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Chromatin remodeling by GAGA factor and heat shock factor at the hypersensitive *Drosophila* hsp26 promoter *in vitro*

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The chromatin structure at the *Drosophila* hsp26 promoter *in vivo* is characterized by two DNase I-hypersensitive (DH) sites harboring regulatory elements. Proximal and distal DH sites are separated by a positioned nucleosome. To study the contribution of transcription factors to the establishment of this specific chromatin configuration we assembled nucleosomes on the hsp26 promoter using a cell-free reconstitution system derived from fly embryos. Both DH sites were readily reconstituted from extract components. They were separated by a nucleosome which was less strictly positioned than its *in vivo* counterpart. The interactions of GAGA factor and heat shock factor with their binding sites in chromatin occurred in two modes. Their interaction with binding sites in the nucleosome-free regions did not require ATP. In the presence of ATP both factors interacted also with nucleosomal binding sites, causing nucleosome rearrangements and a refinement of nucleosome positions. While chromatin remodeling upon transcription factor interaction has previously been interpreted to involve nucleosome disruption, the data suggest energy-dependent nucleosome sliding as main principle of chromatin reorganization.

Key words: chromatin dynamics/heat shock gene/nucleosome positioning/promoter architecture/transcription factors

Introduction

The requirement for a local unfolding of eukaryotic chromatin and the dissociation of structural proteins to allow the functional association of the transcription machinery with promoters constitute first levels of transcriptional control (Felsenfeld, 1992; Wolffe and Dimitrov, 1993; Workman and Buchman, 1993; Becker, 1994; Paranjape *et al.*, 1994). Different levels of chromatin compaction can be distinguished by their accessibility towards nucleases. DNase I preferentially cleaves the less compacted chromatin domains of active or potentially active genes in isolated nuclei or permeabilized cells. Even more accessible and, therefore, 'hypersensitive' towards DNase digestion are regulatory sequences such as promoters and enhancers. These sites are devoid of classical nucleosome cores which often impede the binding of transcription factors to DNA (Hayes and Wolffe, 1992). The operational definition of open spots in chromatin as 'DNase I-hypersensitive sites' (DH sites) has been employed for more than a decade to describe these active sites (Wu, 1980; Elgin, 1988), however, little is known about either the structural basis of local chromatin accessibility or the mechanisms that generate it. Rapid changes in chromatin structure accompany some gene activation events in response to stimuli pointing to the intrinsic plasticity of local chromatin organization. By contrast, constitutive DH sites usually characterize the promoters of housekeeping genes, genes transcribed at low basal levels and the heat shock genes (Elgin, 1988). These active sites need to be maintained or re-established after replication and mitosis in each cell cycle. According to the nomenclature of Wallrath *et al.* (1994) the first type of promoters require 'remodeling' whereas the constitutively hypersensitive ones are 'preset'. This classification implies that the mechanisms leading to DNase I hypersensitivity during interphase upon rapid induction or after replication and mitosis are different.

The *Drosophila hsp26* gene provides an example of a 'preset' promoter that is established during early embryonic development and maintained in all cells (Lowenhaupt *et al.*, 1983; Elgin *et al.*, 1993; Wallrath *et al.*, 1994). Hsp26 RNA accumulates rapidly in *Drosophila* cells upon heat shock or other environmental stresses (Lis and Wu, 1994). It is also expressed in the absence of heat shock in a small number of adult tissues, including spermatocytes, nurse cells and ovaries, but nothing is known about the promoter structure in these tissues (Cohen and Meselson, 1985; Glaser *et al.*, 1986; Glaser and Lis, 1990). In embryos and larvae the promoter is characterized by DNase I hypersensitivity over important proximal and distal regulatory sequences: the TATA box and two Heat Shock Elements (HSEs) between −40 and −70, binding sites for Heat Shock Factor (HSF) (Lis and Wu, 1994) and upstream HSEs around −350 (Figure 1; Elgin *et al.*, 1994). *In vivo* footprinting has identified a positioned nucleosome between proximal and distal transcription factor binding sites which contributes to the specific promoter architecture (Thomas and Elgin, 1988).

