
<tr><td>Start at exon 2</td><td>3' GAATGTTTTACACACTGCTTCTTCA 5'
|||||||||||||||||||||||||
5' CUUACAAAAUGUGUGACGAAGAAGU 3'
MetCysAspGluGlu--></td><td>140nt</td><td>++</td><td>++</td><td>++</td></tr>
<tr><td>Start in exon 2</td><td>3' GAATGTTTTACACACTGCTTCTTCA 5'
|||||||||||||||||||||||||
5' CUUACAAAAUGUGUGACGAAGAAGU 3'
MetCysAspGluGlu--></td><td>130nt</td><td>++</td><td>++</td><td>++</td></tr>
<tr><td>88F</td><td>3' GAATGTTTTACACACTGCTTCTTCA 5'
|| ||| ||||||||||| || |
5' CUGCCAAGAUGUGUGACGAUGAUGC 3'
MetCysAspAspAsp--></td><td>205nt</td><td>+</td><td>+</td><td>+</td><td>1, 2, 5</td></tr>
<tr><td>42A</td><td>3' GAATGTTTTACACACTGCTTCTTCA 5'
|||||||||||||||||||| ||
5' UCUACAAAAUGUGUGACGAAGAGGU 3'
MetCysAspGluGlu--></td><td>119nt</td><td>+</td><td>+</td><td>+</td><td>1, 2, 4, 6</td></tr>
<tr><td>57B</td><td></td><td></td><td></td><td></td><td></td><td rowspan="3">1, 2, 7</td></tr>
<tr><td>1.8 kb t/cript</td><td>3' GAATGTTTTACACACTGCTTCTTCA 5'
|||||||||||||||| |||||
5' AAAACAAAAUGUGUGACGAUGAAGU 3'
MetCysAspAspGlu--></td><td>?</td><td>+++</td><td>+++</td><td></td></tr>
<tr><td>1.85 kb t/cript</td><td>3' GAATGTTTTACACACTGCTTCTTCA 5'
|||||||||||||||| |||||
5' AAAACAAAAUGUGUGACGAUGAAGU 3'
MetCysAspAspGlu--></td><td>? + 50nt</td><td>+++</td><td>+++</td><td></td></tr>
</table>

<table>
<tr><th rowspan="2">Actin</th><th rowspan="2">Hybridisation and homology of 5C actin primer (top) to binding site on actin mRNA (bottom) from database search</th><th rowspan="2">Calculated extension product</th><th colspan="3">3rd instar expression</th><th rowspan="2">Refs.</th></tr>
<tr><th>E</th><th>M</th><th>L</th></tr>
<tr><td>79B</td><td>3' GAATGTTTTACACACTGCTTCTTCA 5'
|| ||||||||||||||||
5' AACCAAACAUGUGUGACGAAGAAGC 3'
MetCysAspGluGlu--></td><td>161nt</td><td></td><td></td><td></td><td>1, 2, 5</td></tr>
<tr><td>87E</td><td>3' GAATGTTTTACACACTGCTTCTTCA 5'
||| ||||||||||| ||| |
5' UAGCCAAGAUGUGUGACGAUGAGGU 3'
MetCysAspAspGlu--></td><td>99nt</td><td>+</td><td>+</td><td>+</td><td>1, 2, 7, 8</td></tr>
</table>

Table 3.1: *5C actin* primer hybridisation to *Drosophila* actin mRNAs showing expected primer extension product lengths and relative expression throughout third larval instar (E, early; M, middle; L, late). Genes and hybridisation regions were identified in a BLASTN search of the Berkeley *Drosophila* genome project database. Although the 5' end of *57B actin* message has not been characterised, Northern analysis has shown that two transcripts produced from this gene, that differ in length by 50nt, are coordinately downregulated during the third larval instar (7). In a primer extension analysis of *Drosophila* third instar larval RNA using the *5C actin* primer, two extension products which are downregulated during third instar and differ by 55nt were detected (Fig 3.10). If these products correspond to the *57B actin* message then the 5' untranslated regions of the two transcripts would be 147nt and 95nt.

For brevity references have been anotated as follows for this figure;

1. Fyrberg *et al.*, 1981
2. Fyrberg *et al.*, 1983
3. Vigoreaux and Tobin, 1987
4. Andres *et al.*, 1993
5. Sanchez *et al.*, 1983
6. Couderc *et al.*, 1987
7. Tobin *et al.*, 1990
8. Manseau *et al.*, 1988

Beckendorf and Kaftos, 1976; Akam *et al.*, 1978; Muskavitch and Hogness, 1980). The protein products encoded by these genes are components of a glue that is expelled from the salivary glands as the prepupae forms to attach the pupal case to a solid surface (Fraenkel and Brookes, 1953).

The message levels produced from one glue gene, *Sgs-4* , were examined here to determine the relative induction of a gene activated mid way through the third instar in w^{1118} and $His2AvD^{810}$ null mutant larvae. Primer extension analysis of RNA extracted from w^{1118} third instar larvae, using primer complementary to the *Sgs4* message (3.3.9(i)), showed that transcript was undetectable at 14h, reached maximal levels at 40h and then decreased in abundance by 50h (Fig 3.10). In $His2AvD^{810}$ null mutants, no *Sgs4* transcript was detected during third instar (Fig 3.10). Primer extension analysis of *Sgs4* transcript levels at other time points throughout the protracted third larval instar of $His2AvD^{810}$ null mutants also failed to detect any transcript (data not shown). These results are consistent with the observation that the salivary glands of $His2AvD^{810}$ null mutants do not become bloated with glue at any stage during their extended third instar.

The lack of *Sgs4* transcript in $His2AvD^{810}$ larvae supports the suggestion, derived from results obtained with a *5C actin* primer, that development of null mutants is disrupted before they reach a stage which is analogous to 40h after the second moult in w^{1118} larvae. To further investigate this possibility, the behaviour of $His2AvD^{810}$ larvae was examined. Normally, larvae cease feeding 30-40h after the second moult and begin to "wander" across the walls of the culture container. $His2AvD^{810}$ null mutants do not exhibit wandering behavior.

(iii) *hsp70 and hsp23* transcript levels in w^{1118} and $His2AvD^{810}$ larvae

To investigate whether shock gene activation was disrupted in $His2AvD^{810}$ null mutants, *hsp23* and *hsp70* message levels were analysed by primer extension in shocked and unshocked w^{1118} and $His2AvD^{810}$ third instar larvae. Larvae were selected for analysis of heat shock gene induction 50h after the second moult so the production of heat shock transcripts could be analysed after the time where expression of developmental genes is disrupted in $His2AvD^{810}$ larvae (above).

The heat shock genes are maintained in a transcriptionally competent state (reviewed in Wallrath *et al.*, 1994). In *Drosophila*, the GAGA transcription factor pre-sets chromatin in the *hsp26* and *hsp70* promoter regions to facilitate the binding of heat shock factor (HSF) to its cognate element (HSE) (Lu *et al.* 1992; Tsukiyama *et al.* 1994). The

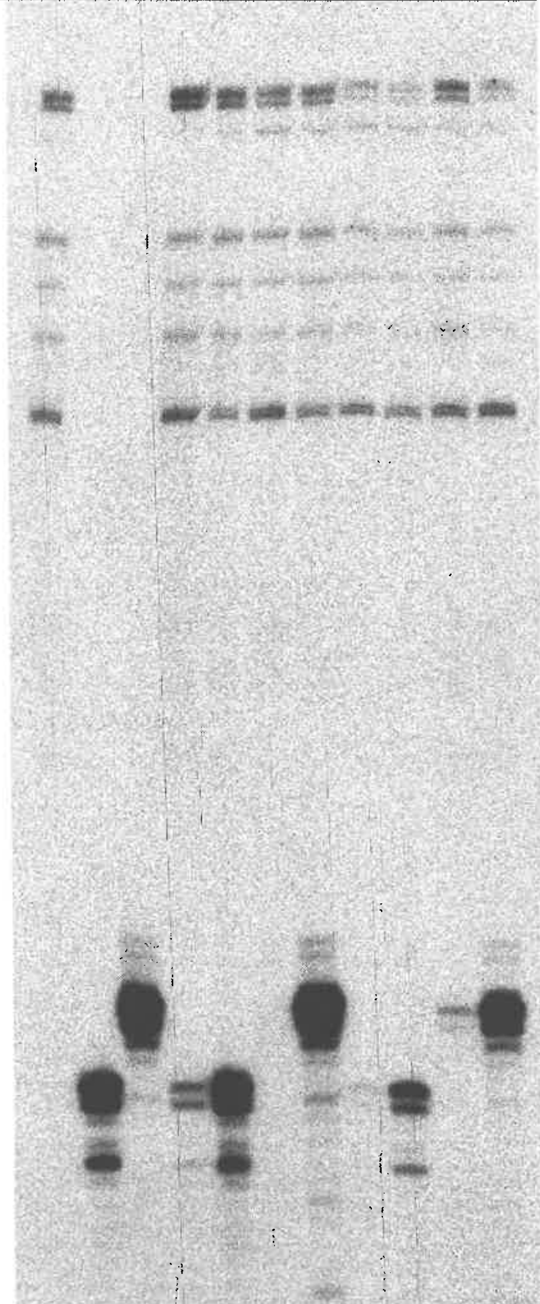
uninduced *hsp70*, *hsp26* and *hsp27* genes also have a transcriptionally engaged RNA polymerase II which pauses after synthesising a short transcript of 20-50 bases (Rougvie and Lis, 1990, 1988; O'Brien and Lis, 1991; Giardina *et al.* 1992; Rasmussen and Lis, 1993). Following heat shock or environmental stress, HSF trimerises and binds to HSEs in the promoters of heat shock genes (Clos *et al.*, 1990; Westwood *et al.*, 1991; Rabindran *et al.*, 1993; Zhong *et al.*, 1998). This binding causes the polymerase to elongate through the pause and increase the rate of transcript reinitiation (O'Brien and Lis, 1991; O'Brien *et al.*, 1995; Brown *et al.*, 1996).

While the *Drosophila hsp70* gene appears to be activated almost exclusively in response to environmental stress, the small heat shock genes are also expressed in a temporal- and tissue-specific manner throughout development (Ireland and Berger, 1982; Sirotkin and Davidson, 1982; Zimmerman *et al.*, 1983; Mason *et al.*, 1984; Ayme and Tissieres, 1985; Thomas and Lengyel, 1986; Glaser *et al.*, 1986; Pauli and Tonka, 1987; Pauli *et al.*, 1988, 1989). The *Drosophila* small heat shock genes (*hsp22*, *hsp23*, *hsp26* and *hsp27*) are clustered at the chromosomal locus 67B (Southgate *et al.*, 1983; Ayme and Tissieres 1985). At the end of third larval instar, elevated titres of the hormone ecdysone activate expression from these four heat shock genes through the action of the *Broad-Complex* (*BR-C*) group of transcriptional regulators (Dubrovsky and Zhimulev, 1988; Dubrovsky *et al.*, 1994). This ecdysone dependent induction of small heat shock genes coincides with extensive chromatin rearrangements in the 67B region which are not observed after heat shock (Cartwright and Elgin, 1986; Kelly and Cartwright, 1989; Dubrovsky *et al.*, 1994). Thus, different mechanisms appear to be utilised for heat shock and developmental induction of genes at this locus.

Primer extension analysis of *hsp23* and *hsp70* transcript levels illustrates that these genes are induced to similar levels in w^{1118} and $His2AvD^{810}$ null mutant larvae 50 hours after the second moult (Fig 3.11). Consistent with previous reports that *hsp23* is developmentally expressed during this stage, *hsp23* transcripts were detected in unshocked w^{1118} and $His2AvD^{810}$ null mutant larvae. It is difficult to ascertain whether this result indicates that, unlike *Sgs4*, the developmental induction of *hsp23* is not affected because *hsp70* is also activated in $His2AvD^{810}$ null mutant larvae in the absence of heat shock. Thus, the detection of *hsp23* and *hsp70* transcripts in $His2AvD^{810}$ null mutant larvae might reflect the general expression of heat shock genes in response to stress experienced by the null mutant rather than an example of a developmentally expressed gene that is not affected by the absence of His2AvD protein.

Figure 3.11: Primer extension analysis demonstrating that *hsp23* and *hsp70* gene expression is induced in w^{1118} and $His2AvD^{810}$ null mutant larvae following heat shock 50h after the second moult. Primer extension products produced using the 5C actin primer are discussed in section 3.3.2 (i) and the legend to Fig 3.10. Primer extension reactions containing primers complementary to *hsp23* and *hsp70* transcripts produced products of 47nt and 53nt respectively following heat shock in both w^{1118} and $His2AvD^{810}$ null mutant larvae. These sizes are consistent with the calculated lengths of primer extension products produced from the *hsp23* and *hsp70* transcripts (Southgate *et al.*, 1983; McGary and Lindquist, 1985) with the primers used here.

Strain		Wild type							*His2AvD* null			
Primer	*5C Actin*	+	−	−	+	+	+	+	+	+	+	+
	hsp23	−	+	−	+	+	−	−	+	+	−	−
	hsp70	−	−	+	−	−	+	+	−	−	+	+
Heat shock		−	+	+	−	+	−	+	−	+	−	+



3.3.3 Selection of phenotypic markers for analysis of *His2AvD*^{810} null mutant rescue by mutant *His2AvD* transgenes

Analysis of *His2AvD*^{810} development determined that nulls are phenotypically normal during embryogenesis, first larval instar and second larval instar then they undergo a protracted third instar and die without entering pupation. The observation that null mutant larvae do not exhibit wandering behaviour suggests that their development is arrested some time before mid third instar. This is supported by the demonstration that expression of developmentally regulated genes is disrupted in *His2AvD*^{810} null mutants by 40h after the second larval moult. Since heat shock genes can still be induced after this time, it appears that disrupted expression of developmental genes is not due to a disruption in the function of the transcription apparatus. As *His2AvD* message is maternally loaded into the oocyte (van Daal and Elgin, 1992) it seems more likely that the arrest of development in null mutants reflects a depletion of maternally derived protein to subcritical levels at this stage than to the lack of a prior requirement for His2AvD protein.

This characterisation of *His2AvD*^{810} null mutant development was conducted so that mutant versions of *His2AvD* could be analysed for their ability to restore His2AvD function to null mutant larvae. Since it appears that the development of *His2AvD*^{810} null mutants arrests some time before mid third instar, progression into pupation was chosen as the first marker to indicate the presence of functional His2AvD protein.

3.4 Domains of *His2AvD* required for *Drosophila* development

By demonstrating that lethality arising from a combination of a 311bp deletion in *His2AvD* and a deficiency in this region could be rescued with a *His2AvD* transgene, van Daal and Elgin (1992) showed that *His2AvD* function is essential in *Drosophila*. The 311bp deletion in the *His2AvD* was generated by ems mutagenesis. Other potentially detrimental mutations carried on this chromosome were removed by recombination with a marked chromosome so it was homozygous viable in the presence of the *His2AvD* transgene (section 3.3). Characterisation of null mutant individuals that were homozygous for the *His2AvD* null mutation showed that their development was disrupted early in the third larval instar stage (3.3). Here, the 4.1kb DNA fragment containing *His2AvD* that can rescue null mutant lethality when present as a transgene (van Daal and Elgin, 1992) was mutagenised to identify regions of the His2AvD protein that functionally distinguish it from H2A. To this end, sequences that encode residues unique to His2AvD on the 4.1kb rescue fragment were changed so that they encoded

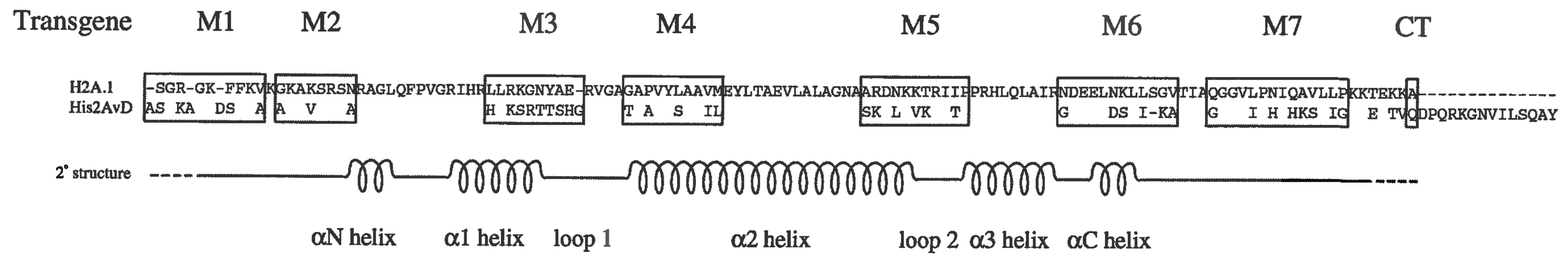
the equivalently positioned H2A residues (Fig 3.12). To examine whether the mutagenised genes produced functional His2AvD protein *in vivo* they were transformed into *Drosophila*, crossed into a *His2AvD*^{810} null background and scored for development into pupae and adults.

3.4.1 Rescue of *His2AvD*^{810} null mutant lethality by *His2AvD* mutants

Flies with *His2AvD* transgenes on the X or second chromosome were crossed as outlined in Fig 3.13a and b respectively to generate stocks homozygous for the transgene in the presence of *His2AvD*^{810}/TM6b. To test the function of mutant *His2AvD* genes these stocks were then self crossed to produce progeny with the *His2AvD* transgene in a *His2AvD*^{810} null mutant genetic background. When normal *His2AvD* function is provided by the transgene, one third of the offspring will be homozygous for the null mutation and the remaining two thirds will be heterozygous (the TM6b marker chromosome is homozygous lethal). For analysis of results, rescue by the wild type *His2AvD* transgene was given a relative value of 100%. For each *His2AvD* gene the relative degree of rescue was determined for at least three independent transgenic lines. Larvae and flies homozygous for *His2AvD*^{810} were scored by the absence of the *Tubby* and *Humeral* dominant markers, respectively, which are carried on the TM6b chromosome.
Strikingly, all of the *His2AvD* containing different H2A replacement cassettes could rescue null lethality to the pupal stage with the exception of M6 (Fig 3.14a). This non rescuing transgene contains H2A sequences that lie at the C-terminal end of the protein and not in the histone fold or in the N-terminal tail (see Fig 3.12). Specifically, transgene M6 encodes a version of His2AvD that has a cassette of H2A residues found in the C-terminal α-helix of H2A. Compared with wild type His2AvD, the M6 mutant contains 6 amino acid differences and one amino acid deletion.

In addition to the M6 domain, which is required to reach the pupal stage, three regions of *His2AvD* also appear to be important for development to adulthood (Fig 3.14b). There is a relatively mild reduction in the rescue to adulthood provided by the M1 and M4 transgenes to 57.2% and 71.7%, respectively. The development of individuals that rely on product produced from the transgene M7 is particularly compromised and on average only 9% of flies eclose. Since cassette 6 and cassette 7 are imediately adjacent to each other it can be concluded that most of the C-terminal region of His2AvD is important for its function. This does not extend to the C-terminal tail region of His2AvD as the 14 terminal amino acids can be removed with little effect on survival.

Figure 3.12: Mutagenesis of the *His2AvD* coding sequence. The amino acid sequence of *Drosophila* H2A.1 (top) and the amino acids unique to *His2AvD* (bottom) are shown. Coding sequences on the rescuing *His2AvD* fragment were mutated to encode the equivalently positioned H2A.1 amino acid residues in one of seven different regions (M1-M7). An eighth mutant transgene (CT), which encoded a version of His2AvD that lacked the 14 C-terminal amino acids, was also produced. A schematic representation of the secondary structure of H2A.1 (Luger *et al.*, 1997) is shown below the sequences.



Transgene
M1
M2
M3
M4
M5
M6
M7
CT
H2A.1
His2AvD
-SGR-GK-FFKVKGKAKSRSNRAGLQFPVGRIHRLLRKGNYAE-RVGAGAPVYLAAVMEYLTAEVLALAGNAARDNKKTRIIPPRHLQLAIRNDEELNKLLSGVTIAQGGVLPNIQAVLLPKKTEKKA--------------
AS KA DS A A V A H KSRTTSHG T A S IL SK L VK T G DS I-KA G I H HKS IG E TVQDPQRKGNVILSQAY
2° structure
αN helix
α1 helix
loop 1
α2 helix
loop 2
α3 helix
αC helix

a.

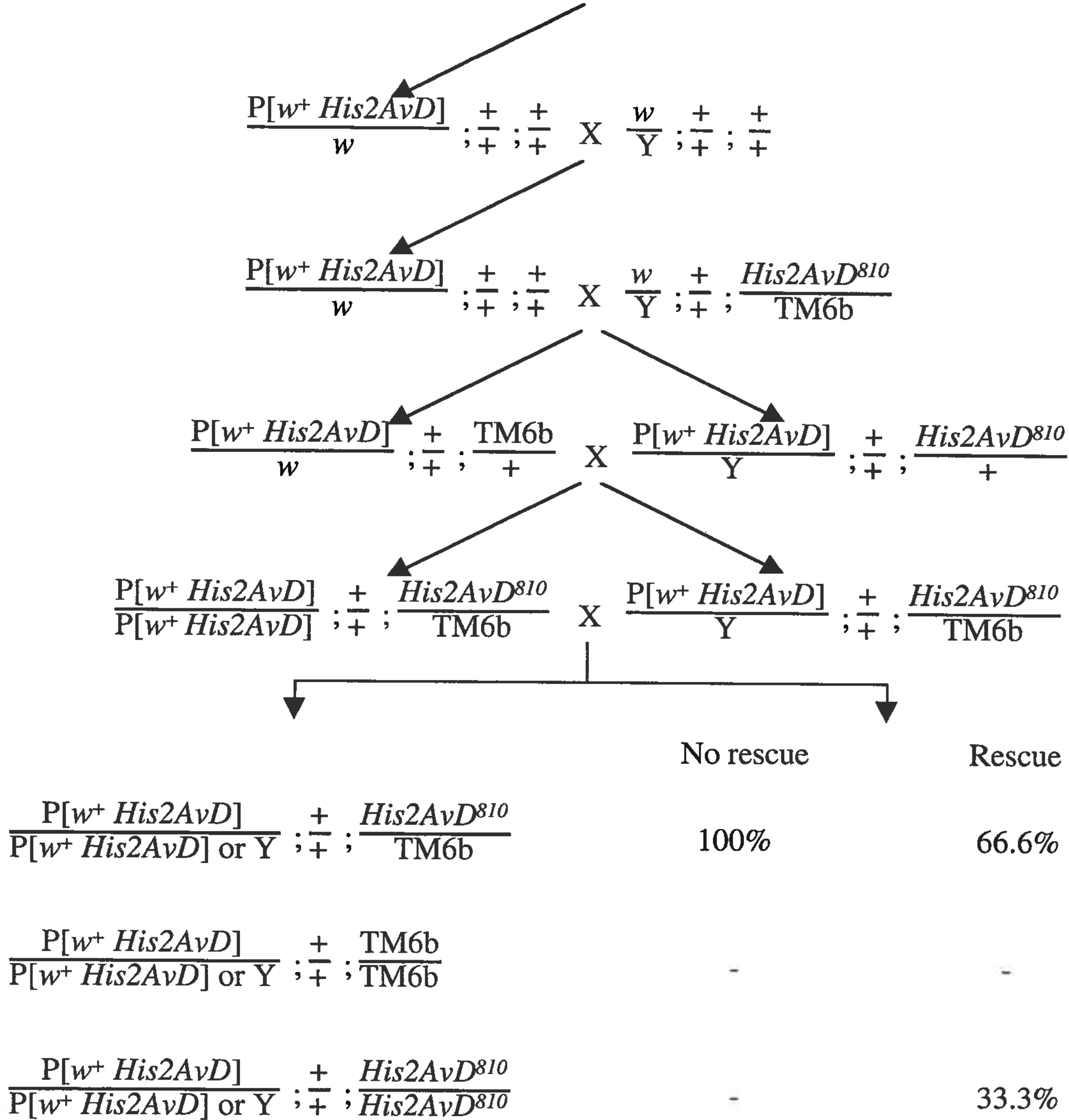
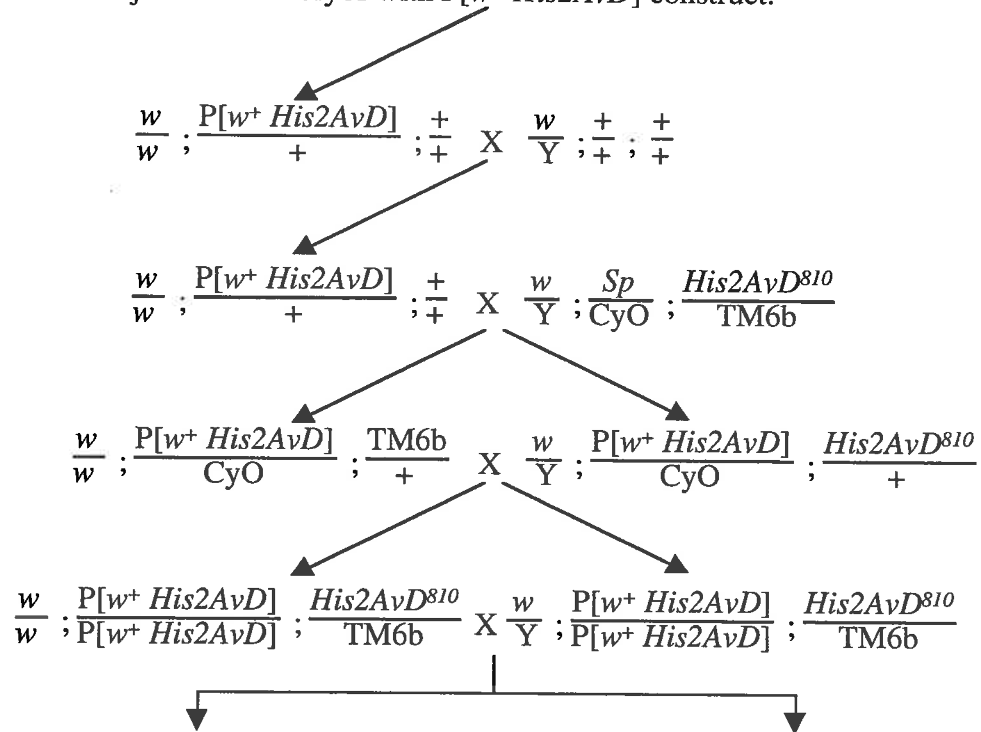


Figure 3.13: Crosses performed to investigate the degree of rescue by *His2AvD* transgenes integrated on the X (**a**) or 2nd (**b**) chromosomes. DNA was integrated into the germline chromosomes of w^{1118} *Drosophila* embryos by coinjecting, within 60min after egg deposition, *His2AvD* transgenes with a plasmid containing the P-element transposase (Δ2-3). The progeny of flies that developed from injected embryos with pigmented eyes were selected as transformants. Flies with *His2AvD* transgenes on the X or 2nd chromosome were crossed to generate stocks homozygous for the transgene in the presence of $His2AvD^{810}$/TM6b. To test the function of mutant *His2AvD* genes these stocks were then self crossed to produce progeny with the *His2AvD* transgene in a

b.

Inject w^{1118} embryos with P[w^+ *His2AvD*] construct.

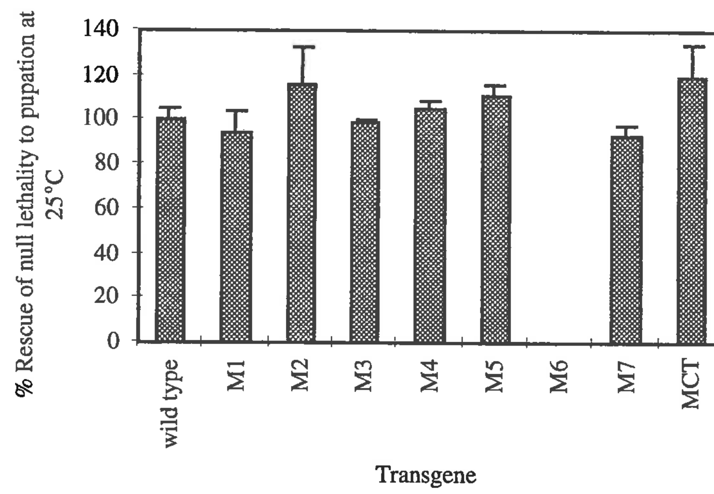


	No Rescue	Rescue
w/*w* or Y ; P[w^+ *His2AvD*]/P[w^+ *His2AvD*] ; *His2AvD*^{810}/TM6b	100%	66.6%
w/*w* or Y ; P[w^+ *His2AvD*]/P[w^+ *His2AvD*] ; TM6b/TM6b	-	-
w/*w* or Y ; P[w^+ *His2AvD*]/P[w^+ *His2AvD*] ; *His2AvD*^{810}/*His2AvD*^{810}	-	33.3%

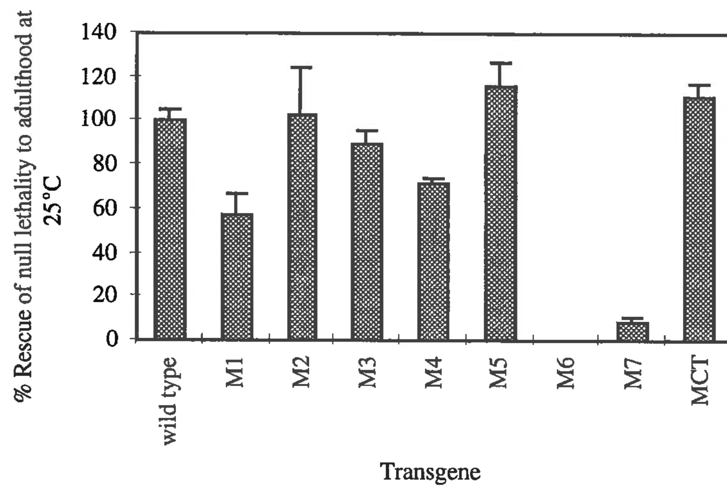
His2AvD^{810} null mutant genetic background. Since the TM6b marker chromosome is homozygous lethal, where normal *His2AvD* function is provided by the transgene, one third of the offspring will be homozygous for the null mutation and the remaining two thirds will be heterozygous. Progeny were scored as pupae and adults by the presence (*His2AvD*^{810}/TM6b) or absence (*His2AvD*^{810}/*His2AvD*^{810}) of the dominant TM6b markers *Tubby* and *Humeral* respectively.

Fig 3.14: Rescue of *His2AvD*^{810} lethality by *His2AvD* transgenes. *His2AvD* transgenes (M1-M7 and CT) were transformed into *Drosophila* and tested for their ability to rescue *His2AvD*^{810} null lethality. The relative rescue provided by each *His2AvD* transgene was determined form three independent transgenic lines normalised against wild type (100%). **a.** The percentage of homozygous *His2AvD*^{810} individuals reaching pupation. **b.** The percentage of homozygous *His2AvD*^{810} individuals reaching adulthood at 25°C. **c.** The percentage rescue of *Drosophila* reaching adulthood at 29°C. The actual level of rescue by the wild type gene, with 33% being the expected value, was 30.8±2.7, 30.6±1.8 and 23.9±3.5 for (a.-c.) respectively.

