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COMMENTARY

Molecular mechanisms of cellular determination: their relation to chromatin structure and parental imprinting

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INTRODUCTION

We have learnt much by looking at the eyes of flies. No more so than when looking at the distinctive mottling within the eyes that can be brought about by chromosomal position-effect. In the classical example of so-called position-effect variegation (PEV) the dominant allele of the *white* gene, w^+, is brought into close proximity to heterochromatin by chromosomal rearrangement. In heterozygotes for the translocation this novel juxtaposition results in the inactivation of w^+ in some cells, with the concomitant phenotypic expression of the recessive allele (Lewis, 1950; Baker, 1968; Spofford, 1976). Thus, white mutant sectors within the eye are placed in stark contrast next to bright vermilion sectors where w^+ remains active (Fig. 1). Careful inspection of the patterns of mottling found in several hundred eyes has shown that, in general, the sectors are continuous rather than a random salt-and-pepper distribution, with many of the geographical zones within the patterns being shared and, most strikingly, that both positive and negative images of the same pattern can be observed. The integration of these patterns with lineage analysis derived from somatic recombination studies (Becker, 1957) reveals that the time at which the determinative event occurs - that is, the decision of whether or not to inactivate w^+ - is during the first larval instar stage of development within the eye disc cells (Fig. 2). Since both positive and negative images of the same pattern are obtained this decision is arrived at by a stochastic process, with no predisposition of the disc cells to, or not to, inactivate w^+. Once the decision is made, however, it is essentially irreversible and stored for many cellular generations as the eye disc cells multiply and fill the sectors that will differ with regard to the activity of the rearranged genes. In fact, *white* mottling only becomes phenotypically manifest some seven days after the determinative event, mid-way through metamorphosis, at the time *white* is normally transcribed and pigment is first observed within the eye (Baker, 1963) (Fig. 2). These simple, yet elegant, experiments have served to bring home the notion that the acquisition of a potential to express a pattern of genes (determination) precedes the overt expression of this potential (differentiation), and that these two states are separable in developmental time.

Insight into the mechanism of inactivation of w^+ in the eye disc cells has come from the observation that within polytene nuclei variegating breakpoints assume the condensed morphology of heterochromatin (Henikoff, 1981). This result has been crucial, since it has suggested that determination, as it relates to PEV, is a consequence of a change in chromatin packaging - a change that can be followed for a variegating euchromatic gene as a cytological transition from a euchromatic conformation to one that is decidedly heterochromatic. In short, the determined state in PEV is a chromatin state.

A MODEL FOR HETEROCHROMATIN ASSEMBLY: ROLE OF THE *HP1* GENE

The ease with which changes in *white* mottling can be scored has also enabled a variety of genetical and environmental factors to be identified that can modify the determined state (Fig. 3). There are, for example, two sensitive periods for the modulation of PEV by temperature (Fig. 2); generally, an increase in temperature suppresses variegation while cooling enhances (Fig. 3; Gowan and Gay, 1933a). The first sensitive period is before the time of determination during the first few hours of embryogenesis (Spofford, 1976), and the second is during the mid-pupal stage of development (Chen, 1948; Becker, 1961). Assuming - prophetically in retrospect - that PEV was the consequence of a self-assembly process that involved many proteins that form a macromolecular complex, Spofford (1976) suggested that, like all macromolecular complexes, its stability would be temperature dependent. At lower temperatures it would be more stable and thus enhance variegation. Heating would have an opposite effect, as thermal energy would be likely to disturb an ordered structure and lead to a suppression of variegation. In light of this prescient explanation, the time at which the sensitive periods occur during development might be understandable. The first is around the time when heterochromatin becomes recognisable within the nuclei (Cooper, 1959) and a temperature shift at this time could have an effect on the initial steps involved in heterochromatin assembly. The second is at the time when the genes responsible for pigment formation are transcribed and heat shock at this time might allow transcription factors to have access to the otherwise heterochromatinised *white* gene. A key feature is that both these sensitive periods represent windows within which the determined state can be changed.

Spofford's assumption has been turned into fact through the analysis of genetic modifiers of PEV, which can have two effects on variegation (Grigliatti, 1991; Reuter and Spierer; 1992). *Suppressors of PEV* (*Suvar*) convert the mottled

Key words: cell determination, position-effect variegation, homeotic gene, mating-type repression, parental imprinting

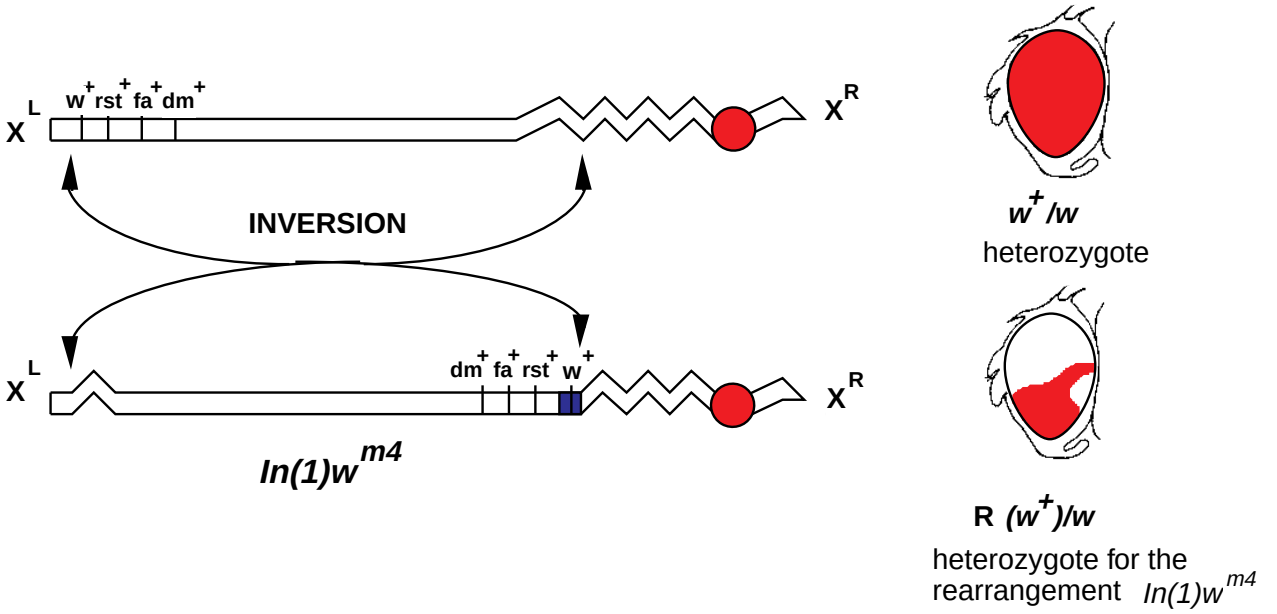

Fig. 1. A schematic illustration of *white* variegation in the X chromosome inversion *In(1)w*^{m4}. The distal region of the X chromosome contains a cluster of genes, including *white* (*w*$^+$), *roughest* (*rst*$^+$), *facet* (*fa*$^+$) and *diminutive* (*dm*$^+$), that have been useful for studying variegation of eye phenotypes. In strains that possess the *In(1)w*^{m4} chromosome these genes are brought close to heterochromatin (zig-zag line) by chromosomal rearrangement, such that the *white* gene is now only 25 kb away from constitutive heterochromatin (Tartof et al., 1984). In heterozygotes for the rearrangement, where the rearranged chromosome bears the dominant allele of *white*, *w*$^+$, the eye has a distinctive mottled appearance. This is because in some cells *w*$^+$ has been repressed by its proximity to heterochromatin, and the recessive allele carried on the normal unrearranged chromosome has gained phenotypic expression. In all figures, the vermilion parts of the eye represent pigmented wild-type coloration, while the colourless parts represent non-pigmented, mutant, areas.

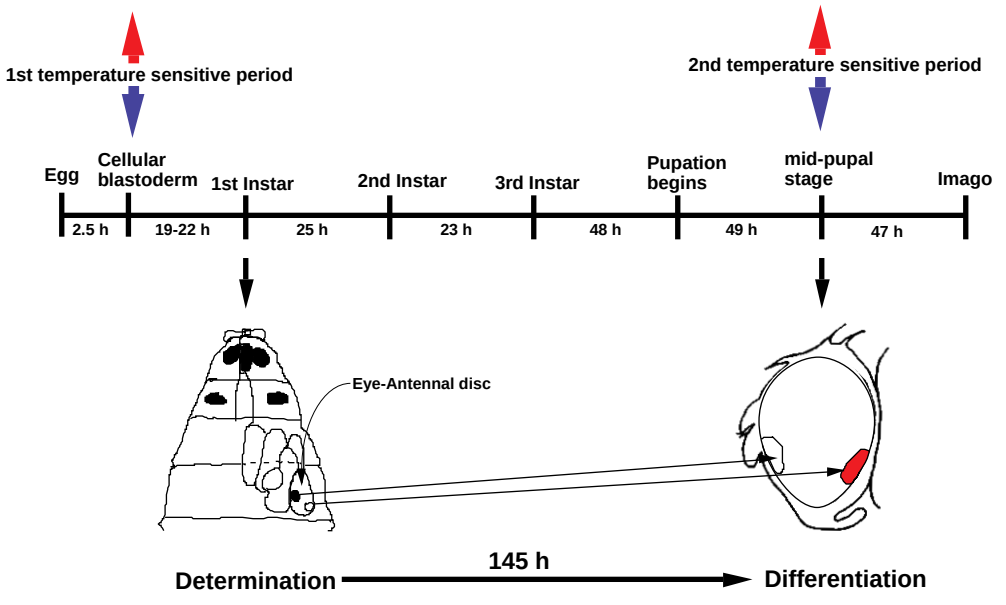

Fig. 2. In *Drosophila* PEV, determination and differentiation are widely separated in developmental time. Determination occurs in the eye disc cells of the first instar larva. Inactivation of the *w*$^+$ gene occurs in some eye disc cells (shown as a black cell), and in others *w*$^+$ remains active (shown as a white cell). Once the decision is made it is mitotically stable, while the two cells divide and multiply to fill the sectors that form in the eye during metamorphosis. It is only when the *white* gene is transcribed in the eye, mid-way through metamorphosis, that the determinative event becomes phenotypically manifest. In the clonal progeny of the disc cell that inactivated *w*$^+$ there is no transcription and the sector has the white, mutant, coloration. In the eye disc cell that did not inactivate *w*$^+$ the sector is the typical wild-type coloration.