A hallmark of the preset hsp26 promoter is the constitutive binding of TFIID (Thomas and Elgin, 1988) and transcription initiation of a polymerase molecule, halted some 20 nucleotides downstream of the cap site (Rougvie and Lis, 1988, 1990). These features define a state of alarm: the functional HSEs are always kept accessible to HSF, which acquires DNA binding properties upon stress induction. Activation of transcription by HSF includes release of the 'poised polymerase' obviating the time-consuming preinitiation complex formation.

Intact binding sites for the GAGA factor (GAF) (Gilmour *et al.*, 1989; Soeller *et al.*, 1994) are critical for

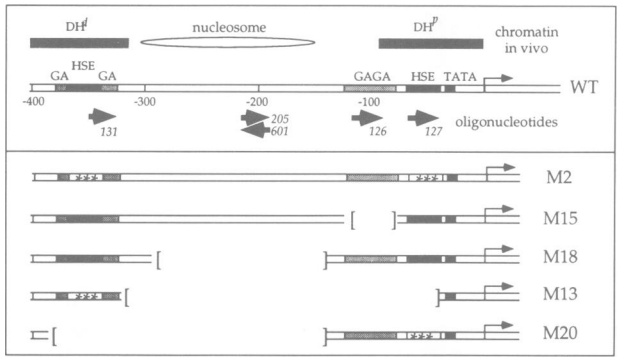

Fig. 1. Schematic representation of the wt hsp26 promoter, mutant promoters and oligonucleotide probes used in the study. Upper panel: the wild-type hsp26 promoter up to position −400 (relative to the transcription start site at +1 (small arrow) is represented by a double line, the known regulatory elements by shaded boxes: TATA, TATA box; HSE, Heat Shock Element; GAGA, GAGA box, binding site for GAGA factor. Horizontal arrows indicate positions of oligonucleotide probes. Central features of native chromatin (Elgin *et al.*, 1993) are depicted above: Dh^p and DH^d, proximal and distal DNase I-hypersensitive sites. Lower panel: mutant promoters. Asterisks, HSEs inactivated by triple point mutations; M15, proximal GAGA element (40 bp) deleted.

the establishment of DNase I hypersensitivity (Lu *et al.*, 1992, 1993), the poised polymerase (Lee *et al.*, 1992) and, therefore, heat shock inducibility (Glaser *et al.*, 1990). GAGA boxes overlap or reside adjacent to the functional HSEs. They flank the positioned nucleosome, thus demarcating the accessible area (Figure 1). GAF has been assigned a key role in pre-setting the hsp26 promoter because GAGA box mutations in *hsp26* transgenes have strong effects on chromatin structure and transcription (Wallrath *et al.*, 1994).

In order to unravel the contribution of transcription factors to promoter architecture in chromatin we assembled chromatin on the hsp26 promoter in *Drosophila* embryo extracts (Becker and Wu, 1992). The recent finding that the transcriptional activity of the hsp26 promoter can be uncoupled from the establishment of essential chromatin features (Lu *et al.*, 1994) indicates that we may be able to break down the complexities of hsp26 transcriptional regulation into amenable areas of investigation. Recently, a related study revealed the property of GAF to trigger an energy-dependent remodeling of chromatin at the hsp70 promoter leading to DH site formation (Tsukiyama *et al.*, 1994). The data suggested a nucleosome disruption over GAGA boxes, however, the precise nature of the structural changes remained unclear. Our analysis of nucleosome rearrangements by transcription factors at the hsp26 promoter indicates that nucleosomes are not disrupted. Rather the data point to energy-driven nucleosome sliding as the basis for the observed chromatin rearrangements. Interestingly HSF, like GAF, can cause nucleosome rearrangements which lead to a refinement of nucleosome positions. Nucleosome remodeling by both factors is ATP dependent.