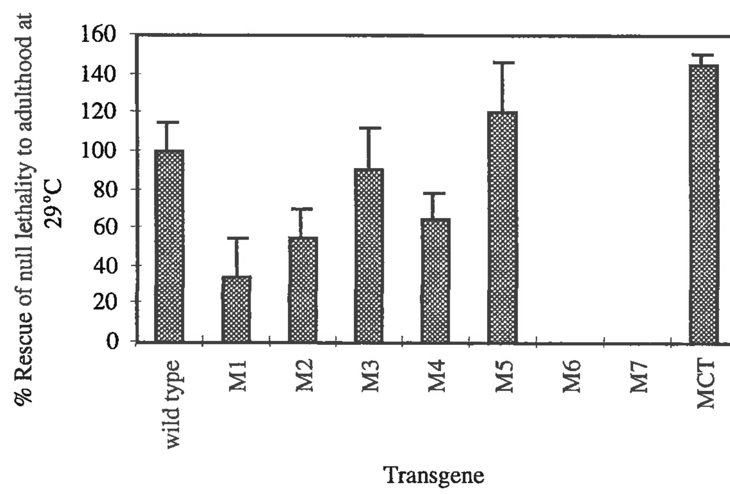
a.



b.



c.



To investigate whether the function of mutated His2AvD transgenes was temperature sensitive, rescue from null mutant lethality was tested at 29°C. At elevated temperatures the overall trend is very similar to the results obtained at non-elevated temperatures, although the rescue ability of some transgenes is affected more than others (compare Fig 3.14b with 3.14c). One marked difference is that the M7 transgene did not rescue null lethality at all. A second difference is that the relative rescue of *His2AvD* null lethality at elevated temperatures is higher for the CT mutant which does not contain the last 14 amino acids.

3.5 The effect of *His2AvD* mutants on position effect variegation (PEV)

The effect that *His2AvD* mutants have on PEV was investigated here because previous studies have shown that the degree of PEV silencing is influenced by chromatin structure. For example, PEV is suppressed in *Drosophila* strains that lack half of the histone gene cluster (Moore *et al.*, 1983), contain mutations in the gene encoding Heterochromatin protein 1 (HP1) (Eissenberg *et al.*, 1990, 1992), have additional heterochromatin (reviewed in Spofford, 1976) or increased levels of acetylated histone H4 (Dorn *et al.*, 1986) and is enhanced by the loss of RPD3 histone deacetylase function in the eyes (De Rubertis *et al.*, 1996). Since His2AvD is enriched in regions containing actively transcribing chromatin (see section 1.4) it is possible that mutations that affect its function could modify PEV. The influence of *His2AvD* mutations on PEV was analysed in flies that were homozygous for the $His2AvD^{810}$ null mutation and contained a mutated *His2AvD* transgene in the presence of the PEV marker $white^{mottled\ 4}$ (w^{m4}). The *white* gene encodes a protein which is required for the entry of pigment precursors into ommatidia (Ewart *et al.*, 1994). In the w^{m4} line, the endogenous *white* gene has been translocated by an X-ray induced chromosomal rearrangement so that it is adjacent to a breakpoint within centromeric heterochromatin (reviewed in Reuter and Spierer, 1992; Karpen, 1994). At this location, repressive chromatin structures encroach on the *white* gene and silence it in a cell specific manner to produce an eye with mottled pigmentation.

3.5.1 Design of the transformation vector, pONIX, for assessing the effect of *His2AvD* mutants on PEV

The vector pONIX was designed to transform mutant *His2AvD* genes into *Drosophila* to test for both rescue of null mutant lethality and modification of PEV. pONIX was produced by modifying the *Drosophila* transformation vector pCaSpeR4. The pCaSpeR series of (Pirrotta, 1988) contain sequences necessary for both recombinant

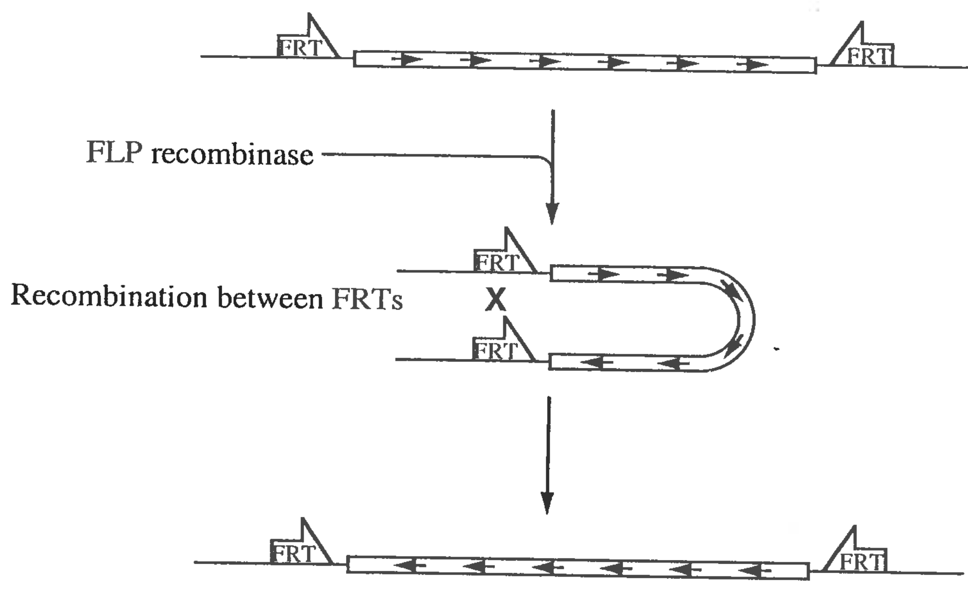
DNA manipulation in *E.coli* and P-element mediated transformation into *Drosophila melanogaster*. Sequences required for plasmid replication and ampicillin selection in bacteria were derived from the pUC vector. The *Drosophila melanogaster* transformation component consists of a polylinker and a *mini-white* gene flanked by two P-element inverted terminal repeats.

In the pCaSpeR series of vectors, the *mini-white* gene has been included between P-element ends to provide a phenotypic marker for the identification of transformant lines. Injected embryos and flies used in the cross for transformant selection are mutant in the endogenous *white* gene (the w^{1118} allele) and therefore have unpigmented (white) eyes. Flies that express the *mini-white* marker in this genetic background have eye pigmentation that varies from yellow to bright red depending on the site of transgene insertion.

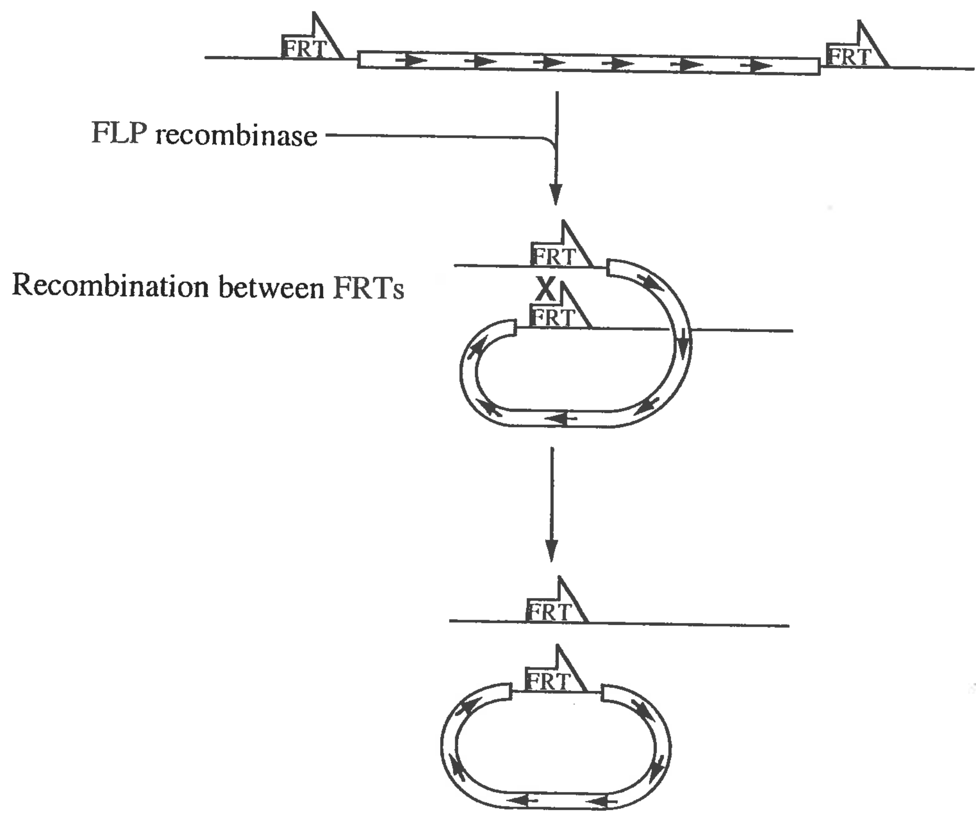
pCaSpeR4 was modified so that it was suitable for examining whether the mutant versions of His2AvD influenced PEV. The w^{m4} allele of *white* is widely used for identifying modifiers of PEV because it can be easily scored by visual examination of eye pigmentation. The caveat with use of w^{m4} for this investigation is that a *mini-white* gene was also used as a marker for transgenesis and, as mentioned above, pigment production in independent transgenic lines is highly variable. To overcome this problem, the vector pONIX was designed to allow the *white* transgene marker to be removed after transformation, as required, using the FLP recombinase system derived from the yeast 2μ plasmid (Golic and Lindquist, 1989). In this system the FLP protein catalyses a recombination reaction between two FRT (FLP recombinase target) sites. FLP protein catalysed intramolecular recombination reactions can occur between inverted or direct repeat FRT sites to invert or excise the intervening DNA sequences respectively (Jayaram *et al.*, 1983 and Fig 3.15). The excision reaction leaves an FRT at the site of recombination and an FRT on a circle containing the intervening sequences (Fig 3.15). pCaSpeR4 was modified to produce pONIX by cloning two FRT sites as direct repeats either side of the *mini-white* gene (section 3.2.4 and Fig 3.1). This allows the *mini-white* to be excised, as required, from developing germ cells by supplying them with FLP recombinase. In this case, FLP recombinase was produced from a heat shock-induced transgene (section 3.2.6 and Fig 3.3). Fly lines produced from germ cells after the action of FLP recombinase then contain the integrated transgene without the *mini-white* marker and are suitable for analysis of PEV in a w^{m4} genetic background.

Figure 3.15: FLP catalysed intramolecular recombination events. **a.** When FRT sites are present as inverted repeats, FLP recombinase directs the inversion of the intervening sequence. **b.** FLP catalysed recombination between two direct repeat FRT sites results in excision of the intervening sequence. The reverse reaction, involving intermolecular recombination has also been demonstrated.

a.



b.



3.5.2 Analysis of the w^{m4} phenotype in flies containing mutant *His2AvD* transgenes

To examine whether non-essential mutant versions of *His2AvD* modified PEV in a w^{m4} genetic background the FRT flanked *mini-white* transgene marker was first removed from integrated *His2AvD* P-element constructs using FLP recombinase. For this step, fly lines containing the integrated mutant transgenes in a $His2AvD^{810}$/TM6b genetic background were crossed to lines containing an integrated *hsFLP* transgene and allowed to lay in vials overnight. Vials were cleared, incubated at 25°C until the appearance of wandering third instar larvae and then partially submersed in water at 37°C for 1h to induce larval expression of FLP recombinase. After heat shock, vials were returned to 25°C to allow the developing *Drosophila* to mature to adulthood. Flies derived from the heat shocked larvae were then backcrossed to the $His2AvD^{810}$/TM6b line to remove the *hsFLP* transgene. Progeny derived from this cross with unpigmented eyes were selected for usein the PEV assay. These flies were then crossed individually with lines containing the w^{m4} mutation in a $His2AvD^{810}$/TM6b genetic background to investigate the effect of PEV modification by different His2AvD mutant transgenes (crosses outlined in Fig 3.16).

Flies produced from the cross to investigate the effect of *His2AvD* transgenes on PEV all had the same intensity of pigmentation in the eye. Unexpectedly, in all cases the eye pigmentation was also homogeneous (Fig 3.17). This phenotype was observed in all lines of flies containing both mutant and wild type *His2AvD* transgenes in a $His2AvD^{810}$ null mutant background but not in siblings heterozygous for the $His2AvD^{810}$ null mutation. The suggestion from these results is that there could be a recessive mutation on the $His2AvD^{810}$ chromosome which is responsible for abolishing or masking PEV. This phenomena has not been previously reported. Since the $His2AvD^{810}$ chromosome used in this case was created by recombining markers from a third chromosome carrying the markers; *scarlet* (*st*), *stubbloid* (*sbd*), *ebony sooty* (e^S), *rough* (*ro*) and *claret* (*ca*) onto the original ems mutagenised $His2AvD^{810}$ chromosome (section 3.3, Fig 3.9) it seemed possible that this recessive mutation might also be found in *st, sbd*, e^S, *ro*, *ca* stocks. Unfortunately, these lines were lost following a thermostat breakdown in the stock housing area. However, lines with the *ru, st*, e^S, *ca* chromosome from which they were generated (Richard Tearle, pers comm.) were available. As a first step in characterising this phenomena the *ru, st*, e^S, *ca* stocks were crossed to lines containing the w^{m4} allele in a $His2AvD^{810}$/TM6b genetic background. The abolishion of PEV was also observed in w^{m4} ; $His2AvD^{810}$/*ru, st* ,e^S,*ca* progeny of this cross but *st* and ca^4; $His2AvD^{810}$/TM6b and w^{m4} ; *ru, st*, e^S, *ca*/TM6b siblings had mottled eyes.

a.

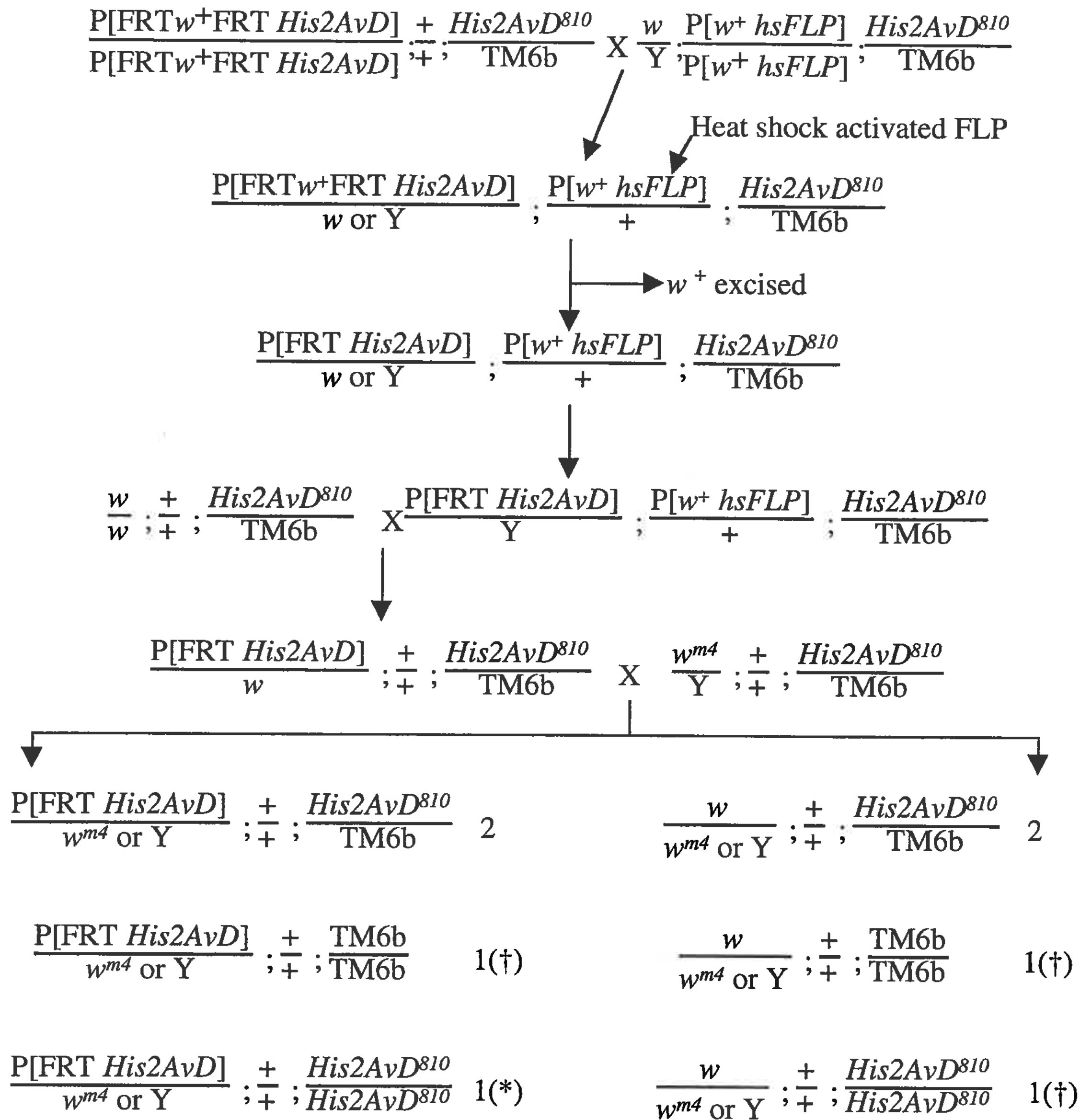
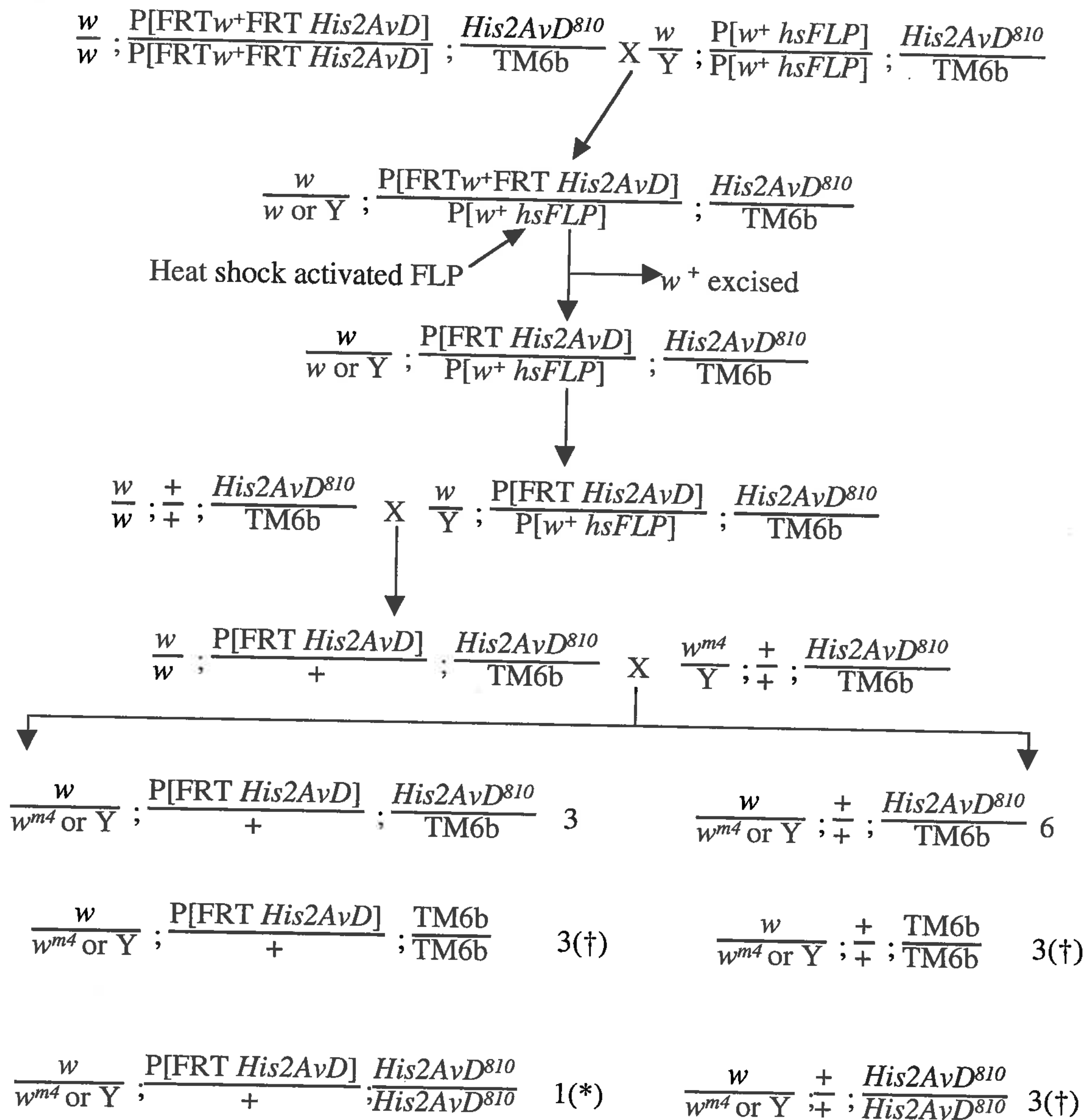


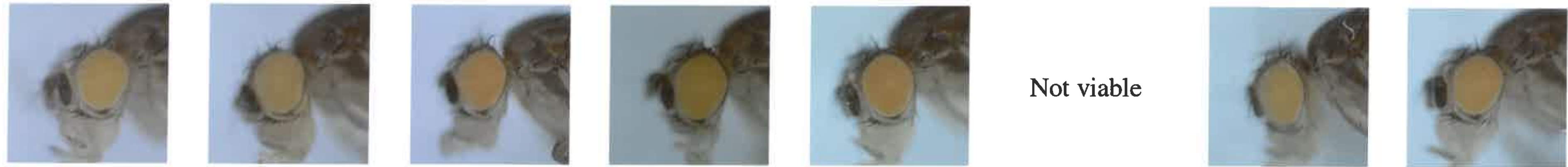
Figure 3.16: Crosses conducted to investigate the effect of *His2AvD* transgenes on PEV modification. Transgenes integrated on the X (**a**) or 2nd (**b**) chromosomes in a *His2AvD*^{810}/TM6b genetic background were crossed with flies homozygous for an integrated *hsFLP* construct, also in a *His2AvD*^{810}/TM6b genetic background. The resultant progeny were heat shocked during third larval instar to induce expression of FLP recombinase to excise the w^+ transgene marker from the integrated *His2AvD* construct in developing germ cells. Adult flies that developed after heat shock

b.



treatment were backcrossed to *His2AvD*^{810}/TM6b lines to remove the *hsFLP* transgene. Progeny arising from this cross with unpigmented eyes were crossed to flies containing w^{m4} in a *His2AvD*^{810}/TM6b genetic background to investigate the effect of *His2AvD* transgenes on modification of PEV (*). Numbers to the right of progeny produced from analytical crosses indicate the ratio that is produced by gamete combination. Lethality prior to adulthood is marked (†).

Figure 3.17: The effect of *His2AvD* transgenes on PEV. *His2AvD* transgenes were tested for modification of PEV by examining their influence on the mottled eye phenotype produced by the w^{m4} allele in a $His2AvD^{810}$ null mutant background (see section 3.5.2 of text and Fig 3.16). Eye pigmentation was the same in lines which contained either wild type or mutant transgenes. In all cases, eye pigmentation was homogeneous and did not exhibit the familiar mottling pattern that is associated with the w^{m4} allele. Photographs of female individuals were taken 1 day after eclosure. The M6 mutant was not included in this investigation because it was not capable of rescuing *His2AvD* null lethality to adulthood.



Transgene M1 M2 M3 M4 M5 M6 M7 CT

```
H2A.1    -SGR-GK-FFKVKGKAKSRSNRAGLQFPVGRIHRLLRKGNYAE-RVGAGAPVYLAAVMEYLTAEVLALAGNAARDNKKTRIIPPRHLQLAIRNDEELNKLLSGVTIAQGGVLPNIQAVLLPKKTEKKA--------------
His2AvD  AS KA   DS   A A  V   A            H KSRTTSHG    T A  S  IL                 SK L VK   T            G     DS I-KA    G   I H HKS IG  E TVQDPQRKGNVILSQAY
```

2° structure

αN helix α1 helix loop 1 α2 helix loop 2 α3 helix αC helix

Wild type transgene

Both the *His2AvD*^{810} and *ru, st,* e^S, *ca* chromosomes contain the markers *st* and *ca*. These markers are non functional alleles of genes which, like the *white* gene, are involved in the production of eye pigment (Lindsley and Zimm, 1992). The possibility that the *st* and *ca* alleles reduce pigment production in the eye to levels that obscure w^{m4} mottling is currently being investigated.

To conduct the intended analysis of PEV modification by mutant forms of His2AvD, the *His2AvD*^{810} mutation is currently being recombined onto a wild type third chromosome. This procedure is similar to the process used to generate the *st, stb,* e^S, *His2AvD*^{810}, *ca* lines (section 3.3, Fig 3.9) except that recombinants are selected on the basis of marker loss rather than gain.

3.6 Summary and discussion

3.6.1 His2AvD is required for gene activation

Prior to the commencement of this project the *His2AvD*^{810} null mutant had been identified in *Drosophila melanogaster* (van Daal and Elgin, 1992). This, in conjunction with amenable genetics and relatively easy transformation procedures made *Drosophila* the most suitable system for investigating the function of *His2AvD*.

Since the phase of lethality often provides some indication of the function of a gene the progress of *His2AvD*^{810} null mutants was characterised. Homozygous *His2AvD*^{810} null mutant progeny, derived from heterozygous null mutant parents, are phenotypically normal during the developmental stages of embryogenesis, first larval instar and second larval instar but they undergo a protracted third instar and die without entering pupation. Analysis of developmentally regulated gene expression and behaviour in *His2AvD*^{810} null mutants indicates that development in these individuals does not progress beyond early third instar. This arrest occurs just prior to an ecdysone pulse that triggers the production of "early" puffs on polytene chromosomes and induction of the *Broad Complex* (*BR-C*), *E74* and *E75* genes. These genes encode transcription factors that direct the production of other "secondary response" transcription factors that are required for metamorphosis (reviewed in Thummel, 1996). Since it has been shown in other organisms that H2A.F/Z histones are associated with actively transcribing chromatin (see section 1.4), the demonstration here that *His2AvD*^{810} null mutant development arrests at a stage when there are substantial changes in gene expression suggests that His2AvD could be required for expression of developmentally regulated genes. As His2AvD message loaded into the oocyte during embryogenesis (van Daal

and Elgin, 1992), it seems likely that this arrest is probably due to a depletion of protein to subcritical levels at this stage rather than lack of a prior requirement.

Expression of the developmentally regulated gene, *Sgs4*, is disrupted in *His2AvD*^{810} null mutants by 40h after the second larval moult, however, *5C actin* continues to be expressed and heat shock genes can still be induced after this time. This result, in conjunction with a previous immunolocalisation study which detected His2AvD on polytene chromosomes at developmentally activated puffs but not at puffs induced by heat shock (Donahue *et al.* 1986), supports the possibility that His2AvD is necessary for the expression of some, but not all, genes. However, *5C actin* and heat shock genes are activated or primed for activation in response to environmental stress, respectively, during embryogenesis when His2AvD is abundant in null mutants (Graziosi, *et al*, 1980; Fryberg, *et al*, 1983; Vogareaux and Tobin, 1987; Andres, *et al*, 1993). Thus, His2AvD could be involved in establishing the transcriptional competence of *5C actin* and heat shock genes before levels of His2AvD become depleted to critical levels during third larval instar. If this were the case, then the lack of His2AvD on heat shock activated puffs would suggest that His2AvD is required for the establishment of transcriptional competence but not for transcription *per se*.

Characterisation of H2A.F/Z and the heat shock response may explain why His2AvD is incorporated into developmentally activated puffs but not heat shock puffs. Two pieces of experimental data have lead to the proposal that H2A.F/Z replaces core histone H2A lost from chromatin as part of normal metabolism. Firstly, expression studies with synchronised human and chicken tissue culture cells have shown that H2A.F/Z is synthesised throughout the cell cycle (see section 1.4). Secondly, H2A.F/Z histones are present at higher concentrations in chromatin fractions containing transcribed sequences (see section 1.4), where the rate of histone turnover is higher than in bulk chromatin (Jackson, 1990). Since His2AvD is an intron containing gene, and the synthesis of proteins encoded by intron containing genes is inhibited during heat shock (Yost, *et al*, 1990 and refs therein) it is therefore possible that His2AvD cannot replace H2A on heat shock puffs because it is not produced during heat shock.

3.6.2 His2AvD differs from the core H2A in a function provided by its C-terminal region

The demonstration that null mutant lethality in *Drosophila* can be rescued with a 4.1kb genomic fragment containing the His2AvD gene (van Daal and Elgin 1992) created a unique opportunity to characterise the functional domains of His2AvD. The domains of

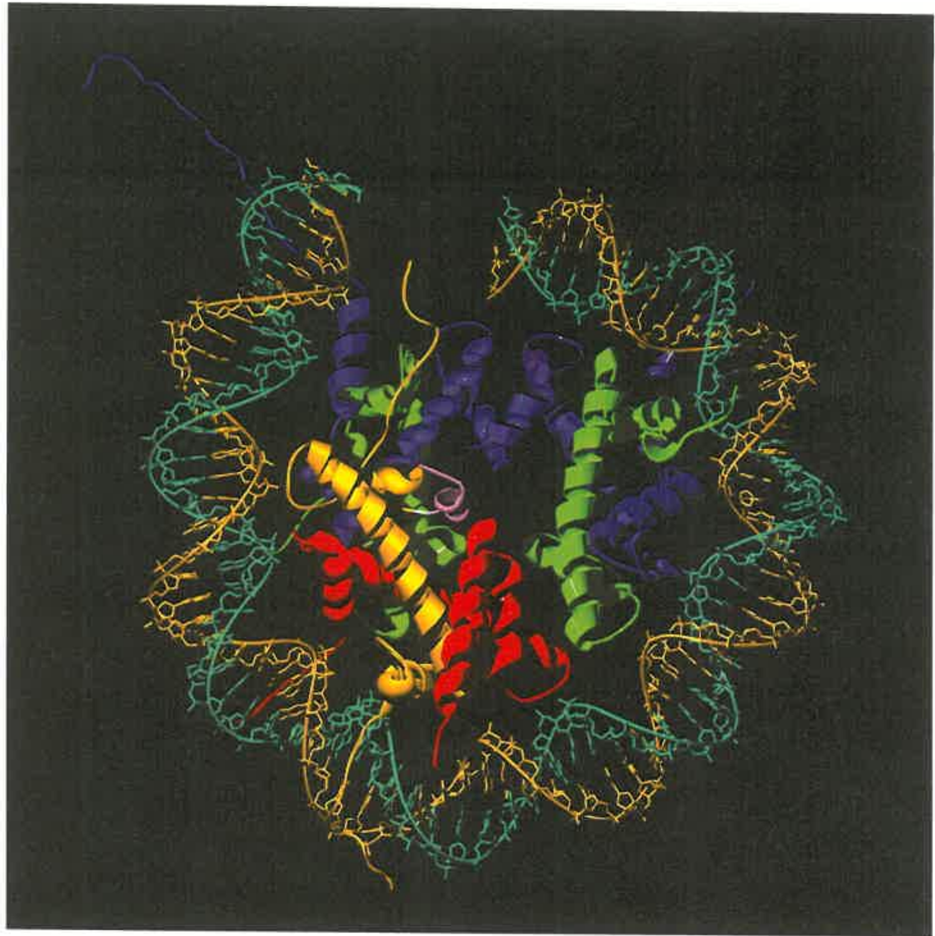
His2AvD that distinguish its function from its core histone counterpart, H2A were identified here by mutating regions or "cassettes" encoding amino acids in the His2AvD rescue fragment to encode the equivalently positioned H2A.1 residues (M1-M7 in Fig 3.12). An additional mutant was also produced (Q127->stop) to examine the functional contribution of the carboxy-terminal 14 amino acids which are only found in the *Drosophila* H2A.F/Z homologue (CT in Fig 3.12). P-element mediated transformation of *Drosophila* was used to generate lines of flies containing stably integrated wild type and mutant *His2AvD* transgenes. These transgenes were then tested for their ability to overcome *His2AvD*^{810} null developmental arrest and lethality. All of the *His2AvD* transgenes containing different H2A replacement cassettes could rescue *His2AvD*^{810} null lethality up to pupation with the exception of M6. This non-rescuing transgene contains H2A sequences that lie at the C-terminus of the protein, not in the histone fold region. The remaining His2AvD transgenes permit survival to adulthood, however the M7 mutant is severely compromised in its ability to do so.