phenotype to one that is essentially indistinguishable from wild-type, while *enhancers of PEV* (*Evar*) have the opposite effect, and alter the phenotype to a more mutant form (Fig. 3). The reciprocal effects of the *Suvar* and *Evar* suggest that they have opposite effects on heterochromatin formation; *Suvar* are thought to be involved in the formation of heterochromatin, through encoding either components of the compact state or enzymes that post-translationally modify the components, whereas the *Evar* promote the euchromatic state, perhaps by binding hypothetical boundary elements (Tartof et al., 1984) that stop the spreading of heterochromatin. Analysis of the *Suvar* has provided firm evidence for a connection between

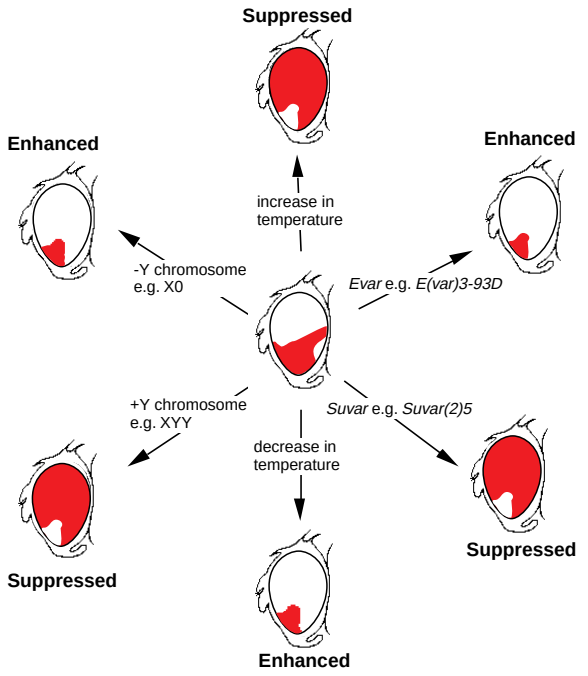

Fig. 3. Both genetical and evironmental factors can affect the process of determination and therefore the degree of variegation within a tissue. The central eye illustrates an idealised variegation of the *white* gene in an XY male. In general, an increase in temperature or addition of heterochromatin to the genome via an extra Y chromosome leads to a reduction in variegation. The reverse, that is cooling or the removal of heterochromatin, has the opposite effect of enhancing variegation. Euchromatic modifiers of position-effect variegation have two types of effect. Suppressor mutations such as *Suvar(2)5* convert the mottled phenotype to one that is almost wild-type, whilst enhancer mutations such as *E(var)3-93D* have the opposite effect and convert the mottled phenotype to one that is more mutant. Not shown here is the modifying effect of butyrate (Reuter and Spierer, 1992), which has a suppressing effect on variegation that has been explained by an increase in histone acetylation.

PEV and chromatin, since some of the first *Suvar* to be described were the genes encoding the core histones (Moore et al., 1979), a result that hinted strongly that repression seen in PEV involves the packaging of, at the very least, nucleosomal if not chromatosomal (Simpson, 1978) DNA.

A model based on the sensitivity of PEV to changes in the concentration of several modifier loci has suggested that the assembly of heterochromatic domains obeys the laws of mass-action, with the amount of assembled heterochromatin being directly proportional to the concentration of the constituent (structural) components (Fig. 4; Locke et al., 1988; Tartof et al., 1989). The mass-action model also provides an explanation for the observation that extra Y chromosomes present in the genome suppresses variegation of the euchromatic *white* gene (Fig. 3; Gowen and Gay, 1933b). This effect can be explained as a titration of components necessary for heterochromatin assembly by the Y chromosome (Zuckerkandel, 1974), which in the somatic tissues of *Drosophila* is entirely heterochromatic. Thus the Y chromosome acts as a 'sink' for components that would otherwise be used to repress genes by the formation of heterochromatin at the variegating breakpoint.

Some of the structural components of heterochromatin, apart from the histones, have now been characterised at the molecular level (Fig. 4). The first to be identified and consequently the best studied, is heterochromatin protein 1 (James and Elgin, 1986). Studies on the overexpression of the *HP1* gene have shown that an increase in concentration of HP1 protein leads to an enhancement of variegation (Eissenberg et al., 1990) while its natural mutation, *Suvar(2)5*, suppresses variegation. HP1 protein is also found to localise to the majority of euchromatic regions that become cytologically heterochromatinised by their translocation next to constitutive heterochromatin (Belyaeva et al., 1993). Thus, the dosage-dependent effects of the *HP1* gene on PEV can be directly related to the compaction of euchromatin into heterochromatin that occurs at variegating breakpoints and, more importantly, HP1 is a component of this repressive, heterochromatic, environment. The finding that HP1 does not localise to all variegating breakpoints confirms the previous observation (Belyaeva and Zhimulev, 1991; Bishop, 1992) that heterochromatin itself is made up of domains consisting of different combinations of proteins, some of which are shared between domains and others that are unique. The common feature is that all domains share the same cytological characteristics of constitutive heterochromatin.

Since PEV is a result of aberration and is not part of a cell's normal physiology, the true function of HP1 cannot be as a modifier of PEV, and must therefore lie elsewhere. A role in centromere function for HP1 has been suggested from a detailed analysis of the null mutant phenotype in *Drosophila* embryos (R. Kellum and B. M. Alberts, unpublished data). The principal effect of the *Suvar(2)5* null mutation is observed during anaphase B of the cell cycle where there is a problem in disjunction, characterised by a lack of separation of the segregating sister chromatids. These observations support a previous suggestion, based on the conserved localisation of mammalian HP1-like proteins to centromeric heterochromatin (Fig. 5; Wreggett et al., 1994), that HP1, as part of highly condensed constitutive heterochromatin, might provide structural support for proteins involved in chromosome segregation. While these observations suggest an understandable role for HP1 in centromere function, especially in light of its known centromeric localisation on polytene chromosomes, it does not account for the fact that HP1 also binds to a number of euchromatic sites including the entire length of the cytologically euchromatic (banded) fourth chromosome. The localisation of HP1 to euchromatic sites has suggested that mechanisms similar to those proposed for the formation of heterochromatin domains (Fig. 4; Tartof et al., 1989) might also exist for domains within euchromatin, and that such heterochromatin-like domains might determine the transcriptional fate of euchromatic genes (Singh et al., 1991). It is the finding that HP1 shares a region of homology with another *Drosophila* protein, namely *Polycomb* (Pc) (Paro and Hogness, 1991), a protein also involved in the heritable inactivation of genes, which has added support to this notion.