Results

Reconstitution of DNase I hypersensitivity at the hsp26 promoter in vitro

We reconstituted chromatin on the *hsp26* gene in the context of 5 kb of *Drosophila* sequence using an extract from *Drosophila* embryos (Becker and Wu, 1992; Becker *et al.*, 1994). Primary structure details of the hsp26 promoter and mutant variants as well as relevant features of native chromatin (Elgin *et al.*, 1993) are shown in Figure 1. Reconstituted chromatin was treated with increasing concentrations of DNase I and DH sites were analyzed by indirect endlabeling (Figure 2M; Wu, 1980). Chromatin-specific cuts were derived from a comparison with the DNase I digestion pattern of protein-free DNA (Figure 2F). Modulations in the continuum of fragments created on free DNA reflect sequence preferences of the enzyme. Reconstituted chromatin yielded a distinct cleavage pattern with two regions of pronounced hypersensitivity over the hsp26 promoter region (Figure 2A, B and G). We mapped the positions of the DH regions relative to the regulatory sequences in six independent experiments. The extent of the hypersensitivity varied somewhat between different experiments such that the extreme values deviated by ~30 bp from the average indicated here and in Figure 5A. The proximal DH sites (DH^p) fell between positions −90 and +5 (relative to the transcription start at +1), the distal site (DH^d) between positions −365 and −275. These positions resemble the reported *in vivo* sites (Figure 1; Lu *et al.*, 1993): the reconstituted DH^p coincides with the genomic counterpart. It begins close to or within the proximal GAGA sequences, contains the proximal HSEs, the TATA box and the transcription start site. The DH^d appears similar but shifted downstream by ~30 bp compared with the genomic site. It overlaps with distal GAGA boxes and HSEs but appears not to contain the most upstream GAGA sequences.

The DH sites were not due to the presence of HSF, inadvertently activated during extract preparation, because they also occurred on promoter M2 (Figure 2B) containing mutated HSEs that abolish HSF binding to both proximal and distal HSEs (C.Mitchelmore, R.Sandaltzopoulos, E.Bonte and P.Becker, in preparation). Deletion of sequences between the DH sites reported to be nucleosomal in nuclei (Thomas and Elgin, 1988) moved the DH sites closer together (M18, Figure 2J). Therefore hypersensitivities map to specific sequences and are not due to boundary effects of a presumed positioned nucleosome in between them. While the DH sites (within the error limits) did not include the long proximal GAGA box ($GAGA^p$), the presence of these sequences influenced DH site formation. Their deletion resulted in a narrower and less intense DH^p, but did not abolish it completely (M15, Figure 2H). Constructs containing only proximal or distal GAGA boxes upstream of the TATA box created only one broad DH site around the GAGA sequences (M20, M13, Figure 2K and L).

In addition to the two most prominent DH sites an array of sites within the flanking sequences are detected, each 60–80 bp wide, with centers around positions −800, −560, +270, +430 and +640 (Figure 2). The distances between the sites are irregular, but large enough to accomodate a single nucleosome in each case. The interesting possibility that DNase I digestion reveals arrays of phased nucleosomes flanking the promoter boundary requires further testing in the future.

GAF and HSF modulate the fine structure of DH sites

Reconstituted DH sites were not only accessible to DNase I but also to recombinant HSF and GAF. When added to