3.6.3 The essential region of His2AvD may influence transcription through specific dimer-tetramer interactions

Results obtained here from the analysis of gene expression in *Drosophila His2AvD* null mutants are consistent with studies in other organisms which have demonstrated that H2A.F/Z and transcriptionally active chromatin have a similar spatial and temporal distribution (Gabrielli *et al.*, 1981; Wenkert and Allis, 1984; Allis *et al.*, 1986; Donahue *et al.*, 1986; Ridsdale and Davie, 1987; Stargell *et al.* 1993). This data supports a role for H2A.F/Z in cellular processes associated with transcriptional activity. As outlined in the introduction, His2AvD could promote the activation of transcription directly by forming chromatin structures which are less refractory to the binding of transcription factors and/or by facilitating the processivity of RNA polymerase. Alternatively, or in addition, His2AvD may promote transcription indirectly by recruiting activating factors and/or excluding inhibitory factors. To determine which of these aspects of transcription His2AvD would be able to influence, the location of the M6 and M7 regions was examined in the X-ray crystal structure of the nucleosome (Fig 3.18 and Fig 3.19). In H2A containing nucleosomes, both of these regions contribute to intranucleosomal interactions at the dimer-tetramer interface as part of a larger domain called the docking domain. To further characterise the specific contribution of amino acids in the M6 and M7 domains to nucleosome structure and function, histone residues in close proximity to these regions were identified.

Figure 3.18: a. The location of the M6 domain of H2A in the nucleosome. Ribbon trace diagram of DNA (brown and turquoise) and histone proteins (H3: blue; H4: green; H2A: yellow; H2B: red) in the nucleosome viewed down the superhelical axis with the dyad region at the top of the image. For clarity, the H2A-H2B dimer on the back face of the nucleosome and one superhelical turn of DNA have been omitted. The M6 region is shown here in pink. In this view it is apparent that the M6 region of H2A is both exposed to solvent and buried within the nucleosome core. **b.** Proximity of the M6 domain of H2A to H4 Y98 (rendered as space filling molecule) visualised from the same point as (a.).

a.



b.

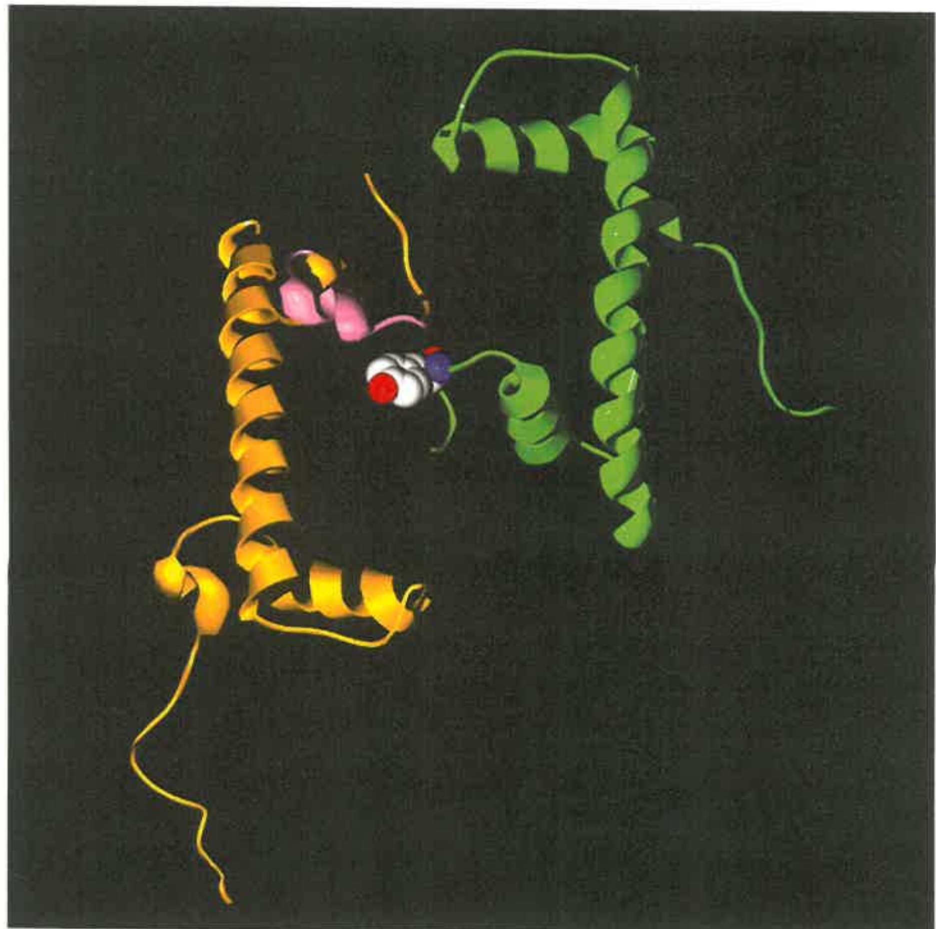
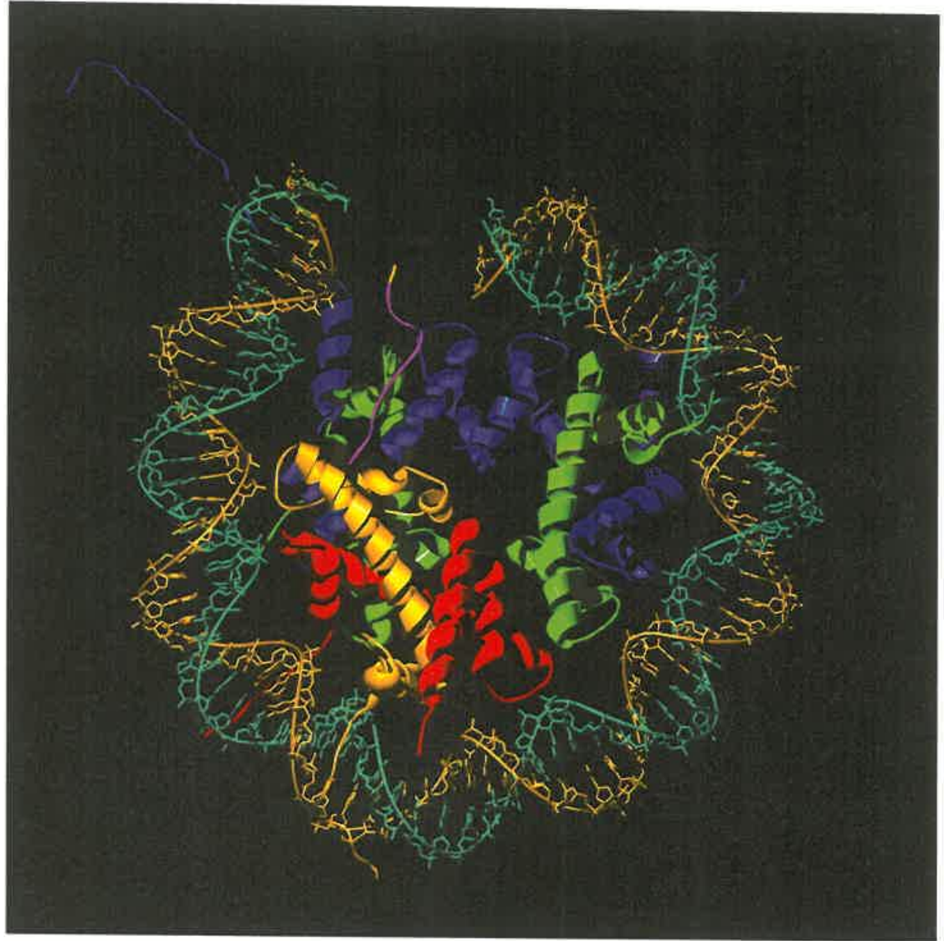
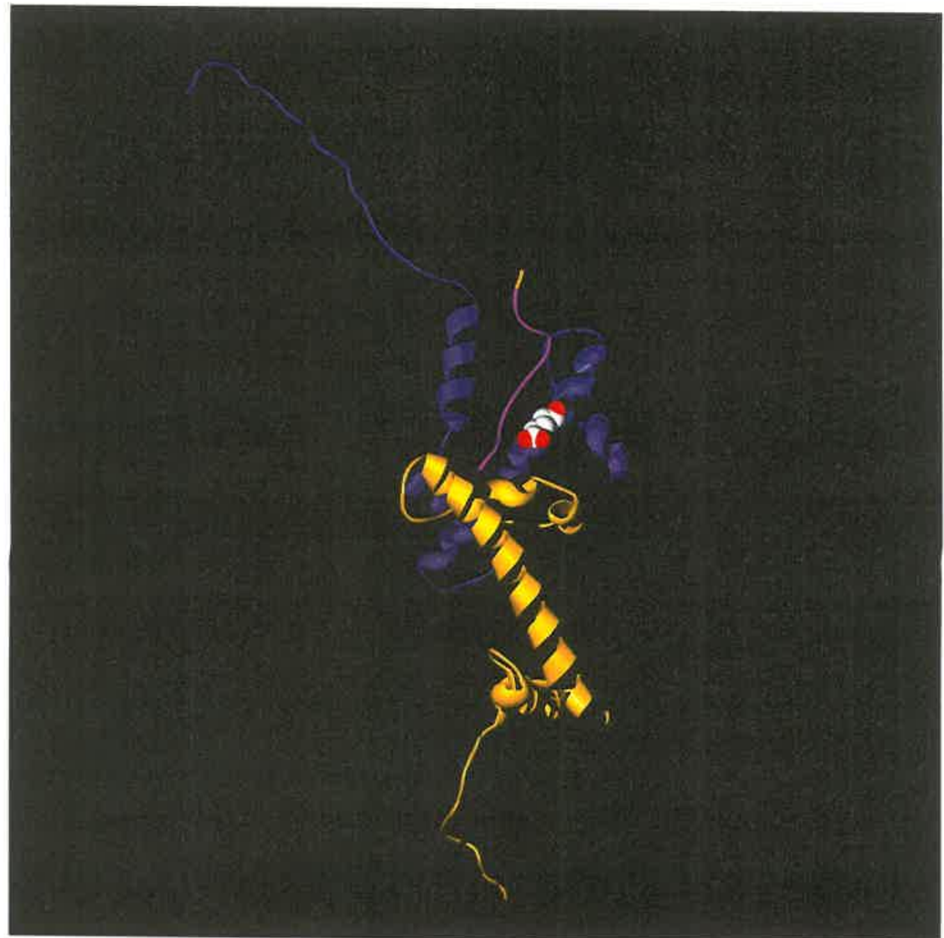


Figure 3.19: **a.** The location of the M7 region (cyan) of H2A in the nucleosome (complementary DNA phosphodiester backbones: brown and turquoise; H3: blue; H4: green; H2A: yellow and H2B: red). This extended coil region lies between, and slightly out of the plane of, the H3 αN and α2 helices. **b.** Proximity of the M7 domain of H2A to H3 E105 (rendered as space filling molecule) visualised from the right of the image in (a.).

a.



b.



The only non-H2A residue within 3.3Å of the non-rescuing M6 domain is histone H4 Y98 (Fig 3.18). The function of histone H4 Y98 has been examined in a yeast study of three tyrosines in H4 that are involved in dimer-tetramer interactions (Santisteban *et al.*, 1997). In the nucleosome, H4 Y98 interacts with one H2A-H2B dimer and the Y72 and Y88 residues interact with the other H2A-H2B dimer. The tyrosil ring of Y98 is inserted into a cleft in the dimer surface while Y72 and Y88 contribute to a large hydrophobic domain in conjunction with Y83 from histone H2B. Mutation of the histone H4 Y98 residue is lethal when substituted with G, is temperature sensitive lethal when substituted with H, and has no phenotype when mutated to a W. Mutation of two other H4 tyrosines involved in dimer-tetramer interactions at positions 72 and 88 to G was not lethal but cells with these versions of H4 grew slower than wild type cells. Histone H4 Y72-G, Y88-G and Y98-H mutants also have partial Sin phenotypes in that they are able to suppress the effect of a deletion mutation in the *snf2* subunit of the SWI chromatin remodelling complex on the transcription of *INO1*, but not *SUC2*, under derepressing conditions. Interestingly, it has been shown that similar phenotypes to the H4 Y72-G, Y88-G mutants (expression of *INO1* but not *SUC2*) can be generated by mutating the H2B tyrosine (Y83-G) that interacts with these residues in histone H4 (Recht and Osley, 1999). Thus, particular interactions at the dimer-tetramer interface on the surface of either the dimer or the tetramer can have similar effects on transcription. These results highlight the importance of interactions involving H4 Y98 for survival and support the possibility that His2AvD might alter the function of the nucleosome by modifying dimer-tetramer interactions that are directed through the Y98 residue.

Unfortunately, the mechanism by which incorporation of the Y98-H version of H4 partially overcomes the requirement for the SWI chromatin remodelling complex has not been identified. The only structural analysis conducted, to date, on chromatin from cells containing H4 Y98-H found that the MNase digestion profile was the same as that produced by digestion of chromatin isolated from cells containing wild type H4 (Santisteban *et al.*, 1997). Clearly however, this mutation must alter interactions between the dimer and tetramer in the nucleosome. An additional important feature of interactions occurring between this region of H4 and the M6 region of H2A is that they direct a dimer-tetramer alignment which produces a continuous helical ramp of DNA binding sites around the octamer.

The M7 domain, which is adjacent to the essential M6 region and contributes significantly to function of histone His2AvD, is in close proximity to the E105 residue of histone H3 (Fig 3.19). In yeast, substitution of E105 with K, like the H4 Y98-H

mutation, confers a Sin phenotype. The close proximity of the E105 residue of histone H3 to the M7 domain of histone H2A suggests that interactions in this region could be important for maintaining repression in the absence of SWI. The influence of E105-K and two other Sin versions of H3 (R116-H and T118-I) on nucleosome core structure and function have been analysed *in vitro* using chromatin assembled with these forms of histone H3 (Kurumizaka and Wolffe, 1997). The R116-H and T118-I versions of H3 have an increased sensitivity to MNase and DNase1 which indicates that nucleosomes containing these Sin mutants have a reduced affinity for DNA. Since no increase in MNase or DNase1 sensitivity was detected in nucleosomes containing H3 E105-K, this version of H3 must package DNA in a similar manner to nucleosomes containing wild type H3. Analysis of the helical periodicity of DNA bound to nucleosomes assembled with these Sin versions of histone H3 using hydroxy radical footprinting demonstrated that periodicity was only disrupted in nucleosomes containing H3 R116-H. None of the H3 mutants affect the supercoiling of plasmid DNA therefore they don't appear to affect the ability of nucleosomes to restrain supercoils. They also don't substantially influence the binding of transcription factors or the RNA polymerase III transcription machinery to chromatin templates. Since nucleosomes containing the E105-K version of H3 do not exhibit any distinguishing characteristics it is difficult to use information derived from analysis of the H3 E105-K mutant to speculate how the M7 region might distinguish the functions of H2A and His2AvD. Interestingly, however, the M7 domain also interacts with the N-terminal helix of H3. This helix interacts with, and directs the path of, DNA as it enters and leaves the nucleosome. This raises the possibility that differences between H2A and His2AvD in the M7 region could alter the trajectory of DNA at the periphery of the nucleosome. Interactions between the M7 domain of H2A and the N-terminus of H3 also form part of the docking domain that stabilises the dimer-tetramer interface. While these observations are consistent with a role for the M7 region of His2AvD in complementing the function of the essential M6 region there are other reasons why this mutation might disrupt viability. These considerations are presented later, in conjunction with results obtained in chapter 6.

Data obtained here from mapping the location of the functional sites of His2AvD suggests that this histone variant might differ from H2A in the way that it participates in the interactions occuring at the dimer-tetramer interface or it could alter the trajectory of DNA in nucleosomes. Analysis of histone mutants in the vicinity of the M6 and M7 domains has shown that alterations in this region of nucleosomes can have a profound effect on processes that are influenced by chromatin structure. This characterisation of histone mutants did not, however, identify how these changes might direct their effect. In other studies it has been demonstrated that this region of the nucleosome is involved

a number of aspects of chromatin structure and function. The implications of these studies in relation to the possible function of His2AvD will be considered below.

3.6.4 His2AvD containing nucleosomes could promote transcription by destabilising the dimer-tetramer interface

Interactions at the dimer-tetramer interface have the potential to influence transcription, histone metabolism and the formation of higher order chromatin structures. For example, it has been demonstrated that histones H2A and H2B are turned over much more rapidly than histones H3 or H4 in both proliferating and non proliferating cells (Djondjurov *et al.*, 1983; Grove and Zweidler, 1984; Louters and Chalkley, 1985; Tsvetkov *et al.*, 1989; Wunsch and Lough, 1989; Jackson, 1990). In addition, nucleosomes lacking a H2A-H2B dimer have a higher affinity for RNA polymerase II (Baer and Rhodes, 1983), are less refractory to the binding of transcription factors (Tremethick *et al.*, 1990; Hayes and Wolffe, 1992) and are less able to assemble into higher order chromatin structures (Hansen and Wolffe, 1994) than nucleosomes with a full complement of histones (see also Chen *et al.*, 1994; Walter *et al.*, 1995). Promotion of nucleosome displacement and transcription factor binding by nucleoplasmin and NAP1 also requires disruption of the dimer-tetramer interface (Walter *et al.*, 1995). Since, residues in the M6 and M7 regions are involved in dimer-tetramer interactions, then an attractive hypothesis for the mechanism of His2AvD function is that it could promote transcription by destabilising the dimer-tetramer interface.

3.6.5 His2AvD containing nucleosomes may adopt, or stabilise, a nucleosome conformation that facilitates transcription.

Information derived from the X-ray crystal structure of H2A containing nucleosomes suggests that interactions between the H2A M6 region and the short C- terminal tail of H4 stabilises a dimer-tetramer alignment so that there is a continuous helical ramp for DNA to follow around the octamer (Luger *et al.* 1997). Since this region distinguishes the function of H2A from His2AvD histones it is possible to speculate that nucleosomes containing His2AvD could adopt, or stabilise, a particular dimer-tetramer alignment that promotes transcription. The M7 domain is also located where it could influence the conformation of the nucleosome. As outlined in section 3.6.3, this domain interacts with the N-terminal helix of H3 which, in turn, interacts with DNA as it enters and exits the nucleosome.

A study that examined the stability of acetylated histones and histone variants in chromatin on isolated from chicken erythrocytes is also worth considering in relation to the conformation that His2AvD nucleosomes may adopt. This study found that the chicken His2AvD homologue, H2A.F, dissociates from isolated chicken chromatin at 0.9M NaCl whereas H2A dissociates at < 0.7M NaCl (Li *et al.*, 1993). From this result it is possible to speculate that His2AvD/H2B dimers could be more stable than H2A/H2B dimers in a nucleosome conformation that occurs as an intermediate during disassembly (an alternate explanation for the increased stability of H2A.F/Z histones is discussed below in section 3.6.6).

By adopting a different conformation to H2A containing nucleosomes, His2AvD containing nucleosomes could promote transcription through increased affinity for transcriptional activators or decreased affinity for inhibitors of transcription. Alternatively, or in addition, His2AvD containing nucleosomes could be a better substrate for enzymes that promote transcription by modifying chromatin structure. The possibility that incorporation of H2A.Z into chromatin could influence the activity of chromatin remodellers is supported by a recent demonstration that remodellers are influenced by at least one change in the composition of chromatin, namely acetylation. Remodelling of nucleosome arrays by SWI/SNF and RSC is diminished, to similar extents, on arrays which contain acetylated histones (Logi *et al.*, 1999). While this result suggests that SWI/SNF and RSC remodel acetylated nucleosome arrays less effectively than unacetylated nucleosome arrays, further examination of this phenomena has shown that this is not necessarily the case. Over time, the activity of RSC diminishes faster in the presence of arrays containing unacetylated histones whereas SWI/SNF function is protected against time-dependent loss of activity by incubation with acetylated nucleosome arrays. Therefore, SWI/SNF remodelling activity is inhibited by acetylation, and RSC activity is decreased on arrays containing acetylated histones because it is inactivated to a greater extent by this substrate than by arrays containing unacetylated histones (Logi *et al.*, 1999). Thus, incorporation of His2AvD into chromatin could promote the activity of chromatin remodellers either directly, by being a better substrate than H2A containing nucleosomes or indirectly, by protecting the remodeller from inactivation.

3.6.6 Histone H2A.Z has the potential to alter internucleosomal interactions.

In nucleosome crystals, basic residues in the H4 amino terminal tail (K16 to N25) make multiple ionic and hydrogen bonds with a patch of acidic residues on the surface of the H2A-H2B dimer (derived from H2A residues E56, E61, E64, D90, E91, E92 and H2B

E110) in the adjacent nucleosome core particle (Luger *et al.*, 1997). In His2AvD containing nucleosomes, this acid patch is extended extended slightly by replacement of the H2A residues N93 and K95 with D and S respectively. Increased acidity in this region might therefore increase its affinity for the basic H4 amino-terminal tail. Thus, if the dimer-tetramer interface was disrupted in nucleosomes containing His2AvD, a more stable interaction between the N-terminal tail of H4 and the acid patch could be important for stabilising the association of His2AvD with chromatin. Indeed, this might explain why H2A is displaced from isolated chicken chromatin at < 0.7M NaCl while H2A.F dissociates at 0.9M NaCl, co-incident with H4 (Li *et al.*, 1993).

Although the interaction between N-terminal tail of H4 and the acid patch on adjacent nucleosomes was detected in X ray crystals, it has been proposed that this interaction may also occur in chromatin (Luger *et al.*, 1997). This proposal arises from demonstrations that histone tails are involved in the compaction and oligomerisation of nucleosome arrays into higher order chromatin structures (Schwarz *et al.*, 1996; Moore and Ausio, 1997; Tse and Hansen, 1997). Conditions that favor compaction inhibit transcription by RNA polymerase III on chromatin templates (Hansen and Wolffe, 1992). These results suggest that a more extensive acid patch on H2A.F/Z containing nucleosomes might favor chromatin compaction and have an inhibitory affect on transcription. This might not be the case, however, if the acid patch increased the affinity of His2AvD containing nucleosomes for proteins or complexes involved in the activation of transcription. As well as doing this directly, it could produce this effect, indirectly, by changing the ability of the H4 tail of adjacent nucleosomes to interact with regulators of transcription. Interactions between the H4 tail and both positive and negative transcriptional regulators have been characterised. *In vitro* binding studies have demonstrated that the H4 N-terminus interacts with the bromodomain of transcription co-factor and histone acetylase, Gcn5 (Ornaghi *et al.*, 1999). Bromodomains are also found in the SWI/SNF and RSC chromatin remodelling subunits in yeast humans and *Drosophila* (see Kingston and Narlikar, 1999). Although it is unclear whether the bromodomain is required for the function of SWI/SNF (see Bourachot *et al.*, 1999) it has been demonstrated that histone tails are required for the full activity of SWI/SNF and RSC (Logie *et al.*, 1999). SWI/SNF and RSC have an inhibited capacity to catalytically remodel, and a higher affinity for, nucleosome arrays assembled with histones predigested with trypsin to remove their amino and carboxy termini than nucleosome arrays assembled with intact histones (Logie *et al.*, 1999). This result has lead to the proposal that histone termini are involved in the transfer of SWI/SNF and RSC chromatin remodelling complexes from one nucleosome array to another (Logie *et al.*, 1999). Negative regulators of transcription that interact with the

H4 N-terminus include the yeast proteins Tup1 (Edmonson *et al.*, 1996), Sir3 and Sir4 (Hecht *et al.*, 1995). Thus, the incorporation of His2AvD into chromatin could indirectly facilitate transcription either by promoting or reducing the binding of positive or negative regulators of transcription respectively, to the N-terminus of H4. Of these two possibilities, the most obvious way that His2AvD containing nucleosomes could promote transcription is by sequestering the N-terminus of H4 so that it reduces the binding of inhibitors. However, it is also possible that His2AvD might influence the binding of both activators and repressors of transcription by stabilising the H4 N-terminus in a particular conformation.

3.6.7 Models for the promotion of transcription by His2AvD

Results obtained in this chapter support a role for His2AvD in the generation of transcriptionally competent chromatin. His2AvD provides this function through a domain at the C-terminus of the protein, not through changes in the histone fold region. Analysis of the location of this domain in the crystal structure of the nucleosome found that incorporation of His2AvD into chromatin could alter both inter- and intra-nucleosomal interactions. The part of this domain that participates in intra-nucleosomal interactions is involved in stabilising and aligning the dimer-tetramer interface so that the periodicity of DNA binding sites on the surface of the octamer is maintained. An alteration in these interactions could, therefore, promote transcription either by destabilising the dimer-tetramer interface or altering the alignment of the dimer and tetramer. The part of the essential domain of His2AvD that participates in inter-nucleosomal interactions increases the size of an acid patch on the surface of the nucleosome. In nucleosome crystals, this region binds the H4 N-terminal tail of adjacent nucleosomes. It has been postulated that interactions between the N-terminal tail of H4 and the acid patch are involved in chromatin compaction. This suggests that the incorporation of His2AvD might actually inhibit transcirption by promoting chromatin compaction. However, differences in this acid patch could also promote transcription by affecting the binding of transcriptional regulators to nucleosomes containing His2AvD or to the N-terminal tail of H4. From this data three models have been generated to explain how differences between His2AvD and H2A might alter the properties of chromatin to promote transcription.

Model 1: His2AvD could destabilise the dimer-tetramer interaction so that chromatin containing His2AvD is less refractory to the binding of transcription factors. Although this model suggests that His2AvD would be less stable than H2A in chromatin, it has been demonstrated that H2A.F/Z is actually less susceptible to displacement from

chicken chromatin than H2A (Li *et al*., 1993). However, a more extensive acid patch on the surface of nucleosomes containing His2AvD could stabilise its association with chromatin by binding the N-terminal tail of H4 molecules in adjacent nucleosomes with a higher affinity than nucleosomes containing H2A.

Model 2: His2AvD containing nucleosomes could adopt, or be more stable in, a conformation that promotes transcription. The location of the essential region of His2AvD suggests that it could change the dimer-tetramer alignment.

Model 3: A more extensive acid patch on the surface of His2AvD containing nucleosomes could influence the binding of transcriptional regulators to chromatin. This effect could be mediated either directly through the acid patch region or indirectly through specific interactions between the H4 N-terminal tail and nucleosomes containing His2AvD. By having a higher affinity for the H4 N-terminal tail than H2A containing nucleosomes, His2AvD containing nucleosomes could promote transcription by sequestering the H4 tail so that it is refractory to the binding of inhibitors of transcription like Tup1, Sir3 or Sir4. Alternatively, the acid patch on His2AvD containing nucleosomes might stabilise the binding or action of activators like the Gcn5 histone acetylase or the SWI and RSC chromatin remodellers.

Chapter 4: In *Drosophila*, His2AvD is associated with chromatin throughout the cell cycle and is imported into embryonic nuclei at the onset of transcription in nuclear cycle 9.

4.1 Introduction

This chapter reports the distribution of His2AvD protein in *Drosophila* tissues throughout the cell cycle and during development. For this investigation the rescuing *His2AvD* genomic fragment was modified to encode a fusion of the His2AvD protein and the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* (Chalfie *et al.*, 1994; Heim *et al.* 1995; Cubitt *et al.* 1995; Yeh *et al.*, 1995). After demonstrating that the fusion gene was capable of rescuing null mutant lethality, lines of flies containing the fusion gene were used to examine the localisation of His2AvD on chromosomes *in vivo* and in real time.

As discussed previously, two experimental observations support an association between the presence of histone H2A.F/Z protein and transcriptionally active chromatin. Firstly, chromatin fractions enriched in actively transcribed sequences contain higher concentrations of histone H2A.F/Z than bulk chromatin (Gabrielli *et al.*, 1981; Ridsdale and Davie, 1987). Secondly, a histone H2A.F/Z homologue in the ciliate *Tetrahymena*, *hv1*, temporally and spatially colocalises with regions of transcriptional activity in the nucleus (Wenkert and Allis, 1984; Allis *et al.*, 1986; Stargell *et al.* 1993). *Drosophila* embryogenesis also offers an opportunity to investigate whether the presence of H2A.F/Z protein in chromatin coincides with the activation of transcription. Following fertilization in *Drosophila* there are 14 rapid nuclear divisions in a syncitial blastoderm. During this time no transcription has been detected prior to nuclear cycle 8. In this chapter the distribution of His2AvD-GFP fusion protein was examined prior to, during and after the onset of transcription in embryogenesis.

Another aspect of His2AvD function that is addressed here is its distribution during the cell cycle. This is an important consideration in relation to the involvement of His2AvD protein in relation to transcription and chromosome dynamics as transcription is shut down and chromosomes undergo major structural transitions during mitosis.

4.2 Methods

4.2.1 Creation of *His2AvD-GFP*.

The coding region of the S65T mutant green fluorescent protein (GFP) (Heim *et al.*, 1995; Cubitt *et al.*, 1995) was cloned into a *Stu*I restriction enzyme site in the third last codon of *His2AvD* in pONIX.AvD to create a *His2AvD-GFP* fusion gene as detailed below and in Fig 4.1.