THE STEP WISE PROGRESSION TOWARDS CELL FATE DETERMINATION: ROLE OF THE *Polycomb* GROUP COMPLEX

The characteristics of PEV emphasised in the previous section,

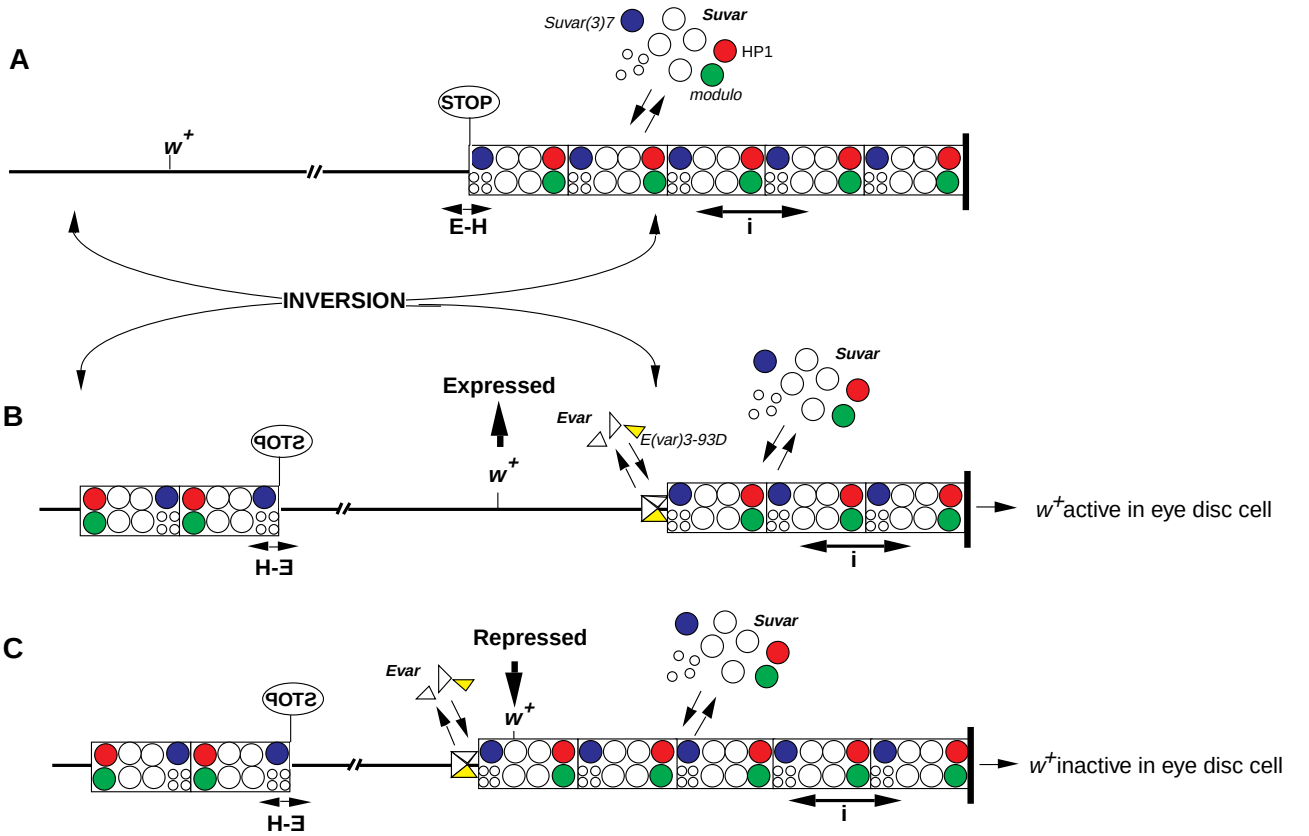

Fig. 4. A highly schematic diagram illustrating the *cis*- and *trans*-acting factors that are likely to be involved in heterochromatic position effects, based on the mass-action model of Tartof and his school (Locke et. al., 1988; Tartof et al., 1989). (A) The normal chromosome where heterochromatin components (circles) encoded by *Suvar* such as HP1 (James and Elgin, 1986), *Suvar(3)7* (Reuter et al., 1990) and *modulo* (Grazino et al., 1993) assemble into a core complex (boxes with circles in them) that reiterates along the chromosome forming a condensed heterochromatin domain. Genetical analysis also shows that the core complex may include nucleosomal DNA (Moore et al., 1979), which is represented by the line passing through the middle of the boxes. While no argument is made for a direct interaction between the modifiers given here, some evidence does exist for an interaction of HP1 with *modulo* (Grazino et al., 1993). *Suvar(3)7* (Reuter et al., 1990) has not been localised to centric heterochromatin in polytene nulcei and is only placed in the core complex on the basis of its clear dosage effects. The domain is propagated *in cis* from an initiation site (i) that may consist of arrays of middle repetitive sequences such as Dr D (Miklos and Costell, 1990). Termination of the complex occurs at a natural boundary or stop-signal and forms the euchromatin-heterochromatin (E-H) junction. (B) A chromosomal inversion, which has one breakpoint between i and the stop-signal and permits the spreading of heterochromatin into a normally euchromatic region of the chromosome. According to the mass-action model the final degree of spreading is dependent on a variety of factors, which include both the concentration of the *Suvar* components that form the heterochromatin itself and the *Evar* (triangles in the figure), such as *E(var)3-93D* (Dorn et al., 1993). The *Evar* have an antagonistic effect to the *Suvar*, in stopping the spreading of heterochromatin, and promoting euchromatin. The sensitivity to concentration changes is hightened if any of the components are required in more than one copy (e.g. the small circles). It is the outcome of the equilibrium brought about by these interactions that regulates the degree of spreading; as the concentration of any one of the many components in this equilibrium is likely to vary from cell to cell, the spreading also varies from cell to cell. For example, if, in one of the eye disc cells the spreading terminates before the *white* gene, the gene remains transcriptionally competent (expressible) and is transcribed in the progeny of the cell - at around the mid-pupal stage of development - giving rise to a sector that has the normal, wild-type, coloration. On the other hand, if the spreading encapsulates the *white* gene, the gene is heterochromatinised (non-expressible) and remains inactive, eventually giving rise to a colourless, mutant, sector. In polytene nuclei, variegation caused by changes in chromatin structure may be compounded by under-replication or elimination of DNA sequences at the variegating breakpoint (Karpen, 1994). Not given here are the modifiers that are either enzymes (*Suvar(3)6*; Dombardi and Cohen, 1992) or activities that are thought to remodel chromatin during replication (*mus209/Pcna*; Henderson et al., 1994) or mitosis (*Suvar(2)16*; Grigliatti, 1991).

namely the early determination and clonal inheritance of gene expression patterns, are intrinsic features of normal development in *Drosophila*. A clear and well documented example that illustrates these features is to be found in the many studies that have been made on the homeotic genes (Akam, 1987; Duncan, 1987). Homeogenes are determinants of cellular fate in flies, in mice and, by extrapolation, in man (for review, see McGinnis and Krumlauf, 1992). It is through their spatially restricted patterns of expression that the homeogenes set up the identities of the structures and appendages along the anterior-posterior (A-P) axis of the body. When the expression of the homeogenes is aberrant or missing, through mutation, and they are expressed inappropriately, dramatic shifts in cellular fate can take place (Lewis, 1978). Moreover, mis-expression of the

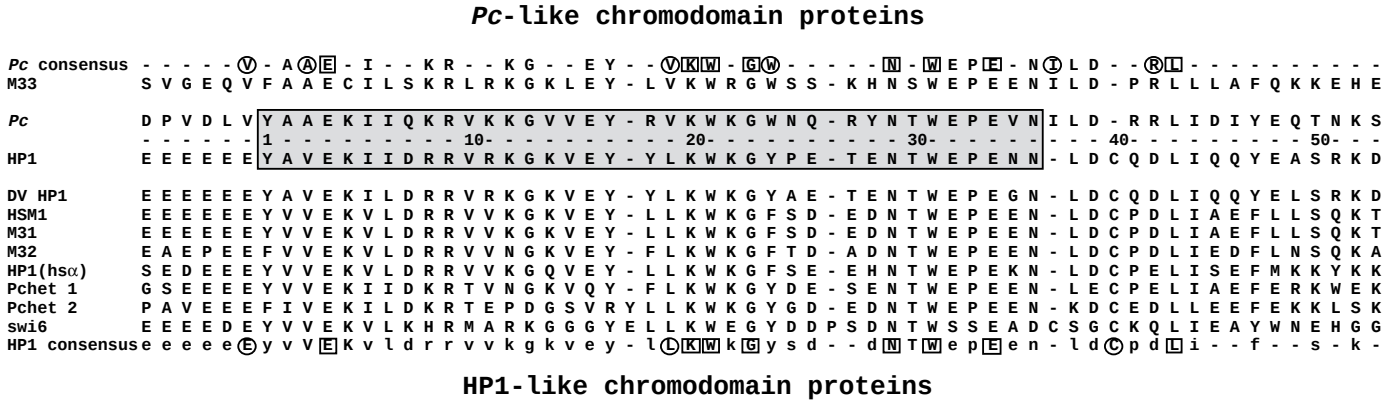

Fig. 5. The HP1 and *Pc* chromodomain subfamilies. The shaded box represents the minimal chromodomain with the sequence of Pc given above the HP1 sequence. All sequences are aligned with respect to this sequence with gaps in the sequences, required for alignment, represented by dashes. The Pc consensus sequence is derived from the comparison of M33 (Pearce et al., 1992), the murine homologue of Pc, and Pc (Paro and Hogness, 1991). The upper case letters represent residues that remain invariant in the Pc subfamily but can sometimes be found in members of the HP1 subfamily. Boxes around the upper case letters represent residues that are invariant across both subfamilies; circles around the residues denote subfamily specific residues. Enough HP1 subfamily members have now been cloned from *Drosophila melanogaster* (James and Elgin, 1986), *Drosophila virilis* (Clarke and Elgin 1992), mouse (Singh et al., 1991), man (Singh et al., 1991; Saunders et al., 1993), mealy bug (Epstein et al., 1992) and *S. pombe* (Lorentz et al., 1994) for a more complete consensus sequence of the HP1 chromodomain to be given. Lower case letters in the consensus sequence denote conservation in at least 5 out of 9 sequences. A dash in the consensus denotes that fewer than 5 out of 9 residues at this location are conserved. The designation for the upper case letters is as for the Pc consensus.

homeogenes can change cellular fate at any time during development (Struhl, 1981). Thus, for completion of normal development homeogene expression (and therefore their spatially-restricted patterns) must be maintained, i.e. homeogenes must be active within their normal realms of expression and be repressed outside of them. These two states must also be maintained through many mitotic divisions until development is complete.

In *Drosophila*, the *trithorax* group (*trx*-G) (Kennison, 1993) and the *Polycomb* group (*Pc*-G) (Jurgens, 1985) of genes play a crucial role in maintaining, respectively, the active and repressed state of the homeotic genes. Some clues to the means whereby the *Pc*-G might heritably repress homeogene activity has come from the isolation and sequencing of the group's namesake, Pc. Pc shares a 37 amino acid domain, called the chromodomain, with HP1 (Fig. 5; Paro and Hogness, 1991). Identification of this motif brought together two different classes of genes that are involved in the repression of gene activity and, most importantly, suggested that the *Pc*-G of genes might exert their effect through heritable changes in chromatin structure. By analogy with the mass-action model for heterochomatin assembly (Locke et al., 1988; Tartof et al., 1989) the *Pc*-G genes are also thought to encode proteins that form a multimeric complex (Gaunt and Singh, 1990; Paro, 1990) (Fig. 6D), which has recently been confirmed by elegant immunoprecipitation experiments (Franke et al., 1992).

The enormous efforts that have been made over the years to understand the proper regulation of the homeogenes have enabled a fairly rapid elucidation of the *cis*-acting sequences and *trans*-acting factors required to direct the assembly of the *Pc*-G complex to specific sites within the fly genome. That the *Pc*-G complex does indeed bind specific sites comes from the observation that anti-Pc antibodies localise Pc protein to over 100 reproducible sites on polytene chromosomes (Zink and Paro, 1989), many of which are shared by other members of the *Pc*-G (Rastelli et al., 1993). The latter finding is a prediction of the models (Gaunt and Singh, 1990; Paro, 1990).

cis-ACTING DNA SEQUENCES REQUIRED FOR ASSEMBLY OF THE *Pc*-G COMPLEX

Indirect evidence that sequences surrounding the homeogenes are required for *Pc*-G assembly have come from transgenic experiments using reporter constructs consisting of the regulatory sequences of the *Ubx* (*bxd*; Muller and Bienz, 1991; Simon et al., 1993) and *abd-A* (*iab-2 and iab-3*, Simon et al., 1993) homeogenes coupled to *LacZ*. In transgenic flies harbouring these constructs the anterior boundaries of transgene expression correspond to those found for the respective homeotic genes and are dependant on the activity of the *Pc*-G; in the absence of any one of eight *Pc*-G genes, *LacZ* was expressed indiscriminantly in parasegments anterior to the normal realm of expression of the homeogenes (Simon et al., 1993). Thus, the *bxd*, *iab-2* and *iab-3* regulatory regions appear to contain sequences that can nucleate the assembly of the *Pc*-G complex. An interesting feature of these experiments is that the sites for initial regulation of the homeogenes, which may represent binding sites for the gap and pair-rule segmentation genes, can be separated from the sequences required for maintenance of homeogene expression, i.e. the *Pc*-G binding sites (Simon et al., 1993).

