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Review

Chromatin organization and transcriptional control of gene expression in *Drosophila*

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Abstract

It is increasingly clear that the packaging of DNA in nucleosome arrays serves not only to constrain the genome within the nucleus, but also to encode information concerning the activity state of the gene. Packaging limits the accessibility of many regulatory DNA sequence elements and is functionally significant in the control of transcription, replication, repair and recombination. Here, we review studies of the heat-shock genes, illustrating the formation of a specific nucleosome array at an activatable promoter, and describe present information on the roles of DNA-binding factors and energy-dependent chromatin remodeling machines in facilitating assembly of an appropriate structure. Epigenetic maintenance of the activity state within large domains appears to be a key mechanism in regulating homeotic genes during development; recent advances indicate that chromatin structural organization is a critical parameter. The ability to utilize genetic, biochemical and cytological approaches makes *Drosophila* an ideal organism for studies of the role of chromatin structure in the regulation of gene expression.

Keywords: Cellular memory; Chromatin remodeling complexes; Gene silencing; Heat shock genes; PcG and trxG proteins

1. Introduction

Eukaryotic genomes are enormous in size; the packaging of the DNA within the nucleus (and cyclically in metaphase chromosomes) is in itself a formidable task. Moreover, the packaging must at least accommodate, if not contribute to, a system of regulated gene expression that supports development of a multicellular organism, with extensive specialization of cell types. Thus, while in prokaryotes the ground state for transcription appears to be non-restrictive, one might anticipate that in eukaryotes the ground state would be restrictive; indeed, in higher eukaryotes, most of the genes are not expressed in any given cell type. A further defining characteristic of eukaryotes is the presence of histones, small basic proteins found in the nucleus in a 1:1 mass ratio with DNA. The old hypothesis that histones might serve not only to package DNA in a chromatin structure, but also function as general repressors of gene expression, has now been largely substantiated, leading to a new appreciation of the differences in gene regulation between prokaryotes and eukaryotes (Struhl, 1999).

The nucleosome model of chromatin structure, proposed 25 years ago, has stimulated a tremendous amount of research activity and has led to a much clearer picture of the packaging of DNA at the primary level (reviewed by Kornberg and Lorch, 1999). The basic structural results may be summarized as follows. The nucleosome, the primary subunit of eukaryotic chromatin, consists of an octamer of core histones with 146 bp of DNA wrapped around the outside in 1 2/3 left-handed turns;

Abbreviations: ACF, ATP-utilizing chromatin assembly and remodeling factor; BX-C, bithorax complex; CHRAC, chromatin-accessibility complex; DH sites, DNase I hypersensitive sites; HSE, heat-shock element; HSF, heat-shock factor; HS sites, hypersensitive sites; ISWI, Imitation Switch; NURF, nucleosome remodeling factor; PcG, Polycomb group; PRC1, Polycomb repressive complex 1; PRE, Polycomb response element; RCAF, replication-coupling assembly factor; TRE, trithorax response element; trxG, trithorax group; X-CHIP, cross-linking followed by chromatin immunoprecipitation.

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a linker of ~20–50 bp of DNA connects one core to the next in a chain of repeating subunits, making up the 100 Å chromatin fiber. The histone core consists of an $(H3+H4)_2$ tetramer and two (H2A + H2B) dimers. One molecule of histone H1 is associated with each repeating unit, interacting with the linker DNA as well as the core. The 100 Å chromatin fiber is further coiled into a 300 Å fiber; both histone–histone and histone–DNA interactions within and between core particles and linkers are potentially involved in stabilizing this structure. Micrococcal nuclease cuts the linker DNA between subunits preferentially, generating a series of DNA fragments representing monomers, dimers, trimers, etc.; by using a restriction enzyme to define a fixed endpoint, the positions of the nucleosomes along the DNA can be mapped.

The presence of nucleosomes on DNA generally restricts gene expression. Nucleosomes inhibit both the binding of RNA polymerase II to initiation sites and transcriptional elongation (Kornberg and Lorch, 1999). Mapping of chromatin structure using a variety of nucleases has shown that the 5′ regulatory regions of active genes appear to be nucleosome-free; such regions are referred to as 'DNase I hypersensitive sites' (DH sites) or simply 'hypersensitive sites' (HS sites) (Elgin, 1988). In some cases, including the heat-shock genes discussed in detail below, the inducible gene is maintained in this configuration; such genes are referred to as 'pre-set', as no change in the nucleosome array at the promoter is required for activation. Most housekeeping genes might fall into this group, although relatively few studies have been done on such genes. In many cases, 5′ regulatory regions, including the promoter, are packaged into a nucleosome array when the gene is in an inactive state. In this case, a 'remodeling' of the chromatin structure is an integral part of the process of specific gene activation (Wallrath et al., 1994). During the last several years, several protein complexes with ATP-dependent remodeling activity have been identified and characterized (see Kingston and Narlikar, 1999; Tyler and Kadonaga, 1999; and discussion below).

In addition to the repressive role of nucleosomes in the primary chromatin fiber, higher-order packaging is of critical importance. While higher-order structures are not well defined, it is clear that the genome is subdivided by boundaries that limit the regulatory effects of positive and negative elements such as enhancers and repressors (Bell and Felsenfeld, 1999). Further, one can identify large domains that are, or are not, permissive for gene expression, and this level of gene regulation apparently depends on packaging (Gasser et al., 1998). Such larger-scale organization appears to be critical in defining and maintaining the developmentally regulated expression pattern of complex loci such as the bithorax complex (BX-C), which includes three homeotic genes. The homeotic genes are typically found in clusters with a conserved organization; the bulk of the DNA at such loci does not code for protein but is required for the intricate pattern of regulation (Duncan, 1987; Lewis et al., 1995; Martin et al., 1995).

The ATP-dependent remodeling machines present in the nucleus can shift nucleosomes to an altered conformation, can cause nucleosomes to 'slide,' altering their local position, and in some cases can transfer the histone core from one DNA molecule to another, thus providing 'fluidity', the means to change from one state to another. However, on the whole, the chromatin structure is remarkably stable; once the pattern of cell-specific gene expression is established in a differentiated cell, it is maintained through subsequent generations. Recent findings suggest that the altered state might be 'locked in' by modification of the histones; in particular, histones H3/H4 in active domains have high levels of lysine acetylation, while those in inactive domains are hypoacetylated (for a review, see Grunstein, 1997). Post-translational modification of the other histones, as well as the utilization of variants of both the linker and core histones, could have a profound effect on the packaging of the 100 Å fiber, altering nucleosome stability and spacing. In turn, this could affect the ability of the nucleosome array to unfold, accommodating passage of RNA polymerase, or conversely, the ability of the nucleosome array to fold into a stable 300 Å (or higher-order) fiber, blocking access.

The multiplicity of packaging steps, and the number of possible targets for regulation, indicate the complexity of the system. The participation of nucleosomes and higher-order chromatin structures in gene regulation has been studied extensively in *Drosophila melanogaster* using a combination of biochemical, cytological and genetic methods. This organism offers an opportunity for an in-depth look at the processes leading to activation and inactivation of genes, and at the mechanisms for propagating these states through multiple rounds of cell division.

2. Establishing the chromatin structure at promoters

2.1. Chromatin structure of an active/inducible gene: the heat-shock genes

Studies of the *Drosophila* heat-shock genes have revealed the specialized chromatin structure at the 5′ regulatory region of a pre-set gene. The family of heat shock proteins belongs to the class of 'molecular chaperones'; one function of these proteins is to bind to, and re-fold, partially denatured proteins to prevent their degradation following environmental stress (for example, heat shock) (Morimoto et al., 1994). These genes show a similar response to heat shock in most cell types throughout most of development. Synthesis of heat-

shock proteins must be quickly induced in response to stress to ensure survival. In *Drosophila*, it takes 30 s to bind the specific transcriptional activators involved, notably the heat-shock factor (HSF) (Wu et al., 1994), and about 2 min to reach full 100–200-fold activation of the *hsp70* gene (O'Brien and Lis, 1993; O'Brien et al., 1995). The ability to respond rapidly appears to be due to the 'advance preparation' both of the HSF and of the chromatin structure of the heat-shock gene promoters, allowing an almost immediate start of transcription (Lis and Wu, 1995).

2.1.1. Chromatin structure of the heat-shock genes prior to activation: the role of GAGA factor

The well-characterized *Drosophila hsp26* and *hsp70* genes are organized and function in a very similar fashion (Fig. 1). Both have a highly defined nucleosome array, with HS sites (nucleosome-free regions) encompassing the key regulatory elements — the TATA box and heat-shock elements (HSEs). Both show binding of a preassembled TFIID complex and one molecule of paused RNA polymerase. Both have several GAGA factor binding sites, clustered small repeats of (CT) dinucleotides, upstream of the transcription start site (for a review, see Lis and Wu, 1995). The $(CT)_n$ repeats are located in the immediate vicinity, or even overlap, with the HSEs, essential for HSF binding and heat-shock inducibility. DNA footprinting of the endogenous *hsp26* gene showed that the two upstream clusters of GAGA factor binding sites and HSEs are separated from each other by a precisely located nucleosome (Cartwright and Elgin, 1986; Thomas and Elgin, 1988). (Note that folding of the DNA around this nucleosome will bring the two regulatory regions together.) The chromatin structure is organized so that the GAGA factor binding sites, HSEs, and TATA box appear to be nucleosome-free, lying within HS sites in the inactive (but rapidly inducible) gene. At the *hsp70* genes, specifically positioned nucleosomes are found flanking the GAGA factor and HSF binding regions (Lis and Wu, 1995). Deletion or mutation of the $(CT)_n$ elements, but not of the HSEs, has a drastic effect on the chromatin structure of these genes, abolishing or decreasing accessibility of the promoter to DNase I or appropriate restriction enzymes (Lu et al., 1993; Weber et al., 1997). The results suggest that in vivo, placement of the nucleosomes is dictated by the presence of binding sites for GAGA factor, which is thus a major player in the creation of this well-organized chromatin structure.