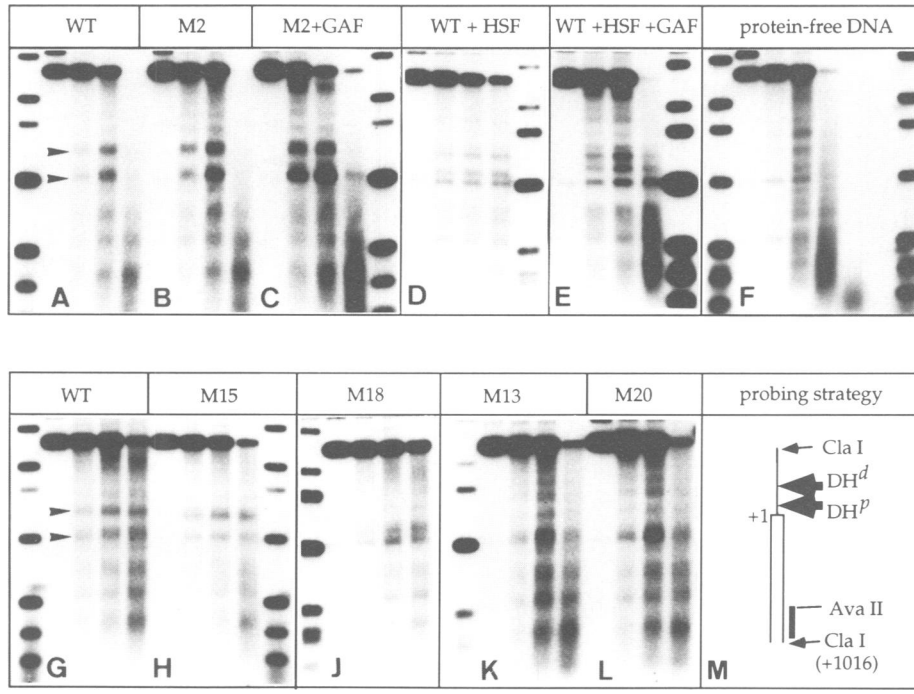

Fig. 2. Mapping of DNase I-hypersensitive sites by indirect endlabeling. Reconstituted chromatin was digested with 0, 1.5, 3 and 6 U of DNase I. Purified DNA fragments were cleaved with *Cla*I and DH sites were mapped by indirect endlabeling following the strategy indicated in panel M: Southern blots were probed with the indicated *Cla*I–*Ava*II fragment. The location of DH^p and DH^d (arrows in A and G) is illustrated in M. Plasmid and transcription factors added after assembly are indicated above each panel. Size marker: 1 kb ladder (BRL).

assembled chromatin, they caused characteristic changes in the fine structure of the DH pattern. The interaction of GAF with reconstituted chromatin resulted in an intensification of DH^p and a broadening of DH^d which now appeared as a closely spaced doublet (Figure 2C). The extent of DH^d in the presence of GAF appeared more similar to the genomic counterpart reported to contain bound GAF (Lu *et al.*, 1993). HSF was also able to interact within the DH site, protecting DNA over the HSEs from DNase I digestion (Figure 2D). The binding of GAF and HSF together protected a broader area within the hypersensitive region, flanked by new upstream cuts (Figure 2E). A similar change in DH site fine structure has been reported for the native promoter upon heat shock (Cartwright and Elgin, 1986). The association of GAF with the plasmid in the absence of chromatin did not result in pronounced hypersensitivity (data not shown). The fact that exogenous GAF and HSF produce distinct changes in DH site appearance supports the notion that neither of the two factors is responsible for the prior formation of these accessible sites.