Antibody studies have provided direct evidence that sites for binding of *Pc*-G proteins exist within the regulatory regions of the homeogenes. When sequences flanking the homeogenes are introduced into a fly via transgenesis they form new sites of binding for Pc protein (Zink et al., 1991), and the product of another *Pc*-G gene, *Polyhomeotic* (*Ph*) (Fauvarque and Dura, 1993). Immunoprecipitation experiments have shown that Pc protein is found at specific sites within the chromatin of the

bithorax complex, such as *Mcp* and *Fab-7* (Orlando and Paro, 1993). These sites had previously been designated as boundaries (Eissenberg and Elgin, 1991) that insulate homeogenes from the possible promiscuous activities of parasegment-specific enhancers (Peifer et al., 1987; Galloni et al., 1993). Pc protein was also found in chromatin immunoprecipitated from the *Antp-P1* and *Abd-B* promoters (Orlando and Paro, 1993), a result that suggests that the sequences required for *Pc*-G silencing of these genes may be closely associated with the sequences normally required for their activation. Transgenic experiments have also shown that as little as 2.9 kb of flanking sequence linked to a *white* marker gene is sufficient to assemble the *Pc*-G complex, when assayed by antibody binding to the product of *Ph* (Fauvarque and Dura, 1993). Interestingly, the *white* gene exhibited noticeable variegation, indicating that the realm of action of the *Pc*-G complex did not always extend the full 2.9 kb required for repression.

Taken together, these experiments provide formal proof that *cis*-acting sequences are necessary for the binding of *Pc*-G proteins and can specify the assembly of a repressed chromosomal domain. They also suggest that the assembly of a heterochromatin-like complex can regulate gene expression locally, on a gene-by-gene basis, and may only need to complex sequences required for activation (promoters or enhancers) in order to silence a gene effectively. The assembly of the *Pc*-G complex also appears to be able to act as a boundary that can insulate a gene from *cis*-acting enhancer elements (Galloni et al., 1993).

trans-ACTING FACTORS REQUIRED FOR ASSEMBLY OF THE *Pc*-G COMPLEX

The identification of *trans*-acting factors involved in the assembly of the *Pc*-G complex has come from mutational analysis of a gap segmentation gene, *hunchback* (*hb*), a gene known to be involved in setting up the homeogene expression patterns (White and Lehmann, 1986). Particular emphasis has been placed on the repression of the *Ubx* gene, whose normal realm of activity encompasses parasegments 6 to 13 (Akam, 1987) (Fig. 6B). Anterior to these parasegments it has been proposed that initial *Ubx* repression is by the hb protein (Zhang and Bienz, 1992) and it is this initial binding of hb that may direct the specific assembly of the *Pc*-G complex (Fig. 6B). One of the most interesting features of this 'imprint' provided by the hb protein, is that it is developmentally labile and stable for only a few cell divisions, since the maternal and segmentation genes in *Drosophila* are only expressed maximally for a brief period during embryogenesis - for the two hours before the cellular blastoderm stage of development, whereupon their levels fall rapidly (Akam, 1987). At the time when the level of hb is falling, cells along the A-P axis have already begun to express homeogenes or sets of homeogenes that will determine their fates (Wilcox and Sang, 1983). It is this pattern of homeogene expression that must be maintained and thus the transient repression of *Ubx* mediated by hb protein has to be transduced into a more permanent form of repression.

It is likely that the initial binding of hb is transduced by an intermediary molecule, of which the most likely candidate is the product of a *Pc*-G gene *extra sex combs* (Struhl, 1981, 1983; *esc*). *esc* is the only *Pc*-G gene for which zygotic expression is not required for proper development. In fact, *esc* activity is only required up to the extended germ-band stage of development, whereupon there is no further need for its gene product (Struhl, 1981, 1983). The early requirement for *esc* is also specific for the selector genes within the homeogene clusters as *esc* is not required for the stable, restricted, expression of another selector gene, *engrailed* (*en*) (Moazed and O'Farrell, 1992). The restriction of *en* to the posterior compartment of each segment does, however, require the other members of the *Pc*-G complex (Heemskerk et al., 1991). No direct interaction of esc and hb is envisaged, since the sites for for initiation and maintenance of *Ubx* repression are known to be separable (Simon et al., 1993), instead esc may recognise local, transient, changes in chromatin structure that accompany the repression of *Ubx* by hb protein. Accordingly, esc recognises the binding of hb (Fig. 6B) and then targets the assembly of the rest of the *Pc*-G repressor complex. Recruitment of the *Pc*-G proteins and/or maintenance of the complex may involve an RNA moiety that is known to be associated with Pc (Paro et al., 1993).

So far, four gene products of the *Pc*-G genes have been immunolocalised to the homeotic genes: they include *Pc* (Zink and Paro, 1989), *Ph* (DeCamillis et al., 1992), *Posterior sex combs* (*Psc*; Martin and Adler, 1993) and *Suppressor (2) of zeste* (*Su(2)z*; Rastelli et al., 1993). Assembly of the *Pc*-G complex is likely to occur with, or soon after, the binding of esc - around the late blastoderm stage - since mutation in many members of the *Pc*-G of genes show maternal effects (Breen and Duncan, 1986; Lewis, 1978; Martin and Adler, 1993; Phillips and Shearn, 1990; Ingham, 1984; Duncan, 1982; Weischaus and Noell, 1986). Once assembled, the requirement for esc protein after the extended germ-band stage is removed (Fig. 6C) and the complex re-nucleates on the site(s) identified by hb and esc, during further rounds of DNA replication (Fig. 6D). Once the activities of the *trx*-G and *Pc*-G genes are brought into place the fate of cells is sealed and, as in PEV, remains so through many mitotic divisions to the end of development. Indeed, the ability of imaginal discs (Fig. 6D) to differentiate appropriately even after the time between determination and differentiation has been greatly expanded (Hadorn, 1965), in some cases from days to years, implies that the state of determination as regulated by the *trx*-G and *Pc*-G genes, is very stable. The occasional lapses from stability (transdetermination) may be due to changes in the epigenetic regulation of homeogene expression by the *trx*-G and *Pc*-G genes, leading to inappropriate expression, or repression, of homeogenes within imaginal disc cells (Gaunt and Singh, 1990).

In summary, detailed molecular and genetical analyses have shown that, for developmentally regulated genes such as the homeogenes, there is a step wise progression towards a final epigenetic, yet heritable, state of expressibility that is mitotically stable through to the end of development, and into adulthood. For the homoeogenes in *Drosophila* both the *cis*- and *trans*-acting factors involved in these steps are becoming well defined and they, it would seem, provide the specificity for more permanent changes in expressibility. It is also becoming clear that these heritable states of expressibility are chromatin states (Fig. 6). Essentially irreversible states of determination directed by developmentally labile interactions are also likely to exist in other organisms. For example, a *Pc*-

like gene has been isolated in the mouse (Pearce et al., 1992) and, moreover, mutation of the murine homologue of *Psc* (*bmi-1*) gives rise to embryos that are posteriorised (van der Lugt et al., 1994), a feature typical of mutations in members of the *Pc*-G family of genes (Jurgens, 1985).

DNA REPLICATION AND CELLULAR DETERMINATION: ROLE OF THE *SIR1* GENE

The signals for more permanent changes in the expressibility of *Antp* and *Ubx* within parasegment four are likely to come from temporary changes in transcriptional regulation, directed by *ftz* and *hb*, respectively (Fig. 6). However, good evidence exists to show that origins of DNA replication can also delineate the site and initiate the assembly of a repressed heterochromatin-like domain. In the budding yeast, *Saccharomyces cerevisiae*, each of the two donor mating-type loci are flanked by silencing elements that are required for repression (Brand et al., 1985). Molecular dissection of these silencers, particularly *HMR-E*, has shown that they are composite structures (Fig. 7A; Laurenson and Rine, 1992), containing RAP1 and ABF-1 binding sites and an autonomously replicating sequence (ARS). The activity of the ARS is crucial for silencing, as derepressed cells require at least one round of DNA replication for silencing to be restored (Miller and Nasmyth, 1986). The ARS is known to be bound by an origin of replication complex (ORC), which is a heteromeric complex that contains six polypeptides (Foss et al., 1993; Bell et al., 1993). Mutational analysis has shown that one of the proteins, ORC2, is required for both silencing and repression, while evidence exists that another, ORC6, is at least required for replication. The finding that the ORC binds the ARS and is required for silencing suggests that this binding might be the signal for assembly of the repressed chromosomal domain. Some clues as to the mechanism whereby ORC binding to the ARS might be transduced into the assembly of such a domain has come from the study of hybrid fusions of the SIR1 protein to the GAL4 DNA-binding domain (Cheng-ting et al., 1993). *SIR1* is required for repression of the donor loci (Laurenson and Rine, 1992). When the SIR1 hybrid is directed to GAL-4 binding sites within *HMR*, *HMR* is silenced. The repression directed by the targeted SIR1 hybrid is typical in that it requires four of the *trans*-acting factors, including *SIR2, SIR3, SIR4* and either of the two genes encoding histone H4, which are known to be necessary for silencing. Thus experimentally targeting SIR1 to the donor loci can bypass the requirement for the silencing element *HMR-E*. Taken together, these results support a model where proteins, such as a complex of ORC, RAP1 and ABF-1 at the silencer, recruit SIR1 and that this recruitment is sufficient for the assembly of a repressed chromosomal domain, which silences the donor copies (Fig. 7B-E). Interestingly, the SIR1 protein is only required for the establishment of the repression, since inheritance of the repressed state does not require its activity (Pillus and Rine, 1989).

The pivotal role of *SIR1* in silencing is reminiscent of the role *esc* plays in the assembly of the *Pc*-G complex (Fig. 6), although here it is specific binding of ORC, RAP1 and ABF-1 to the silencer that is translated into a stable, epigenetic, state of gene repression. However, the analogy of *SIR1* with *esc* appears only to be mechanistic rather than biological, as repression of the donor copies is part of the differentiative process that occurs when mating types are switched. After mating-type switching, repression of the donor copies ensures the sexual phenotype of a cell by allowing only one mating-type allele to be expressed; that is, the allele present at the constitutively active true mating-type locus, *MAT* (Laurenson and Rine, 1992). Biologically, therefore, *SIR1* activity has more in common with the *Pc*-G complex in that it commits a cell to a particular developmental pathway that only becomes phenotypically mainfest upon differentiation. An elegant series of experiments (Pillus and Rine, 1989) appears to bring this second analogy closer. Strains carrying *sir1* mutations do, infrequently, switch their donor loci from derepressed to repressed states and a study of pedigrees has shown that this switch occurs simultaneously in all four progeny of a grandparental cell (Fig. 8). The parallel of such a pedigree with the determinative events we have described above for developmental lineages is noteworthy and suggestive. Perhaps, in the normal life cycle of *S. cerevisiae* repression is determined two cell divisions before it actually has an effect. Since the switch from derepressed to repressed donor loci occurs much more frequently in *SIR1* strains than in the *sir1* mutant, *SIR1* is likely to be critically involved in this determinative event, which may occur at the time of DNA replication.