Interestingly, inversion, duplication, deletion, or replacement by random sequence (in transgenic constructs) of the endogenous DNA underlying the precisely positioned nucleosome within the *hsp26* promoter does not affect either maintenance of the HS sites, or heat-shock inducibility (Lu et al., 1995). This suggests that not only the internal nucleosome but also the flanking nucleosomes are positioned by the presence of GAGA factor (Lu et al., 1995). These results are in contrast with an analysis of the regulatory region of the *Xenopus* vitellogenin B1 promoter, where the precise positions of nucleosomes in the regulatory region appear to be defined solely by the underlying DNA sequence (Schild et al., 1993). In the general case, one should anticipate that both specific DNA-binding proteins and the underlying DNA sequence can contribute to the organization of the nucleosome array in regulatory regions.

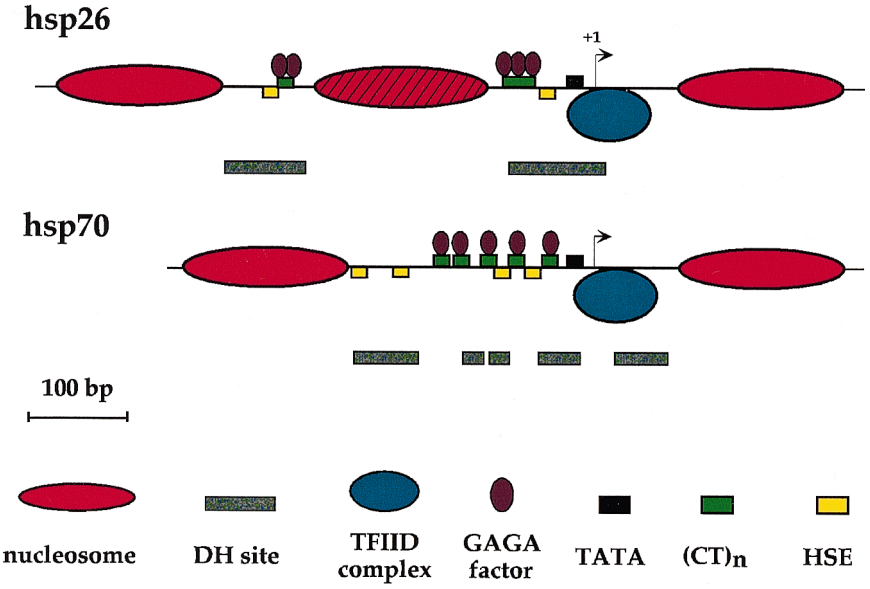


Fig. 1. Chromatin structure of the *hsp26* and *hsp70* promoters in *Drosophila*. Both promoters are characterized by the presence of positioned nucleosomes prior to activation, leaving the HSEs accessible for HSF binding. The positioned nucleosome within the *hsp26* regulatory region is hatched. For details, see Section 2.1.

The potential chromatin-organizing role of the TFIID complex and paused RNA pol II located on the heat-shock genes prior to heat shock has been less well studied. In the presence of GAGA factor binding sites, a mutation in the TATA box of the *hsp26* promoter that decreased TFIID binding in vitro decreased the accessibility of upstream sequences to nucleases in vivo (Lu et al., 1994). Furthermore, a replacement of the complete TFIID/RNA pol II binding region with random sequence practically abolishes the access of nucleases to their respective targets (Leibovitch et al., 1999). Mutations in the TFIID binding region of the *hsp70* gene decrease the extent of HSF binding at the HSE after heat shock (Shopland et al., 1995). These results indicate that TFIID/RNA pol II may act synergistically with GAGA factor to create the HS sites.

The data cited above allow one to conclude that the chromatin structure of the heat-shock genes is 'pre-set', keeping the DNA-binding sites for HSF accessible and nucleosome-free, i.e. in an HS site. HS sites are also observed at the 5′ regulatory regions of those 'housekeeping' genes that have been studied (Gross and Garrard, 1988), implying a similar strategy. The chromatin structure of the heat-shock genes must be either maintained or quickly re-established following cell division. In fact, preservation of the characteristic HS sites in mitotic chromosomes has been shown for the human *hsp70* gene (Martinez-Balbas et al., 1995). Given that the ground state in eukaryotic genomes is maintenance of the 'off' configuration (in particular, using nucleosomes to block TFIID binding), this maintenance of accessibility can be considered an example of epigenetic inheritance. Further study of the formation and maintenance of this preset organization of the nucleosome array will be essential if we are to understand the epigenetic regulation of developmentally programmed genes (see Lyko and Paro, 1999 and part 4).

2.1.2. Chromatin structure of the heat-shock genes following activation

Following heat shock, several changes occur in the *hsp26* and *hsp70* promoter regions. Monomers of HSF shift to form trimers and bind to their target HSE sites (Wu et al., 1994; Lis and Wu, 1995). Bound HSF interacts with the TBP-subunit of TFIID; this may change the interaction between TFIID and RNA pol II, allowing the paused RNA pol II to escape into elongation (Mason and Lis, 1997). The profound change in the cleavage pattern with MPE.Fe(II), showing less-defined positions of nucleosomes, and increased overall sensitivity to DNase I indicates that there is a change in the histone–DNA interactions in the downstream transcribed region (Cartwright and Elgin, 1986). GAGA factor may have a role in facilitating this nucleosome displacement, as it was found to be progressively associated with the transcribed region, advancing in parallel with RNA pol II (O'Brien et al., 1995). Multiple CTC trinucleotides are scattered along the coding regions of *hsp70* and other genes (O'Brien et al., 1995); such trinucleotides are sufficient for GAGA factor binding in vitro and might be used here (Wilkins and Lis, 1998).

Nothing has been reported concerning histone modifications in vivo during activation of the heat-shock genes. It will be of interest to track changes in the level of and type of acetylation, determining what occurs during creation of the accessible structure of the preset genes, as well as changes at the start of productive transcription. However, while assembly of chromatin in vitro with hyperacetylated histones (particularly acetylated H4) increases HSF binding, it does not facilitate binding of GAGA factor, TBP, TFIIA, TAFII150 or CTD RNA polymerase II to their respective target sites in competition with nucleosomes already present on the template (Nightingale et al., 1998).

2.1.3. GAGA factor plays a role in establishing the nucleosomal pattern on heat-shock gene promoters in vitro

Given the detailed characterization of the in vivo chromatin structure, the heat-shock genes were ideal substrates to attempt to reconstruct precise chromatin assembly in vitro. Nucleosomes can be assembled on a plasmid containing a promoter and a part of the coding sequence of the *hsp70* gene (Tsukiyama et al., 1994; Wu et al., 1998) or the *hsp26* gene (Wall et al., 1995) using an extract from preblastoderm *Drosophila* embryos. The extract contains all of the necessary factors to assemble a nucleosomal array with an average repeat length of $\sim$180 bp and a characteristic $\sim$146 bp core nucleosome (analysis by micrococcal nuclease digestion). The addition of GAGA factor to the assembly mix at any time in the process leads to a local perturbation of the nucleosome array around the *hsp70* promoter; micrococcal nuclease-accessible sites surround the GAGA factor target sequences, coinciding with the TATA box and HSEs. At the same time, clearer and more prominent protection by nucleosomes was observed in the flanking regions, probably due to restriction of the adjacent nucleosomes to a subset of positions. Interestingly, the organizing effect of GAGA factor was evident only over a short distance. Nucleosomes on the coding region of the *hsp70* gene, or the vector sequences, were not affected by GAGA factor. The preblastoderm embryo extract contains little or no linker histone H1. Addition of H1 to the assembly mixture not only increased the nucleosome repeat length (as expected), but also inhibited nucleosome disruption; GAGA factor was still able to facilitate alteration of the nucleosome array if added simultaneously with the other components, but was much less effective when added after assembly (Tsukiyama et al., 1994).

These data demonstrate that assembly and/or remod-

eling of the nucleosomes to achieve a specific, positioned array, including creation of HS sites (nucleosome-free regions), can be achieved by the components of the embryonic extract with the cooperation of a DNA-binding sequence-specific protein, in this case, GAGA factor. The process is energy-dependent and requires the addition of ATP. However, GAGA factor binding to DNA does not require ATP and GAGA factor does not have ATPase activity. The results suggest that GAGA factor might target a 'nucleosome exclusion activity', or might serve as a natural boundary, limiting the position of nucleosomes mobilized by some component(s) of the embryonic extract.