A localized nucleosome between proximal and distal DH sites

The sequences between proximal and distal DH sites are occupied by a positioned nucleosome in nuclei (Thomas and Elgin, 1988). *In vitro* this DNA is also largely nucleosomal (see below). We mapped the translational positions of reconstituted nucleosomes in this area by micrococcal nuclease (MNase), an enzyme with high preference for linker DNA between nucleosomes. Reconstituted chromatin was progressively digested with MNase to produce DNA fragments protected by nucleosomes from further degradation. Radiolabeled oligonucleotides were annealed to the purified fragments between proximal and distal DH sites (Figure 1) and extended with Vent (Exo^-) polymerase to either end. Mapping of the fragment ends on a denaturing gel allowed the identification of proximal and distal nucleosome boundaries (Figure 3). Protein-free DNA was progressively degraded by MNase through a series of fragment intermediates reflecting the sequence preference of the enzyme (Figure 3, 'DNA'). When chromatin is analyzed in this assay a uniquely positioned nucleosome would give rise to a single resistant fragment on either side, whereas a random nucleosome positioning would result in a profile resembling protein-free DNA. The experiment revealed a series of fragments that resisted even extensive MNase digestion ('chromatin' lanes, marked areas in Figure 3) indicating a non-random positioning of nucleosomes. On the promoter proximal side (Figure 3A) dominant MNase cuts were confined to a region of 90 bp including the proximal HSEs and GAGA boxes. Resistant fragments on the distal side (Figure 3B) ended within ~60 bp of DNA close to or overlapping the distal *cis* elements. The finding of a series of fragments identifies a corresponding range of alternative nucleosomal positions (Butinelli *et al.*, 1993), some of which reach into and partially cover the regulatory elements. Prominent nucleosome boundaries *in vitro* (clamps in Figure 3) coincided with the reported edge of the positioned nucleosome in nuclei (Thomas and Elgin, 1988). The absence of a unique nucleosomal position was also inferred from our failure to observe a 10–11 bp repeated cleavage pattern with DNase I, indicative of rotational positioning, on a significant number of templates (data not shown). In the absence of clear rotational positioning, unique translational restrictions are not expected. We will refer to this nucleosome as a 'localized' nucleosome, to indicate the non-

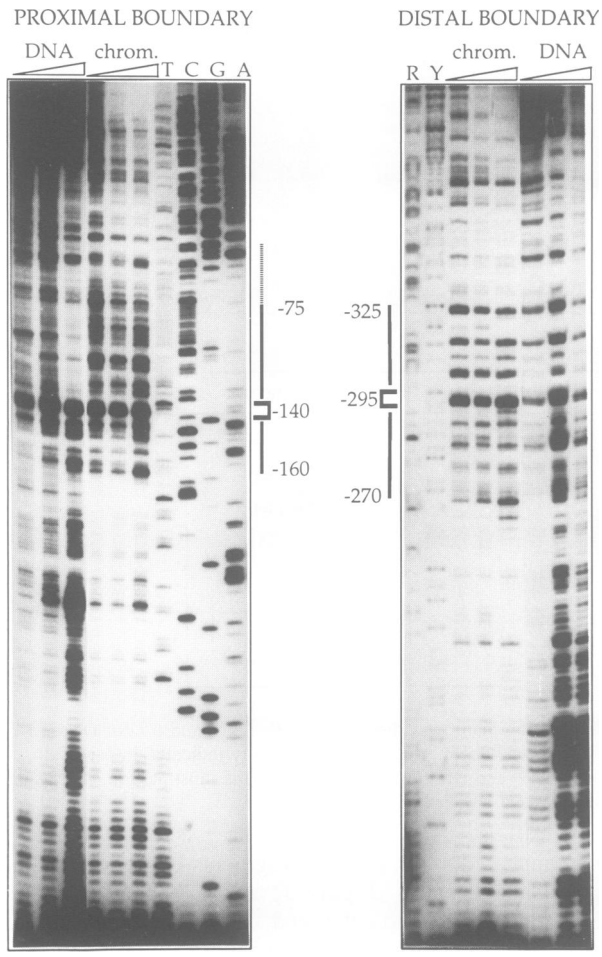

Fig. 3. Mapping of nucleosome boundaries. Reconstituted chromatin ('chrom.') was digested with increasing amounts of MNase to finally yield mainly mononucleosomal DNA. Protein-free DNA ('DNA') was digested, to yield fragments of the same lengths. Two purified DNA primers 205 or 601 (see Figure 1) were annealed and extended with Vent-Exo$^-$ polymerase to the fragment ends, mapping the proximal or distal nucleosome boundaries respectively. Fragments after linear amplification were resolved on an 8% sequencing gel and mapped with respect to dideoxy sequencing reactions using the same primer (T,C,G,A; R = purine-, Y = pyrimidine-specific sequencing reactions). Vertical bars: MNase-resistant fragments obtained from chromatin reactions (numbering with respect to the start site at +1) resistant to MNase. Clamps: nucleosome boundary in nuclei.