The requirement for the positively charged N-terminal residues of histone H4 in silencing the donor loci (Johnson et al., 1992) confirms that higher-order packaging of chromatin is likely to be the cause of the repression. This is also supported by the observation that the reversible acetylation (a marker of gene activity; Turner, 1991) of the lysine residues contained within this region correlates well with the position of the mating-type alleles within the genome. At *MAT* the lysine residues are acetylated, while at the silent loci, *HMR* and *HML*, they are not (Braunstein et al., 1993). Further insight into the nature of the packaging of the silent mating-type loci has come from the isolation of the *swi6* gene in the distantly related yeast *Schizosaccharomyces pombe* (Lorentz et al., 1994). The *swi6* mutation allows expression of the silent loci and relieves the recombination suppression of the *mat2*-K-*mat3* region (Klar and Bonaduce, 1991; Lorentz et al., 1992). The striking homology of the negatively charged group of amino acids adjacent to the chromodomain places *swi6* in the *HP1* subfamily of chromobox genes (Fig. 5). While a *swi6* homologue has not yet been identified in *S. cerevisiae*, the similarity of the nature of the silencing and that there are more components to be identified (Sussel et al., 1993) suggest that the utility of the mass-action model might soon be extended to explaining silencing of the donor mating-type loci in both yeasts.

REGULATION OF DETERMINATION BY PARENTAL IMPRINTING

Before a cell becomes determined and committed to a particular developmental pathway its fate is still pliant and can be modified by cell extrinsic factors (reviewed by Slack, 1983). For example, environmental factors such as heat shock during the first temperature-sensitive period can change the state of determination (Figs 2 and 3; and Spofford, 1976). Also,

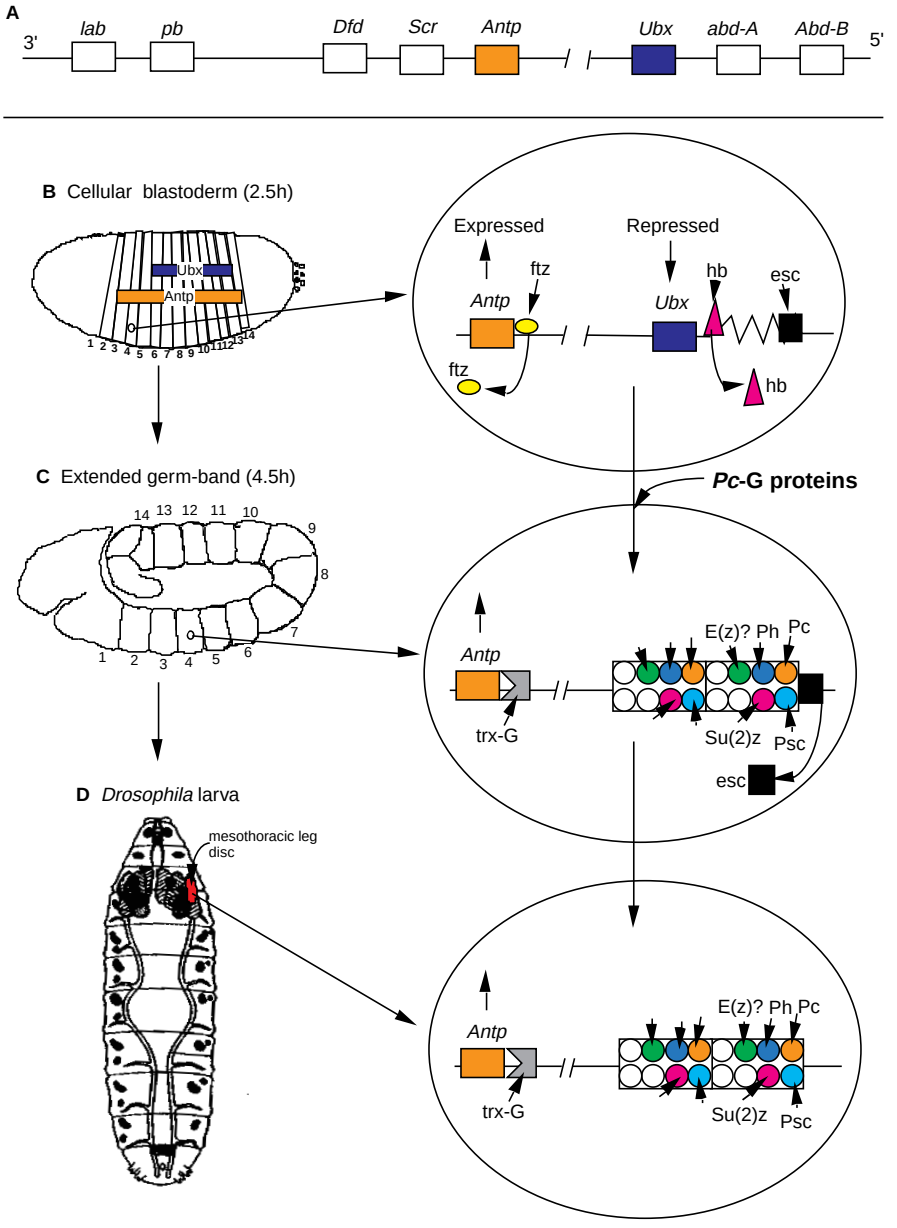

Fig. 6. A schematic diagram illustrating the step wise progression towards cell fate determination. (A) The homeobox genes in *Drosophila* are divided into two clusters known as the *bithorax* and *Antennapedia* complexes. The homeobox genes are ordered (5′-3′) as follows: *Abdominal-B* (*Abd-B*), *abdominal-A* (*abd-A*), *Ultrabithorax* (*Ubx*), *Antennapedia* (*Antp*), *Sex combs reduced* (*Scr*), *Deformed* (*Dfd*), *proboscipedia* (*pb*), *labial* (*lab*). (B) At the cellular blastoderm stage of development the parasegmentally restricted pattern of homeogene expression determines the fate of cells along the A-P axis. Here I focus only on the *Antp* and *Ubx* homeotic genes whose realms of expression encompass parasegments 3-14 and 6-13, respectively. In a cell taken from parasegment 4 of the cellular blastoderm the *Antp* gene is active, and it is through the exclusive expression of the *Antp* gene in parasegment 4 that the cell is fated to become part of the mesothoracic leg. Also, since this domain is anterior to the normal realm of expression of the *Ubx* gene the *Ubx* gene is inactive and must remain so if the fate of the cell is to be sealed. The initial repression of the *Ubx* gene is through the binding of a transcriptional repressor, *hunchback* (*hb*). The hb protein acts as an imprintor that imprints a site on the DNA that will direct the assembly of the *Pc*-G complex. The recognition of hb binding by esc is not direct, instead esc is envisaged to recognise local, transient, changes in chromatin stucture (zig-zag line), which result from binding of hb to its recognition sequence. These chromatin changes, which temporarily repress *Ubx*, may affect nucleosome position (Roth et al., 1990) and be dependent upon the local anisotropic flexibility of DNA (Travers and Klug, 1990). The *Pc*-G may assemble at, or around, the same time as esc binds. (C) In a cell (progeny of the cell above) taken from parasegment 4 of the extended germ-band embryo the *Pc*-G complex has already assembled on the site marked by *esc*. Note the similarity of the *Pc*-G complex and the heterochromatin complex described in Fig. 4. Polycomb (Pc), Polyhomeotic (Ph), Posterior sex combs (Psc) and Suppressor (2) of zeste (Su(2)z) have been immunolocalised to the homeotic genes on polytene chromosomes. The mutant phenotype of *Enhancer of zeste* (*E(z)*) suggests that its gene product may be part of the *Pc*-G complex (Rastelli et al., 1993), although it has yet to be shown to localise to the homeotic genes. Immunochemical studies have also shown that the *Pc*-G complex does not contain H1 histone (Franke et al., 1992) and may therefore only contain nucleosomal DNA. The activation of *Antp* in parasegment 4 by the fushi tarazu (ftz) protein (Ingham and Martinez-Arias, 1986) is also translated into a more permanent form of activation by the *trithorax*-group of genes (*trx*-G). The *trx*-G (reviewed by Kennison, 1993) keeps the chromatin surrounding the *Antp* in an ‘open’ expressible state that maintains expression of *Antp* throughout embryogenesis. Beyond the extended germ-band stage of development the need for *esc* is removed. (D) Cells that expressed the *Antp* gene in parasegment 4 of the early embryo have greatly expanded and now form part of the mesothoracic leg disc within the *Drosophila* larva. This disc will differentiate into the mesothoracic leg during metamorphosis. A cell taken from this disc possesses the final, heritable, state of chromatin that determines the transcriptional fate of the *Antp* and *Ubx* genes: the *Antp* gene is maintained in an expressible state by the *trx*-G of genes, while the *Ubx* gene is rendered inexpressible by the *Pc*-G of genes.

exposure of blastoderm embryos to ether vapours appears to change the state of determination of the homeotic genes as regulated by the *trx*-G and *Pc*-G genes (Capdevila and Garcia-Bellido, 1978). These effects may reflect a modulation of early-acting labile interactions - such as *hb*, *ftz*, *esc* and *mechanistically* in yeast, *SIR1* - which normally direct more permanent changes later in development, whereupon the fate of cells becomes cell intrinsic, and largely independent of environment.