3. Establishing and changing nucleosomal patterns in *Drosophila*

3.1. Multiplicity of chromatin remodeling complexes

In vitro chromatin assembly systems from *Drosophila* embryonic extracts have now been used to identify and purify several activities capable of establishing and/or changing the specific pattern of nucleosomes in the promoter regions of heat shock and other genes. Different research groups, using different criteria for the analysis of chromatin assembly/remodeling, have characterized three different multimeric complexes — nucleosome remodeling factor (NURF), chromatin-accessibility complex (CHRAC) and ATP-utilizing chromatin assembly and remodeling factor (ACF) (see Table 1 and description below). In addition, a genetic screen for dominant modifiers of Polycomb mutations identified mutations in *brahma* and *kismet* (Kennison and Tamkun, 1988); the protein products of these genes have ATPase domains homologous to ATPase domains in subunits of the yeast SWI/SNF complex (Tamkun et al., 1992; Daubresse et al., 1999). The SWI/SNF complex participates in regulated chromatin remodeling of a subset of yeast genes. *Drosophila* Brahma protein has been found in a large complex (BRM) of ~2 MDa (Papoulas et al., 1998); the subunits of this complex are homologous to the subunits of yeast SWI/SNF, human hSWI/SNF (hBRM and hBRG1), and mouse mBRG1 complexes (for reviews, see Cairns, 1998; Kingston and Narlikar, 1999; Muchardt and Yaniv, 1999). Much more is known about the first three complexes, as reported below.

3.1.1. Chromatin remodeling complex NURF

The first multimeric chromatin remodeling complex purified from *Drosophila*, NURF, has a size of ~500 kDa (Tsukiyama and Wu, 1995). Purified NURF, like the unfractionated embryonic extract, disrupts nucleosomal structure over the *hsp70* promoter. The perturbation is dependent on GAGA factor and on the number of GAGA factor binding sites, given a stoichiometry of about 1 NURF per ~20–50 nucleosomes (Tsukiyama and Wu, 1995; Wu et al., 1998). This points to a catalytic function for NURF, implying that it works by a 'hit-and-run' mechanism, i.e. that its continued interaction is not required for maintenance of the altered chromatin structure. In higher ratios relative to nucleosomes, NURF acts less specifically, and is able to disrupt nucleosomes in the absence of GAGA factor, affecting both promoter regions and distant parts of the plasmid. The observed activity of NURF is not specific for functional interaction with GAGA factor; analogous results are obtained with other DNA-binding proteins, including HSF (Tsukiyama and Wu, 1995) and GAL4 derivatives (Mizuguchi et al., 1997). Both proteins result in evident NURF activity in the vicinity of their corresponding DNA-binding sites. However, it is not yet clear whether NURF (or its subunits) has any tendency to 'work' together with some DNA-binding proteins in preference to others, as has been suggested for some remodeling complexes that are targeted by interaction with transcription factors (Vignali et al., 2000).

NURF has an ATPase activity that is significantly induced by the presence of nucleosomes but not by DNA or by purified histones. It appears that NURF recognizes the N-terminal tails of histones on nucleosomes as the enzymatic removal of the tails, or their addition to the reaction as recombinant fusion proteins,

Table 1
Comparison of chromatin remodeling complexes in *Drosophila*[a]

Protein complex	Size (MDa)	Number of known subunits	Subunits with an ATPase domain	ATPase activity is induced by	Effect on nucleosome array	Nucleosome assembly activity	Nucleosome sliding
NURF	~0.5	4	ISWI	Nucleosome	Perturbation	No	Bidirectional
CHRAC	~0.7	5	ISWI; DNA topoII	Nucleosome and DNA	Regular spacing	Yes	Monodirectional
ACF	~0.4	3	ISWI	DNA	Regular spacing	Yes	ND
BRM	~2.0	8	Brahma	ND	ND	ND	ND

[a] For references, see reviews by Cairns (1998) and Muchardt and Yaniv (1999), and original publications cited in the text. ND: not determined. Complexes closely related to BRM have been identified in yeast, human and mouse. These complexes contain an ATPase stimulated by DNA and perturb the nucleosome array. The yeast SWI/SNF complex gives bidirectional sliding (Whitehouse et al., 1999). Several ISWI proteins have been identified in yeast (Tsukiyama et al., 1999).

decreases NURF activity. Hyperacetylation of the histones in nucleosomes does not change NURF activity (Georgel et al., 1997). These findings suggest that NURF should be able to recognize any nucleosome in vivo, independent of the functional status of the gene.

NURF consists of four subunits (Tsukiyama and Wu, 1995); three of them have been cloned and characterized. The 55 kDa NURF subunit is also found in the chromatin assembly factor histone chaperone (dCAF-1 complex) (Martinez-Balbas et al., 1998). The latter complex has been shown to stimulate in vitro replication (Tyler et al., 1996). The 55 kDa protein appears to interact with acetylated $(H3+H4)_2$ tetramers as part of the complex that deposits them on newly synthesized DNA. The 38 kDa subunit, surprisingly, is inorganic pyrophosphatase (Gdula et al., 1998), an enzyme that plays an important role in nucleotide metabolism, including transcription as well as replication (Kornberg, 1962). The pyrophosphatase activity of NURF is not important for its remodeling activity. One subunit, the ~140 kDa Imitation Switch protein (ISWI), can perform all of the functions of NURF in vitro, including GAGA factor-stimulated nucleosome reorganization on the *hsp70* promoter, albeit with a much lower efficiency (Tsukiyama et al., 1995; Corona et al., 1999; Ito et al., 1999). In contrast to NURF, ISWI alone is capable of generating regular nucleosome arrays on plasmid templates with irregularly deposited nucleosomes (Corona et al., 1999). The differences between NURF and the 140 kDa ISWI indicate that the other subunits affect the function of the protein complex. ISWI is a very abundant nuclear protein (~100 000 molecules/nucleus in 3–6 h embryos); while the amount of ISWI declines at later stages, it is still detected in nuclei (Elfring et al., 1994; Tsukiyama et al., 1995; Ito et al., 1999).

ISWI is the only subunit of NURF that has ATPase activity. ATPase activity is critical for remodeling complexes, presumably supplying energy for destabilizing nucleosomes. The ISWI ATPase domain is homologous to the ATPase domain of yeast SWI2, a component of the SWI/SNF complex. In fact, the presence of a *Drosophila* DNA sequence with homology to the yeast SNF/SWI2 ATPase domain was identified independently by Elfring et al. (1994). Recently, homologues of *Drosophila* ISWI have been identified in yeast (Tsukiyama et al., 1999), and one suspects that as the sequence of the *Drosophila* genome is completed, additional ISWIs may be identified.

3.1.2. NURF induces nucleosome 'sliding'

Wu and colleagues have carried out a detailed analysis of the mononucleosomes assembled by high salt on a ~350 bp DNA fragment from the *hsp70* gene promoter to examine the possible mechanism(s) of NURF function (Hamiche et al., 1999). Mononucleosomes will assemble at several favorable sites on this DNA fragment, reflecting the underlying DNA sequence; the different subspecies can be separated by gel electrophoresis. In the presence of NURF and ATP, shifts in the frequency of occupancy of different positions were observed. Control experiments showed that such 'relocations' of core position can best be explained by bidirectional 'sliding' of the mononucleosome along the DNA fragment, progressing through numerous intermediate positions in increments of a few base pairs. This indicates that NURF is capable of mobilizing a nucleosome in the absence of any additional factors in some energy-dependent way. The efficiency of this nucleosome 'sliding' depends on the stability of the histone interactions with the particular DNA sequence. A very stable mononucleosome that assembled on a sea urchin 5S rRNA gene fragment (Shrader and Crothers, 1989) was not susceptible to NURF action.

ISWI alone is capable of inducing nucleosome 'sliding' in a similar fashion (Hamiche et al., 1999; Langst et al., 1999). This, as well as the fact that ISWI is the only subunit with ATPase activity, emphasizes that the main chromatin remodeling features of NURF are dependent on ISWI, albeit the activities are modulated by the presence of the other subunits.

3.1.3. ISWI is a member of other chromatin remodeling complexes

Drosophila protein complexes CHRAC (Varga-Weisz et al., 1997) and ACF (Ito et al., 1997, 1999) also contain ISWI; both are capable of performing chromatin remodeling (Table 1). CHRAC contains five different subunits; only ISWI and DNA topoisomerase II, both ATPases, have been characterized. Topo II is active in this complex but not critical for its chromatin remodeling activity. CHRAC ATPase activity is stimulated by DNA and by nucleosomes. The activity induces regular spacing of nucleosomes and increases restriction enzyme accessibility within assembled chromatin (Varga-Weisz et al., 1997). CHRAC is also capable of inducing the 'sliding' of mononucleosomes along DNA fragments, but moves the nucleosomes in a different manner to NURF, or the ISWI subunit alone (Hamiche et al., 1999; Langst et al., 1999), suggesting a possible difference in the mechanism by which the movement is catalyzed. All of these features clearly make CHRAC functionally distinct from NURF; given that the ISWI subunit is common to the two activities, the differences must be directed by the other subunits. To explain the increase in accessibility to restriction enzymes in the presence of CHRAC, Varga-Weisz et al. (1997) have suggested that CHRAC induces very unstable, frequently oscillating nucleosomes that periodically open target sites for these enzymes. Theoretically, such brief exposure would create the same opportunities for DNA-binding proteins to find their targets. However, GAGA factor has a minor effect, if any, on chromatin remodel-

ing induced by CHRAC using *hsp70* DNA as the substrate (Varga-Weisz et al., 1997). Perhaps such DNA-binding proteins cannot serve as natural barriers for CHRAC action; this would explain why CHRAC action results in regular arrays of nucleosomes. Note, however that GAGA factor can self-associate into larger multimers, a transition that could limit its activity in this assay (Espinas et al., 1999; Katsani et al., 1999; Wilkins and Lis, 1999).