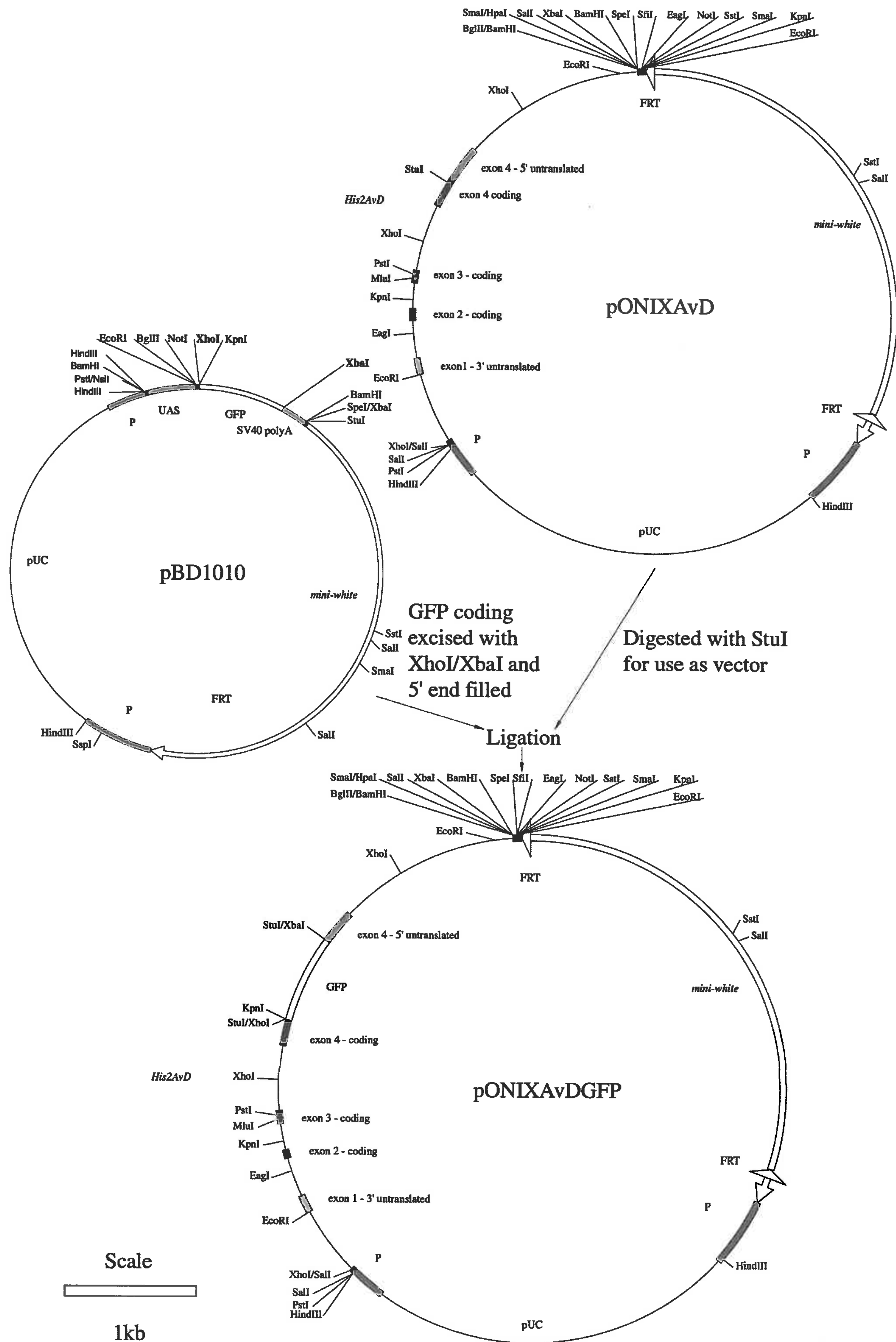
A DNA fragment containing GFP coding sequences was excised from the vector pBD1010 (a gift from by Barry Dickson) by restriction with the endonucleases *Xho*I and *Xba*I. This digest created 5' overhangs at either end of the fragment which were end filled with Klenow polymerase (2.3.8(ii)b). The blunt ended GFP coding fragment was then cloned into a *Stu*I restricted pONIX.AvD to produce pONIX.AvD-GFP. This protocol was designed so that when the GFP coding sequence was cloned into the *Stu*I site of pONIX.AvD, in the correct orientation, the translational reading frame is maintained to produce a gene that encodes a His2AvD-GFP fusion protein. The ligation process removed the last two codons of *His2AvD*, encoding Ala and Tyr, of the 141-codon His2AvD open reading frame (ORF) and introduced five additional codons between this and the 5' end of the GFP ORF. A *Kpn*I restriction endonuclease recognition site present at the 5' end of the *Xho*I/*Xba*I GFP fragment (see Fig 4.1) was used to determine the orientation of GFP. Restriction of the *His2AvD-GFP* fusion gene in pONIX with *Kpn*I produced fragments of 9.5kb, 2.5kb and 1kb whereas clones containing GFP in the opposite orientation produced a 9.5kb fragment and two 1.8kb fragments. Sequencing of a clone with GFP inserted in the correct orientation across the 5' and 3' cloning junctions with the primers M7 and 2374 (2.2.9(i)) respectively confirmed that the reading frame was maintained in pONIX.AvD-GFP.

4.2.2 Generation of transgenic *Drosophila melanogaster* containing the *His2AvD-GFP* fusion gene

The *His2AvD-GFP* fusion gene was transformed into *Drosophila melanogaster* using the same techniques outlined in section 3.2.8 for the generation of flies containing mutated *His2AvD* transgenes. The chromosome incorporating the transgene was determined using the protocol outlined in section 3.2.9 for characterisation of mutated *His2AvD* transgenes.

Figure 4.1: Production of the *His2AvD-GFP* construct. **a.** Outline of how the cloning strategy maintained the reading frame between the C-terminus of *His2AvD* and GFP coding sequences. Boldface type has been used to indicate sequences associated with the ORFs of His2AvD and GFP. **b.** Plasmid diagram illustrating the protocol used for the creation of pONIXAvD-GFP. The coding region of the S65T mutant green fluorescent protein (GFP) (Heim *et al.*, 1995; Cubitt *et al.*, 1995) was cloned into a *Stu*I restriction enzyme site in the third last codon of *His2AvD* in pONIX.AvD to create a *His2AvD-GFP* fusion gene (see section 4.2.1 of text for details).

a. C-terminus of *His2AvD* — N-terminus of GFP


pONIXAvD
SmaI/HpaI
SalI
XbaI
BamHI
SpeI
SfiI
EagI
NotI
SstI
SmaI
KpnI
BglII/BamHI
EcoRI
EcoRI
XhoI
FRT
StuI
exon 4 - 5' untranslated
exon 4 coding
His2AvD
XhoI
PstI
MluI
exon 3 - coding
KpnI
exon 2 - coding
EagI
exon1 - 3' untranslated
EcoRI
SstI
SalI
mini-white
FRT
P
XhoI/SalI
SalI
PstI
HindIII
P
HindIII
pUC
pBD1010
EcoRI
BglII
NotI
XhoI
KpnI
HindIII
BamHI
PstI/NsiI
HindIII
UAS
GFP
SV40 polyA
P
XbaI
BamHI
SpeI/XbaI
StuI
pUC
mini-white
SstI
SalI
SmaI
FRT
P
HindIII
SspI
SalI
GFP coding excised with XhoI/XbaI and 5' end filled
Digested with StuI for use as vector
Ligation
pONIXAvDGFP
SmaI/HpaI
SalI
XbaI
BamHI
SpeI
SfiI
EagI
NotI
SstI
SmaI
KpnI
BglII/BamHI
EcoRI
EcoRI
XhoI
FRT
StuI/XbaI
exon 4 - 5' untranslated
GFP
KpnI
StuI/XhoI
exon 4 - coding
His2AvD
XhoI
PstI
MluI
exon 3 - coding
KpnI
exon 2 - coding
EagI
exon 1 - 3' untranslated
EcoRI
SstI
SalI
mini-white
FRT
P
P
XhoI/SalI
SalI
PstI
HindIII
HindIII
pUC
Scale
1kb

4.3 Results

4.3.1 The *His2AvD-GFP* fusion gene is capable of rescuing *His2AvD*^{810} null mutant lethality

To determine whether the *His2AvD-GFP* fusion gene produced functional His2AvD protein, it was tested for its ability to rescue *His2AvD*^{810} null mutant lethality as a transgene. Lines of flies containing the His2AvD-GFP transgene on the second or X chromosomes were crossed to establish stocks that contained the fusion gene in a *His2AvD*^{810}/TM6b genetic background, as per section 3.4.1. These lines were then self crossed and their progeny examined for the presence of *His2AvD*^{810} homozygotes. Flies homozygous for *His2AvD*^{810} were scored by the absence of the *Humeral* dominant marker, which is carried on the TM6b chromosome. *His2AvD*^{810} homozygotes were observed, indicating that the fusion gene, like the genomic fragment from which it was derived, produced functional His2AvD protein.

4.3.2 His2AvD-GFP is nuclear and is associated with chromatin throughout the cell cycle

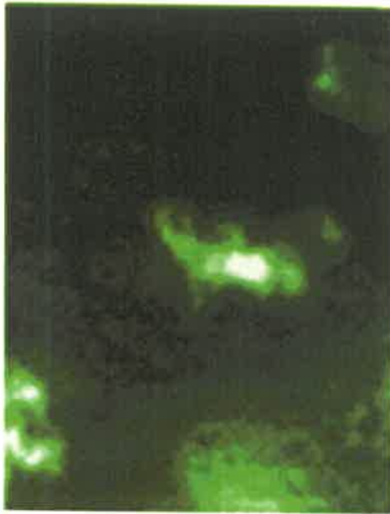
Tissues at various stages of development were examined using standard fluorescence and confocal microscopy. Strong fluorescence was detected in cycle 14 interphase nuclei of blastoderm stage embryos and on prophase, metaphase and anaphase chromosomes of cycle 14 mitoses (Fig 4.2a-f). Similarly, in living cells of the brain outer proliferative centre, His2AvD-GFP derived fluorescence was visualised on chromosomes during mitosis and within all interphase nuclei (data not shown). Nuclear and chromosomal fluorescence persisted in all tissues throughout development and adult life. On salivary gland polytene chromosomes, His2AvD-GFP fluorescence was present at a large number of discrete sites (Fig 4.3)

4.3.3 Nuclear localisation of His2AvD correlates with the start of transcription in the cleavage embryo

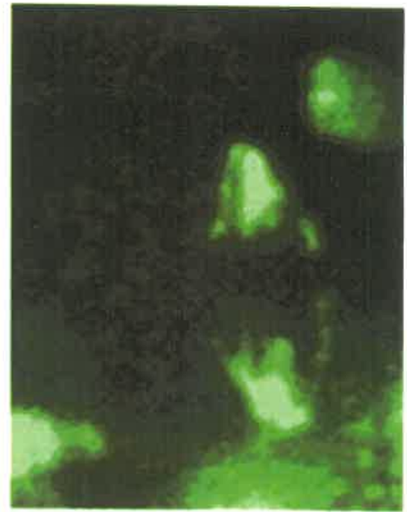
To investigate the association of His2AvD with transcriptional activity, the location of His2AvD-GFP fusion protein was analysed before and during the activation of transcription in the early embryo. To date, the first zygotic transcripts that have been detected are that of the *snail* and *sis A* genes during the prophase of nuclear cycle 8 (Pritchard and Schubiger, 1996). Transcription is activated gradually in the early embryo with specific mRNAs initially being detected only in some nuclei in some

Figure 4.2: A series of images showing the metaphase to telophase transitions of a single mitotic event during cycle 14 of embryogenesis, taken by manual image capture using a cooled CCD camera at approximately 45s intervals. Note that in addition to the obvious mitotic division, a second mitotic figure, partially obscured at the left hand edge of the images, progresses from prophase to metaphase while another at the top right progresses through telophase. Image produced by Robert Saint and Peter Kolesick.

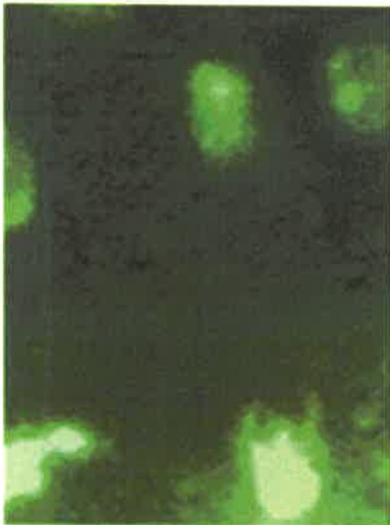
a.



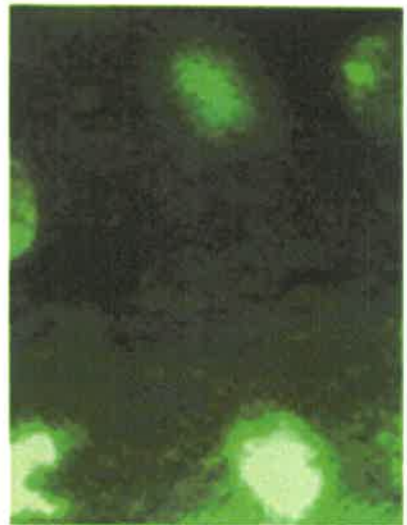
b.



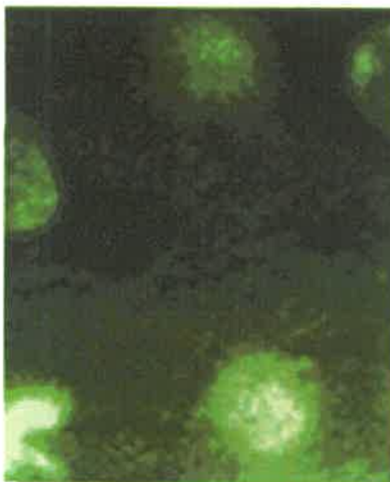
c.



d.



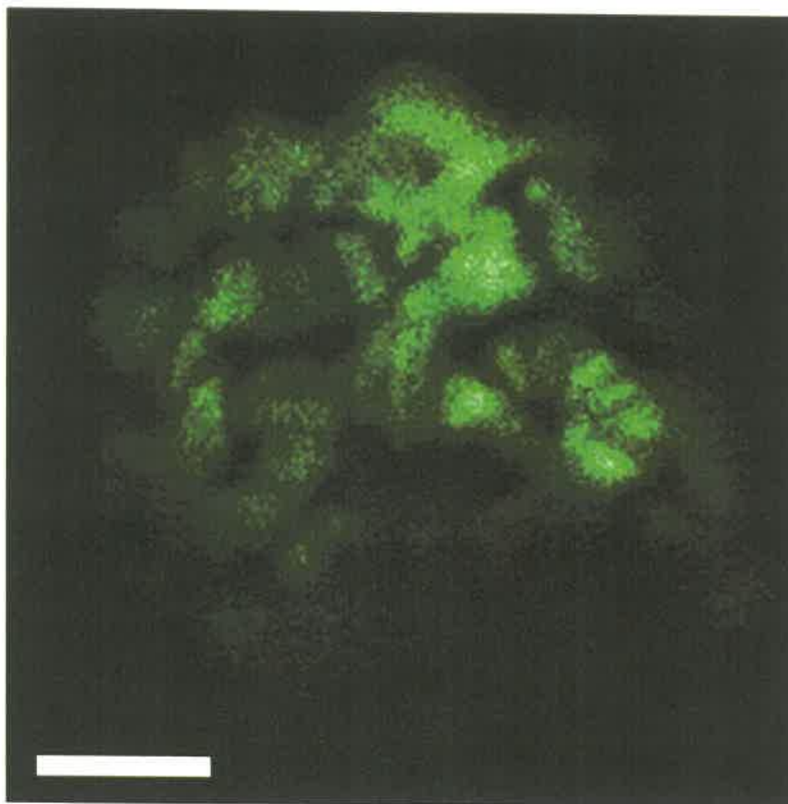
e.



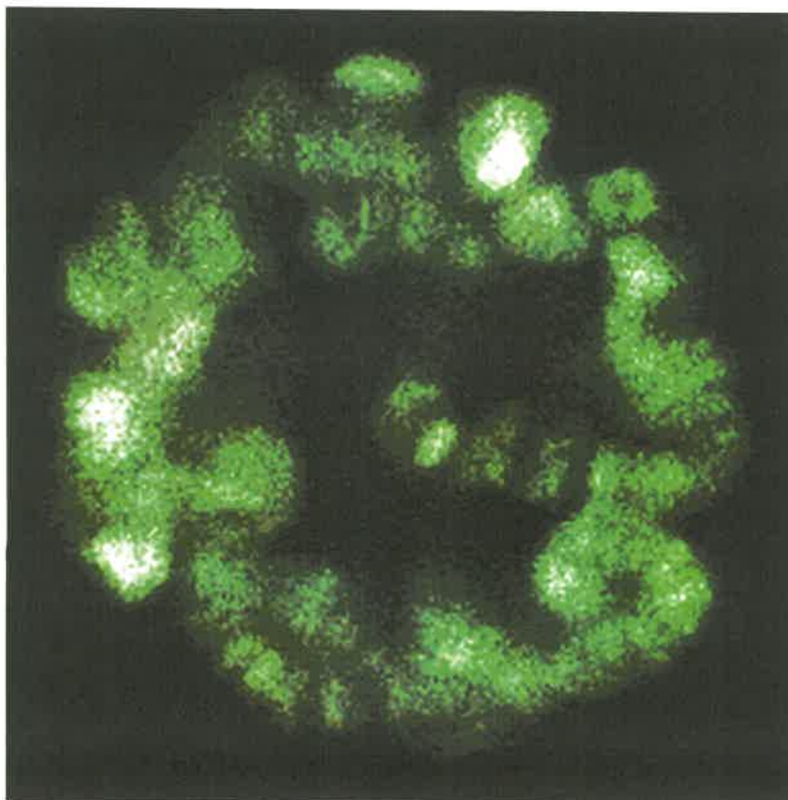
f.

Figure 4.3: Two confocal images, at different nuclear depths, of a salivary gland polytene nucleus within a live disected salivary gland. Note the appearance of a banded pattern of flourescece on the polytene chromosomes. Scale bar = 10μm. Image produced by Robert Saint and Peter Kolesick.

a.



b.



embryos. As development proceeds transcripts were detected in more embryos and in a greater proportion of nuclei in individual embryos (Pritchard and Schubiger 1996). His2AvD-GFP associated fluorescence was homogeneously distributed throughout early cleavage embryos but was not present in nuclei until the 9th mitosis. At this stage, His2AvD-GFP fluorescence was observed at the nuclear periphery in a striated pattern (Fig 4.4). As development progressed cytoplasmic fluorescence in the syncitium faded and, by the end of mitosis 10, His2AvD-GFP was only visible in nuclei. By this stage the nuclear fluorescence became more widely distributed but some regions of the nucleus remain free of His2AvD-GFP. A similar heterogeneous distribution of His2AvD-GFP protein was observed in all diploid nuclei after mitosis ten.

4.4 Discussion

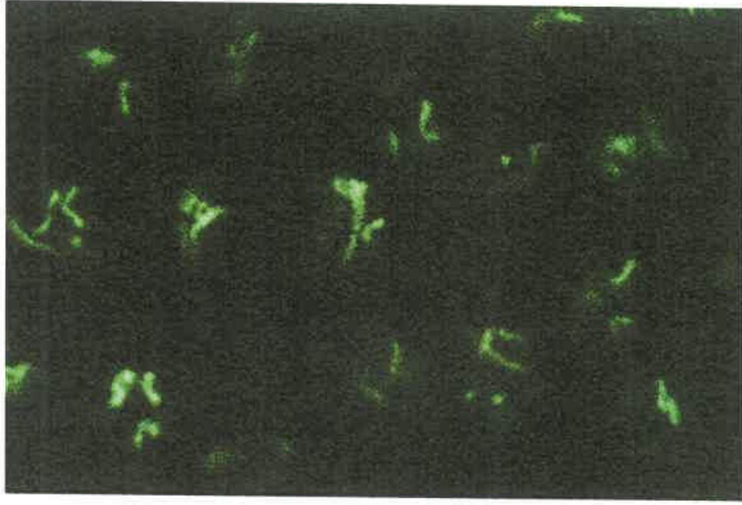
A His2AvD genomic rescue fragment was used to generate a construct that encoded a fusion protein composed of *Drosophila* H2A.Z/F-class variant histone, His2AvD, with GFP from the jellyfish *Aquorea victoria* fused to the C-terminus. In complementation studies it was shown that the fusion gene was capable of rescuing *His2AvD*^{810} null mutant lethality. Thus, the fusion of GFP to the C-terminus of His2AvD does not disrupt the function of His2AvD *in vivo.*

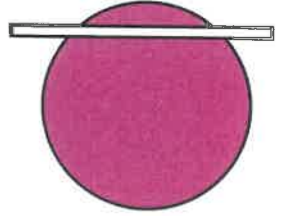
His2AvD-GFP fluorescence was associated with chromatin throughout all stages of mitosis and in polytene nuclei. On the highly polytenised chromosomes of the larval salivary gland, His2AvD-GFP fluorescence was associated with a large number of discrete sites. These observations are consistent with previous reports of His2AvD distribution characterised in studies using antibodies directed against the His2AvD protein (Donahue *et al.*, 1986; van Daal and Elgin, 1992). Unfortunately, GFP fluorescence is destroyed by acid which is used in standard chromosome squash preparations to simultaneously weaken the nuclear membrane, mechanically strengthen the chromosomes and fix the sample. To overcome this problem microdissected "native" chromosomes need to be prepared to further examine the distribution of His2AvD-GFP (see Hill and Watt, 1978; Mott *et al.*, 1980; Mott and Hill, 1986).

Although the precise function of His2AvD has not been identified, fractionation of nuclear chromatin using a number of different protocols has shown that H2A.F/Z is enriched in fractions which are also enriched in transcribed sequences (Gabrielli *et al.*, 1981; Ridsdale and Davie, 1987). Studies conducted in *Tetrahymena thermophila* have also supported the association of H2A.F/Z class histones with transcriptionally active chromatin. In this organism, immunolocalisation studies have shown that the H2A.F/Z

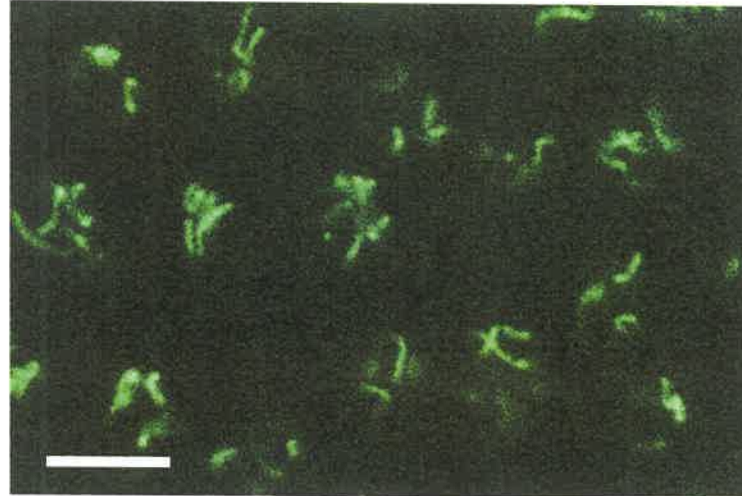
Figure 4.4: Confocal images focused at three depths (indicated at right) within the nuclei of a live cycle 9 syncitial embryo during prophase. Prior to this stage no His2AvD-GFP associated florescence was observed in nuclei and in later stages the fluorescence became more widely distributed throughout nuclei. Scale bar = 10µm. Image produced with the assistance of Roger McCart.

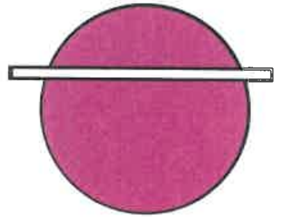
a.



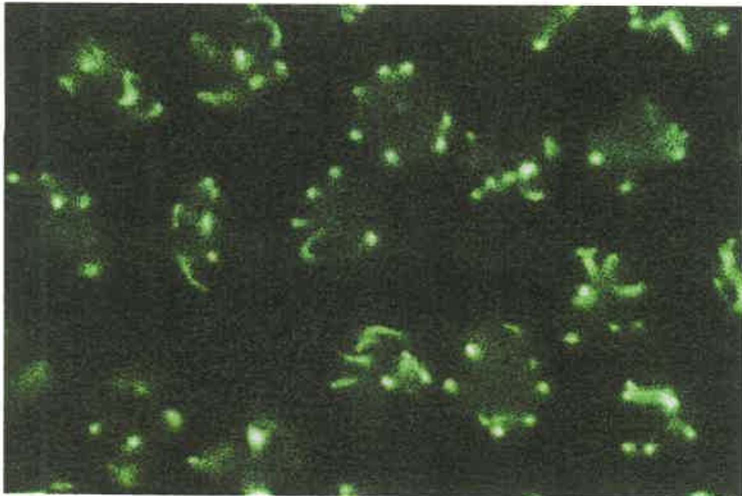


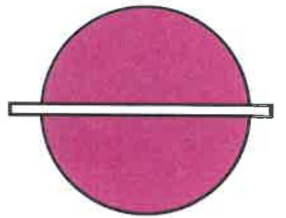
b.





c.





histone, hv1, is found in the transcriptionally active macronucleus and in the micronucleus specifically during a brief period during conjugation when transcription takes place (Wenkert and Allis, 1984; Allis *et al.*, 1986; Stargell *et al.*, 1993). In this context, the nuclear import of *Drosophila* His2AvD fusion protein into nuclei during cycle 9 of the syncitial divisions during embryogenesis is significant, as this coincides with the time that transcription is initiated in the zygote (Pritchard and Schubiger, 1996). Thus, the temporal distribution of His2AvD in *Drosophila* and of hv1 in *Tetrahymena* correlates with transcriptional activity.

One aspect of His2AvD distribution that does not correlate with transcription is its persistence on mitotic chromatin. During mitosis, transcription is attenuated through a combination of inhibition and displacement of the proteins involved in transcription (see section 1.6.7). Transcription by RNA polymerase II and III is inhibited by a mitosis specific phosphorylation event which has been reproduced *in vitro* (White *et al.*, 1995; Segil *et al.*, 1996). Immunocytochemical analysis of protein localisation has shown that a number of transcription factors and transcriptional coactivator proteins are displaced from chromatin during mitosis (Gonda *et al.*, 1985; Kovesdi *et al.*, 1986; Ye *et al.*, 1993; Ghysdeal *et al.*, 1986; Bohman *et al.*, 1987; Fletcher *et al.*, 1987; Scheidereit *et al.*, 1988; Hsu *et al.*, 1992; Luscher and Eisenman, 1992; Martinez-Balbas *et al.*, 1995; Muchardt *et al.*, 1996; Segil *et al.*, 1996; Falciola *et al.*, 1997; Hock *et al.*, 1998). Shortly after the decondensation of metaphase chromosomes, early in G1, transcribed genes are rapidly reactivated (see Michelotti *et al.*, 1997; John and Workman, 1998). At this time, most of the chromatin in the nucleus is still condensed, however, nucleoprotein particles containing putative RNA polymerases have been observed in regions of decondensed chromatin (Belmont and Bruce, 1994). It appears that these regions might be marked throughout mitosis for decondensation and reactivation of transcription in G1 because actively transcribed sequences are an order of magnitude more sensitive to the endonuclease activity of DNase I than bulk chromatin in both interphase and metaphase (Gazit *et al.*, 1982; Kerem *et al.*, 1984; see also Michelotti *et al.*, 1997; John and Workman, 1998). Given that His2AvD is enriched in transcribed and endonuclease sensitive chromatin (Gabrielli *et al.*, 1981; Ridsdale and Davie, 1987), it is feasible to speculate that, during mitosis, this variant histone could promote the rapid reactivation of genes during metaphase chromosome decondensation. Acetylated histones are also enriched in transcribed and endonuclease sensitive chromatin. Histones in mitotic chromosomes, however, are underacetylated relative to those in interphase chromatin (Chahal *et al.*, 1980; D'Anna *et al.*, 1983; Turner, *et al.*, 1989; Turner and Fellows, 1989; Jeppesen *et al.*, 1992).

During the course of these studies, the generation of human core histone-GFP fusion proteins was reported (Kanda *et al.*, 1998). The approach described here differs in that the fusion protein was shown to be functional *in vivo* using a genetic complementation assay. It is clear that this construct will be very useful for observing chromosome behaviour *in vivo* in *Drosophila melanogaster*. This marker will be particularly convenient in the analysis of phenotypes in a variety of cell-cycle and chromosome condensation mutants. In addition, derivatives of this construct could have wider applications. His2AvD is a member of a highly conserved family of variant histones. The amino acid sequence of genes encoding H2A.F/Z family of variant is more extensively conserved than that of the core histones (van Daal *et al.*, 1990). This extraordinary degree of conservation suggests that equivalent fusion proteins made with other H2A.F/Z proteins would function equally well as a marker for chromosome dynamics in other species.

Chapter 5: Production of recombinant histone proteins and antibodies to further characterise the function of H2A.F/Z variants in mammals and *Drosophila*.

5.1 Introduction

It is well established that the packaging of DNA into chromatin has an inhibitory effect on transcription *in vivo* and *in vitro* (see section 1.6). Activities have been characterised within eukaryotic cells that circumvent the inhibitory influence of chromatin. These activities are directed by large protein complexes that modify the structure of chromatin by ATP-dependent remodelling of histone-DNA contacts or by acetylating histone tails (see section 1.6). *In vitro* experiments with acetylation and remodelling complexes has demonstrated how they influence chromatin structure and the consequences that this has for the activation of transcription. The first step in these structural and functional assays is reconstitution of chromatin on suitable DNA templates. With a view to conducting similar assays on chromatin templates reconstituted with either H2A.F/Z or H2A.1, recombinant His2AvD, H2A.Z and H2A.1 were produced and purified here.

During the course of this thesis, a number of very powerful techniques were developed to characterise the DNA sequences with which a nuclear protein is associated *in vivo* (Hebes *et al.*, 1988, 1994; O'Neill and Turner, 1995). For this type of analysis, chromatin is isolated from nuclei and immunoprecipitated with antibodies directed against the protein of interest. DNA sequences isolated from the precipitated fraction can then be recovered and analysed. Here poly and monoclonal antibodies were developed against *His2AvD*, H2A.Z and H2A.1 proteins to similarly characterise the *in vivo* distribution of H2A.F/Z proteins.

5.2 Methods

5.2.1 Chemical decorionation of *Drosophila* embryos.

Embryos were dechorionated by immersion for 90sec in 50% (v/v) bleach (White King – 4% (v/v) sodium hypochlorite) at room temperature. Immediately following decorionation embryos were washed extensively with DEPC treated water and allowed to drain.

5.2.2 RNA isolation from *Drosophila* embryos.

1ml settled volume dechorionated *Drosophila* embryos was homogenised in 2ml of 0.2M sodium acetate pH 4.0 saturated phenol. To the homogenate, 3.6ml of 0.2M sodium acetate pH 4.0 and 400μl 10% (w/v) SDS was added. Samples were then incubated at 65°C for 5min, vortexed and allowed to cool to room temperature before the addition of 2ml of chloroform. After centrifugation of this material at 10Krpm for 10min (HB-4 rotor in Sorval RB-5C centrifuge) the aqueous phase was transferred to a fresh tube and extracted with a mixture of 2ml of 0.2M sodium acetate pH 4.0 saturated phenol and 2ml of chloroform. Aqueous and organic phases were separated, as before, by centrifugation at 10Krpm for 10min (HB-4 rotor in Sorval RB-5C centrifuge). The aqueous layer was removed, extracted with 4ml of chloroform and transferred to a fresh tube containing 10μl of 20mg/ml glycogen (not added to liver samples) and 2.5-3 volumes of ethanol. RNA was then pelleted from the samples by a 16h incubation at -20°C and centrifugation at 15Krpm for 15min (SS-34 rotor in Sorval RB-5C centrifuge). 3ml of 80% (v/v) ethanol was used to wash the pellet and was then aspirated. The RNA pellet was air dried and resuspended in 400μl of water.