Another effect that must also occur early, before the time of

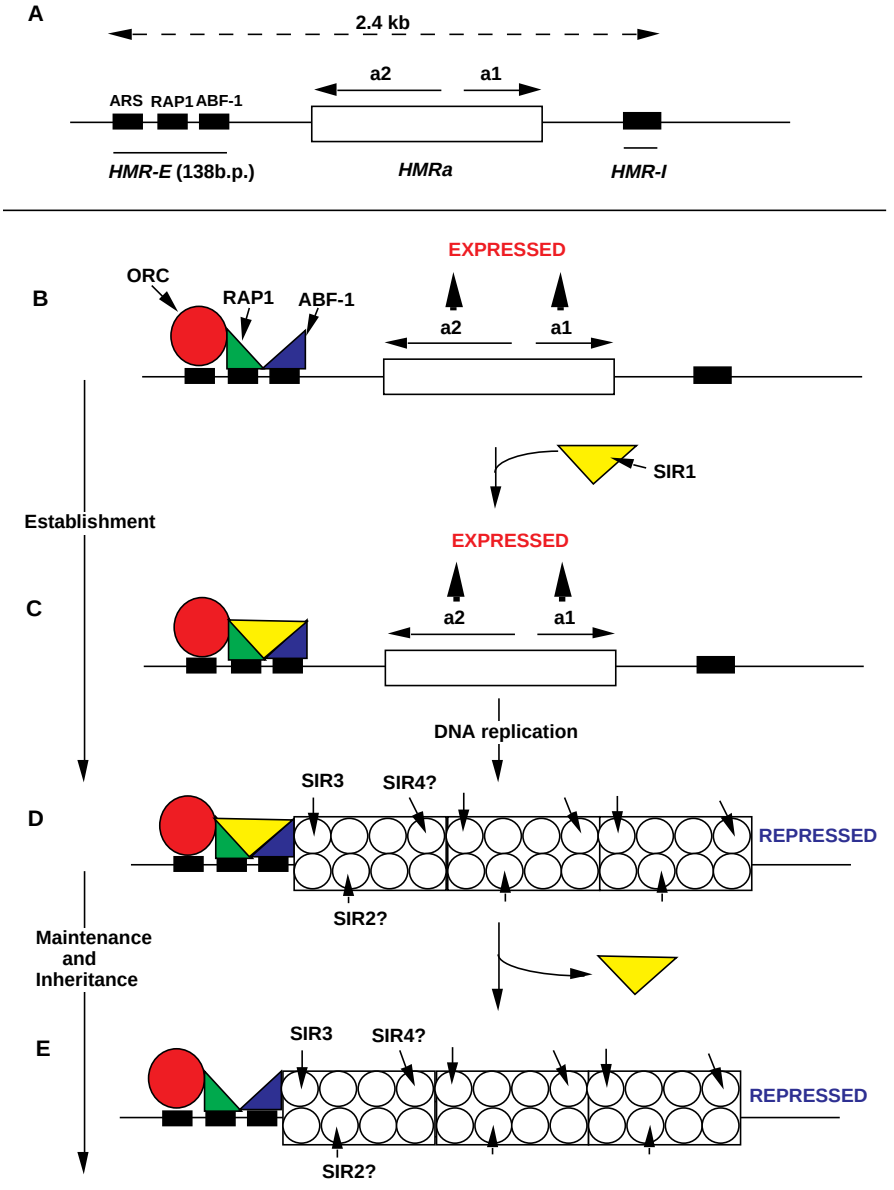

Fig. 7. A possible scheme for silencing derepressed donor mating-type loci in *S. cerevisiae*. (A) Elements that are involved in repression of *HMRa*. *HMRa* contains two transcripts, a1 and a2, which confer all of the phenotypic characteristics peculiar to the *a* sexual type. The silencers, *HMR-E* and *HMR-I* are both required for complete silencing of *HMRa*, although *HMR-E* is essential for this repression, whilst *HMR-I* is only important. The 138b.p. contains three sub-elements, an ARS consensus sequence and two regions footprinted by RAP1 and ABF-1. (B) The specificity for the repression at the silent loci is provided by the binding of ORC, RAP1, ABF-1. (C) The binding of the three proteins recruits SIR1, and establishes the requirements for changes in chromatin structure that will repress the donor loci after DNA replication. (D) After DNA replication the donor loci on the two daughter chromatids (only one given here) are packaged into a heterochromatin-like complex. This complex is likely to contain SIR3, which, like the modifiers of PEV, exhibits dosage effects in a phenomenon related to mating-type repression, namely telomeric position effect (Gottschling et al., 1990; Renald et al., 1993). SIR4 localises to the same sub-nuclear region as SIR3 (Pallidino et al., 1993) and is also a good candidate for the complex. The evidence is weaker for SIR2, although the *sir2* mutation not only relieves repression at the donor loci (Laurenson and Rine, 1992), but also relieves the recombination suppression at the ribosomal genes (Gottlieb and Esposito, 1989); the ribosomal genes are usually embedded within heterochromatin (Ritossa, 1976). Mutations in histone 4 (Johnson et al., 1992) also derepress the silent loci, suggesting that the complex includes nucleosomal DNA, shown by the line running through the boxes. (E) After the donor loci have been repressed by packaging into a heterochromatin-like complex, repression can be maintained and inherited in the absence of SIR1. Not given here are the other mutations *NAT1* and *ARD1* (Mullen et al., 1989), which encode enzymes that may modify components involved in the repression.

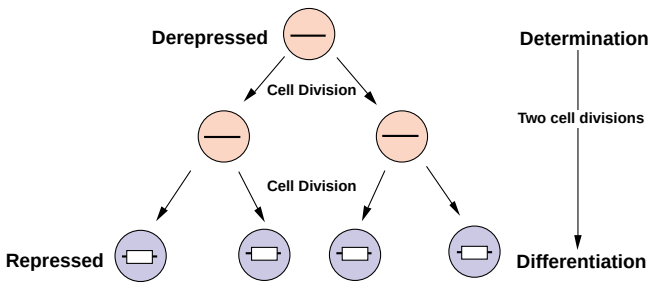

Fig. 8. Role of *SIR1* in determination of repression in *S. cerevisiae*. In *sir1* mutant strains there are infrequent switches at the *HML* donor locus, from derepressed to repressed states. These switches are non-random in that pedigree analysis shows that all four progeny of a grandparental cell switch at the same time (Pillus and Rine, 1989). The decision to switch therefore occurs two cell divisions before the switch itself. Since the switch occurs much more frequently in *SIR1* strains than in the mutant, *SIR1* is likely to be crucially involved in the determinative event. Thus the role *SIR1* plays in establishing the repression is akin to determination in multicellular organisms, whilst the maintenance and inheritance of the repression (Fig. 7) is like differentiation.

determination, but has as yet no explanation results from the finding that determination in PEV can be affected by the parental origin of the rearrangement; that is, the degree of variegation is dependent upon whether the rearranged chromosome is derived from the sperm or the egg (Morgan et al., 1937; Noijdin, 1944; especially, Spofford, 1959, 1961; Hessler,

1961). While the early experiments provided proof of the phenomenon they were flawed to a small degree by the fact that the parental-origin effects could be due to the conditioning of egg cytoplasm by the pre-meiotic presence or absence of the rearrangement in the mother. This caveat was lifted by a tightly controlled experiment (Baker, 1963) where *both* parents harbour the rearrangement $Dp(1;3)N^{264\text{-}58a}$ (Fig. 9A), and in fathers the non-rearranged homologue is marked with the dominant mutation *Wrinkled* (*W;* wrinkled wings). Since males homozygous for $Dp(1;3)N^{264\text{-}58a}$ die, the parental origin of the rearrangement can be determined with certainty in sons (Fig. 9B). Thus, *W* sons have the maternal rearrangement, whilst the rearrangement in non-*W* sons is paternally derived. All other factors being equal, the results from the cross $Dp(1;3)N^{264\text{-}58a}/+$ (mothers) × $Dp(1;3)N^{264\text{-}58a}/W$ (fathers) were striking and reproducible. Comparison of the degree of variegation showed that non-*W* sons have four to five times as much pigment as *W* sons (Fig. 9B). Moreover, this effect only lasts for one generation as it is reversed when the duplication is inherited from daughters of the cross. These features are entirely consistent with the definition of parental imprinting (Crouse, 1960).

As emphasised by Baker (1963), since the number of pigmented sectors within the mottled eyes of the *W* sons is different from non-*W* sons (Fig. 9B) it can be concluded that the parental-origin effect acts via the normal mechanism of determination (cf. Fig. 2). More specifically, the rearrangement possesses an 'imprint' (see Appendix) that can affect the number of eye disc cells (and ultimately sectors) in which the *white* gene is inactivated by heterochromatinisation. The direction in which the imprint can affect the determinative event is such that paternal derivation of the rearrangement makes it less likely that *white* will be inactivated in an eye disc cell, while maternal derivation makes it more likely. By analogy with the explanation for the known effect of heat shock during the first temperature-sensitive period (Fig. 2; Spofford, 1976), and bearing in mind that the imprint must act very early, I suggest that the imprint might modulate - perhaps by being part of - the initial steps involved in heterochromatin assembly at the variegating breakpoint.

While we know very little about the steps that specify and initiate the assembly of a heterochromatin domain in PEV, I have discussed in some detail the role of *hb* and *esc* in the assembly of the *Pc*-G complex (Fig. 6). In short, parental imprints may have much in common with proteins like hb; they may be developmentally labile and could change during gametogenesis or embryogeny via intermediary molecules like esc, before becoming permanent later in development through heritable changes in chromatin structure, including those brought about by DNA methylation in mammals. Put into a locution typically associated with parental imprinting, the hb protein can be thought of as an 'imprintor' (see Appendix) that specifically marks out a region of the genome that will be changed in its expressibility at some later stage during development when the fate of a cell is determined.

Some evidence that is consistent with a labile parental imprint that is stored during early embryogeny and only later transduced into a more permanent form comes from recent studies on autosomally imprinted genes. Of the mammalian imprinted genes the first discovered and the best studied is *Igf2* (DeChiara et al., 1991). *Igf2* is co-dominantly expressed within the pre-implantation mouse embryo (Latham et al., 1994), yet only expressed from the paternal genome in foetal and adult mice (DeChiara et al., 1991). Allelic differences in methylation of *Igf2* are consistent with the expression studies and arise only at the blastocyst stage of development; methylation patterns of *Igf2* also show no difference in the respective parental germ cells (Brandeis et al., 1993). Thus DNA methylation - a heritable modification - appears not to represent the primary imprint on the *Igf2* gene. Likewise, the closely linked *H19* gene shows no allelic differences in methylation up to the blastocyst stage and no differences in the respective parental germ cells (Brandeis et al., 1993). A third gene, *Igf2r*, is, like *Igf2*, co-dominantly expressed during early embryogeny (Latham et al., 1994); however, it is different from the other two imprinted genes in one important aspect. The *Igf2r* gene has an unconventional CpG-rich intron which is highly methylated on the expressed maternal allele in adults and, moreover, some of this intronic methylation is present in oocytes, suggesting that it might be the maternal imprint (Storger et al., 1993).