The third complex, ACF, consists of three subunits, ISWI and two isoforms of the protein encoded by the *Acf-1* gene (Ito et al., 1997, 1999). This novel protein contains several conserved motifs (PHD, bromodomain and others) found in many transcription factors. In the presence of the non-specific histone chaperone nucleosome assembly protein 1 (NAP-1), ACF facilitates the deposition of nucleosomes on DNA; the resulting nucleosomes are regularly spaced (Ito et al., 1997, 1999), as observed with CHRAC activity. However, in contrast to NURF and CHRAC, the ATPase activity of ISWI in ACF is stimulated by DNA, and not by nucleosomes. Neither of the Acf-1 subunits shows remodeling activity by itself. Both are found in vivo predominantly in the complex with ISWI. Curiously, one of the Acf-1 isoforms is sufficient to induce ISWI activity in the abridged complex in vitro.

Despite the recent efforts to identify and characterize chromatin assembly/remodeling complexes in *Drosophila* and other organisms, the in vivo functions of these complexes are not yet well understood. The fact that several complexes can share the same subunit (e.g. ISWI is a critical component of NURF, CHRAC and ACF) complicates genetic analysis; further, work in yeast suggests that these complexes may have overlapping functions (Tsukiyama et al., 1999). The chromatin assembly/remodeling function of each given complex is likely to be modulated by other proteins, either interacting with the complex itself, or with DNA, and/or with other chromosomal proteins. While assembly following replication is a general function, subsequent remodeling is almost certainly targeted by such interactions (see Kingston and Narlikar, 1999 for a review); control of the activity state through the establishment of appropriate chromatin structure is essential to both establish and maintain the needed patterns of gene expression. Above, we described the specific nucleosomal pattern in the promoter region of two *Drosophila* heat-shock genes; this pattern is dependent on the activity of a DNA sequence-specific binding protein, GAGA factor. The question is when and how this pattern is established.

3.2. Establishing the nucleosome array

Replication of the genome requires not only replication of the DNA, but also replication of the chromatin structure; nucleosomes must be disassembled from the parental DNA strand, and then rapidly reassembled on both daughter DNA molecules (reviewed in Adams and Kamakaka, 1999; Krude, 1999). What is the state of the newly assembled nucleosome array? Resolution of this question is important for consideration of the stepwise processes required to achieve the final state, with the distinctive nucleosome distribution required for activity (with appropriate HS sites) or silencing.

In vitro experiments have shown that highly irregular nucleosomal arrays are assembled on DNA using any of several non-specific means of histone deposition, including dialysis from 2 M NaCl, delivery by polyanions (such as polyglutamate), and delivery by non-specific histone chaperones such as CAF-1, NAP-1, nucleoplasmin and nucleoplasmin-like proteins (Pazin and Kadonaga, 1998; Adams and Kamakaka, 1999). [CAF-1 and NAP-1 are members of protein complexes that are required during replication and repair (Adams and Kamakaka, 1999).] Under at least some in vitro experimental conditions, nucleosomes are capable of 'sliding' slowly along a DNA fragment (reviewed in Workman and Kingston, 1998; Widom, 1999). The rate of 'sliding' is dependent to some extent on the underlying DNA sequence, since some positions are more stable than others, reflecting the pattern of histone–DNA contacts. In extreme cases, some sequences are unable to assemble into nucleosomes in vitro, while others have a very high affinity for the histone core and form a very stable nucleosome (for reviews, see Travers and Drew, 1997; Widlund et al., 1999). However, these activities appear insufficient to achieve either the specific irregular pattern of nucleosomes seen at active/inducible genes or the regularly spaced array associated with heterochromatin (Wallrath and Elgin, 1995; Cryderman et al., 1999a), implying that additional factors are required to regenerate the chromatin structure present before passage of the replication fork.

In vivo studies of viral and cellular DNA replication in somatic cells have shown that behind the replication fork, the nascent chromatin has an irregular ('immature') nucleosomal array, spanning from several hundred base pairs to 25 kb of DNA (for reviews, see Sogo and Laskey, 1995; Wolffe, 1999). Newly synthesized regions have an increased nuclease sensitivity, apparently reflecting the stepwise assembly of nucleosomes from 'old' pre-replicative and newly synthesized histones, randomly distributed on the two daughter DNA strands. At later stages, the nuclease sensitivity decreases, with concomitant establishment of a more regular nucleosome array (see Wolffe, 1999). Analysis of early *Drosophila* embryos has provided an illustration of the changes in the nucleosomal array linked to replication. The early *Drosophila* embryo remains a syncitium (lacking cellularization) during the initial rounds of rapid, synchronous nuclear replication and division. After the first 10 replication rounds, the cycle begins to slow, and

a cellular blastoderm is formed after the fourteenth replication cycle; thereafter, the cells divide more slowly (approximately once per hour) during embryogenesis (Foe et al., 1993). Analysis of the nucleosome array following digestion with micrococcal nuclease, using both a highly repeated satellite DNA and the *hsp70* gene as probes, showed a distinct set of DNA fragments (representing nucleosome monomers, dimers, trimers, etc. of a relatively uniform size) in nuclei from older embryos, but a 'smeared' nucleosomal array from preblastoderm embryos (Lowenhaupt et al., 1983). This suggests the formation of an irregular nucleosomal array in rapidly replicating nuclei.

In all species studied, newly synthesized histones H3 and H4 are post-translationally acetylated by a cytoplasmic histone acetyltransferases and are deposited as such on newly synthesized DNA. A recently identified chromatin assembly complex in *Drosophila*, replication-coupling assembly factor (RCAF) contains H3 acetylated at lysine 14 and H4 acetylated at lysines 5 and 12 (Tyler et al., 1999). Shortly after deposition, the histones are deacetylated by multiple histone deacetylases (Krude, 1999; Wolffe, 1999). Histone acetylation (which occurs primarily on lysine residues within the N-terminal ends) decreases the net charge, potentially reducing interactions with DNA, and diminishes the interactions between histones of neighboring nucleosomes. Thus, acetylation is likely to make postreplicative nucleosomes more vulnerable to competition from other DNA-binding factors and may make them more accessible to further modification and/or movement. It has been suggested that replication may provide a brief 'window of opportunity' to determine the chromatin structure in daughter cells. Preblastoderm embryos of *Drosophila* (with rapidly dividing nuclei) have an increased level of di-acetylated H4 (Giancotti et al., 1984); the embryos contain maternally loaded regulatory proteins, including various DNA-binding activators and repressors that are non-uniformly distributed (see Bate and Arias, 1993). The interaction of such regulatory proteins with their target DNA sequences within this period is likely to be critical to establishing the patterns of differential gene activity evident after the start of zygotic transcription.

The transient enrichment of hyperacetylated histones in nucleosomes assembling behind the replication fork raises an intriguing question concerning possible mechanisms for establishing specific patterns of histone acetylation. Immediately, postreplicative H4 is di-acetylated at lysines 5 and 12 (see Krude, 1999; Tyler et al., 1999). In *Drosophila*, histone H4 is di-acetylated at lysine residues 5 and 8 in nucleosomes of active genes and is acetylated at lysine 12 in nucleosomes located in heterochromatin (Turner, 1998). While, in general, it appears that newly assembled histones are deacetylated and selectively reacetylated, selective deacetylation might contribute to establishing this pattern. (See Strahl and Allis, 2000, for a recent review of histone modification.)

3.3. Creating specificity in nucleosome arrays: possible mode of NURF action in vivo

Several different mechanisms might play a role in specifying the location of nucleosomes. During the course of evolution, one might select for DNA sequences that have different 'affinities' for nucleosome formation, particularly in critical regions such as promoters (for a discussion, see Travers and Drew, 1997; Ioshikhes et al., 1999; Widom, 1999). Such patterns, if significant, might facilitate formation of HS sites. Another possibility is the presence of sequence-specific DNA-binding proteins having a higher affinity for a given site than the histone core. In this case, the DNA-binding protein will simply block core histones from deposition on the given DNA sequence (e.g. Pazin et al., 1997), or serve as a boundary for 'sliding' nucleosomes (either moving by diffusion or with the aid of remodeling factors). As discussed above, *Drosophila* GAGA factor, a protein that binds the $(CT)_n$ repeats associated with many promoters, may play such a role. Such proteins may also recruit machinery to remodel or maintain active status.

The data available suggest that in vivo, NURF (and other remodeling complexes) can interact briefly with nucleosomes and mobilize them, disrupting DNA–histone contacts using the energy of ATP. This activity might effectively 'push' nucleosomes around; thus, in the presence of sequence-specific DNA-binding proteins, NURF action might allow the proteins to compete efficiently for DNA binding as the oscillating nucleosome periodically leaves the target site open. The result will be binding of the sequence-specific protein, with the nucleosomes 'trapped' at flanking sites. In this context, at least some of the sequence-specific DNA-binding proteins, irrespective of their role in transcriptional regulation, may serve as natural boundaries to define arrangements of nucleosomes. This would create a highly specific nucleosome pattern at any site where an appropriate DNA-binding protein and a chromatin remodeling complex, such as NURF, are simultaneously present in reasonable stoichiometric amounts. The stability and specificity of such a 'construct' would be further determined by DNA–histone interactions in the resulting nucleosomes. The potential effect on transcription of generating an HS site by this mechanism will of course reflect the sequences thereby made accessible, potentially either for a transcriptional activator or for a repressor.