5.2.3 Reverse transcription PCR.

10μg of RNA and 1ng of oligonucleotide were ethanol precipitated (2.4.2) and resuspended in 10μl primer extension hybridisation buffer (10mM Tris pH 8.3, 200mM NaCl). Samples were reverse transcribed, extracted with phenol/chloroform and ethanol precipitated as described in primer extension analysis of RNA (3.2.3). Following ethanol precipitation pellets were resuspended in 25μl of water. PCR analysis was conducted as outlined in 2.3.14 using 1μl of the reverse transcribed material as template.

5.2.4 Production of expression vectors containing *His2AvD*, H2A.Z and H2A.1 coding sequences.

Recombinant histone proteins were made in *E.coli* using the T7 RNA polymerase system developed by Studier *et al.* (1990). The plasmid, pET.AZ, containing the mouse H2A.Z coding sequence in the T7 expression vector pET8H3 was provided by V. Thonglairoam (1996). The *Drosophila His2AvD* and H2A.1 cDNA sequences were generated for cloning into the T7 expression plasmids pET3b and pET8H3 respectively by RTPCR from *Drosophila* embryo RNA (5.2.3). *His2AvD* message was reverse transcribed (5.2.3) with the primer His2AvD 3' (2.2.9(ii)) and then PCR amplified

(2.3.14) using the primers *His2AvD* 3' and *His2AvD* 5' (2.2.9(ii)). These primers incorporated *Nco*I and *BamHI* at the 5' and 3' ends of the amplified product, respectively. The RTPCR-amplified *His2AvD* fragment was restricted with the endonucleases *Nco*I and *BamHI* and then cloned into a similarly digested pET3b vector to create pET.AvD. Independent pET.AvD clones were digested with *XbaI/Hind*III to obtain DNA fragments containing the *His2AvD* sequence. These fragments were then subcloned into similarly digested pBluescript vector and sequenced (2.3.10) using the primers USP and RSP (2.2.9(i)). Further experiments using pET.AvD refer to a clone that was selected for having a cDNA insert with the same sequence as the *Drosophila* His2AvD coding region (van Daal *et al.*, 1990). A DNA fragment containing the H2A.1 coding sequence was obtained by reverse transcription (5.2.3) of *Drosophila* embryo RNA with the primer H2A.1 3' (2.2.9(ii)) followed by PCR amplification (2.3.14) with the primers H2A.1 3' and H2A.1 5' (2.2.9(ii)). The H2A.1 RTPCR fragment was restricted within the 5' and 3' primer sequences with the endonucleases *Nco*I and *Hind*III repectively so that it could be cloned into a similarly digested pET8H3 vector to create pET.A1. The RTPCR amplified coding sequence of individual pET.A1 clones was subcloned as an *XbaI/Hind*III fragment into a similarly digested pBluescript vector and sequenced (2.3.10) using the primers USP and RSP (2.2.9(i)). Further experiments using pET.A1 refer to a clone that was selected for having a cDNA insert with the same sequence as the *Drosophila* H2A.1 coding region (Goldberg 1979).

5.2.5 SDS polyacrylamide gel electrophoresis.

Gels for protein electrophoresis were prepared in 1XSDS gel buffer and had separating and stacking gels that were 15% (w/v) acrylamide:bisacrylamide (38:1), 1% (w/v) APS, 0.1% (v/v) TEMED and 4% (w/v) acrylamide:bisacrylamide (38:1), 1% (w/v) APS, 0.1% (v/v) TEMED respectively. The samples were added to an equal volume of 2XSDS loading buffer, incubated at 100°C for 3min and loaded onto the gel. Electrophoresis was conducted in a Biorad miniprotean II apparatus in 1XSDS gel running buffer at 185V. SDS PAGE gels were stained in 0.2% (w/v) amido black, 5% (v/v) acetic acid, 25% (v/v) isopropanol and destained in 5% (v/v) acetic acid, 25% (v/v) isopropanol. After destaining gels were dried between two pieces of cellophane.

5.2.6 Production of polyclonal H2A.1, H2A.Z and His2AvD antibodies.

H2A.1, H2A.Z and His2AvD crude anti-sera were generated by Chiron mimotopes. This sera was raised in sheep against a synthetic peptide equivalent to the C-terminal 19 amino acids of H2A.1 and the C-terminal 16 amino acids of histones H2A.Z and

His2AvD (Fig 5.4, 2.2.10). Briefly, the synthetic peptide was covalently coupled to the Diptheria Toxoid via a non-histone cysteine at the N-terminus of the peptide. The peptide-carrier conjugate was injected into two sheep at 100nM of peptide per injection in Freunds complete adjuvant (1:1). This was followed two weeks later by a booster injection with the same amount of peptide-carrier in Freunds incomplete adjuvant (1:1). Three weeks after the second injection sheep were bled and sera pooled to obtain the hyperimmune anti-peptide sera. Chiron mimotopes provided 5ml of preimmune serum and 50ml of hyperimmune serum on dry ice. This was defrosted, dispensed into small volumes (0.5-5ml) and stored at -20°C.

5.2.7 Affinity purification of antibodies directed against H2A.1 and H2A.Z using recombinant protein bound to nitrocellulose strips

To affinity purify H2A.1 or H2A.Z antibodies, 90μg of purified recombinant protein was subject to SDS PAGE and transferred to nitrocellulose as outlined for Western analysis in section 5.2.7. Recombinant H2A.1 or H2A.Z was located on the filters by western analysis of a 0.4cm wide strip cut from one edge following transfer. An 8cm long, 0.5cm wide region containing the histone was cut from the remaining membrane and incubated for 16h at room temperature with 200 μl of crude serum diluted 1:1 in 3% (w/v) BSA in PBS. After the membrane was washed, bound H2A.1 or H2A.Z specific antibodies were eluted with 200μl of ice cold 200mM Glycine pH 2.0, 1mM EGTA which was neutralised immediately with an equal volume of ice cold 100mM Tris base. Sodium azide was added to the affinity purified antibodies as a preservative to a final concentration of 0.02% (w/v). Affinity purified antibodies were stored at -20°C in small aliquots.

5.2.8 Affinity purification of antibodies directed against His2AvD using biotinylated peptides and streptavidin agarose.

A 400μl slurry of 50% (w/v) streptavidin agarose (Gibco BRL) was washed with 40ml of PBS and pelleted by centrifugation at 1.8Krpm for 5min (Jouan C312 benchtop centrifuge). Washing was repeated and streptavidin agarose was resuspended to a final volume of 400μl in PBS. Half of this material was used to pretreat the antibody sample and the other half was complexed with biotinylated His2AvD peptide for affinity purification of antibodies. For pretreatment, 200μl of streptavidin agarose (equilibrated in PBS) was added to 1ml of crude serum diluted to 10ml with 3% (w/v) BSA in PBS. After incubating at room temperature with gentle agitation for 30min the streptavidin

agarose was pelleted by centrifugation at 1.8Krpm for 5min (Jouan C312 benchtop centrifuge) and the pretreated sample was transfered to another tube.

Lyophilised, biotinylated peptide was resuspended to 1mg/ml in PBS. Following resuspension, 1mg of this material was added to 200µl of streptavidin agarose and allowed to bind for 1h at room temperature. Peptide conjugated agarose was pelleted by centrifugation in a microfuge (6Krpm for 5min), resuspended to 200µl with PBS, added to pretreated antibody sample and incubated overnight at room temperature with gentle agitation. Antibody/biotin peptide complexed streptavidin agarose was then washed three times by pelleting and resuspension in 10ml PBS. Antibodies were eluted from pelleted agarose by adding 300µl ice cold 200mM Glycine pH 2.0, 1mM EGTA and incubating on ice for 3min with gentle agitation. After pelleting the agarose by centrifugation at 1.8Krpm for 5min (Jouan C312 benchtop centrifuge) the glycine solution was transferred to another tube on ice. The pelleted agarose was treated again, this time with 300µl of Tris base which was ultimately added to the glycine eluate as a neutralisation solution. Sodium azide was added to the affinity purified antibodies to a final concentration of 0.02% (w/v) as a preservative. Affinity purified antibodies were stored at -20°C in small aliquots.

5.2.9 ELISA.

Elisa's were conducted in 96 well polyvinylchloride trays (Costar). Each well was coated by incubation with 50µl of antigen diluted in PBS for 1h at room temperature. After aspiration of the antigen solution wells were blocked by incubation with 200µl of 1% (w/v) BSA in PBS for 1h at room temperature. Blocking solution was aspirated and 50µl of antibody, either a 1:500 dilution of sera with 1% (w/v) BSA in PBS or neat aspirated hybridoma media, was allowed to bind to each well for 1h at room temperature. Antibody solution was removed and wells were washed with 200µl of PBS. After washing, 50µl of secondary antibody diluted 1:500 with 1% (w/v) BSA in PBS was added to each well and allowed to bind for 30min at room temperature. Wells were washed three times with 200µl of PBS each. ELISA was developed by adding 100µl of 100mM sodium citrate pH 6.5, 1mg/ml o-phenyline diamine, 0.03% (v/v) hydrogen peroxide to each well until colour had reached a desirable intensity (5min at room temperature was usually sufficient). Reactions were stopped by the addition of 100µl of 1M H_2SO_4.

5.2.10 Monoclonal antibody production.

H2A.Z and His2AvD monoclonal antibodies were generated in conjunction with Joe Wrin (University of Adelaide Department of Biochemistry). Monoclonal antibodies were raised against the same Diptheria Toxoid coupled synthetic peptides of the C-terminal 16 amino acids of histones H2A.Z and His2AvD used for generation of sheep antisera (Fig 5.4, 2.2.10, 5.2.6). The peptide-carrier conjugate was injected intraperitoneally into six female mice at 100nM of peptide per injection in Freunds complete adjuvant (1:1). Mice recieved intraperitoneal booster injections with the same amount of peptide-carrier in Freunds incomplete adjuvant (1:1) two weeks and five weeks after the primary immunisation. Sera collected from tail bleeds one week after the second boost were tested in an ELISA and the best two respondants were boosted again one week later. Spleens were removed from these mice three days after the final boost and were gently homogenised into 20ml of DMEM between the frosted surface of two etched microscope slides. Cells from each spleen were pelleted by centrifugation (1.2Krpm for 5min in a Jouan C312 benchtop centrifuge) and washed twice by resuspension and centrifugation in 20ml of DMEM. The pelleted cells from each spleen were again resuspended this time with 20ml of DMEM containing $1X10^8$ myeloma cells that had been harvested while growing rapidly and washed once. Cells were pelleted by centrifugation (1.2Krpm for 5min in a Jouan C312 benchtop centrifuge) and fused by the addition over 1min of 1ml 50% (w/v) PEG in DMEM with constant stirring. Over the next minute, 1ml of DMEM was added with stirring and then a further 8ml of media was added, again with stirring, over the next 2min. Cells were pelleted by centrifugation (1.2Krpm for 5min in a Jouan C312 benchtop centrifuge), resuspended in 10ml of DMEM containing 20% (v/v) FBS, 1XOPI, 1XAH and then diluted to 200ml in the same media. Aliquots of 100µl were dispensed into each well of 96 well microtitre plates and grown at 37°C in a CO_2 incubator. After 1 week an ELISA was conducted using recombinant proteins and 50µl of media aspirated from wells containing colonies. Where a signal was detected colonies were expanded for freezing in liquid nitrogen and selection of clones derived from a single hybridoma.

Single cell clones were selected in 96 well plates by limiting dilution of rapidly growing hybridoma cells in suspension. A 96 well plate was set up with 100µl of media (DMEM containing 20% (v/v) FBS, 2XOPI) in each well. To the 100µl of media in the top left hand well, 100µl of a hybridoma cell suspension was added and mixed by pipeting up and down. After mixing, 50µl of this material was added to the media in the well below and mixed. This serial dilution procedure was repeated down the entire column. Serial dilutions were then conducted across the rows starting with 50µl of

diluted hybridoma suspension from the left most well of each row. After incubating the plates for 1 week at 37°C in a CO_2 incubator, trays were inspected by light microscopy. ELISAs were performed using recombinant proteins and 50µl of media aspirated from wells containing only one colony. From each limiting dilution one clone which secreted antibodies that cross reacted with recombinant protein was expanded for freezing in liquid nitrogen and to produce media for use in Western analysis. A hybridoma clone was selected for ascites production on the basis of its titration in ELISAs and its detection of protein in Western analysis.

For production of ascites, mice were primed by injecting 500µl of Freunds incomplete adjuvant into the peritoneum. After 14 days $1X10^6$ hybridoma cells, resuspended to 500µl in PBS, were injected into the peritoneum. Ascites was collected 4 weeks and 7 weeks after the injection of hybridoma cells. Following each collection ascites was treated by a 1h incubation at 37°C, an overnight incubation at 4°C, centrifugation (2.0Krpm for 5min in a Jouan C312 benchtop centrifuge) and addition of sodium azide to a final concentration of 0.02% (w/v). Monoclonal antibodies were stored at -20°C in small aliquots.

5.2.11 Western analysis.

Protein samples electrophoresed on 15% (w/v) acrylamide:bisacrylamide SDS PAGE gels were electrotransfered to nitrocellulose with a semi-dry transfer apparatus (Pharmacia LKB Multiphor II) in Western transfer buffer at $3mA/cm^2$ (membrane) for 45min. After transfer the nitrocellulose filter was washed (three times for 5min each) in PBS before being blocked for 16h in 5% (w/v) non fat skim milk powder in PBS. The blocked filter was washed and the primary antibody was applied at a 1:500 dilution in 3% (w/v) BSA in PBS ($1ml/cm^2$ membrane). After allowing the primary antibody to bind for three hours at room temperature with agitation the filter was washed. The secondary antibody (donkey anti sheep/goat conjugated to horse-radish peroxidase, Silenus or rabbit anti mouse conjugated to horse-radish peroxidase, Dako) was added at the same dilution as the primary and allowed the same time to bind. Excess secondary antibody was removed by washing. Filters were developed with diaminobenzidine (Harlow and Lane 1988).

5.2.12 Histone isolation from *Drosophila* embryos.

Drosophila embryo nuclei were isolated essentially as outlined in protocol 106, method 2 of Ashburner (1989b). Embryo nuclear isolation buffers 1 and 2 in this protocol were

replaced with 1X Hewish and Burgoine buffer A containing 0.2% (v/v) βme, 0.2mg/ml PMSF and 1% (v/v) thiodiglycol. Histones were isolated from nuclei as described in 6.2.4.

5.2.13 Histone isolation from mouse spleen.

Histones were prepared from mouse spleen as outlined in section 6.2.4.

5.3 Results

5.3.1 Production and purification of recombinant histones His2AvD, H2A.Z and H2A.1.

Recombinant histone proteins were made in *E.coli* using the T7 RNA polymerase system developed by Studier *et al.* (1990). pET vectors containing the coding sequences of *D. melanogaster* H2A.1 (pET.A1) and His2AvD (pET.AvD) were constructed as outlined in 5.2.4 and the H2A.Z expression clone, pET.AZ was provided as a gift from V. Thonglairoam. These clones were transformed (2.3.9) into the *E.coli* strain BL21(DE3) (2.2.7) and grown on L-agar with ampicillin (50µg/ml) and chloramphenicol (30µg/ml). A 1ml overnight culture, derived from a single bacterial colony, was used to innoculate 1l of 2YT broth containing ampicillin (50µg/ml) and chloramphenicol (30µg/ml) in a fermenter. Cultures were grown at 37°C to mid log phase and induced with IPTG to a final concentration of 0.1mM.

Western analysis of samples taken from cultures for three hours following induction found that, relative to total protein, the amount of H2A.Z and H2A.1 increased over this period whereas His2AvD levels reached a maximum after 1h then decreased dramatically (data not shown). In light of these results, for the preperation of recombinant histone proteins H2A.1 and H2A.Z cultures were induced for 3h while His2AvD cultures were induced for 1h only. Inspection of cells by phase contrast light microsopy during the induction period revealed that cells containing H2A.Z and H2A.1 contained inclusion bodies but cells containing the His2AvD expression construct did not (data not shown). Further analysis of His2AvD expression indicated that the protein was associated with the insoluble fraction following cell lysis by sonication (data not shown). Since recombinant H2A.1 and H2A.Z were produced as inclusion bodies and His2AvD was not, 2 different methods were employed for further processing and purification.

(i) Isolation and purification of histones H2A.1 and H2A.Z from inculsion bodies.

Cells pelleted by centrifugation at 6Krpm for 10 min (GS-3 rotor in a Sorvall RB-5C centrifuge) were resuspended in 1l 1XTE and lysed by 6 passages through a 15M homogeniser at 9000psi. Cell free lysates from *E.coli* expressing H2A.1 and H2A.Z as inclusion bodies were centrifuged at 9Krpm for 10min (GS-3 rotor in a Sorvall RB-5C centrifuge). The inclusion body pellet was resuspended to 20Xpellet (w/v) in 10mM KH_2PO_4 pH 7.8, 1M urea, 30mM NaCl, 1% (v/v) NP40 and left at room temperature for 15min. Inclusion bodies were then pelleted by centrifugation at 10Krpm for 10min (SS-34 rotor in a Sorvall RB-5C centrifuge), washed in 20X pellet (w/v) with 10mM KH_2PO_4 pH 7.8, 30mM NaCl, pelleted again and freeze dried for storage at -70°C.

H2A.1 and H2A.Z were purified by FPLC using a 1ml Resource S column (Pharmacia). 12.5mg of freeze dried inclusion body was dissolved in 1ml of 6M GuHCl, added to 50ml of buffer A (20mM maleic acid pH 2.0, 10% (v/v) acetonitrile) and filtered. This material was loaded onto a Resource S column equilibrated in buffer A and eluted with a linear gradient of buffer B (20mM maleic acid pH 2.0, 2M NaCl, 10% (v/v) acetonitrile). The elution profiles of H2A.Z (Fig 5.1) and H2A.1 (Fig 5.2), monitored at 280nm, revealed a single major peak in each case at 55% (v/v) B (1.1M NaCl) and 62.5% (v/v) B (1.25M NaCl) respectively. Fractions asociated with these peaks were shown to contain protein with the same mobility in SDS PAGE analysis as either native H2A.Z (Fig 5.5a) or H2A.1 (Fig 5.6a) and were greater than 90% pure. In a Western analysis, these proteins cross-reacted with antibodies directed specifically against H2A.Z (Fig 5.5b, c, d) or H2A.1 (Fig 5.6b, c).

(ii) Isolation and partial purification of histone His2AvD from the insoluble fraction of bacterial lysates.

Cells were pelleted in 6X500ml rotor buckets by centrifugation at 6Krpm for 10 min (GS-3 rotor in a Sorvall RB-5C centrifuge) and treated separately. Each pellet was resuspended in 10ml 1XTE, 0.2mg/ml PMSF and lysed by 4 X10sec pulses of a sonicator (Cell disruptor B30, Branson Sonic Power Co.). Cell free lysates were centrifuged at 15Krpm for 30min (SS-34 rotor in a Sorvall RB-5C centrifuge), the supernatant was aspirated and the pellet was resuspended by sonication for 10 sec in 10ml of 0.5XTE, 250mM H_2SO_4, 0.2% (v/v) βMe, 0.2mg/ml PMSF. This material was left at 4°C for 10min and then centrifuged at 15Krpm for 30min (SS-34 rotor in a Sorvall RB-5C centrifuge). The supernatant was added to 3 volumes of acetone in a fresh tube, placed at -70°C for 3h and then spun at 15Krpm for 30min (SS-34 rotor in a

Figure 5.1: Purification of recombinant H2A.Z protein. 12.5mg of freeze dried inclusion bodies were dissolved in 1ml of 6M GuHCl, added to 50ml of buffer A (20mM Maleic acid pH 2.0, 10% acetonitrile) and filtered. This material was loaded onto a Resource S column (Pharmacia) equilibrated in buffer A, eluted with a linear gradient from 0-100% buffer B (20mM Maleic acid pH 2.0, 2M NaCl, 10% acetonitrile) over 20min at 1ml/min and fractions were collected at 30s intervals (0.5ml). Monitoring of the eluate at 280nm revealed a single major peak that corresponded to 55% (v/v) B (1.1M NaCl).

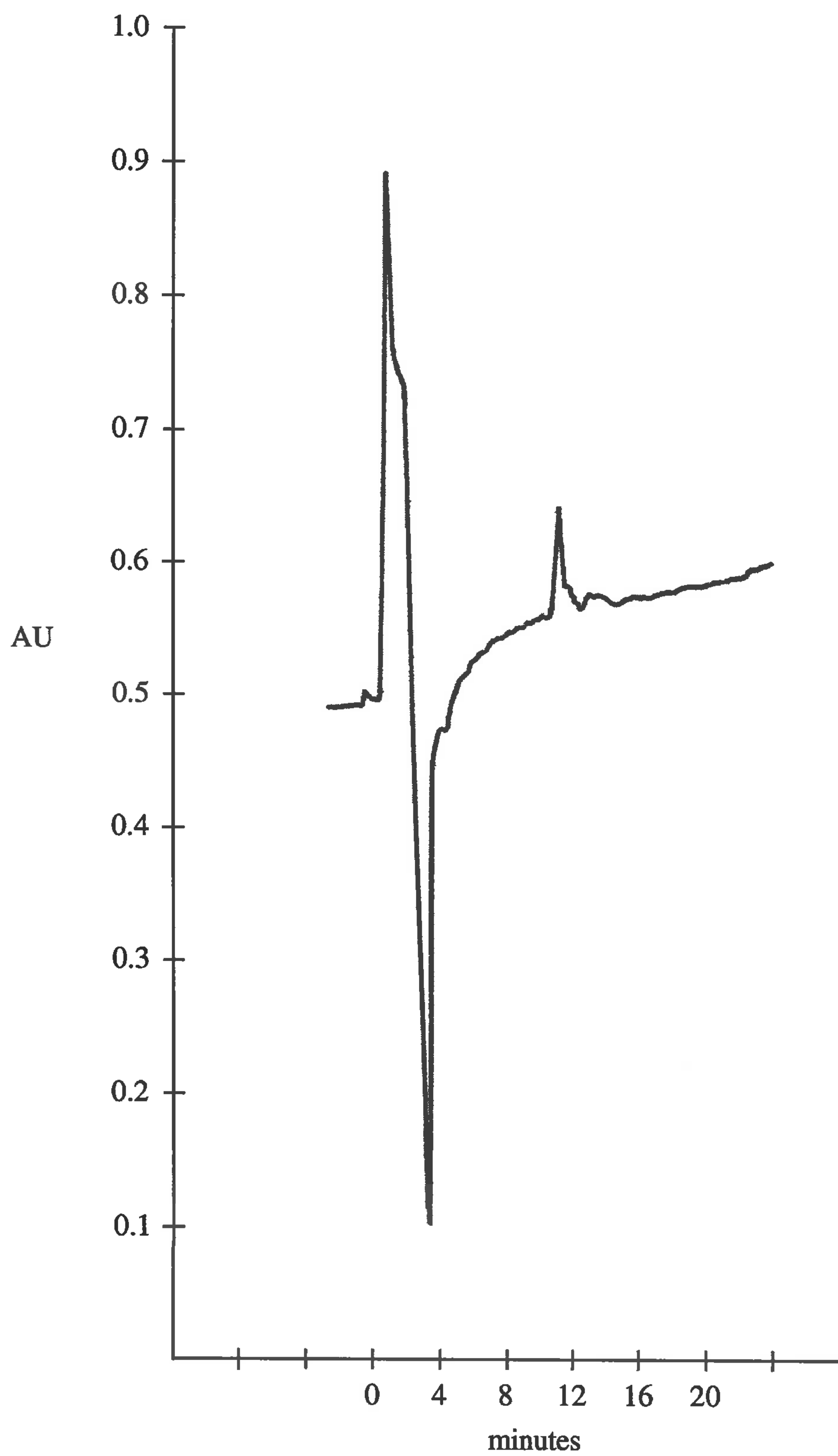


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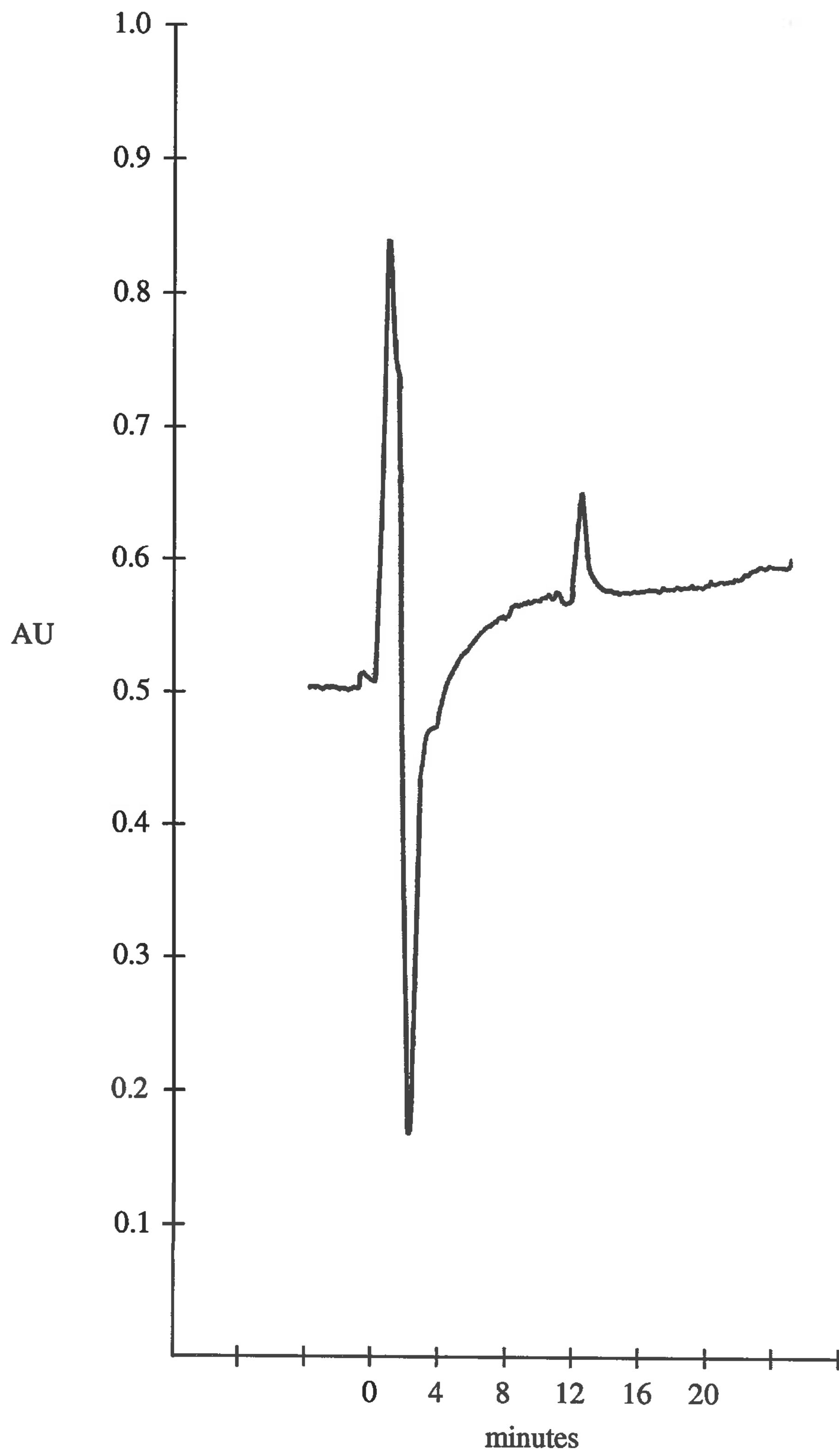
Figure 5.2: Purification of recombinant H2A.1 protein. 12.5mg of freeze dried inculsion bodies were dissolved in 1ml of 6M GuHCl, added to 50ml of buffer A (20mM Maleic acid pH 2.0, 10% acetonitrile) and filtered. This material was loaded onto a Resource S column (Pharmacia) equilibrated in buffer A, eluted with a linear gradient from 0-100% buffer B (20mM Maleic acid pH 2.0, 2M NaCl, 10% acetonitrile) over 20min at 1ml/min and fractions were collected at 30sec intervals (0.5ml). Monitoring of the eluate at 280nm revealed a single major peak that corresponded to 62.5% (v/v) B (1.25M NaCl).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20
minutes

Sorvall RB-5C centrifuge). Pellet was air dried and resuspended in 2ml buffer A (50mM sodium acetate pH 4.5, 6M urea).

This material was further purified by FPLC using a Resource S column (Pharmacia) equilibrated in buffer A and eluted with a linear gradient of buffer B (50mM sodium acetate pH 4.5, 6M urea, 1M NaCl). The FPLC elution profile, monitored at 280nm, revealed a number of peaks between 20% (v/v) B (0.2M NaCl) and 65% (v/v) B (0.6M NaCl) (Fig 5.3). Analysis of fractions collected across this region by SDS PAGE and Western blotting demonstrated that His2AvD eluted at 47.5% (v/v) B (0.475M NaCl) (Fig 5.7a-d).