A possible role for the methylation within the second intron of *Igf2r* has been posited: methylation on the maternal chromosome may inhibit the binding of proteins to a silencer within the intron and thus enable the maternal allele to be expressed (Storger et al., 1993). This view is supported by the observation that in the methyl-transferase-deficient mouse, both maternal and paternal alleles of *Igf2r* are silenced (Li et al., 1993). Insight into the silencing of *Igf2r* on the paternal chromosome (where the intron is not methylated; Storger et al., 1993) may come from *Drosophila* where the *iab-2* regulatory sequence, found within the fourth intron of the *abd-A* gene of the *bithorax* complex, can nucleate the assembly of the *Pc*-G complex, resulting in repression of the *abd-A* gene (Simon et al., 1993).

Findings from the study of boundary elements within the *bithorax* complex may also be applicable to models of the reciprocal imprinting of the *Igf2* and *H19* genes by competition of enhancers (Bartolomei et al., 1993; Efstradiatis, 1994). In the most recent model (Efstradiatis, 1994), whose elements lie in the order, *H19* enhancers - *H19* - *Igf2*, on chromosome 7, the suggestion is that the selective usage of the *H19* enhancers by either *H19* or *Igf2* is regulated by a postulated boundary element that lies between the structural genes. The boundary is assembled on the maternal chromosome as a consequence of a maternal imprint (that appears not to be methylation; Brandeis et al., 1993) and its role, when assembled, is to direct the activities of the enhancers to the *H19* gene alone. The *Igf2* gene is therefore effectively silenced by proxy, by being insulated from the *H19* enhancers. Inhibiting the assembly of the boundary element on the paternal chromosome allows the enhancers to act upon the *Igf2* gene (Efstradiatis, 1994). As for the *Igf2r* gene discussed above, methylation is thought to inhibit the assembly of the boundary element on the paternal chromosome, since in the methyl-transferase-deficient mouse both alleles of the *Igf2* gene are silenced (Li et al., 1993). A feature of this model is that the *Igf2* gene remains in an expressible conformation throughout and in this way explains the low, but detectable, levels of transcription of the *Igf2* gene on the maternal chromosome (Sasaki et al., 1992). Again, a precedent for controlling enhancer activity through the formation of a physical boundary has come from *Drosophila* where the *Fab-7* boundary element regulates the activities of

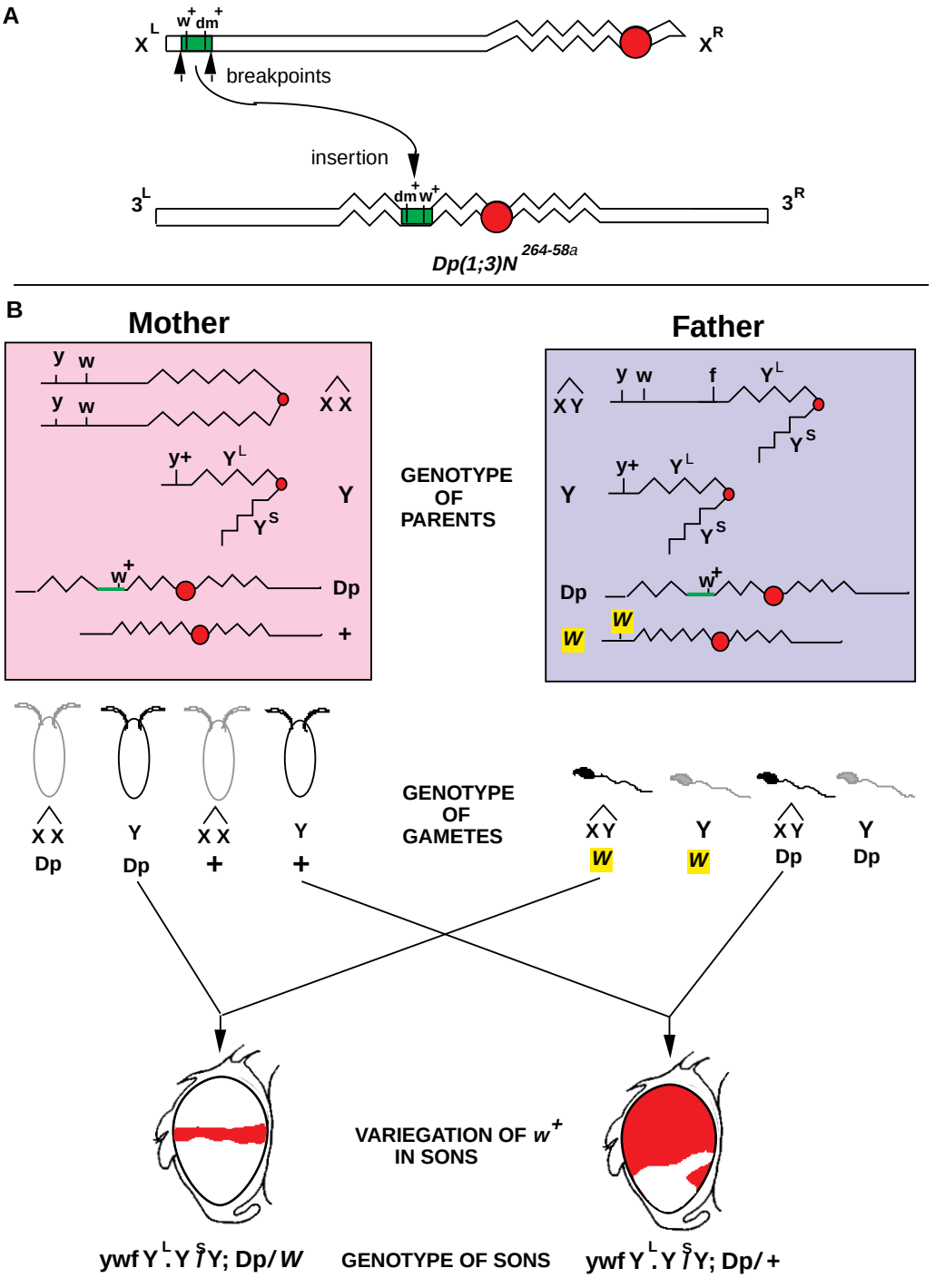

Fig. 9. The degree of variegation in PEV is affected by parental origin of the rearrangement. (A) The *Dp(1;3)N*$^{264\text{-}258a}$ chromosome is an inverted insertion of 20 chromosome bands from the X chromosome into the proximal heterochromatin (zig-zag line) of the left arm of chromosome 3. (B) Cytological and genetical constitution of the flies used to show that variegation caused by position effect of *Dp(1;3)N*$^{264\text{-}258a}$ can be affected by parental origin. Both parents contain *Dp(1;3)N*$^{264\text{-}258a}$. (This situation is directly analogous to that of endogenous imprinted genes, where each parent has a copy.) In fathers the normal unrearranged chromosome bears a dominant marker, *W* (*Wrinkled wings*), which can be used identify the parental origin of the normal, unrearranged third chromosome. The *W* marker does not affect variegation itself. Both parents also possess a Y chromosome, which suppresses variegation and makes it easier to score differences between the offspring. The attached-Y and attached-X chromosomes reduce the number of segregating elements in the crosses. The gametes that give rise to the informative progeny are drawn as a continuous line. The effect of parental origin can be unequivocally shown in sons, since males homozygous for the duplication die. In sons that inherit *Dp(1;3)N*$^{264\text{-}258a}$ from the father the mutant areas within the eye are much less extensive than when *Dp(1;3)N*$^{264\text{-}258a}$ is inherited from the mother. Daughters are not given here, both for clarity and because the homozygotes are viable, making it impossible to identify unambiguously the parental origin of the rearrangement without breeding tests.

the *iab-6* and *iab-7* *cis*-regulatory sequences within the abdominal region of the *bithorax* complex (Galloni et al., 1993). *iab-7* is normally active in parasegment 12 and regulates the *Abd-B* gene in this parasegment; however, deletion of *Fab-7* results in the ectopic activation of *iab-7* in parasegment 11, where the *iab-6* regulatory element normally functions (Galloni et al., 1993). Thus, the *Fab-7* element acts as a boundary (Eissenberg and Elgin, 1992) that directs the activities of enhancer elements to their appropriate targets within a parasegment. *Fab-7* also binds Pc protein (Orlando and Paro, 1993), suggesting that the boundary is the *Pc*-G repressor complex (Fig. 6). In both the *Igf2r* and *H19/Igf2* models the postulated function of methylation is essentially passive and is to inhibit the active formation of a repressed chromosomal domain, a domain that may be something like the *Pc*-G complex. This function of DNA methylation is distinct from the more typical role of methylation in gene repression (Bird, 1992).

In summary, the mechanisms underlying the stable, epigenetic, states of some autosomally imprinted genes are likely to involve methylation (Li et al., 1993). However, it seems unlikely that methylation is the sole imprint - except perhaps

for the active maternal copy of *Igf2r* - that is retained during early (especially preimplantation) embryogenesis. Instead, the molecular mechanisms that lead to the final epigenetic, yet heritable, state of an imprinted gene or chromosome may have much in common with the regulatory interactions that provide the specificity for consequent heritable changes in gene expressibility (Figs 6 and 7). I have described some of these interactions and they include transcriptional activation (e.g. *ftz*), repression (e.g. *hb, esc*) and DNA replication (e.g. ORC, *SIR1*). An important route for the effects of parental imprints might be the latter, as recent studies have shown that allelic differences in replication timing exist between imprinted regions in mice (Kitsberg et al., 1993).

The difference between parentally imprinted and non-imprinted genes is that in the former the initial imprintor acts very early, when the parental genomes are separate. Only thus can the maternal and paternal alleles (or chomosomes) be imprinted differently and therefore be subject to different states of determination, leading to stable differences in expression observed later in development and adulthood. This first step may take place in the respective germ-lines (Crouse, 1960) or be completely under maternal control - the imprintor acting during female meiosis and/or during the period when the sperm is being assembled into the paternal pro-nucleus by the ooplasm (Chandra and Brown, 1975).