The data discussed above allow us to propose a specific example, citing the promoters of the *hsp26* and *hsp70* genes. In vivo and in vitro analyses indicate that GAGA factor, in conjunction with RNA polymerase II, and presumably aided by NURF or a related activity, creates accessibility specifically for HSF at these promot-

ers. While GAGA factor may be sufficient to initiate the process, the presence of both GAGA factor and the paused RNA pol II is a prerequisite for successful binding of HSF after heat shock (Shopland et al., 1995). In *Drosophila* it appears that as many as 10–20% of the genes may have a molecule of paused RNA polymerase II (Law et al., 1998), corresponding to the widespread distribution of the hypophosphorylated form of the enzyme on polytene chromosomes (Weeks et al., 1993). At the same time, a statistical analysis of sequences in 252 *Drosophila* promoters indicates that nearly 15% of the genes have $(CT)_n$ repeats (Arkhipova, 1995). Immunofluorescence analysis using polytene chromosomes indicates that GAGA factor is associated with many euchromatic sites (Tsukiyama et al., 1994; Granok et al., 1995; Benyajati et al., 1997). The cooperation of RNA polymerase II with abundant DNA-binding proteins such as GAGA factor may be a common mechanism for establishing the open chromatin structure required for housekeeping and/or other preset genes. The presence of multiple GAGA factor binding sites in the regulatory regions of many genes (Granok et al., 1995; Wilkins and Lis, 1997) suggests that such multiplicity may be important to insure formation and stable maintenance of the HS site. The efficient replication of this chromatin structure, in a genome that does not use marks such as DNA methylation, may require redundant signals.

NURF can also alter nucleosome structure directly when present at high concentrations; this observation raises the very interesting possibility that remodeling complexes might perform differently when concentrated in a small volume. The suggested regulatory role of nuclear compartmentalization (Lamond and Earnshaw, 1998) may be based on local deviations in stoichiometric ratios between different regulatory molecules and/or their target genes. Immunostaining reveals that GAGA factor, and many other proteins participating in chromatin organization and/or function (e.g. see Buchenau et al., 1998; Gerasimova and Corces, 1998; Platero et al., 1998), are distributed non-uniformly in diploid interphase nuclei, often in large 'speckles'. Unfortunately, similar studies of the remodeling complexes will be difficult or impossible to do because of the sharing of subunits and potential overlap of activities. None the less, the existence of nuclear sites with high concentrations of different proteins may point to the possibility of creating distinct nucleosomal patterns reflecting the stoichiometric ratios of the participants. Recent studies in mammalian nuclei have shown instances in which gene silencing is correlated with a change in nuclear position (e.g. Brown et al., 1997); many classical studies have shown that chromosomal rearrangements can result in a gain or loss of silencing, as seen in Position Effect Variegation (reviewed by Weiler and Wakimoto, 1995). Analysis of the chromatin structure of test transgenes has shown that the silencing observed in heterochromatic domains is reflected in the loss of HS sites and in the generation of a nucleosome array with very regular spacing (Wallrath and Elgin, 1995; Cryderman et al., 1999a,b). Changes in the local concentration of various chromosomal proteins, and of access to remodeling complexes, could result in such differences.

4. Maintaining differential gene expression

4.1. Role of chromatin structure in the regulation of larger domains

While many genes must be continuously maintained in an active or inducible state, the majority of the genes must be active in only a few cell types, and transcriptionally silent in others. To achieve the extremely complex pattern of gene expression seen in higher eukaryotes, it is necessary both to initiate a differential transcription pattern and to preserve this information through multiple cell-division cycles, maintaining specific 'reminders' of the earlier defined state. Previous studies have shown that epigenetic mechanisms, operating on large domains rather than on individual promoters, are often used in maintaining and stably transmitting chromatin states. In *Drosophila*, these types of mechanisms are involved in the silencing of genes juxtaposed or within pericentric and telomeric regions by rearrangement or transposition (Weiler and Wakimoto, 1995; Wallrath, 1998), as well as in developmental regulation; for the latter, the best-studied example is the regulation of the homeotic genes in the bithorax complex (reviewed by Pirrotta, 1999). A large number of the loci involved have been identified by genetic means (Kennison and Tamkun, 1988; for reviews, see Kennison, 1993, 1995). In many cases, the protein products of these loci are thought to be chromosomal proteins or to affect chromatin functions. Recent work in *Drosophila* has defined specific chromosomal elements as organizers of 'switching' events; the same elements can be responsible for 'fixing' the trancriptional state, enabling the cells to remember the predetermined developmental program through generations of cell division (reviewed in Hagstrom and Schedl, 1997; Lyko and Paro, 1999).

Despite the importance of epigenetic mechanisms in propagating the transcriptionally determined state and the effort invested in the subject over the last few years, relatively little is understood about the underlying molecular mechanisms at the nucleosomal level. Recent data, however, have begun to advance our understanding significantly.

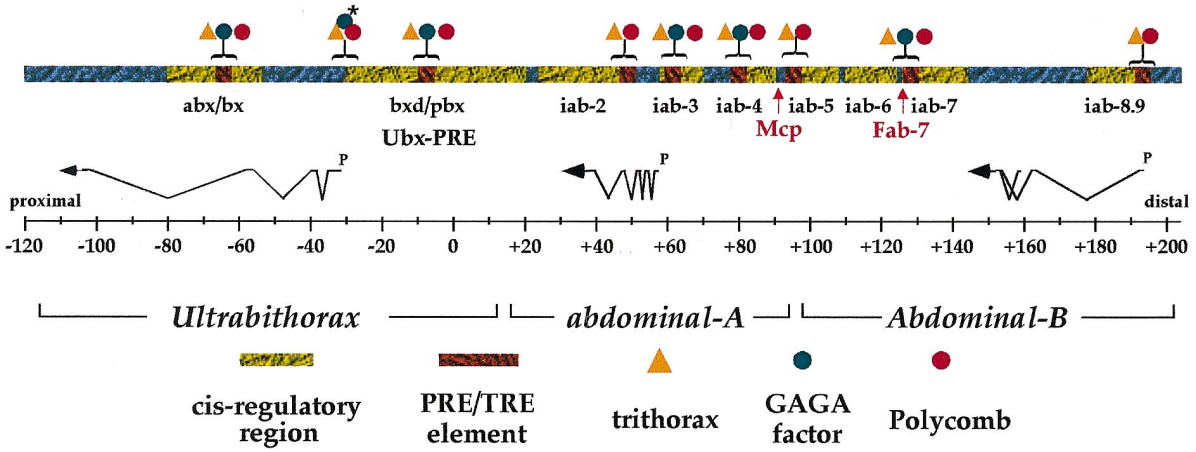
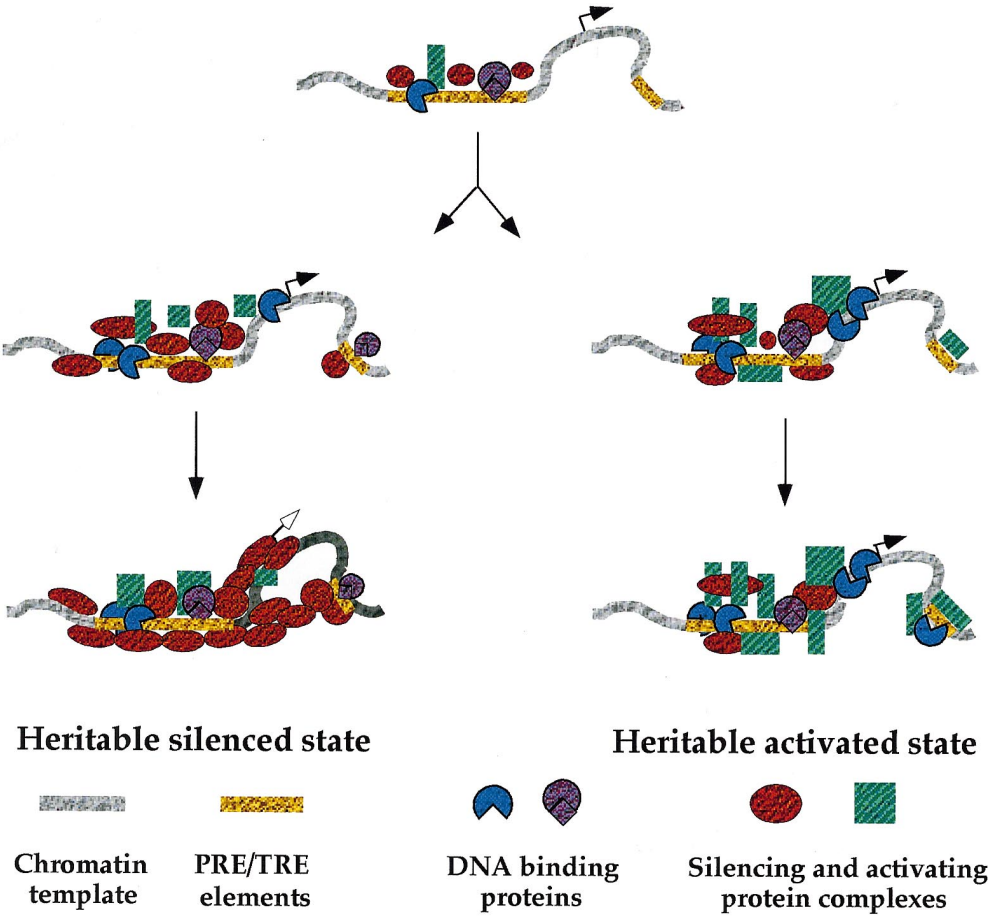