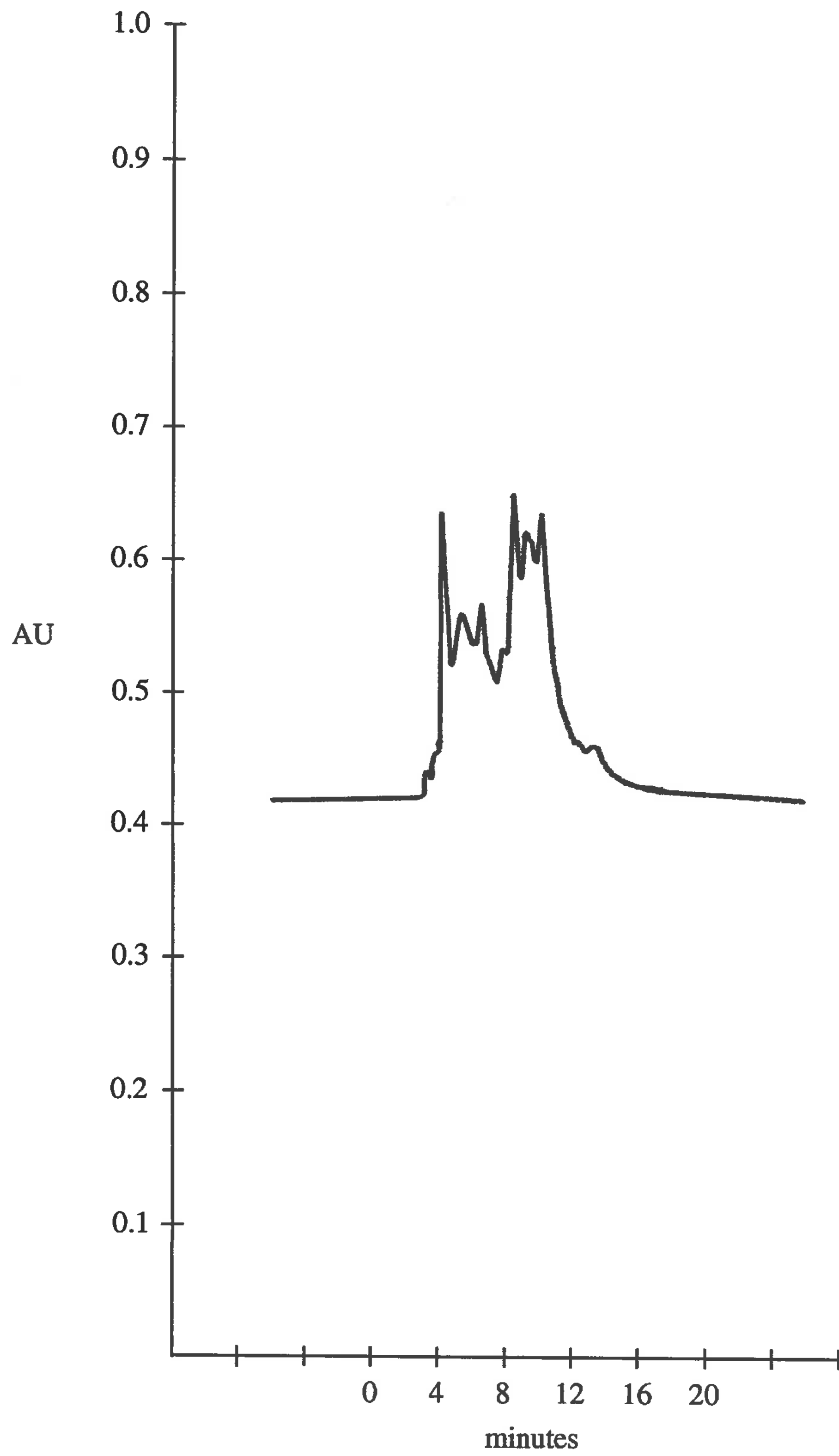
5.3.2 Production of sheep sera containing antibodies directed against the C-terminus of His2AvD, H2A.Z and H2A.1.

His2AvD, H2A.Z and H2A.1 crude anti-sera were generated for our laboratory by Chiron mimotopes. These sera were raised in sheep against synthetic peptides equivalent to the C-terminal 16, 16 and 19 amino acids of histones His2AvD, H2A.Z and H2A.1 respectively (Fig 5.4). Histone termini were chosen for antibody generation because they protrude from the nucleosome core and the amino acid sequences of core and variant proteins are very different in this region. Specifically, C-terminal sequences were used as antigens because lysine residues in the histone amino termini are subject to post-translational acetylation that could interfere with antibody recognition. To improve immunogenicity, the synthetic peptide was covalently coupled to Diptheria Toxoid via a non-histone cysteine at the N-terminus of the peptide. Immunisation details are provided in section 5.2.6.

5.3.3 Affinity purification of polyclonal antibodies from crude sheep sera.

Polyclonal antibodies directed against the C-termini of histones H2A.1 and H2A.Z were affinity purified by binding and elution off recombinant protein immobilised on nitrocellulose. This method was unsuitable for affinity purification of antibodies directed against histone His2AvD because, unlike histones H2A.1 and H2A.Z, μg quantities of recombinant His2AvD was not available. Anti-His2AvD antibodies were instead purified by binding and elution off biotinylated peptides of the C-terminal 16 amino acids immobilised on streptavidin agarose. Details of these protocols follow.

Figure 5.3: Partial purification of recombinant *His2AvD* protein. Acid soluble proteins were isolated from the insoluble fraction of bacterial cell lysates and precipitated with acetone. This material was resuspended in 2ml buffer A (50mM NaAc pH4.5, 6M urea) and filtered before being loaded onto a Resourse S column (Pharmacia) equilibrated in buffer A. Protein was eluted with a linear gradient from 0-100% buffer B (50mM NaAc pH4.5, 6M urea, 1M NaCl) over 20min at 1ml/min and fractions were collected at 30sec intervals (0.5ml). The elution profile was monitored at 280nm. Analysis of fractions by SDS PAGE and Western blotting revealed that *His2AvD* protein eluted at 47.5% (v/v) buffer B (0.475M NaCl).



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minutes

a. Epitopes

H2A.1	VLPNIQAVLLPKKTESHHKAKG
H2A.Z	VIPHIHKSLIGKKGQQKTV
His2AvD	VIPHIHKSLIGKKEETVQDPQRKGNVILSQAY

b. Homology.

H2A.1	VLPNIQAVLLPKKTESHHKAKG
H2A.Z	VIPHIHKSLIGKKGQQKTV
His2AvD	VIPHIHKSLIGKKEETVQDPQRKGNVILSQAY

Figure 5.4: a. Epitopes selected for the generation of antibodies directed against the H2A.1, H2A.Z and His2AvD proteins. These epitopes were produced by Chiron Mimotopes as synthetic peptides with a C residue at the N-terminus to facilitate coupling to diptheria toxin and biotin. Peptides were conjugated to diptheria toxin to improve immunogenicity. **b.** Amino acid sequence homology between H2A.1, H2A.Z and His2AvD within the regions selected for production of synthetic peptides (boxed).

(i) Affinity purification of antibodies directed against the C-terminius of H2A.Z and H2A.1 proteins

H2A.Z and H2A.1 antibodies were affinity purified from sera by binding and elution from recombinant protein immobilised on nitrocellulose as outlined in section 5.2.7. In a Western analysis of histones derived from mouse tissue, *Drosophila* embryos or bacteria, affinity purified H2A.Z and H2A.1 antibodies had a high titre and lower non-specific background cross reactivity than crude sera (compare b and c in Figs 5.5 and 5.6 repectively).

(ii) Affinity purification of antibodies directed against the C-terminus of His2AvD protein.

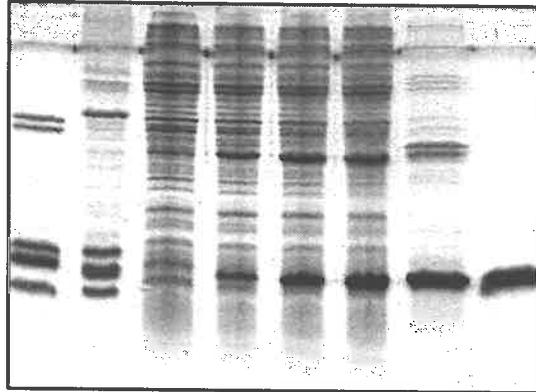
Polyclonal antibodies directed against the C-termini of histone His2AvD were affinity purified by binding and elution off biotinylated immunogen peptide immobilised on streptavidin agarose as outlined in section 5.2.8. The affinity purified antibodies had a high titre and a much lower level of non-specific background staining than crude sera in a Western analysis of histones produced in bacteria or isolated from *Drosophila* embryos (compare b and c in Fig 5.7).

5.3.4 Production of monoclonal antibodies directed against the C-terminus of His2AvD and H2A.Z protein.

H2A.Z and His2AvD monoclonal antibodies were produced in conjunction with Joe Wrin (University of Adelaide Department of Biochemistry). Monoclonal antibodies were generated with the same Diptheria Toxoin coupled synthetic peptides of the C-terminal 16 amino acids of histones H2A.Z and His2AvD that were used for production of sheep antisera in section 5.2.2 (Fig 5.4). Hybridoma cells from mice immunised with these peptides were prepared using standard procedures as outlined in section 5.2.10. Hybridomas that produced antibodies against H2A.Z and His2AvD proteins were identified in an ELISA assay (5.2.9) using media aspirated from tissue culture wells containing growing hybridomas and recombinant histone proteins. Single cell clones were isolated by limiting dilution of rapidly growing hybridoma cells in suspension. H2A.Z and His2AvD hybridoma clones were chosen for ascites production on the basis of their titration in ELISAs and their detection of recombinant and isolated protein in Western analysis. H2A.Z and His2AvD ascites was produced from the hybridoma clones 4F1-1H8 and 2C10-1F11 respectively as outlined in section 5.2.10.

Figure 5.5: SDS PAGE and Western analysis of native and recombinant histone proteins. **a.** Amido black stained SDS PAGE of; native mouse histone (lane 1); native *Drosophila* histone (lane 2); total cell lysate from cells containing the H2A.Z expression construct, pET.AZ, at 0h (lane 3) 1h (lane 4), 2h (lane 5) and 3h (lane 6) relative to induction; solubilised total inclusion bodies (lane 7) and; FPLC purified recombinant histone H2A.Z (lane 8). The FPLC purified recombinant histone shown here is a sample of material that eluted from a Resource S column at 1.1M NaCl in 20mM Maleic acid pH 2.0, 10% (v/v) acetonitrile (see Fig 5.1 and section 5.3.1 (i)). SDS PAGE gels equivalent to that shown in (a.) were transferred to nitrocellulose and probed with crude anti-H2A.Z sheep sera (**b.**), affinity purified H2A.Z antibodies (**c.**) or monoclonal H2A.Z antibodies (**d.**). All immunoblots were conducted with a 1:500 dilution of primary and HRP conjugated secondary antibodies (in 1% BSA 1XPBS). Blots were developed with diaminobenzidine (Harlow and Lane, 1988).

a.



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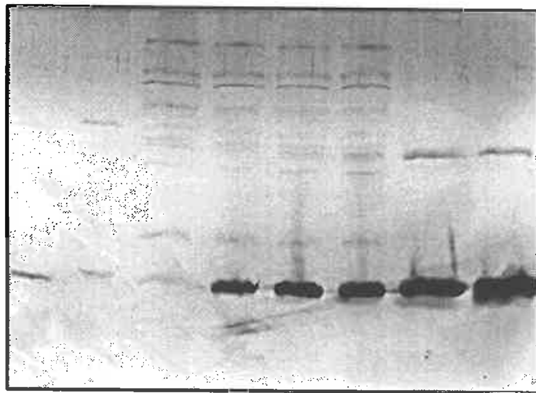

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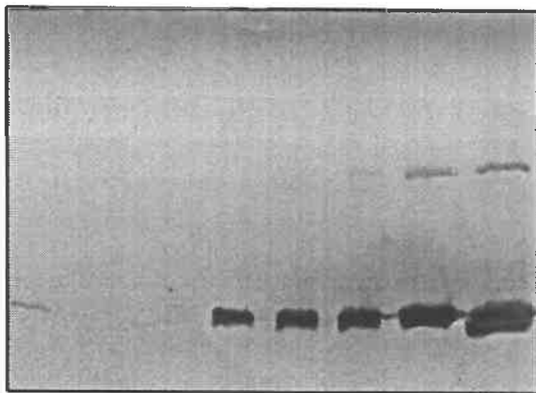

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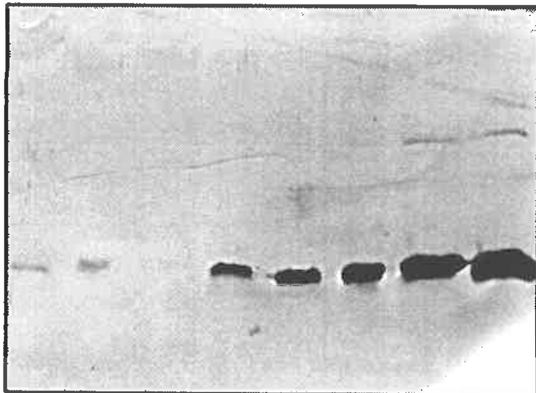
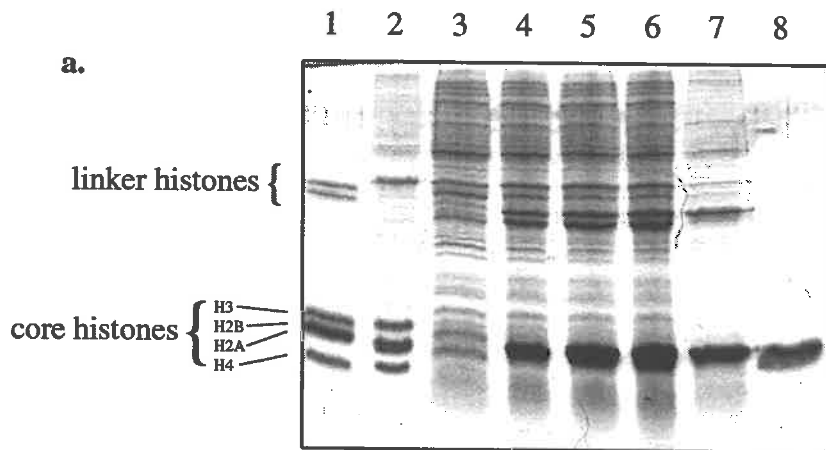


Figure 5.6: SDS PAGE and Western analysis of native and recombinant histone proteins. **a.** Amido black stained SDS PAGE of; native mouse histone (lane 1); native *Drosophila* histone (lane 2); total cell lysate from cells containing the H2A.1 expression construct, pET.A1, at 0h (lane 3) 1h (lane 4), 2h (lane 5) and 3h (lane 6) relative to induction; solubilised total inclusion bodies (lane 7) and; FPLC purified recombinant histone H2A.1 (lane 8). The FPLC purified recombinant histone shown here is a sample of material that eluted from a Resource S column at 1.25M NaCl in 20mM Maleic acid pH 2.0, 10% (v/v) acetonitrile (see Fig 5.2 and section 5.3.1 (i)). SDS PAGE gels equivalent to that shown in (a.) were transferred to nitrocellulose and probed with crude anti-H2A.1 sheep sera (**b.**) or affinity purified H2A.1 antibodies (**c.**). **d.** The SDS PAGE gel used in (b.)stained with amido black after the transfer of proteins to nitrocellulose. All immunoblots were conducted with a 1:500 dilution of primary and HRP conjugated secondary antibodies (in 1% BSA 1XPBS). Blots were developed with diaminobenzidine (Harlow and Lane, 1988).



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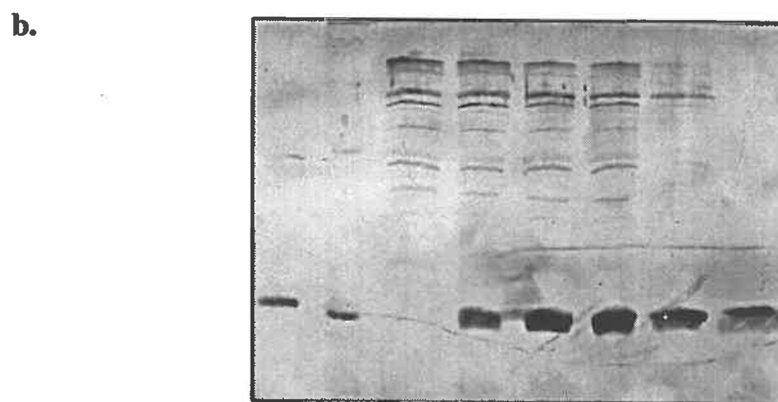

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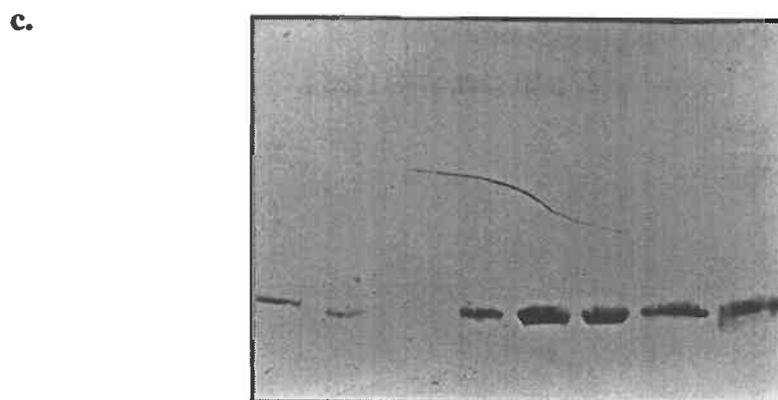

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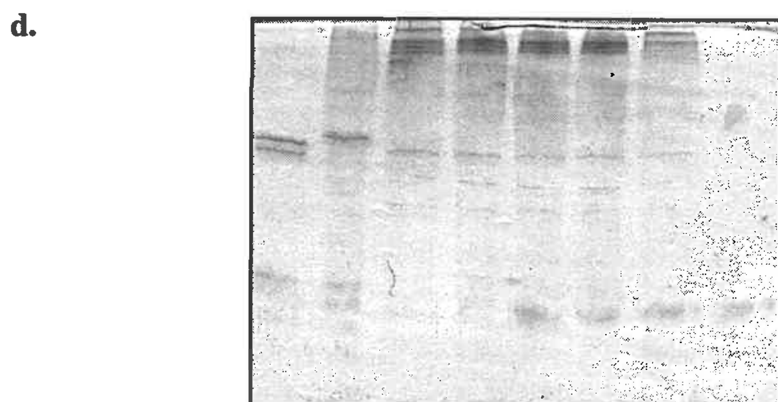

Figure 5.7: SDS PAGE and Western analysis of native and recombinant histone proteins. **a.** Amido black stained SDS PAGE of; native mouse histone (lane 1); native *Drosophila* histone (lane 2); total cell lysate from cells containing the His2AvD expression construct, pET.AvD, at 0h (lane 3) and 1h (lane 4) relative to induction; FPLC fractions of proteins prepared from the insoluble component of bacterial cell lysates expressing His2AvD that eluted from a Resource S column in 50mM NaAc pH 4.5, 6M urea (see Fig 5.3 and section 5.3.1 (ii)) at 0.45M NaCl (lane 5), 0.475M NaCl (lane 6), 0.5M NaCl (lane 7) and 0.525M NaCl (lane 8). SDS PAGE gels equivalent to that shown in (a.) were transferred to nitrocellulose and probed with crude anti-His2AvD sheep sera (**b.**), affinity purified His2AvD antibodies (**c.**) or monoclonal His2AvD antibodies (**d.**). All immunoblots were conducted with a 1:500 dilution of primary and HRP conjugated secondary antibodies (in 1% BSA 1XPBS). Blots were developed with diaminobenzidine (Harlow and Lane, 1988).

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a.

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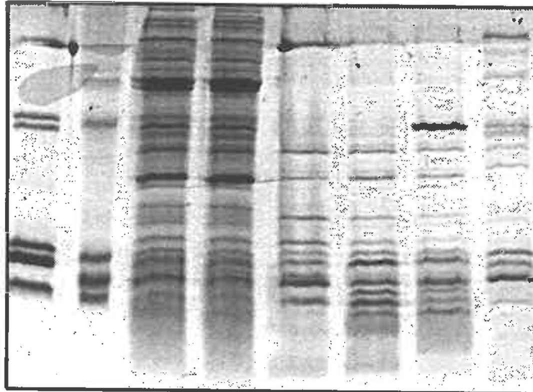
core histones { H3 H2B H2A H4



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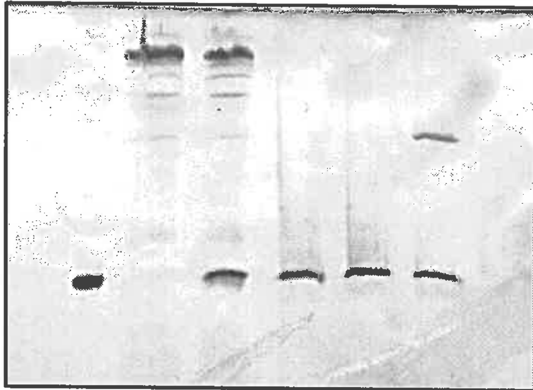

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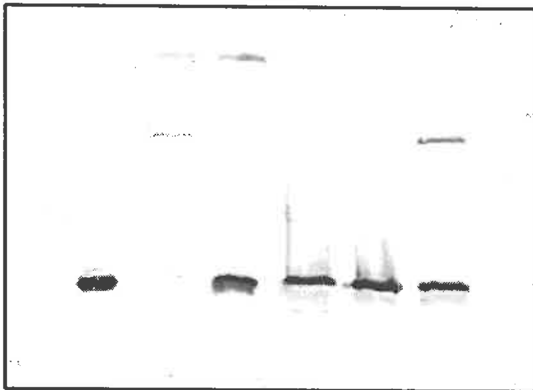

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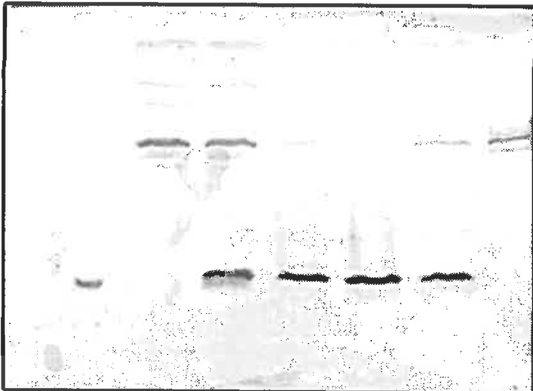


Western blots using H2A.Z or His2AvD ascites showed that these antibodies were highly specific for recombinant and isolated histone proteins (d in Figs 5.6 and 5.7 respectively).

5.4 Discussion.

Recombinant histones were produced and purified here with the eventual aim of analysing the effect of H2A.Z incorporation on chromatin structure and transcription in established *in vitro* assays. To complement these investigations antibodies were also generated to characterise the distribution of H2A.F/Z histones *in vivo.*

Recombinant histones have been used to determine the three dimensional structure of the nucleosome by X-ray crystallography (Arents *et al.*, 1991; Luger *et al.*, 1997) and to assess the effect of Sin mutant versions of histone H3 on nucleosome structure and transcription (Kurumizaka and Wolffe, 1997). Recombinant histone H2A.Z and H2A were produced here as a first step to conducting similar characterisation of nucleosomes containing this variant histone.

Affinity purified antibodies directed against His2AvD, H2A.Z and H2A.1 and monoclonal antibodies directed against His2AvD and H2A.Z were produced here to assist in the further characterisation of the H2A.F/Z class of histone H2A variants in *Drosophila* and mammals. These antibodies have a high titre and are highly specific for the proteins that they were directed against in ELISA and Western assays.

Antibodies have been used to characterise the distribution of nuclear proteins on DNA *in vivo* using two different immunoprecipitation approaches. In one method, cells are initially fixed with formaldehyde then sonicated to disrupt the cell and shear the chromatin. To this extract antibodies are added and allowed time to bind before being precipitated with an insoluble matrix (see Orlando and Paro, 1993; Orlando *et al.*, 1997; Strahl-Bolsinger *et al.*, 1997; Kuo *et al.*, 1998; Cosma *et al.*, 1999; Krebs *et al.*, 1999). In another method, mononucleosomes are isolated from nuclei by micrococcal nuclease digestion and purified by sucrose gradient sedimentation then immunoprecipitated (Hebes *et al.*, 1988, 1994; O'Neill and Turner, 1995; Crane-Robinson *et al.*, 1997). In both of these assays, DNA that coprecipitated with the bound antibodies was hybridised with a series of probes. From the studies detailed here and elsewhere (Gabrielli *et al.*, 1981; Pashev *et al.*, 1983; Wenkert and Allis, 1984; Allis *et al.*, 1986; Ridsdale and Davie, 1987; Donahue *et al.*, 1986; Stargell *et al.*, 1993), DNA probes that would be interesting for similar analysis of DNA that coprecipitated with antibodies directed

against H2A.Z include active and inducible genes, heat shock genes, silent genes, regions of heterochromatin and satellite sequences. In addition, DNA packaged by H2A.Z-containing nucleosomes could be cloned and sequenced to identify whether nucleosomes containing this variant histone have a preference for a particular base composition or contain specific motifs. Motif searches have been used to identify specific sequence features of chromatosomes (nucleosomes containing linker DNA and linker histone) isolated from bulk chromatin (Travers and Muyldermans, 1996).

It may also be possible to analyse the protein composition of immunoprecipitated chromatin that has been cross linked with formaldehyde to identify whether particular proteins interact with H2A.Z containing nucleosomes. Formaldehyde induced histone-DNA cross links can be reversed by incubation of cross linked material at 37°C for 2 days in 1% SDS, 50mM Tris pH 8.0 (Jackson, 1978). Thus, if there was a protein that specifically interacted with DNA in nucleosomes containing H2A.Z *in vivo* it could be identified after reversal of formaldehyde cross links in chromatin immunoprecipitated with H2A.Z antibodies. Considering that the chicken H2A.F/Z variant, H2A.F, co-elutes from immobilised erythrocyte chromatin with one form of histone H2B, H2B.2 (Li *et al.*, 1993), it would be interesting to see whether H2A.F/Z histones are preferentially associated with particular histone isoforms. Alternatively, or in addition, H2A.F/Z histones might be enriched in nucleosomes containing histones which are specifically modified. To this end, it has been demonstrated that chromatin fractions that are enriched for H2A.F/Z histones are also enriched in acetylated histones (Ridsdale and Davie, 1987). In other fractionation experiments, it has been demonstrated that transcriptionally active chromatin is enriched for the HMG-14/-17 proteins (Gabrielli *et al.*, 1981; Postnikov *et al.*, 1991; Bustin and Reeves, 1996). This observation raises the possibility that H2A.F/Z histones may direct HMG-14/-17 proteins to specific chromosomal domains.

In a preliminary analysis of the suitability of antibodies developed against H2A.Z and H2A.1 to recognise histones in fixed and unfixed chromatin they were used to investigate protein distribution in the nuclei of mouse cells. This analysis is presented in the next chapter in conjunction with a characterisation of histone H2A.Z expression and stoichiometery.

Chapter 6: Characterisation of mouse histone H2A.Z

6.1 Introduction

In this chapter, the levels of H2A.Z mRNA and protein derived from selected adult mouse tissues was investigated and affinity purified antibodies were used to characterise the protein localisation in tissue sections and tissue culture cells.

Previous investigations on H2A.Z gene expression have been conducted in tissue culture cells. These studies have shown that, unlike the major core histones which are produced mainly during S-phase to package the replicating DNA, H2A.Z message and protein are synthesised throughout the cell cycle (Wu and Bonner, 1981; Wu *et al.*, 1982; White and Gorovsky, 1988; Dalton *et al.*, 1989). Hatch and Bonner (1990) have further reported that the H2A.Z transcript levels decrease 20 fold in Chinese hamster ovary cells when they stop proliferating and become quiescent. Here, the amount of H2A.Z message present in total RNA extracted from different tissues of the adult mouse was investigated by Northern analysis. Histone H2A.Z message levels were found to be directly proportional to cell proliferation rate in each tissue. In tissues with a high rate of cell turnover such as the spleen and lung, H2A.Z transcripts are up to two orders of magnitude more abundant than in liver, kidney, heart or skeletal muscle which have little or no cell renewal. Since H2A.Z protein was found at the same concentration, relative to the core histones, in the nuclei of all tissues examined it appears that H2A.Z mRNA synthesis reflects a requirement for maintenance of histone H2A.Z protein levels at 5-10% of the major H2A.

Using an antibody directed against the *Tetrahymena* hv1 protein, Allis *et al.* (1982) reported nucleolar localisation of H2A.Z in several mammalian cell lines. This observation is difficult to interpret, however, because these particular antibodies did not cross react at all against calf H2A.Z or *Drosophila* His2AvD and only reacted very weakly against mouse H2A.Z in immunoblots (Allis *et al.*, 1986). In *Drosophila*, staining of polytene chromosomes using antibodies raised against His2AvD demonstrated that this protein localises to interband regions and developmentally activated puffs but not heat shock induced puffs (Donahue *et al.*, 1986; van Daal and Elgin, 1992). In support of these, and other immunohistochemical studies (Wenkert and Allis, 1984; Allis *et al.*, 1986; Stargell *et al.* 1993), a non-homogeneous distribution of H2A.F/Z protein in the nucleus has also been observed at a biochemical level. Analysis of the protein constituents of fractionated chromatin have shown that H2A.F/Z and transcribed sequences are enriched in fractions that are more accessible to nucleases

than bulk chromatin (Gabrielli *et al.*, 1981; Ridsdale and Davie, 1987). Here, using affinity purified antibodies, chromatin containing histone H2A.Z was shown to be generally distributed throughout the nucleus but was not associated with satellite DNA sequences.

6.2 Methods

6.2.1 RNA isolation from mouse tissues.

RNA was isolated from 1g samples of mouse tissues using the same protocol for isolation of RNA from 1ml settled volume of *Drosophila* embryos (section 5.2.2).

6.2.2 RNA isolation from mouse ES cells

RNA was isolated from $1.5X10^8$ pelleted ES cells using the protocol outlined for isolation of RNA from *Drosophila* larvae (section 3.2.2).

6.2.3 Northern analysis.

RNA samples were made up to 50µl in 1XMOPS, 6.5% (w/v) formaldehyde, 50% (v/v) deionised formamide and heated at 65°C for 5min before being loaded onto a 1% (w/v) agarose gel prepared with 1XMOPS buffer and 1.2% (w/v) formaldehyde. Electrophoresis was conducted at 6V/cm (between electrodes) in 1XMOPS until a bromophenol blue dye marker had migrated approximately three quarters of the length of the gel. Following electrophoresis, RNA was transferred from the gel to NYTRAN nylon membrane (Schleicher and Schuell) by capillary transfer in 20XSSC for 18h and then crosslinked to the membrane by UV irradiation. The membrane was then prehybridised for 4h at 42°C in 1M NaCl, 50mM Tris pH 7.4, 40% (v/v) de-ionised formamide, 10% (w/v) PEG, 1% (w/v) SDS, 0.5XDenhard's solution. Probe (25ng in 50µl)/salmon sperm (300µl) mix was denatured by heating at 100°C for 10 minutes, added to the membrane in prehybridisation mix and hybridised for 16h at 42°C . The H2A.Z probe was a NcoI/HindIII fragment derived from pET.AZ. A plasmid containing the Glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) (Lints *et al.* 1993) was used as a probe for mRNA loading. Probes were radioactively labelled as previously outlined (2.3.13). Filters were washed once in 2XSSC, 0.1% (w/v) SDS for 5 minutes at 42°C (no further washing was required for GAPDH probing) then, (for H2A.Z only) in the same buffer at 65°C for 5 minutes and in 0.2XSSC, 0.1% (w/v) SDS for 5 minutes at 65°C. H2A.Z probing was conducted first and the filter was stripped

for GAPDH probing by boiling in 0.1% (w/v) SDS. Filters were exposed on Kodak phosphorimager screens which were scanned and quantitated on a Molecular Dynamics phosphorimager.