THE CHROMATIN STATES REGULATED DURING CELLULAR DETERMINATION

The foregoing discussion has led to the notion that the transition from a situation where developmental fate is governed by cell extrinsic factors to one where it becomes cell intrinsic and determined is likely to be a transition in chromatin state. Interestingly, there are considerable global changes in chromatin composition in *Drosophila* around the time major determinative events are taking place. A dramatic shift in the ratio of HMG-D:H1 occurs at the blastoderm stage of development (stage 12; Ner and Travers, 1994), which is close to the time that cells begin to express the homeogenes (Akam, 1987) and thus become fated (Simcox and Sang, 1983). This inverse stoichiometric correlation of HMG-D to H1 has suggested that the presence of HMG-D in the early embryo might result in a less compacted intermediate on the pathway to the thick 30 nm fibre, which normally requires H1 histone (van Holde, 1988). A looser structure is envisaged as facilitating the rapid divisions that occur during early embryogeny. In addition to this, HMG-D-containing chromatin might also facilitate the binding of the maternal and early-acting segmentation gene products to their cognate recognition sequences. In this way, the looser chromatin packaging would not only allow easy access of ftz and hb to their binding sites for initial regulation of the homeotic genes but would also serve to target mechanisms, such as the *trx*-G and *Pc*-G complexes, to specific sites within the homeogene clusters (Fig. 6B).

The accumulation of H1 histone in *Drosophila* embryos, to levels normally found in the adult (stage 14A/B; Ner and Travers, 1994), broadly coincides with the time when the *Pc*-G and *trx*-G complexes are being assembled (Fig. 6), and suggests that maintenance of the homeogene expression patterns might involve the regulation of the 30 nm (thick) chromatin fibre. The 30 nm fibre is foremost a morphological definition and various electron microscopy studies have shown that the thick fibre represents the inactive state of chromatin within the nucleus (Ris and Kubai, 1970; Andersson et al., 1984). In vitro and under physiological salt conditions, the energetically favourable conformation of H1-containing chromatin is also a chromatin fibre of around 30 nm in thickness (Thoma et al., 1979; Worcel et al., 1981; Woodcock et al., 1984). The fibres produced in these experiments are, however, far from uniform and they exhibit many bumps and indentations along their length; in vitro, the integrity of the 30 nm fibre is usually poor. I suggest that in vivo the integrity of the 30 nm fibre within euchromatin might be maintained by specialised chromatin complexes such as *Pc*-G. According to this scheme, the repression of a gene could brought about by incorporation into the complex itself or, by proxy, by being assembled into the 30 nm fibre that surrounds and is maintained by the complex. Assembly into the 30 nm fibre might inhibit transcription either by steric hindrance caused by the components of the compact state itself, or by changes in nucleosome positioning that lead to inappropriate placement of sequences required for proper transcription.

Since the default pathway of chromatosomal DNA under physiological salt conditions is, in general, a folding towards a thick fibre, it is also likely that energy might be required - perhaps through the hydrolysis of ATP - for its dissolution in order to provide access to the transcriptional machinery. Interestingly, the *brahma* gene, a member of the *trx*-G genes is a DNA-dependent ATPase (reviewed by Kennison, 1993). The brahma protein might act as a 'molecular matchmaker' (Sancar and Hearst, 1993) that brings a specific activator protein into close proximity to the transcriptional machinery by disrupting the 30 nm fibre. An alternative, though not mutually exclusive, possibility is that brahma acts as a traditional helicase and disrupts chromatin by changing the topology of DNA surrounding a targeted activator protein (Travers, 1992).

In the mouse, methylation changes are likely to be synonymous with chromatin changes, since methylation of CpGs within chromatin is thought to be stabilised by methyl-binding proteins (Bird, 1992). As in *Drosophila*, there is evidence of dramatic changes in chromatin structure during mouse embryogenesis. A global demethylation occurs during preimplantation embryogenesis between the 8-cell and the blastocyst (Kafri et al., 1992; Brandeis et al., 1993) stages of development. CpG islands are also undermethylated in a variety of embryonic cell types (Antequera et al., 1990) and these reduced levels are not inimical to the survival of ES cells, as the latter remain viable in vitro even when the small amount of methylation at the CpG islands is reduced further (Li et al., 1992). ES cells can also tolerate the ablation of a methyl-binding protein, MeCP2, which is thought to stabilise the repression brought about by methylation (Meehan et al., 1992).

The programmed demethylation seen in the pre-implantation embryos may provide a window, like the HMG-D-containing chromatin in *Drosophila*, where the packaging of chromatin is looser and can allow developmentally labile interactions (e.g. *hb* in *Drosophila*) to direct heritable changes in the expressibility of genes at a later stage in development. Some of these heritable changes are likely to involve de novo methylation and the repression of chromatin through binding of proteins such as MeCP2 (Meehan et al., 1992). While the mechanism of

repression involving MeCP2 is unknown, it may function by stabilising the 30 nm fibre. A key feature of this scenario is that the demand for specificity is met, since the labile interactions that direct de novo methylation are specific; it is unlikely that methyl-transferase alone could specify the sites that are to be changed in their expressibility. Since there is a demand for specificity in parental imprinting effects (specific genes are imprinted), it is also unlikely that the initial imprintor (see Appendix) in mammals will be methyl-transferase acting by itself.

VARIEGATED CONCLUSIONS: EVOLUTION AND CONSEQUENCES OF HERITABLE STATES OF DETERMINATION

A conclusion that can be drawn from comparison of the determinative events in yeasts (Figs 7 and 8) with those that are found in multicellular organisms (Figs 2 and 6) is that while only two cell divisions separate determination from differentiation in yeasts, days - or even months - can separate these two phases in Metazoans. As a consequence, perhaps, the determinative events in yeasts involve labile interactions (*SIR1*), whilst determination in Metazoans involves stable changes in chromatin structure (*Pc*-G and *trx*-G) or methylation. Determined states characterised by epigenetic, yet heritable, changes in gene expression are, it would seem, a peculiarly Metazoan phenomenon.

One of the selective pressures for the evolution of determined states that are heritable through many rounds of cell division is likely to have come from the need of cells to remember their fates during growth of the embryo. This is particularly so for organisms undergoing regulative development where patterning of embryos is dependent upon morphogenetic gradients that provide positional information to cells along the embryonic axes (Wolpert, 1969). It is this positional information that is converted into cellular fate by the homeogenes. However, due to their physical properties (molecular gradients diffuse) and the fact that they can only act effectively over short distances, in the region of 100 cell widths (reviewed by Slack, 1983), they cannot on their own continue to specify fate, via the homeogenes, throughout development. It seems likely that, once a decision is made by a cell to express a pattern of homeogenes, according to the position it finds itself in along the A-P axis natural selection would have favoured mechanisms such as *Pc*-G and *trx*-G, which would make this decision final and mitotically stable. These mechanisms may have usurped those already found in yeasts, where heritable changes in gene expression are a feature of differentiation (Fig. 7). This seems all the more likely because of the role of chromodomain proteins in differentiation in yeast (*swi6*) and determination in *Drosophila* (*Pc*).

Another consequence of determinative events that involve heritable changes in chromatin structure is that they may enable the developmentally labile factors that direct such changes to be promiscuous and to be used several times during development (Epstein, 1992). For example, during the first two hours of *Drosophila* development a set of helix-loop-helix (HLH) proteins measure the X/A ratio and determine sexual fate and also dosage compensation (Cline, 1993). At least for dosage compensation these early activities are known to be transformed into a permanent form by changes in chromatin structure. In males, where the X:A ratio is 0.5 the HLH proteins repress the *Sex lethal* gene, which in turn allows the specific targeting of the *male-specific lethal-1* gene to the X-chromosome (Palmer et al., 1994). As a result, there is a twofold increase in expression of the genes on the X in males, which is associated with increased levels of histone H4 acetylation and a more diffuse chromatin packaging (Bone et al., 1994). Having determined sexual fate and dosage compensation, different combinations of the same HLH proteins are again involved in another determinative event, that of forming the nervous system, which takes place later during the third and sixth hours of development (Younger-Shepard et al., 1992). Thus, the HLH transcription factors may be considered to be an example of a functional gene cassette (Jan and Jan, 1993) that may be used time and again during development.

These latter observations are probably related to older somatic recombination studies, which showed that, as development proceeds, determination gradually restricts the developmental potential of cells (Garcia-Bellido, 1975). My suggestion would be that the allocation of cells to distinct lineages (lineage restriction) is likely to be presaged by determinative changes in chromatin structure, similar to those I have described above.

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APPENDIX

A note on definitions used in the text

Imprintor

A molecule (could be protein or nucleic acid) that marks out a region of the genome that will be changed in its expressibility at some later stage of development. An example of an imprintor would be the hb protein (Fig. 6), which targets the assembly of the *Pc*-G complex. The imprintee is the DNA sequence to which the imprintor binds.

Imprint

A combination of imprintor and imprintee. However, in the example discussed the imprintor may change during development (hb to esc; Fig. 6). Nevertheless, it remains that the same region marked out by the initial imprintor will be heritably changed in its expressibility, sometime during development; the imprint must at least be partially stable for a few rounds of DNA replication before it directs more permanent changes. It may constitute a methylation imprint in mammals.

It is emphasised that until the imprint becomes permanent an imprinted gene may be regulated in any way and independently of the final stable change in expressibility that is a consequence of the imprint. For example, preferential inactivation of the paternal *Igf2r* gene occurs despite both alleles being active during pre-implantation embryogeny (Latham et al., 1994). Beyond this point, as with non-imprinted genes, the transcriptional fate of a gene is determined. Only in special cases (e.g. de-heterochromatinisation of the paternal chromosome set in the gut of male coccids (Nur, 1967) and bi-allelic

expression of *Igf2* in choroid plexus and leptomeninges (DeChiara et al., 1991)) can the early determination of gene expression be reversed.

Note added in proof

The imprint on the paternal copy of the mouse *Xist* gene is developmentally labile (Kay et al., 1994). Indeed, the developmental characteristics of this imprint are similar to that of *ftz* in *Drosophila* (Fig. 6B) in that it is required for gene activity during early embryogeny; specifically, up until the 8-cell stage of development. Beyond this stage and in cells that become determined to form the trophectoderm lineage, where the paternal X chromosome is preferentially inactivated, activity of *Xist* is maintained by another mitotically stable mechanism. I suggest that this maintenance mechanism may have similarities to the *trx*-G complex (Fig. 6C).

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