Fig. 2. Regulatory elements of the BX-C. The complex expression patterns of *Ubx, abd-B* and *Abd-B* are generated by a *cis*-regulatory region, which spreads over 300 kb of DNA (blue line). The scale of the map in kb follows the numbering of Bender et al. (1983); proximal is toward the centromere. Some of the transcripts of the three transcription units are indicated 5′ to 3′ from right to left. The genetically defined, parasegment-specific *cis*-regulatory subregions (*abx/bx, bxd/pbx, iab-2* to *iab-9*, green segments) and several functionally important specific elements [PRE, TRE (brown boxes) and boundaries (Mcp, Fab-7)] are also indicated (for review, see Mihaly et al., 1998a, and references in the text). Regions where the association of PC, TRX and GAGA factor have been detected are marked. Data have been compiled from Strutt et al. (1997), Cavalli and Paro (1998), Orlando et al. (1998) and Tillib et al. (1999). Colocalization of GAGA/PC and TRX/PC has been detected by immunoprecipitation. The presence of GAGA factor at the *Ubx* promoter has been shown by DNase I footprinting (Biggin and Tjian, 1988) marked with an asterisk.



4.2. Maintaining stable states of gene expression during development

The homeotic genes of the bithorax complex (BX-C) control the segmental identities of the abdomen and a subset of the thorax of an adult fly (Lewis, 1978). The enormous regulatory region of BX-C spans over 300 kb and is organized into distinct domains, each of which directs expression of the homeotic genes in a specific parasegment (Duncan, 1987). The initial regulatory information is provided by transient signals of the gap and pair-rule genes; after these signals have decayed, the activity state is maintained by negative and positive regulatory factors, which are thought to act by stabilizing the chromatin state. The Polycomb group (PcG) proteins function in maintaining transcriptional repression, while the trithorax group (trxG) proteins play a critical role in maintaining a transcriptionally permissive environment (Kennison, 1993, 1995; Simon, 1995; Gellon and McGinnis, 1998). Both PcG and trxG proteins constitute a heterogenous group, rather than a family of structurally related members. Current molecular data fail to provide an adequate model for how either group of proteins carries out their function. To understand better how the PcG and trxG proteins work, we need to address several questions: where and how are the maintenance complexes established? What is the precise composition of the protein complexes at given sites? What interplay occurs between the contributing proteins? How are the repressive and active structures maintained, e.g. what heritable marks are used to pass the information through multiple rounds of cell division? We report here on several recent experiments addressing these fundamental questions.

4.2.1. Regulatory elements and the distribution of chromatin modifiers within BX-C

Maintaining stable repression of the homeotic genes of BX-C depends on the PcG proteins. The PcG proteins are known to form large multimeric complexes (Franke et al., 1992; Strutt and Paro, 1997; Kyba and Brock, 1998; Shao et al., 1999); immunofluorescence analysis shows patterns of colocalization on the polytene chromosomes of *Drosophila* (Franke et al., 1992; Lonie et al., 1994; Carrington and Jones, 1996). Silencing by PcG proteins is mediated through distinct target sites such as the Polycomb response elements (PREs). The PREs are defined as DNA elements that interact (as shown by biochemical and genetic tests) with the PcG repressive complex; they apparently serve as nucleation sites to recruit PcG proteins. A number of PRE sites have been identified within BX-C (see Fig. 2) and in regulatory regions of other genes in various transgenic assays. This approach is based on the PREs' ability to direct silencing in an artificial construct, a transposon stably integrated into the genome of the fly. Several criteria are used to define a PRE in this type of transgenic assay. (1) PREs maintain the segment-specific repression conferred on the reporter gene by segment-specific enhancers (assuming that both the PRE and the enhancer are present in the construct); the silenced state of the reporter gene is thus maintained in body segments where it was originally silenced during embryogenesis (Simon et al., 1990; Muller and Bienz, 1991; Busturia and Bienz, 1993; Chan et al., 1994; Chiang et al., 1995; Gindhart and Kaufman, 1995; Hagstrom et al., 1997). (2) PREs create a new binding site for the PcG proteins at the transgene insertion site, viewed on *Drosophila* polytene chromosomes (Chiang et al., 1995; Zink and Paro, 1995). (3) PREs can silence heterologous genes, such as the mini-*white* reporter gene, in a transgenic construct. The effectiveness of silencing can differ with the site of transposon insertion, indicating a variable influence of adjacent sequences and/or suggesting that the complexes formed at that genomic site can interact with those at other sites in the genome (Pirrotta, 1997). Silencing of mini-*white* by the PRE can require more than one copy of the transposon (i.e. can require flies homozygous for the insertion), indicating that *trans*-regulatory interactions can mediate the repression of the transgene (Kassis et al., 1991; Chan et al., 1994; Kassis, 1994; Gindhart and Kaufman, 1995; Hagstrom et al., 1997). This pairing-sensitive silencing, mediated by PcG proteins, is observed not only in flies homozygous for the transgene but also in lines carrying transgenes inserted at distant sites, even on different chromosomes (Sigrist and Pirrotta, 1997; Muller et al., 1999).

A key requirement for understanding the function of PRE elements is a knowledge of the composition of the multimeric protein complexes at individual PREs. The localization and composition of such protein complexes in the BX-C regulatory region has been analyzed using formaldehyde cross-linking followed by chromatin immunoprecipitation (X-CHIP), using antibodies raised against specific PcG and trxG proteins. Polycomb (PC) protein is associated with large regions of the BX-C

Fig. 3. Illustration of a possible scenario to initiate and maintain heritable silenced and active states at a hypothetical locus. The hypothetical domain consists of a stronger and weaker PRE/TRE element and a transcription unit. The alternative pathways are initiated by as-yet unexplored mechanisms (e.g. spatial and temporal cues, stochiometric differences in the amount of the components available, enzymatic modifications of proteins changing the pattern of interactions, etc.) to repress or to keep active the transcription unit in a given cell. The 'red complexes' prohibit and the 'green complexes' permit formation of active states. The model suggests considerable flexibility by variation of the components or modification of their function. Once stable silencing has been established, it may no longer be possible to generate a stably inherited active state.

(Orlando and Paro, 1993); however, discrete sites identified as functional PREs show a significantly higher localized concentration of the protein (Strutt et al., 1997; Orlando et al., 1998; Lyko and Paro, 1999). The X-CHIP technique has also been used to show that PcG complexes have different compositions at different target sites (Strutt and Paro, 1997). Surprisingly, the evidence suggests that PcG and trxG proteins can colocalize (or are found in near proximity) in several regulatory regions of BX-C (Chinwalla et al., 1995; Strutt et al., 1997; Orlando et al., 1998; Cavalli and Paro, 1998). Such colocalization in the *Ultrabithorax* (*Ubx*) regulatory region was shown to be essential for proper regulation of the gene (Tillib et al., 1999). The data suggest that some important regulatory regions are composed of separable functional elements responsive to PcG proteins and trxG proteins (Fig. 2). These complex regulatory modules are often referred as PRE/TRE elements; their precise functional anatomy remains to be explored. Colocalization of proteins known to have antagonistic functions at specific elements reinforces the view that these sites might serve to govern the transition between an active and a repressed chromatin configuration, reflecting an interplay between the proteins involved. The fine-tuned balance between the participating components may define the transcriptional fate of a large region, and the properties of a predetermined heritable epigenetic state (Fig. 3).

4.2.2. Targeting repressive and activating complexes to the regulatory elements

How are the PcG and trxG proteins targeted and anchored to their site of action? What is the link between the activity of the relevant DNA-binding proteins (produced by gap and pair-rule genes), defining the initial pattern of gene expression, and the proteins involved in the maintenance of stable expression states? A possible link between Hunchback (HB), the protein product of one of the gap genes, and PcG-mediated silencing has recently been demonstrated. HB functions as a repressor, binding directly to regulatory sequences in BX-C; this activity sets the spatial limits of homeotic gene expression in the organism. HB is required transiently, with the initial repression being maintained by the PcG proteins after production of HB ceases. Using HB as the bait in a yeast two-hybrid screen, Kehle et al. (1998) identified interactions with protein dMi-2. The interacting HB domain is one known to be critical for repression of homeotic genes. Genetic evidence shows synergy between *dMi-2* and *Hb*, as well as between *dMi-2* and selected PcG genes, in that enhanced derepression of homeotic genes is observed in double mutants. These data indicate a possible link between sequence-specific initiation of repression by temporary signals (HB), and long-term, heritable PcG-based maintenance of the established state. Whether or not dMi-2 directly interacts with the repressive complex is not known. The presence of multiple conserved domains in dMi-2 (e.g. an HMG-like motif, PHD-fingers, a chromodomain, a DNA-stimulated ATPase domain and a myb-like domain) suggests that the protein may be involved in a variety of interactions. Interestingly, the *Xenopus* Mi-2 has recently been purified as a part of a nucleosome remodeling activity in a histone deacetylase complex (Wade et al., 1998). It will be of particular interest to determine whether or not the role of dMi-2 in formation of silencing complexes can be linked to creating or maintaining changes in the histone acetylation status. Whether active recruitment of the PcG proteins by HB and dMi-2 occurs, or whether recruitment is the consequence of formation of a transient transcriptionally repressed state, remains to be seen. HB also plays a role in repression of the *Ultrabithorax* (*Ubx*) gene during early development. However, in a test using a reporter transposon, silencing of the *Ubx* imaginal disc enhancers occurred in an HB-independent manner (Poux et al., 1996). It has been suggested that the basic information dictating whether or not to create repressive structures, or to keep a gene potentially active, is the activity state of the gene at the time when stable repression patterns are established.