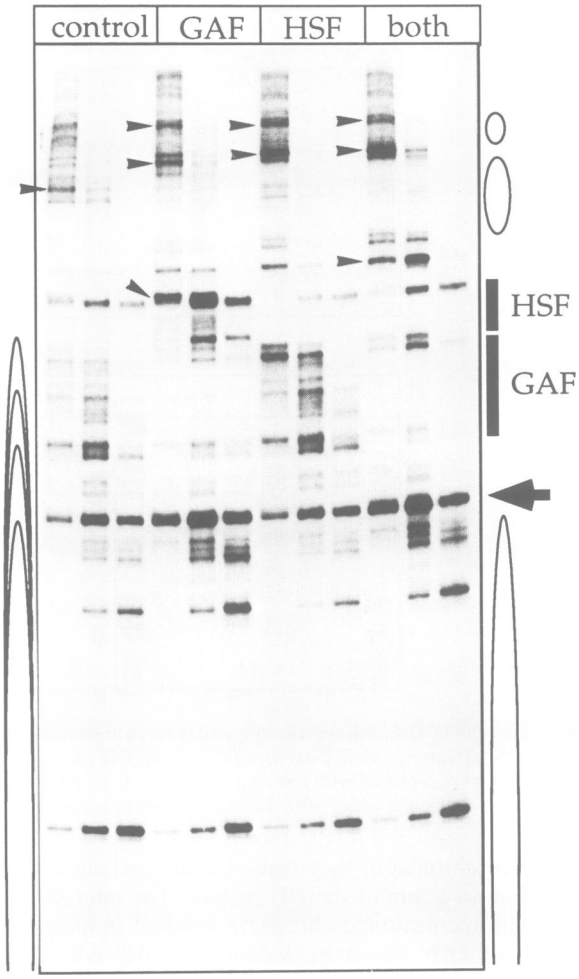

Fig. 4. Binding of transcription factors at the edge of the localized nucleosome. After chromatin reconstitution either no (control) or the indicated transcription factors were allowed to bind for 30 min at 26°C. The samples were then treated with MNase and the proximal boundaries were analyzed as in Figure 3. Arrowheads indicate new boundaries after factor binding. Vertical bars identify areas of protection by HSF and GAF. Arrow, proximal boundary of the nucleosome *in vivo*; ellipsoids, interpretation of the cleavage patterns in terms of nucleosome positions.

random positioning between DH^p and DH^d in the absence of tight positioning *sensu stricto*. While the precise relationship between the DH sites and the MNase linkers cannot be established due to the low resolution in the DH site mapping, it is clear that the DH areas flank the localized nucleosome (see also Figure 5A).

GAF binding at the edge of the localized nucleosome restricts nucleosome positions

The mapping of nucleosomal positions demonstrates that the binding sites for GAF and HSF in reconstituted chromatin are located within sequences flanking the localized nucleosome or on the less tightly associated nucleosomal DNA at entry/exit points. The earlier observation (Figure 2) that both transcription factors were able to interact with the DH sites was confirmed by direct footprinting on chromatin templates with MNase (Figure 4). GAF or HSF were allowed to bind to the assembled chromatin templates before treatment with MNase and primer extension analysis as before. In this experiment the degree of MNase digestion was chosen such that the dominant digestion products were mono- and dinucleosomal fragments and hence also potential neighboring boundaries could be seen. Essentially complete protection of binding sites by the respective transcription factors from MNase attack was observed (Figure 4), confirming the accessibility of target sequences in preassembled chromatin. HSF and GAF bound DNA with no sign of cooperativity (data not shown). The interaction of GAF resulted in an increased MNase cleavage at the site corresponding to the *in vivo* position (arrow in Figure 4) and therefore restricted the range of nucleosome positions such that the configuration in nuclei (Thomas and Elgin, 1988) was approximated. The binding of GAF or HSF also created new boundaries in chromatin (arrowheads in Figure 4). Besides the prominent MNase-hypersensitive cuts flanking the footprints, additional cuts were observed