6.2.4 Histone isolation from mouse tissues.

Liver (Li), kidney (Ki), lung (Lu) and spleen (Sp) nuclei were isolated as per Wallace *et al.* (1971). ES cell (Es), heart (He) and skeletal muscle (Mu) nuclei were isolated according to Neufer *et al.* (1993). All aqueous solutions used in the preparation of nuclei included 0.2% (v/v) βme, 0.2mg/ml PMSF and 1% (v/v) thiodiglycol (to prevent methionine oxidization (Zweidler 1978)). To a suspension of nuclei in 1X Hewish and Burgoine buffer A (Li, Ki, Lu and Sp) or 1X lysis buffer (ES, Mu, He) an equal volume of 0.5M H_2SO_4 was added. Nuclear debris was removed by centrifugation for 5min in a microfuge (12Krpm). The supernatant was transferred to a fresh tube and 6 volumes of acetone:βme:thiodiglycol (100:2:1) was added. After a 16h incubation at -20°C histones were pelleted by centrifugation for 5min in a microfuge (12Krpm), washed with acetone:βme:thiodiglycol (100:2:1) and then desiccated. Pellets were resuspended in 8M urea, 5% (v/v) βme, 1% (v/v) thiodiglycol and quantitated in a Bradford assay (6.2.5).

6.2.5 Quantitation of proteins by Bradford assay.

Proteins were quantitated using Bradfords reagent purchased from Biorad. Using the manufacturers protocol, known concentrations of BSA were used to construct a standard curve from which sample concentrations were extrapolated.

6.2.6 SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was conducted as outlined in section 5.2.5.

6.2.7 Western analysis

Western analysis was conducted as outlined in section 5.2.11.

6.2.8 Immunostaining of tissue culture cells and tissue sections using affinity purified antibodies.

Frozen fresh unfixed tissue embedded in Tissue Tec OCT 4583 compound (Sakura) were cut into 5μm sections on a cryostat and layered onto poly-l-lysine coated microscope slides. Sections were air dried for 2h at room temperature before further processing.

Mouse STO cells were grown in chamber slides to near confluence and were fixed in 100% methanol for 2min before further processing.

Prepared tissue sections or fixed tissue culture cells were washed in PBS (three changes of 5min each) and blocked by incubating with 1% (w/v) BSA in PBS for 10min. Affinity purified primary antibody was applied to the sample at a 1:100 dilution in 1% (w/v) BSA in PBS and incubated at room temperature for 1h. Samples were washed and the secondary antibody (Donkey anti sheep-biotin conjugate, Sigma) was applied at a dilution of 1:100 in 1% (w/v) BSA in PBS and incubated for 1h at room temperature. After washing, a Texas red streptavidin conjugate (Sigma) was applied in 1% (w/v) BSA in PBS and allowed to bind for 30mins. Following removal of the streptavidin Texas red conjugate, samples were incubated with 0.05μg/ml Hoechst 33258 in PBS for 5min, washed with PBS and mounted with 30mM Tris pH 9.0, 70% (v/v) glycerol, 2.5% (w/v) N-propyl-galate (antifade) ready for immunofluorescence and photography. Images were captured on Kodak Ektachrome film using a Ziese microscope.

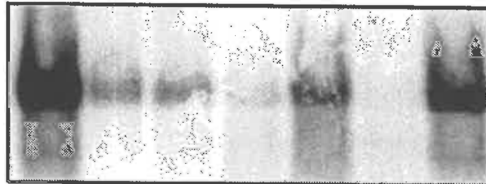
6.3 Results

6.3.1 Northern analysis of H2A.Z transcript levels in adult mouse tissues.

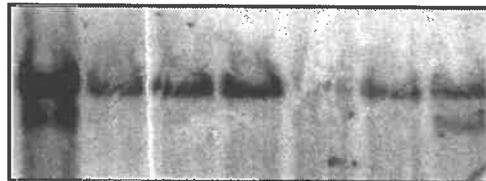
Histone H2A.Z expression was investigated by Northern analysis (6.2.2) of total RNA samples isolated from the selected adult CBA mouse tissues; liver, kidney, heart, lung, skeletal muscle, spleen and mouse E14 ES cells (6.2.1, 6.2.2). H2A.Z message levels were examined by hybridising a Northern blot with an NcoI/HindIII H2A.Z pseudogene probe derived from the plasmid pET.AZ (2.2.11 and Clarkson *et al.* in prep). Following hybridisation and high stringency washing of the filter, a single 1.3kb band was detected (Fig 6.1a). Normalisation of histone H2A.Z message levels against GAPDH message levels (Fig 6.1b) demonstrates that histone H2A.Z mRNA is approximately two orders of magnitude more abundant in spleen and lung than in liver, kidney, heart or skeletal muscle (Fig 6.1c).

Figure 6.1: Northern analysis of message levels in selected adult CBA mouse tissues; liver, kidney, heart, lung, skeletal muscle, spleen and mouse E14 ES cells probed for **a.** H2A.Z and **b.** mGAPDH. **c.** Normalisation of histone H2A.Z message levels against GAPDH message levels demonstrating that histone H2A.Z mRNA is approximately two orders of magnitude more abundant in spleen and lung than in liver, kidney, heart or skeletal muscle.

a.



b.



c.

Tissue	ES	Li	Ki	He	Lu	Mu	Sp
H2A.Z % GAPDH	187	38	19	4	250	11	586

6.3.2 Western analysis of H2A.Z protein levels in adult mouse tissues.

Histone extracts from the adult CBA mouse tissues; liver, kidney, heart, lung, skeletal muscle, spleen and mouse E14 ES cells were subject to a Western analysis (5.2.11) with affinity purified antibodies directed against the carboxy terminus of H2A.Z (5.2.7). Precipitated histones were resuspended in 8M urea, 5% βMe, 1% thiodiglycol and quantitated in a Bradford assay. For each sample 3μg of histone protein per lane was loaded onto two equivalent SDS polyacrylamide gels and subject to PAGE. One gel was stained with amido black (Fig 6.2a) and the other was transferred to nitrocellulose and subject to western analysis with affinity purified anti-H2A.Z antibodies and developed with chemiluminescence (Fig 6.2b). Comparison of the stained gel and Western blot demonstrates that H2A.Z protein is present at essentially the same level, relative to histone H4, in all tissues (confirmed by densitometry - data not shown).

6.3.3 Immunohistochemical analysis of H2A.Z protein localisation in mouse STO cells and adult mouse tissue sections.

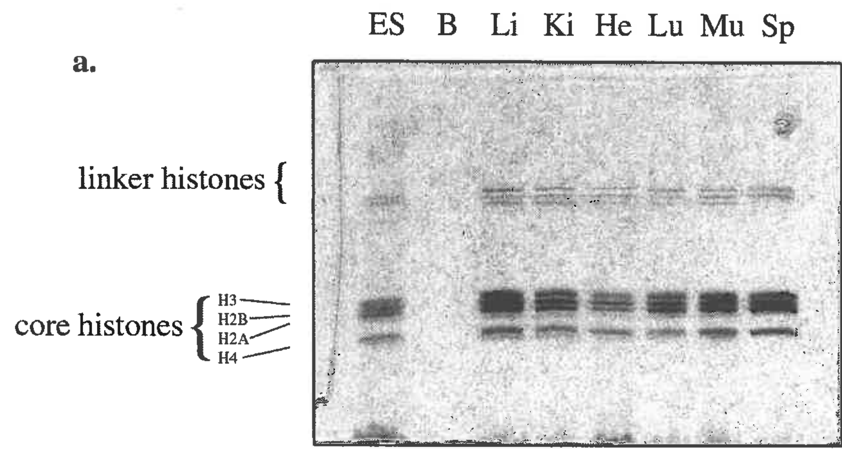
To characterise the subcellular distribution of H2A.Z protein, fixed tissue culture (STO) cells and frozen unfixed mouse tissue sections were probed with affinity purified antibodies directed against H2A.Z (6.2.8). The antibody-antigen complex was detected using a biotinylated secondary antibody and streptavidin conjugated to the fluorophore Texas Red. As can be seen in Figs 6.3 and 6.4, H2A.Z staining is seen in most of the nucleus but is absent from discrete foci that correspond to regions of intense Hoechst 33258 fluorescence. In mouse these AT rich regions correspond to repetitive satellite DNA sequences found at, or near the centromere (Mayfield and Ellison, 1976). It seems likely that this staining pattern reflects the true distribution of H2A.Z rather than a restricted accessibility of antibodies to regions containing satellite sequences because affinity purified H2A.1 antibodies were not excluded from these regions in similarly prepared samples (see Fig 6.3). In support of this result, H2A.Z was not detected in a previous biochemical analysis of proteins associated with satellite DNA sequences (see Fig 3 in Pashev *et al.* 1983).

6.4 Discussion.

In the adult mouse, histone H2A.Z message levels in spleen and lung are approximately two orders of magnitude more abundant than in liver, kidney, heart or skeletal muscle (Fig 6.1). Similar degrees of difference are observed for the cellular turnover rates of these tissues. The amount of H2A.Z protein present, relative to other histones, was

Figure 6.2: Western analysis of histone H2A.Z stochiometry in adult CBA mouse tissues; liver, kidney, heart, lung, skeletal muscle, spleen and mouse E14 ES cells. For each sample 3μg of histone protein per lane was loaded onto two equivalent SDS polyacrylamide gels and subject to PAGE. One gel was stained with amido black (**a**) and the other was transferred to nitrocellulose and subject to western analysis with affinity purified anti-H2A.Z antibodies and developed with chemiluminescence (**b**). Comparison of the stained gel and western blot demonstrates that H2A.Z protein is present at essentially the same level, relative to histone H4, in all tissues.

a.



b.

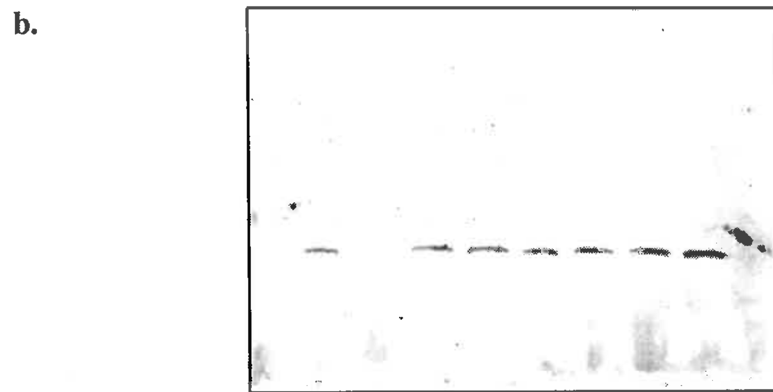
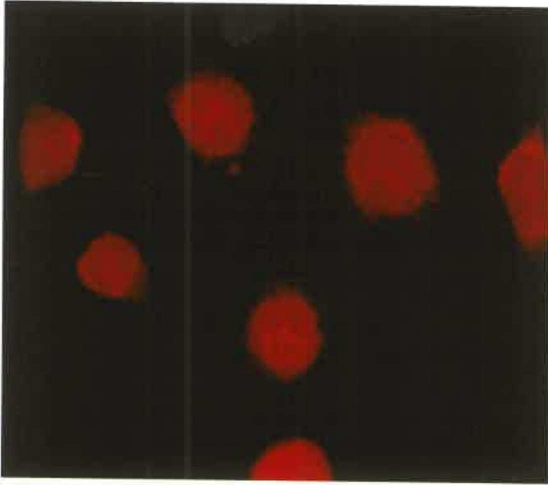


Figure 6.3: Histone H2A.Z is generally distributed throughout nuclei in mouse STO cells but is excluded from regions containing satellite sequences. **a.** Mouse STO cells showing the localisation of H2A.Z protein detected with affinity purified antibodies, counterstained with Hoechst 33258 (**c.**) and visualised by phase contrast (**e.**). Histone H2A.1 antibodies were not excluded from regions containing satellite sequences in similarly prepared samples. **b.** Localisation of H2A.1 in STO cell nuclei detected with affinity purified antibodies. **d.** and **f.** are the same field shown in (b.) counterstained with Hoechst 33258 or visualised by phase contrast respectively. Scale bar = 10μm.

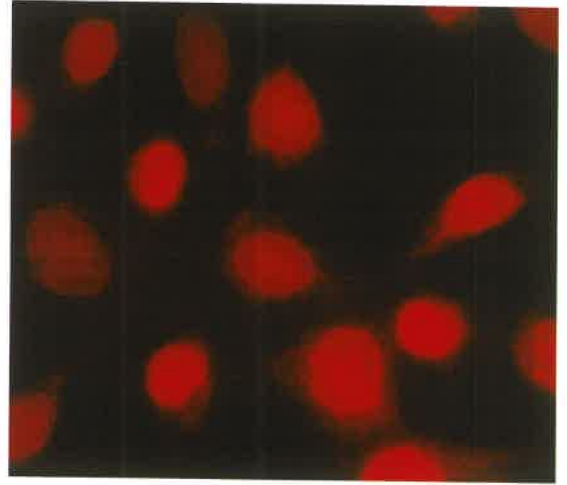
H2A.Z

H2A.1

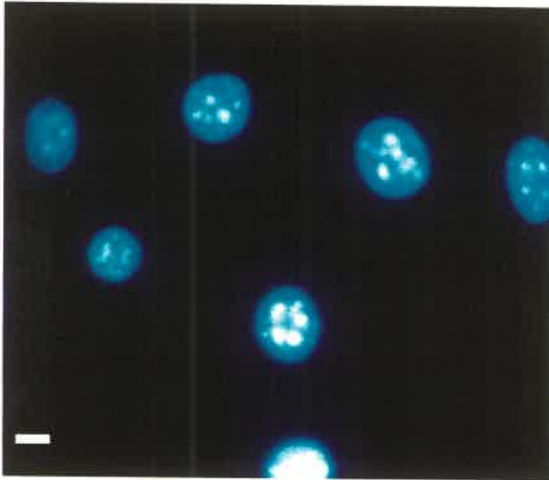
a.



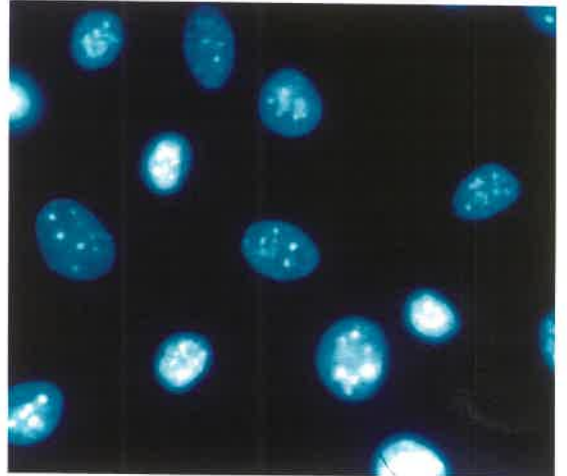
b.



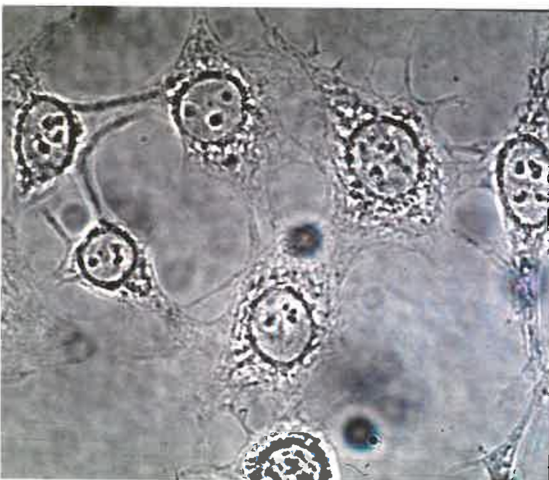
c.



d.



e.



f.

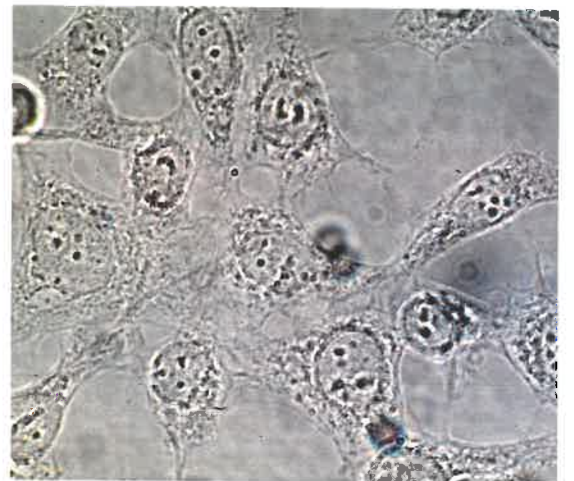
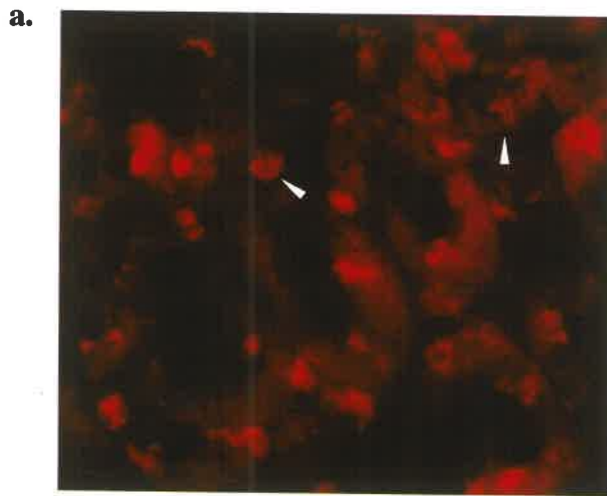


Figure 6.4: In sections of mouse tissue histone H2A.Z is generally distributed throughout the nuclei but is excluded from regions containing satellite sequences. **a.** Kidney tissue sections showing the distribution of histone H2A.Z protein detected with affinity purified antibodies. **c.** The kidney section shown in (a.), counterstained with Hoechst 33258. **e.** The same section simultaneously showing the phase contrast image and Hoechst 33258 counterstaining. **b.** The distribution of H2A.Z in mouse spleen sections detected with affinity purified H2A.Z antibodies. **d.** and **f.** are the same field of cells shown in (b.) visualised under the conditions given for kidney sections in (c.) and (e.) respectively. Scale bar = 10μm.

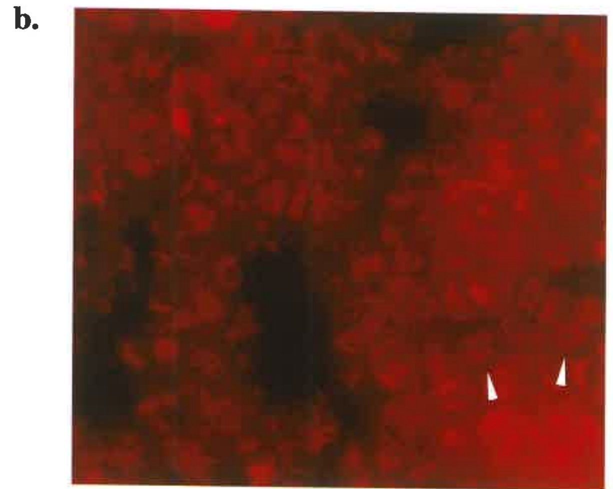
Kidney

Spleen

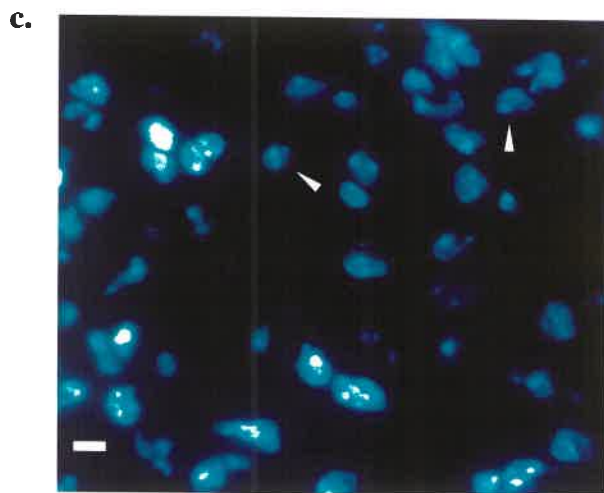
a.



b.



c.



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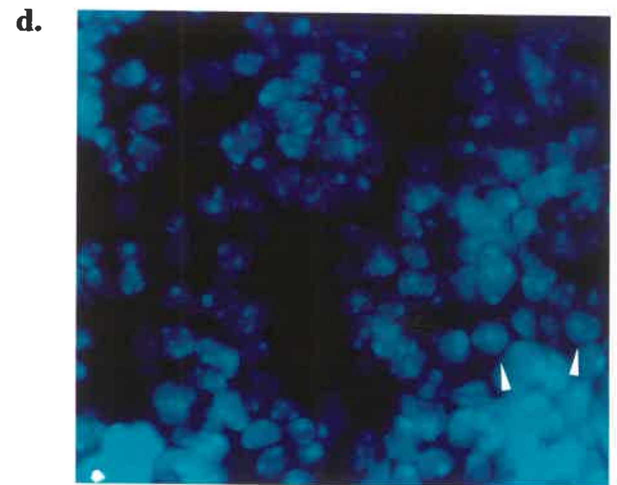
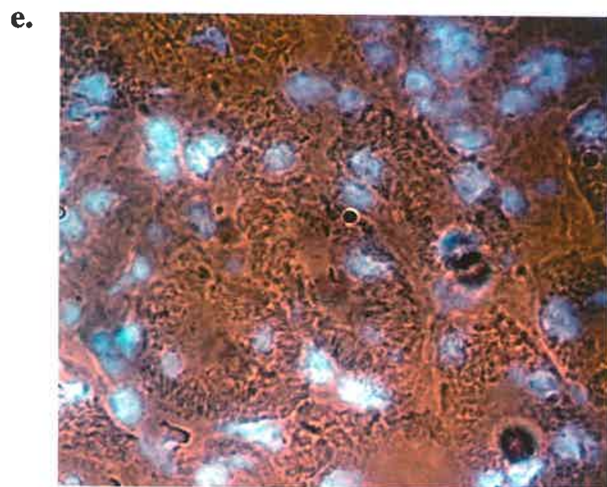
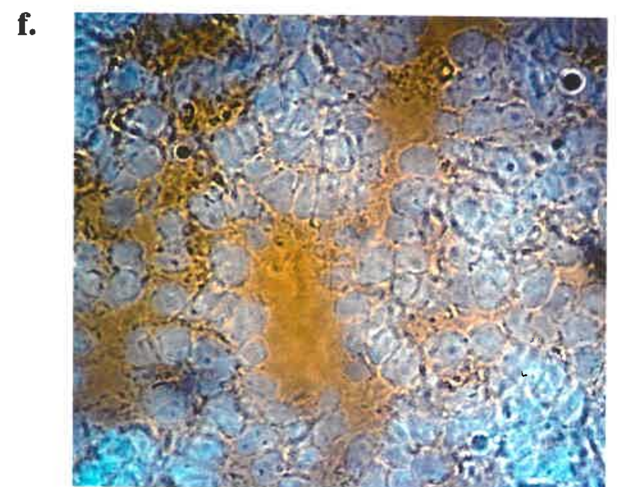


e.



f.



essentially the same in all of these tissues. A similar scenario has also been observed in the comparison of H2A.F/Z message and protein levels during *Drosophila* (van Daal and Elgin, 1992) and chicken (Whiting, 1988) development and in adult chicken tissues (Whiting, 1988). Previous studies have also shown that the production of H2A.Z mRNA is downregulated as tissue culture cells are induced either to enter quiescence or differentiate (Hatch and Bonner, 1990). Thus, it appears that H2A.Z mRNA synthesis reflects a requirement for maintenance of histone H2A.Z protein levels at 5-10% of the major H2A (West and Boner, 1980; Palmer, *et al.*, 1980; Wu and Bonner, 1981; Wu *et al.*, 1982).

The finding that histone H2A.Z protein levels are maintained at a constant level in different cell types suggests that this histone may associate with particular DNA sequences in all nuclei. However, characterisation of another histone variant, which is targeted to particular regions of the nucleus, suggests that this is not likely to be the case for histone H2A.Z. The centromere specific histone H3 variant, CENP-A, is targeted to centromeric sequences through a combination of amino acid differences between it and histone H3, and regulated expression of the gene late in S-phase, coincident with the replication of centromeric DNA (Sullivan *et al.*, 1994; Shelby *et al.*, 1997). Three domains within the histone fold region of the CENP-A protein are required for it to be efficiently targeted to centromeric sequences. Two of these domains are located in regions where, in the crystal structure of the nucleosome, equivalently positioned residues of histone H3 interact with DNA (Shelby *et al.*, 1997). The third targeting region of CENP-A is located where one H3 molecule interacts with another in the nucleosome. Since CENP-A self associates *in vivo*, it has been proposed that this region directs the assembly of homotypic nucleosomes containing two CENP-A molecules (Shelby *et al.*, 1997). Thus, major determinants of CENP-A targeting include synthesis of the protein at a time when centromeric chromatin is being replicated and a cooperative array of specific DNA contact sites in CENP-A nucleosomes. Unlike CENP-A, the synthesis of histone H2A.F/Z occurs throughout the cell cycle (Wu and Bonner, 1981; White and Gorovsky, 1988; Dalton *et al.*, 1989) and thus does not correspond to the replication of particular DNA sequences. In addition, the regions important for H2A.F/Z function in *Drosophila* are not involved in interactions with nucleosomal DNA (see chapter 3).

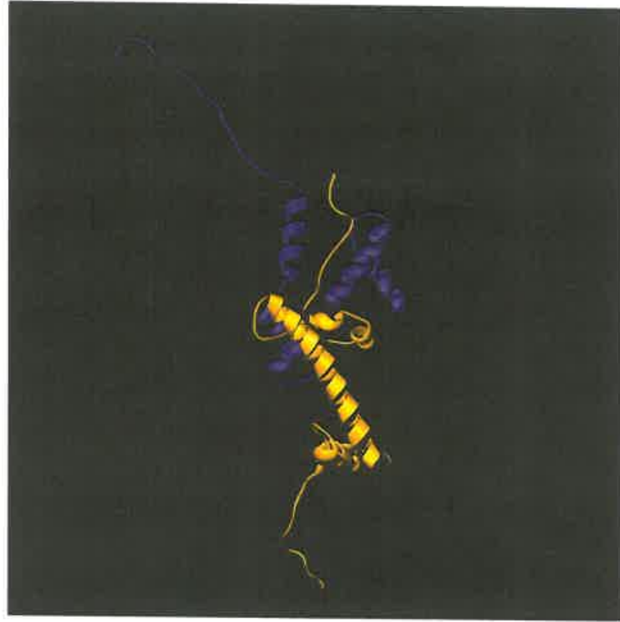
Immunocytological analysis of *Tetrahymena thermophila* hv1 has shown that H2A.F/Z co-localises to regions of transcriptional activity in the macronucleus and in the micronucleus during conjugation (Wenkert and Allis, 1984; Allis *et al.*, 1986; Stargell *et al.* 1993). Although hv1 localises to regions of transcriptional activity in

Tetrahymena thermophila, it appears that this relationship is more complex in other organisms. On the polytene chromosomes in *Drosophila melanogaster* His2AvD localises to interbands and developmentally activated puffs but not heat shock induced puffs (Donahue *et al.*, 1986; van Daal and Elgin, 1992). In chicken, a constant ratio of H2A.F/Z protein, relative to major histones, is observed in transcriptionally active erythroblasts and transcriptionally inactive erythrocytes (Whiting, 1988). Nucleolar localisation of H2A.Z has been reported in several mammalian cell lines using an anti-hv1 antibody. This result is difficult to interpret however, as these antibodies did not cross react with calf H2A.Z or *Drosophila* His2AvD in immunoblots. To resolve this issue, affinity purified antibodies directed against the C-terminus of H2A.Z were generated here to investigate protein localisation in tissue sections from adult mouse liver and spleen and in mouse STO tissue culture cells. The antibodies detected H2A.Z protein throughout the nucleus except at discrete foci that counterstain strongly with Hoechst 33258. In mouse, these regions correspond to AT rich satellite sequences found at centromeres (Pardue and Gall, 1970; Hilwig and Gropp, 1972; Mayfield and Ellison, 1976). Interestingly, in a previous biochemical analysis of histone proteins associated with these sequences, H2A.Z was not detected (see Fig 3 in Pashev *et al.*, 1983). Furthermore, in isolated nuclei it has been demonstrated that H2A.F/Z is enriched in endonuclease sensitive chromatin whereas satellite sequences are packaged into chromatin that is resistant to endonuclease digestion (Gabrielli *et al.*, 1981; Dixon and Burkholder, 1985; Ridsdale and Davie, 1987).

To investigate the possibility that H2A.Z might be excluded from these regions because it is unable to form nucleosomes with the centromere specific histone H3 variant, CENP-A, the points of interaction between histone H2A and H3 were examined in the crystal structure of the nucleosome. The only region of the H2A protein that is in close proximity to H3 includes residues Q112 to P117. These residues are part of the extended C-terminal tail of H2A and are positioned between, but slightly out of the plane of, the histone H3 N-helix (G44 to T58) and the C-terminal half of the $\alpha 2$ helix (A95 to V117). Comparison of the amino acid sequences of H2A.Z and CENP-A with their core histone counterparts in these regions revealed that there are a number of different residues at these sites (Fig 6.5). In addition to sequence differences at the H2A-H3 interaction surface, another feature of CENP-A that makes it a suitable candidate for the exclusion of H2A.Z from centromeric regions relates to the mechanics of nucleosome assembly. On newly replicated DNA, $(H3\text{-}H4)_2$ tetramers are deposited first and this is followed by the slower addition of H2A-H2B dimers (Worcel *et al.*, 1978). Thus, the initial assembly of $(CENP\text{-}A\text{-}H4)_2$ tetramers on centromeric DNA

Figure 6.5: Regions of interaction between H2A and H3 proteins in the crystal structure of the nucleosome highlighting residues which differ in H2A.Z and CENP-A at the interaction surface. **a.** Ribbon trace diagram illustrating the tertiary structures interacting H2A(yellow) and H3 (blue) molecules in the nucleosome. **b.** View of histone H3 in (a.) after removal of histone H2A showing amino acid differences H3 and CENP-A (rendered as space filling molecules) in the region that interacts with histone H2A (N-helix residues G44 to T58 and residues A95 to V117 in the C-terminal half of helix II). **c.** View of histone H2A from the back of (a.) after the removal of histone H3 showing amino acid differences between H2A and H2A.Z (rendered as space filling molecules) in the region that interacts with H3 (residues Q112 to P117 in the C-terminal tail of H2A). This region of the protein corresponds to the M7 region of the mutagenesis study conducted in chapter 3.

a.



b.

c.

after replication could direct the incorporation, or exclusion, of particular dimer combinations.

In summary, the studies detailed here demonstrate that histone H2A.Z message is synthesised at a level that maintains a relatively constant concentration of protein, relative to the core histones, in all tissues of the adult mouse. Immunolocalisation of H2A.Z protein found that it was widely distributed throughout nuclei in tissue culture cells in sections of adult mouse tissues but was excluded from regions containing satellite DNA sequences. Satellite sequences are closely associated with centromeres in mouse (Pardue and Gall, 1970; Hilwig and Gropp, 1972; Mayfield and Ellison, 1976). One way that H2A.Z could be excluded from chromatin is through an inability to form nucleosomes with the centromere-specific histone H3 variant CENP-A. In support of this possibility, the amino acid sequences of H2A.Z and CENP-A differ from their core histone counterparts in domains where H2A and H3 interact in the nucleosome.

Chapter 7: Final Discussion.