This hypothesis suggests that altering the transcriptional status of a gene at the critical time should change the pattern of heritable expression. Indeed, robust transcriptional activation can alleviate PcG-mediated silencing at a transgene; however, the effect is temporary if stable repression had already been established. Transcriptional activation of the same construct during the 'permissive' period in embryogenesis generates a heritable activated state, which persists even in the absence of the activator. The active state is accompanied by an increase in H4 acetylation (Cavalli and Paro, 1998; Cavalli and Paro, 1999, and see text below).

The mechanism that targets PcG and trxG complexes to their site of action is unknown. A few candidate DNA-binding proteins thought to participate in targeting have been identified. Pleiohomeotic (PHO) is the only PcG protein characterized to date that binds specifically to DNA (Brown et al., 1998); a short conserved sequence motif corresponding to the PHO consensus binding site is found in a large number of known PRE elements (Mihaly et al., 1998b). Based on sequence homology, PHO is related to the ubiquitous mammalian transcription factor Yin Yang-1 (YY1), a protein that plays multiple roles in the regulation of gene expression (reviewed in Thomas and Seto, 1999). Point mutations in PHO binding sites in the *bxd* PRE from the *Ubx* gene abolished PcG-dependent repression in vivo in imaginal discs, indicating that the protein plays a role in silencing in larvae (Fritsch et al., 1999). The generality of this function is unknown.

Sequence analysis of the entire BX-C has also revealed that CT and AG repeats are significantly overrepresented (Lewis et al., 1995), suggesting a role for GAGA factor at multiple sites in the BX-C regulatory region. GAGA factor was identified initially as a sequence-specific DNA-binding protein that could stimulate the transcriptional activity of the *Ubx* and *engrailed* (*en*) promoters, suggesting a role as a positive transcription factor (Biggin and Tjian, 1988; Soeller et al., 1988). GAGA factor-binding sites have subsequently been identified in the promoters of numerous *Drosophila* genes (Granok et al., 1995; Wilkins and Lis, 1997); as discussed above, GAGA factor plays an important role in establishing the accessible chromatin structure (HS sites) in the regulatory regions of the heat-shock genes. It now appears that the protein may have a rather global role in regulating a wide variety of chromatin functions. The first genetic evidence indicating that GAGA factor has a role in regulating expression of the homeotic genes in BX-C arose from characterization of the *Trithorax-like* (*Trl*) gene, which encodes GAGA factor. Analysis of this locus shows that GAGA factor is a positive regulator of homeotic genes (a trxG protein); mutations in the gene enhance or cause misexpression of homeotic genes in certain segments of the adult fly, leading to a homeotic phenotype in that body part of mutant animals. This result has led to the conclusion that GAGA factor can assist in generating and/or maintaining an active chromatin configuration not only at promoters, but also at different types of regulatory elements located a considerable distance from the transcription unit, and specific for homeotic gene regulation in a particular segment of the fly (Farkas et al., 1994). The presence of GAGA factor at consensus sites observed within PREs in the BX-C has been experimentally demonstrated; GAGA protein was found by immunoprecipitation to colocalize with PC at these sites, suggesting that they might participate in a macromolecular complex (Strutt et al., 1997; Cavalli and Paro, 1998). In vitro experiments have shown that GAGA factor is indeed a component of at least some PcG complexes and is important for their binding to the *bxd* PRE from the *Ubx* gene (Horard et al., 2000).

Genetic studies have shown a functional relationship; GAGA factor has been shown to facilitate Polycomb action at the PRE element of the Fab-7 region (Hagstrom et al., 1997). The Fab-7 region consists of a chromatin domain boundary and a PRE element; it includes several consensus GAGA factor binding sites, and has several specific HS sites (Karch et al., 1994; Mihaly et al., 1997, 1998a). The data suggest that GAGA factor may play a general role in generating an accessible site within the nucleosome array (as discussed above), allowing action of either the PcG and/or the trxG complex, depending on the regulatory sequences made accessible and local availability of protein. While GAGA factor might act by a mechanism that utilizes the remodeling complexes, it has also been suggested (on the basis of in vitro transcription studies) that it might act as an 'antirepressor', counteracting H1-mediated repression (Croston et al., 1991). Obviously, these suggestions are not mutually exclusive. GAGA factor is also a modifier of heterochromatic position effect variegation (Farkas et al., 1994) and regulates essential chromosome functions through binding heterochromatic sequences during early embryogenesis (Bhat et al., 1996). GAGA factor potentially provides an important link in the selection of specific target sites for a variety of distinct processes.

4.2.3. Maintaining heritable active and silenced states

The concerted action of PcG and trxG proteins at common elements may be the basic determinant of the molecular events defining the heritable state of chromatin at many developmentally regulated genes. Genetic and biochemical analysis of the Fab-7 *cis*-regulatory region of BX-C supports this hypothesis (see Mihaly et al., 1998a). The Fab-7 element participates in proper regulation of *Abdominal-B* (*Abd-B*), which defines the development of several abdominal segments of the fly. Recent experiments using a transposon containing the Fab-7 region and two reporter genes, *mini-white* and *UAS-LacZ*, shed light on some properties of the element (Cavalli and Paro, 1998, 1999). The *white* gene serves as a transformation marker, providing a convenient visual estimation of its activity state. PcG-mediated repression is reflected in a variegated eye phenotype; the extent of repression is indicated by the percentage of pigmented facets. The Fab-7-containing constructs respond to both PcG and trxG mutations, including mutations in PC, trithorax (TRX) and GAGA factor, proteins whose colocalization to this element has been previously demonstrated (Strutt et al., 1997; Orlando et al., 1998; Cavalli and Paro, 1998). The other reporter gene, *LacZ*, is under the control of a potent GAL4 activator. GAL4 was provided upon heat shock from a second transposon containing an *hsp70*-driven copy of GAL4 (Brand et al., 1994). A robust induction of GAL4 resulted in displacement of PcG proteins from the repressed transgene, while driving strong expression of the GAL4-responsive *LacZ* reporter. Induction of GAL4 at various developmental stages, however, had different consequences on the maintenance of the active state of both reporter genes. When GAL4-driven activation was induced by heat shock late in development (e.g. larval stages), a return to normal conditions was quickly followed by restored association of PcG proteins with the element and re-establishment of repression. In contrast, induction of gene expression with a GAL4 pulse during embryogenesis can generate a heritable active state; surprisingly, this event does not seem to result in displacement of PcG proteins.

Mutation in *trx* prevents transmission of the activated state, as shown by the downregulation of the *white* reporter gene (Cavalli and Paro, 1999). Apparently, a strong transcriptional activator alone can alleviate PcG-dependent repression, but stable alteration of the activity state requires additional independent processes, presumably mediated by TRX and/or other trxG gene products. This study demonstrated a requirement for specific developmental timing (a 'window of opportunity') in establishing a committed activity state and suggests a competition between the participating proteins during the critical period. Fab-7 thus acts as a 'cellular memory module' (CMM), insuring that the activated/repressed state of the transgene is transmitted through mitosis, and even through female meiosis (Cavalli and Paro, 1998). Further investigations are needed to determine the role of TRX (and other trxG proteins) in setting the activity state. The trxG is a heterogenous group of proteins that could use a variety of mechanisms to counteract the formation of repressive PcG complexes, including recruiting remodeling complexes or directly activating promoters.

The identification of Fab-7 as a memory element provides an opportunity to address the intriguing question of the nature of the heritable mark(s). Analysis indicates that altered H4 acetylation is linked to the permanently modified chromatin state; maintenance of the derepressed state is associated with H4 acetylation, and lack of maintenance of hyperacetylated H4 in the transgene appears to result in a failure to maintain transcriptional competence (Cavalli and Paro, 1999). We do not yet know how the acetylation pattern is preserved or erased, or how the PcG and trxG gene products might participate in these processes.

4.2.4. Establishing cellular memory

How might the PcG and trxG proteins contribute to introducing readable marks into the chromatin, and how might these marks be preserved through multiple cell cycles? Is there a persistent association of some of these proteins with the chromosomes, or do the complexes reassemble after each round of replication using other signals?

The X-CHIP technique has revealed new details regarding binding of PC and TRX to their targets during embryogenesis. PC and TRX are first observed in association with DNA very early in embryogenesis at PRE/TRE elements. The patterns observed as development proceeds are complex, involving the core PRE/TREs, flanking regions and associated promoters. Interestingly, association of these proteins with regulatory sites appears to begin before transcriptional competence of the gene is required; these early events are potentially important for stabilizing early determined states in chromatin (Orlando et al., 1998).

How is the association of the key participants preserved and/or re-established following replication? Cytological studies of the PcG and trxG proteins indicate a rather dynamic behavior. Some proteins have been shown to remain bound to the chromosomes during the short nuclear cleavage cycles in preblastoderm embryos. For example, GAGA factor appears to be associated with heterochromatic satellite sequences (containing GAGA binding sites) in this early developmental stage throughout the cell cycle (Raff et al., 1994). Mutations resulting in decreased amounts of GAGA factor during early development have been shown to cause a variety of defects in chromosomal function, including asynchrony in nuclear cleavage cycles, failure in chromosome condensation, abnormal chromosome segregation and chromosome fragmentation (Bhat et al., 1996). Later in development, mitosis-specific GAGA factor binding has been detected on chromosomes of larval brain; GAGA factor is dispersed to euchromatic sites during interphase and moves back to heterochromatin in metaphase in every cell cycle (Platero et al., 1998). A different but similarly dynamic behavior has been reported for PcG proteins in embryos. The majority of the Polycomb (PC), Polyhomeotic (PH), and Posterior sex combs (PSC) proteins dissociate from the chromatin during mitosis and disperse into the cytoplasm, reassociating with the chromosomes non-simultaneously at a later stage (telophase) (Buchenau et al., 1998). One cannot rule out the possibility that a small fraction of these proteins remains bound to the chromatin, but the majority appear to be involved in a dynamic dissociation/re-association process.

If trace amounts of PC remain bound to chromosomes, as suggested by in vivo studies with a PC-GFP fusion protein (Dietzel et al., 1999), this association could contribute to generating a persistent signal to define the local assembly of chromatin. Indeed, a potential for direct interaction between PC and the nucleosomal core particle has been demonstrated in vitro (Breiling et al., 1999). PC was shown to bind to the nucleosome core particle through its C-terminal repression domain. The protein might have an affinity for nucleosomal DNA as well. These results raise the possibility that PC might stay linked to the nucleosomes during replication, recruiting other PcG proteins to lock the surrounding nucleosomes into a repressed state in the daughter cells. The direct interaction between nucleosomes and PcG complexes might explain the generation of heritable 'remodeling resistant' chromatin structures, preventing interaction of remodeling complexes with the nucleosomal DNA. Indeed, Shao et al. (1999) have recently shown that PRC1, a Polycomb complex, can stabilize chromatin structure to remodeling in vitro (see below). The mitotic behavior of other PcG and trxG proteins, and their potential to interact with chromatin and/or with each other, needs be explored further to

understand the various roles in establishing stably maintained chromatin structures.

Unfortunately, immunolocalization generally reveals the behavior of the bulk of the proteins in a living cell; given the limitations of sensitivity and resolution, a negative result does not eliminate the possibility of residual binding of a protein that might seed re-formation of the protein complexes. In a multicellular organism such as *Drosophila*, there are also technical limitations to following molecular events in vivo in a single cell. The necessary molecular cues retaining the 'footprints' of chromatin complexes are as yet unobserved. A study of the *hsp70* promoter in human cell culture has found that the characteristic HS sites have been retained on mitotic chromosomes, even though binding of the known transcription factors has been disrupted (Martinez-Balbas et al., 1995). Work monitoring potassium permanganate reactivity of human mitotic chromosomes has suggested that a conformational distortion at transcriptional start sites could be the mitosis-specific mark to label genes that are to be active in the next generation of cells (Michelotti et al., 1997). It will be interesting to see whether similar mechanisms may operate in *Drosophila* not only at promoters but also at memory elements defining epigenetically inherited transcriptional states.

4.3. Possible modes of action of PcG and trxG proteins at their target sites

As discussed above, analysis of BX-C has shown that PcG and trxG proteins reside at distinct sites within regulatory regions, their binding profiles often coinciding (see Fig. 2). The coexistence of PcG and trxG proteins at closely situated sites in some cases suggests the possibility of a broad range of antagonizing and cooperative events to achieve a determined state. The equilibrium between the competitive interactions and the as yet unexplored interplay between the numerous participants may be the essential determinants of an accurately defined chromatin state (see Fig. 3).

Evidence of a competitive mechanism has emerged from an in vitro assay to determine the requirements for 'locking' the nucleosomes into a remodeling-resistant configuration on a nucleosomal plasmid template, using purified *Drosophila* Polycomb repressive complex 1 (PRC1) and an ATP-dependent remodeling complex (SWI/SNF). If PRC1 (which contains at least four known PcG proteins) is present initially, it can inhibit remodeling by SWI/SNF; however, no inhibition of remodeling by PRC1 is observed when SWI/SNF is added at the same time to the assay mixture. Thus, while PRC1 can prevent remodeling, SWI/SNF can interfere with the ability of PRC1 to block remodeling; this activity does not require ATP. Histone tails do not seem to affect formation of the repressive complex in vitro, suggesting that the PRC1 complex interacts with either the nucleosomal templates or with the body of the histones (Shao et al., 1999). A different set of in vitro experiments has demonstrated that PC and PcG complexes are able to interact directly with nucleosomal core particles (Breiling et al., 1999). The data suggest several different modes of PC interaction with the nucleosomal template (e.g. protein–protein interactions, affinity of PC for distorted DNA on the nucleosome core, ability of PC to bind to isolated N-terminal histone tails), which must be resolved by further experimentation.

It will be interesting to learn what specific interactions can be discerned using templates with known PREs and what unique mechanisms might be revealed using the different *Drosophila* ATP-dependent remodeling complexes (see above). Such studies should provide insight into the mechanisms by which PcG and trxG proteins influence each other's function. Participation in remodeling is certainly compatible with the role of trxG proteins in 'opening up' the chromatin structure and could occur either by facilitating remodeling or by localizing remodeling activities to a given site. Among the characterized trxG proteins, Brahma is a SWI2/SNF2 homolog (Papoulas et al., 1998); Moira has been identified as a putative chromatin remodeling factor associated with Brahma in a large complex (Crosby et al., 1999); Kismet has been found to be related to chromatin remodeling factors, sharing a short conserved motif with Brahma and its putative homologs in humans (Daubresse et al., 1999); and Osa shows genetic interaction with components of remodeling complexes (Vazquez et al., 1999).

Competing and opposing functions must be characteristic of some of the trxG and PcG proteins. However, several regulators of homeotic genes have activities that are required for both activation and repression. Recent results have shown that the *Enhancer of zeste* [*E(z)*] and *Additional sex combs*, (*Asx*) genes of *Drosophila* are required not only as PcG genes, as originally classified, but as trxG genes as well (LaJeunesse and Shearn, 1996; Milne et al., 1999). E(z) colocalizes and directly interacts with another PcG protein, the *extra sex combs* (*esc*) product, suggesting that the partnership of the two proteins results in PcG-mediated repression (Jones et al., 1998; Tie et al., 1998). The region of sequence homology between E(z) and TRX suggests that they are interacting with a common target, offering a molecular explanation for the dual character of E(z) (Jones and Gelbart, 1993). It remains to be seen what other interacting partners of E(z) will be discovered. Similarly, we will need to know the partners of the other 'unusual' PcG member, Asx, to explain its activity (Sinclair et al., 1998). It is clear that much work needs to be done to explore the network of interactions between various PcG and trxG proteins to understand their impact on the silenced/active states of entire domains.

5. Conclusions and questions

While many questions remain to be resolved, a general picture of the role of chromatin structure in regulating gene expression is beginning to emerge. In eukaryotes, the default state is repression, the result of nucleosome assembly and higher-order packaging. Multiple assembly/remodeling complexes are present; in conjunction with sequence-specific DNA-binding proteins, these complexes can promote the formation of specific nucleosome arrays that allow access for transcription. In some cases, such as the heat-shock genes, assembly in an activatable form is general, while in other cases, it is highly regulated, occurring only in a subset of cells. Epigenetic mechanisms are used to regulate the homeotic genes, maintaining precise patterns of silencing and activity. To achieve the stable 'off' state in selected cell types, the PcG proteins may create a stable structure that resists remodeling, while the trxG proteins might promote remodeling to the active form. Maintenance of these stable on and off states appears to be linked to maintenance of patterns of histone acetylation.

While this sketch ignores many nuances of the regulatory system, it does provide a broad-brush picture against which one may point to the gaps in our knowledge. How are the preset chromatin structures of the heat-shock genes established following replication? Which of the assembly/remodeling complexes are utilized, and how does GAGA factor play its role? To what extent do the results obtained for the heat-shock genes extend to other housekeeping genes? How do these mechanisms relate to those used in the remodeling process of activation? How are the alternative functional states maintained by PcG or trxG proteins established in response to early activity, and how is this information propagated over large distances? What is the link between the local and large-scale events, the state of the nucleosome and higher-order structure? Do patterns of histone modification serve as the heritable marks of the activity state, and if so, how is that information transmitted from one generation of cells to the next?

Clearly, a great deal of work remains to be done before we can claim an understanding of the mechanisms of eukaryotic gene regulation. Systems such as *Drosophila*, which provide opportunities to synthesize biochemical, genetic, and cytological findings, will continue to play a key role.

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