7.1 Introduction

DNA in the eukaryote nucleus is packaged by histone proteins into chromatin. In addition to their role in DNA packaging, histone proteins are intimately involved in regulating processes that involve DNA like replication, recombination, transcription and mitosis. Recently there has been a great deal of attention focused on understanding the mechanisms of regulatory processes that involve chromatin. From this attention it has become apparent that the structure and function of chromatin can be modified by a number of different mechanisms including; multi-protein complexes which disrupt nucleosome structure; post translational modification of histones; and by incorporation of histone variants (reviewed in Wolffe and Pruss, 1996; Workman and Kingston 1998; Kingston and Narlikar, 1999; Strahl and Allis, 2000).

The central aim of the work conducted in this thesis was to investigate the function of histone H2A variants of the H2A.Z/F class. Isolation and characterisation of the genes encoding H2A.F/Z histones in a variety of eukaryotes has shown that the amino acid sequence of this variant histone is highly conserved across species and is approximately 40% different from the major H2A amino acid sequence (Jackson *et al.* 1996). These features indicate that H2A.F/Z histones perform a different function (or functions) to the H2A histones. This suggestion is supported by two other observations. Firstly, null mutations in the H2A.F/Z genes are lethal in mice (Thonglairoam 1996), *Drosophila melanogaster* (van Daal and Elgin 1992) and *Tetrahymena thermophila* (Liu *et al.* 1996b). This result is significant because it also highlights the fundamental importance of H2A.F/Z function. Secondly, the viability of a yeast strain lacking an endogenous H2A gene could be restored with a *Tetrahymena thermophila* H2A gene but not with the gene encoding the H2A.F/Z homologue, *hv1* (Liu *et al.* 1996a).

7.2 Discussion

7.2.1 His2AvD and transcription

Although the actual function of H2A.F/Z is unknown, characterisation of the distribution of this variant histone has shown that it temporally and spatially colocalises with transcriptionally competent chromatin (Gabrielli *et al.*, 1981; Wenkert and Allis, 1984; Allis *et al.*, 1986, Ridsdale and Davie, 1987; Stargell *et al.*, 1993). Two results presented in this thesis further support an association between H2A.F/Z histones and

transcription. These results, which are detailed in chapters 3 and 4, were generated in two different experimental systems and are considered further here in relation to the implications that they have for the function of H2A.F/Z histones.

In chapter 3, it was shown that the *His2AvD* null mutant undergoes a protracted third instar and then dies without pupating. Analysis of gene expression in null mutants found that during third instar, the *Sgs4* gene, which is normally activated by the middle of this stage, was not expressed. After the time where *Sgs4* would normally be activated, the constitutive *5C actin* gene continued to be expressed and heat shock genes could still be induced in null mutants. These results indicate the arrested development and eventual death of null mutants might occur as a consequence of failure to express developmentally activated genes. In addition, the gene expression profile in null mutants raises the possibility that His2AvD might be required for the expression of developmentally activated genes but not for heat shock inducible or constitutively expressed genes. In support of this, a previous examination of His2AvD distribution, on the highly polytenised chromosomes of *Drosophila* salivary glands, found that His2AvD was present on developmentally activated puffs but not on puffs activated by heat shock (Donahue *et al.*, 1986). If His2AvD is required for the activation of developmental genes then the arrest in null mutant development during third instar suggests that the protein might not be required prior to this stage. However, His2AvD mRNA is maternally loaded into the maturing oocyte (van Daal and Elgin, 1992), therefore, it seems more likely that disrupted development in null mutants occurs because maternally derived His2AvD protein is depleted to critical levels during third instar. Since the *5C actin* and heat shock genes are activated or primed for transcription, respectively, prior to third instar (Grazioso, *et al*, 1980; Fyrberg *et al.*, 1983; Vigoreaux and Tobin, 1987; Andres *et al.*, 1993; Weber, *et al*, 1995), then it is possible that these genes may also require His2AvD to establish transcriptional competence. If this were the case, then the absence of His2AvD on heat shock puffs would indicate that the variant histone could be required for activation but is not necessary for transcription *per se*.

In chapter 4, *His2AvD*^{810} null mutant lethality was rescued with a modified genomic fragment that encoded a fusion of the His2AvD protein with the green fluorescent protein of the jellyfish *Aequorea victoria*. The His2AvD-GFP fusion protein produced from this transgene was used to characterise the positioning of His2AvD on chromosomes *in vivo* and in real time. In *Drosophila* blastoderm embryos, His2AvD-GFP flourescence was first observed in nuclei during nuclear cycle 9. The entry of His2AvD-GFP into nuclei at this stage coincides with the initiation of embryonic

transcription between nuclear cycles 8 and 10 (Prichard and Schubiger, 1996). This observation is consistent with immunolocalisation studies in *Tetrahymena thermophila* which demonstrated that hv1 temporally and spatially associates with with regions of actively transcribing chromatin in the nucleus (Wenkert and Allis, 1984; Allis *et al.*, 1986, Stargell *et al.*, 1993).

When His2AvD first enters nuclei at the onset of zygotic transcription in *Drosophila* embryos, its distribution is initially restricted to the nuclear periphery. Interestingly, at the onset of zygotic transcription in the 2 cell mouse embryo the distribution of RNA polymerase II and histone H4 acetylation on lysines 5, 8, and/or 12 is similarly restricted to the nuclear periphery (Worrad *et al.*, 1995). Although these events were characterised in different organisms they raise the possibility that the initiation of transcription, import of H2A.F/Z histones, and histone acetylation may occur simultaneously. Unfortunately the location of RNA polymerase II or acetylation state of histones during *Drosophila* embryogenesis has not been investigated to date. However, studies conducted in *Xenopus* which have shown that production of a H2A.F/Z homologue, H2A.ZI, and histone hyperacetylation coincide with the initiation of transcription in this organism during the midblastula transition (Newport and Kirschner, 1982; Almounzni *et al.*, 1994; Iouzalen *et al.*, 1996). The conincidence of transcription, acetylation and import of H2A.F/Z would be consistent with a model of H2A.F/Z function where the incorporation of this histone variant would promote the recruitment or action of transcriptional activators. Results obtained from examination of activation at the yeast *HO* promoter, however, indicate that the coincidence of transcription, acetylation and presence of H2A.F/Z histones does not necessarily mean that H2A.F/Z directly promotes the action of acetylases. At this promoter, the transcription factor Swi5p recruits the SWI/SNF chromatin remodeller and then the Gcn5p containing histone acetylase complex, SAGA, associates with the promoter (Cosma *et al.*, 1999). Thus, if His2AvD promoted acetylation it could do so by improving the activity and/or recruitment of the SWI/SNF or Gcn5 complexes or by promoting the binding of transcription factors.

Fly lines containing the His2AvD-GFP fusion construct were also used to examine the distribution of His2AvD throughout the cell cycle. In this investigation it was found that His2AvD remains associated with chromatin throughout the cell cycle including during mitosis when transcription is silenced. The persistance of His2AvD on mitotic chromosomes indicates that the presence of His2AvD does not necessarily correlate with the presence of transcriptionally active chromatin. However, the demonstration that many of the proteins involved in transcription are displaced from chromosomes

during condensation (see section 1.6.7), raises the interesting possibility that one function of His2AvD could be to mark genes for rapid reactivation of transcription after mitosis.

In addition to analysis of *Drosophila* His2AvD, some characterisisation of the mouse homologue, H2A.Z was conducted. In chapter 6, the subcellular location of mouse H2A.Z was investigated in tissue sections and cell culture monolayers using antibodies directed against the carboxy terminus of the protein. H2A.Z protein was detected throughout the nucleus except at discrete foci that counterstain strongly with Hoechst 33258. In mouse, these regions correspond to AT rich, transcriptionally silent, satellite sequences found at centromeres (Mayfield and Ellison, 1976). While this observation does not directly support a role for H2A.F/Z in transcription, it is consistent with demonstrations that H2A.F/Z histones are preferentially incorporated into chromatin containing transcribed sequences (see section 1.4).

7.2.2 Functional domains of His2AvD

From studies of transcriptional activation it has become apparent that the activation of transcription is a multistep process that is initiated by the binding of transcription factors to their consensus recognition sites (reviewed in Hernandez, 1993; Zawel and Reinberg, 1995; Lee and Young, 1998). In chromatin, this binding is influenced by the assembly of nucleosomes on replicating DNA during S-phase, nucleosome positioning, nucleosome remodelling and histone acetylation (see section 1.6; reviewed in Wolffe and Pruss, 1996; Workman and Kingston 1998; Kingston and Narlikar, 1999; Strahl and Allis, 2000). Once bound, the transcriptional activator(s) can recruit the basal transcription apparatus, chromatin remodellers and/or histone acetylases. Where transcription factors recruit chromatin remodellers and/or histone acetylases, the action of these complexes can then facilitate the binding of other factors and/or the basal transcription apparatus. After all of the elements necessary for transcription have been assembled at the promoter, and transcription has been initiated, chromatin can further influence transcription by affecting RNA polymerase processivity.

Studies reported here and elsewhere, support the possibility that H2A.F/Z histones are involved in transcription (see section 1.4 and discussions to chapters 3 and 4). Information derived from the analysis of the role of core histones have indicated that the incorporation of H2A.F/Z histones into chromatin could affect transcription at a number of steps in the initiation process or during transcript elongation. In an effort to obtain a

better understanding of the role that H2A.F/Z histones could play in transcription, the domains of the protein that distinguish its function from the major H2A were identified. Prior to the commencement of this project, it had been demonstrated in *Drosophila* that lethality arising from a null mutation in the H2A.F/Z homologue, *His2AvD*, could be rescued with a 4.1kb genomic fragment containing the *His2AvD* gene (van Daal and Elgin, 1992). To determine the regions of His2AvD that functionally distinguish it from H2A, regions in this *His2AvD* genomic fragment were mutated, *in vitro*, so that they encoded the equivalently positioned H2A amino acids. These mutant transgenes were then transformed into *Drosophila* and assessed for their ability to rescue null mutant lethality. This study found that only the M6 domain is absolutely essential for the function of His2AvD *in vivo*. This region is located at the C-terminus of the protein, not in the histone fold. The function of His2AvD is also severely compromised by substituting His2AvD residues with those found in H2A in the adjacent M7 region. In the crystal structure of H2A containing nucleosomes, residues in these regions are predominantly involved in interactions at the dimer-tetramer interface. Some of the dimer-tetramer interactions directed through the M6 domain appear to be involved in stabilising the alignment of the dimer and tetramer to produce a continuous helical ramp for DNA to follow around the surface of the histone octamer (Luger *et al.*, 1997).

Residues in the M6 domain also contribute to the formation of an acid patch on the surface of the nucleosome that is exposed to solvent. In nucleosome crystals it was revealed that this acid patch binds the H4 N-terminal tail of the adjacent nucleosome (Luger *et al.*, 1997). It has been proposed that this interaction may contribute to the formation of higher order chromatin structures because nucleosome arrays are less able to form these structures when histone tails have been removed by partial digestion with trypsin (Schwarz *et al.*, 1996; Moore and Ausio, 1997; Tse and Hansen, 1997). The function of His2AvD therefore appears to differ from H2A in a region of the protein that is involved in intranucleosomal dimer-tetramer interactions and internucleosomal H2A-H4 interactions. Analysis of core histone mutants has shown that replacement of amino acids which participate in these interactions can have profound effects on transcription, chromatin structure and viability. In chapter 3, consideration of these studies was used to generate three models to explain how His2AvD might facilitate transcription. In model 1, His2AvD containing nucleosomes could have a more labile dimer-tetramer interface than H2A containing nucleosomes which makes them more amenable to transcriptional processes like transcription factor binding and/or RNA polymerase elongation. A larger acid patch on the surface of His2AvD containing nucleosomes may then stabilise its association with chromatin by binding the N-terminal tail of H4 in adjacent nucleosomes with higher affinity than H2A. In model 2,

amino acid differences between His2AvD and H2A, in the essential M6 region, could direct nucleosomes containing His2AvD to adopt, or be more stable than H2A containing nucleosomes, in a conformation that promotes transcription. In model 3, a more extensive acid patch on the surface of His2AvD nucleosomes could influence the binding of regulatory proteins to the acid patch of the N-terminus of H4

7.2.3 H2A.F/Z histones and the composition of transcriptionally competent chromatin.

Experiments which have demonstrated that chromatin fractions enriched in transcribed sequences may also contain higher concentrations of H2A.F/Z than bulk chromatin (Gabrielli *et al.*, 1981; Ridsdale and Davie, 1987) have been used to support a possible role for H2A.Z in transcription. Transcriptionally active chromatin fractions are also enriched in HMG-14/-17 proteins and contain less linker histone than bulk chromatin (Gabrielli *et al.*, 1981; Dimitrov *et al.*, 1990; Kamakaka and Thomas, 1990; Bresnick *et al.*, 1992; Dedon *et al.*, 1991; Postnikov *et al.*, 1991; Bustin and Reeves, 1996). Since HMG-14/-17 and H1 interact with DNA and histones in the nucleosome (see below) it is feasible to speculate that the concentration of these proteins in active chromatin could be due to differences in their affinity for H2A.F/Z and H2A containing nucleosomes.

As H1 is depleted in fractions that contain transcriptionally active chromatin and H2A.Z, then H1 could have a lower affinity for H2A.Z nucleosomes than H2A nucleosomes. Cross-linking studies have demonstrated that the globular domain of histone H1 interacts with H2A (Boulikas *et al.*, 1980, see also Hayes *et al.*, 1994). Further analysis of whether H2A.Z could alter H1 binding to nucleosomes is complicated by the fact that two models exist for the binding of linker histone to the nucleosome (reviewed in Travers, 1999a; Thomas, 1999; see also An *et al.*, 1998a). In one model, the globular domain of linker histone binds across the nucleosome dyad. In the other model linker histone is bound under one of the gyres of the nucleosome and contacts histone H2A. The linker histone in the latter model is located in close proximity to the acid patch region which is consistent with the possibility that the essential (M6) domain of H2A.Z could influence linker histone binding to nucleosomes. If the function of H2A.Z was to produce chromatin which did not bind H1 then it would be expected that H1 null mutations should have a similar phenotype to H2A.Z null mutations. In *Tetrahymena thermophila* it has been shown that deletion of H1 changes the expression of some genes but is not lethal (Lee and Archer, 1998; see also, Dou *et al.*, 1999). Since deletion of H2A.Z is lethal in *Tetrahymena* then it seems that the

function of H2A.Z must be more extensive then just creating chromatin that doesn't bind H1.

If H2A.Z was responsible for the enrichment of HMG-14/-17 proteins in transcriptionally active chromatin then it would be reasonable to expect HMG-14/-17 proteins to have a higher affinity for H2A.Z nucleosomes than H2A nucleosomes. Examination of this possibility shows that there are two ways that it could occur. Firstly, H2A.Z could increase HMG-14/-17 binding by changing the charge on the surface of the nucleosome. Cross linking studies performed with HMGs-14/-17 have indicated that a highly basic region of the protein (residues 17 to 47) is located where it could interact with the acid patch of histone H2A in nucleosomes (Trieschmann *et al.*, 1998). HMG-14/-17 proteins might therefore have a higher affinity for H2A.Z containing nucleosomes because they have a more extensive acid patch. Alternatively, or in addition, structural features of H2A.F/Z nucleosomes might make them more amenable to HMG-14/-17 binding. Here it was shown that the essential region of H2A.F/Z (M6) is involved in stabilising the association and alignment of the dimer and tetramer in the nucleosome. In one structural study of nucleosomes bound by HMG-14/-17 proteins it was shown that HMG-14/-17 binding changes the radius of gyration of the chromatin subunit (Bustin, *et al.*, 1990; Alfonso, *et al.*, 1994; Bustin and Reeves, 1996). It is conceivable that this change could occur if HMG-14/-17 binding altered dimer-tetramer alignment. Thus if H2A.Z containing nucleosomes adopted a conformation which resembled that of nucleosomes bound by HMG-14/-17 then it would be reasonable to expect that HMG-14/-17 might bind to preferentially to H2A.Z containing nucleosomes. In other studies it has been shown that HMG-14/-17 are incorporated into chromatin prior to the completion of chromatin assembly (Crippa *et al.*, 1993; Trieschmann, *et al.*, 1995). Chromatin assembly is a stepwise process where the H3-H4 tetramer is deposited on the DNA first and this is followed by the slower addition of H2A-H2B dimers (Worcel *et al.*, 1978). Since HMG-14/-17 proteins bind to both the dimer and tetramer (Cook *et al.*, 1986; Brawley and Martinson, 1992, Trieschmann *et al.*, 1998) then it seems possible that they might be incorporated into chromatin during assembly because they favour, or possibly stablise, a particular dimer-tetramer alignment. Interestingly, HMG-14/-17 proteins stimulate *in vitro* transcription from chromatin templates when they are included in the nucleosome assembly reaction (Crippa *et al.*, 1993; Trieschmann, *et al.*, 1995). If this increased transcription occured as a result of conformational changes in the nucleosome brought about by HMG-14/-17 binding then, by adopting this conformation, nucleosomes containing H2A.Z could similarly activate transcription. This outcome is consistent with studies that have suggested a role for H2A.F/Z in transcription (see sections 1.4, 3.6 and 4.4).

Analysis of the function of HMG-14/-17 proteins has shown that they can influence the role of H1, chromatin compaction and histone acetylation. If the *in vivo* function of H2A.Z was to recruit HMG-14/-17 then the effects of HMG-14/-17 binding would be particularly pronounced in chromatin containing H2A.F/Z. These effects are considered following in relation to the possible role of H2A.F/Z histones in chromatin structure and function.

In relation to the role of histone H1, it has been shown in an assay system were transcription was repressed by H1, that this effect could be overcome by HMG-14 binding (Ding *et al.*, 1997). As discussed above, modulating the effect of binding of H1, either directly, or through specific interactions with HMG-14, is not likely to be the only function of H2A.Z in chromatin.

HMG-14/-17 proteins direct decompaction of the chromatin fibre through interactions between domains at their C-terminus which are rich in acidic amino acids and the N-terminus of H3 (Trieschmann *et al.*, 1995; Ding *et al.*, 1997). If HMG-14/-17 proteins had a higher affinity for H2A.Z nucleosomes than H2A nucleosomes then chromatin containing H2A.F/Z might be preferentially decompacted. To this effect, it has been shown that chromatin fractions containing these proteins are more susceptible to endonuclease digestion (Gabrielli *et al.*, 1981; Ridsdale and Davie, 1987). Interestingly, while it has been reported that HMG-14/-17 proteins are displaced from chromatin during mitosis it was shown here that histone H2A.Z remains associated with chromatin throughout mitosis in *Drosophila* cells (section 4.3.3). Thus, if H2A.Z functioned by recruiting HMG-14/-17 proteins it could act as a marker for chromatin decompaction by HMG-14/-17 following mitosis.

Recently it has been shown that HMG-17 binds to chromatin and inhibits the acetylation of histone H3 by the human Gcn5 homologue, PCAF (Herrera *et al.*, 1999). Thus, acetylation could be inhibited on H2A.Z containing nucleosomes by HMG-17 binding. This possibility, in conjunction with the demonstration that His2AvD is not incorporated into the chromatin of active heat shock genes (Donahue *et al.*, 1986) presents some interesting considerations for the regulation of heat shock gene expression. By having a restriced His2AvD incorporation, heat shock chromatin could have a lower affinity for HMG-17, which could cause histones at these loci become hyperacetylated. To this effect, Nightingale *et al.* (1998) have reported that acetylation increases the transcription of the *hsp26* gene on chromatin templates assembled with hyperacetylated histones (Nightingale *et al.*, 1998). Since His2AvD may not be

incorporated into chromatin during heat shock because it is not synthesised (see section 3.6.1) then, during recovery, the resumption of His2AvD production might restrict further acetylation and contribute to the attenuation of transcription from these genes.

Although structural homologues of the HMG-14/-17 proteins have only been found in vertebrates it is possible that other proteins in lower eukaryotes might perform equivalent functions. One possible candidate in yeast is the transcriptional regulator Sin1, which like HMG-14/-17 binds to DNA with little or no sequence specificity (Kruger and Herskowitz, 1991). Sin1 is highly charged, containing 20% acidic residues and 25% basic residues. The only other proteins that are this rich in both acidic an basic residues are the HMGs. On the basis of this high proportion of charged residues Sin1 was originally classified as a HMG-1 like protein but it has since been declassified because residues that contribute to the HMG-1 DNA binding domain (HMG-1 box) are not conserved in Sin1 (see Grosschedl *et al.*, 1994).

7.2.4 His2AvD and centromeric chromatin

In addition to being found in transcriptionally active fractions, immunolocalisation studies conducted here on mouse tissue culture cells and tissue sections demonstrated that H2A.Z was excluded from regions of the nucleus containing centromeric DNA. In mouse and humans, centromeric DNA is packaged by the histone H3 variant CENP-A (Palmer *et al.*, 1987; Stoler *et al.*, 1995; Sullivan *et al.*, 1994; Shelby *et al.*, 1997). Since there are amino acid differences between H2A and H2A.Z in the domain that contacts H3 (Fig 6.5) it is possible that H2A.Z is excluded from centromeres because it cannot form nucleosomes with CENP-A.

In the analysis of functional domains of His2AvD protein conducted in chapter 3, a mutant, M7, was created that contained the domain of H2A that interacts with H3. The viability of flies that produced only this mutant form of His2AvD was severely compromised. If H2A.F/Z was similarly excluded from centromeric chromatin in *Drosophila*, by its CENP-A homologue, then reduced vigor of the M7 mutant might reflect inappropriate localisation of His2AvD function to the centromere. This could be the case because the M7 mutant contains the essential functional residues of His2AvD in the M6 region. Interestingly, mutant versions of the CENP-A homologue in yeast, with H3 residues at sites that interact with H2A, exhibit high degrees of chromosome loss (mutants *cse4-279* and *cse4-265* in Keith *et al.*, 1999). Since the amino acid changes in these mutants could conceivably allow them to interact with H2A.F/Z it is possible that their chromosome loss phenotype could also be due to inappropriate

localisation of H2A.F/Z to centromeres. Thus, the reduced vigor of flies containing the M7 mutant could be due to defects in chromosome segregation.

7.3 His2AvD function.

In summary, results obtained from work conducted in this thesis have four implications for the function of H2A.F/Z histones. Firstly, the entry of His2AvD into *Drosophila* embryo nuclei, coincident with the start of transcription, and the disruption of gene expression in His2AvD null mutants supports a role for His2AvD in transcription. Secondly, the persistence of His2AvD on mitotic chromosomes raises the consideration for H2A.F/Z function that it could act as a marker for transcriptionally active regions of chromatin through mitosis. Thirdly, identification of the functional domain of His2AvD indicates that nucleosomes containing this variant histone probably act by changing interactions that occur on the surface of the octamer and/or at the dimer-tetramer interface. Finally, histone H2A.Z is excluded from centromeric chromatin, possibly because it cannot form nucleosomes with the centromeric histone H3 variant, CENP-A.

Note added in proof.

During the preparation of this thesis four reports were published which characterised aspects of H2A.F/Z histone function in both *Drosophila* and *Saccharomyces cerevisiae*.

Leach *et al.* (2000) used antibodies directed against the His2AvD protein to characterise its distribution by indirect immunofluorescence and chromatin immunoprecipitation (ChIP). Immunolocalisation of His2AvD on salivary gland polytene chromosomes found that it was widely, but not generally distributed throughout chromatin. In further agreement with previous results (Donahue *et al.*, 1986), it was also shown that His2AvD is not found at loci containing heat shock genes following activation by exposure to elevated temperatures. The demonstration that His2AvD is found at heat shock loci in individuals that have not been subject to heat stress, or were subject to heat stress earlier in development, indicates that His2AvD is probably only transiently depleted on these genes when they are activated. This is consistent with the proposal that His2AvD functions as a replacement histone and is not incorporated into heat shock loci because its synthesis is inhibited during heat shock. Analysis of the distribution of His2AvD in diploid cells in a ChIP assay found that His2AvD was less abundant than H2A in 1.688 satellite chromatin. These satellite sequences are found in *Drosophila* at centromeres and at other sites in the genome. An exclusion of His2AvD from mouse centromeres was reported in this work. These similar results in two different organisms suggest that the exclusion of H2A.F/Z from centromeric chromatin might be conserved throughout evolution.

Jackson and Gorovsky (2000) showed that disruption of the *Saccharomyces cerevisiae* H2A.F/Z gene, *HTZ1*, was not lethal but did slow growth and increased sensitivity to formamide. In addition, they demonstrated that the phenotype associated with *HTZ1* deletion was not rescued with either of the yeast H2A genes but could be rescued with the H2A.F/Z gene from *Tetrahymena thermophila*. These results provide further evidence that H2A.F/Z histones have a function which is conserved across evolution that is distinct from the major H2A histones (see also Liu *et al* 1996a and b). The demonstration that H2A.F/Z histones have equivalent functions across species has direct implications for results obtained here in chapters 3 and 4. In chapter 3, it was shown that the function of *Drosophila* histone His2AvD is directed through specific amino acid residues in a C-terminal region of the protein. The results of Jackson and Gorovsky (2000) strongly suggest that this region will similarly specify the function of H2A.F/Z histones across species. In chapter 4, fusion of GFP to the C-terminus of *Drosophila* His2AvD did not disrupt its function *in vivo*. Thus, H2A.F/Z histone-GFP

fusion proteins should be similarly useful as a marker for chromosome behaviour and for characterisation of H2A.F/Z function in different species.

Santisteban *et al.* (2000) isolated the yeast H2A.F/Z histone, *HTZ1*, in a screen for genes which, when overexpressed, suppressed temperature sensitivity of strains containing the histone H4 Y98-H mutant. Further characterisation *HTZ1* overexpression demonstrated that it did not rescue the Spt and Sin (expression of *INO1* remains derepressed) phenotypes of H4 Y98-H strains. The authors suggest these results indicate that Htz1p must function in one or more new pathways. Alternatively this phenotype could occur if Htz1p only partially restored the defects of the H4 Y98-H mutation.

Since H4 Y98 is located in a domain that stabilises dimer-tetramer interactions, then it seems likely that defects in the Y98-H mutant strain could be due to altered interactions at the dimer-tetramer interface. In the crystal structure of the nucleosome, the Y98 residue of histone H4 is in close proximity H2A amino acids in a region where there are sequence differences between H2A and Htz1p. It therefore seems possible that these Htz1p residues could overcome H4 Y98-H defects by normalising interactions at the dimer-tetramer interface. The possibility that this region of Htz1p functions differently than H2A is supported by the demonstration, in chapter 3, that a mutant form of *Drosophila* His2AvD with H2A residues in this region is not functional *in vivo*. Results obtained from this particular His2AvD mutant can also be used to formulate an alternate hypothesis for the rescue of H4 Y98-H defects by Htz1p. This non-functional mutant version of His2AvD also has H2A residues in a domain known as the acid patch which is on the surface of the nucleosome exposed to solvent. In nucleosome crystals this acid patch binds the N-terminal tail of histone H4 in the adjacent nucleosome (Luger *et al.*, 1997). Amino acid differences between His2AvD and H2A would mean that this acid patch would be more extensive in His2AvD containing nucleosomes. It is interesting to speculate, therefore, that if the defects seen in H4 Y98-H mutant strains were due to increased dimer displacement caused by disruption of the dimer-tetramer interface, then a larger acid patch on the surface of Htz1p nucleosomes might suppress the mutant phenotype by stabilising the association of dimers with chromatin. In this case, nucleosomes containing Htz1p would suppress defects associated with dimer displacement but not defects associated disruption of the dimer tetramer interface. This possibility is consistent with the demonstration that overexpression of Htz1p does not overcome the Spt and Sin phenotypes of H4 Y98-H mutants.

The viability of double mutant yeast strains where *htz1* and SWI or histone acetylase complex subunits have been disrupted, is more severely compromised than in strains

where only one of these genes has been inactivated. In *Δhtz1/Δsnf2* double mutant strains, disruption of the *sin1* gene did not restore *GAL1* expression. Interestingly, in a previous study Biggar and Crabtree (1999) demonstrated that a *Δsin1* mutant restored *GAL1* transcription in strains lacking SWI chromatin remodelling and Gcn5 histone acetylation activities. Therefore, Htz1p must have functions that overlap, and are distinct from, that of the SWI chromatin remodelling complex and Gcn5 histone acetylase complexes.

Santisteban *et al.* (2000) also performed ChIP assays to characterise the distribution of Htz1p and H2A across the *PHO5* and *GAL1* loci. Htz1p was found at highest concentrations across the promoters of these genes when they were uninduced. This distribution of is consistent with a possible role for Htz1p in marking promoter regions. On induction, Htz1p was displaced. Interestingly, Hirschhorn *et al.* (1995) have identified mutations in H2A that disrupt expression of the *SUC2* gene (but not the *INO1* gene or Ty elements) by attenuating a process in activation that are downstream or independent of SWI function. Thus, it could be necessary for Htz1p to be displaced from chromatin in promoter regions on induction because it might disrupt a step in the activation of transcription like mutations in H2A disrupt transcription. This possibility could be easily investigated by examining the expression of *SUC2* in yeast strains that overexpress *HTZ1*.

Dhillon *et al.* (2000) identified *HTZ1* in a screen for genes that, when overexpressed, suppressed silencing defects in *sir1* mutant yeast strains. In this study, the ability of overexpressed *HTZ1* to restore silencing at an attenuated *HMR* was also investigated. The *HMR* silencer contains three functional silencer elements, namely, binding sites for the Rap1p and Abf1p proteins and an ARS element. Deletion of any or all of these elements results in derepression of gene expression at this loci. Overexpression of *HTZ1* was only able to restore silencing when the Abf1p binding site was abolished. The effect of Htz1p on silencing, was further characterised by analysing the expression of reporter genes at *HMR* and telomeres in *Δhtz1* mutant stains. In these strains, reporter gene expression is mildly derepressed at *HMR* and extensively derepressed at centromeres. While these overexpression and deletion studies support a role for Htz1p in silencing they do not rule out an involvement for Htz1p in both activation and repression of transcription (see Dhillon *et al.*, 2000 for further consideration of this point). In relation to its specific role in transcriptional repression, the results presented in Dhillon *et al.* (2000) are consistent with the possibility that Htz1p could block a step in transcription that requires specific H2A residues (see above and Hirschhorn *et al.*, 1995).

Interestingly, Dhillon *et al.* (2000) also found that a *Δhtz1* mutant was lethal in combination with a mutant version of histone H4 that lacking the N-terminal residues 4-28 (H4 NΔ4-28). Interestingly, it has been shown in previous studies that the H4 NΔ4-28 mutation, like the *Δhtz1* mutation, reduces silencing at *HML* and decreases expression of *GAL1* and *PHO5* (Kayne *et al.*, 1988; Durin *et al.*, 1991). Taken together, these results suggest that functions directed through Htz1p and the N-terminal tail of histone H4 could overlapping and/or be partially redundant. In support of the possibility that Htz1p and the N-terminus of histone H4 could act in the same pathway it was shown in chapter 3 that the functional domain of His2AvD in *Drosophila* includes a region that, in H2A containing nucleosomes, binds the H4 tail of adjacent nucleosomes in X-ray crystals.

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