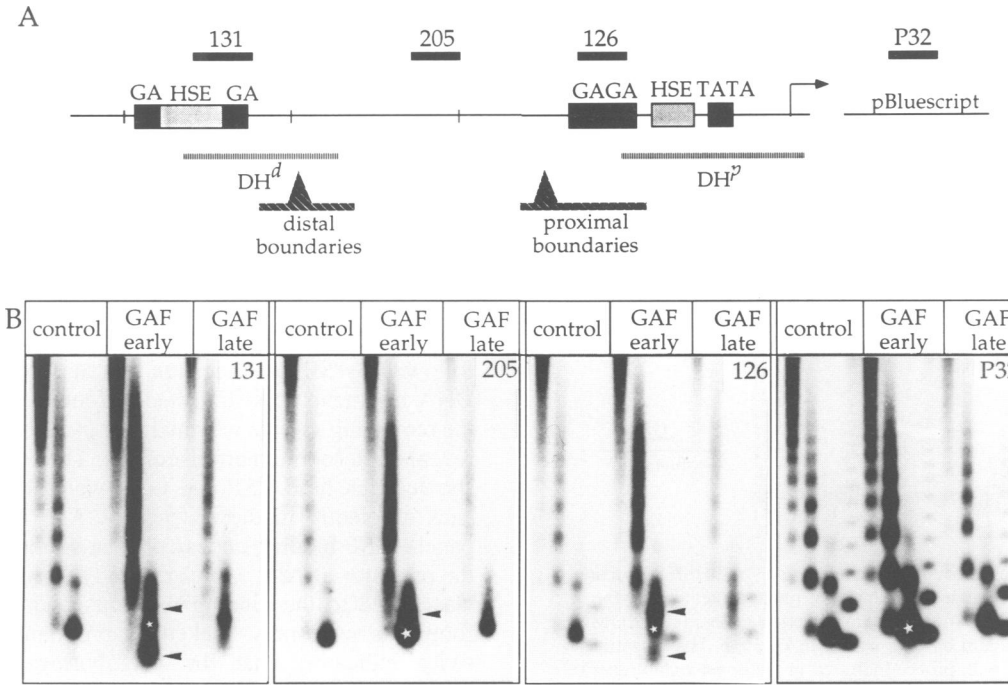

Fig. 5. (**A**) Summary of DH sites and nucleosomal linkers on the hsp26 promoter. The extent of the proximal (DH^p) and distal (DH^d) DH as determined in Figure 2 are indicated. Hatched lines symbolize the boundaries of the localized nucleosome at the proximal or distal side *in vitro*. Arrowheads show the position of the native position in nuclei. Bars with numbers show the position of oligonucleotide probes used in (B). (**B**) Changes in nucleosomal organization upon GAGA factor binding. Chromatin was assembled in the absence of factor (control) or with GAF added at the onset of assembly (early) or after reconstitution was completed (late). Chromatin was cleaved with MNase for 0.5, 1 and 5 min. Purified DNA was resolved on an agarose gel, blotted onto a nylon membrane and hybridized successively with probes P32, 126, 205 or 131 (see also A). Autoradiographs of the respective hybidizations are shown. White asterisks indicate DNA fragments of nucleosomal size. Arrowheads, fragments arising after GAF interaction. In the panel 'P32' the radioactive 123 bp ladder (BRL) is also visible between the MNase samples.

at a considerable distance. We interpret these cleavages, which are not induced in the absence of chromatin (data not shown), as the linkers of the neighboring nucleosomes which line up with respect to the new, transcription factor-created boundary.

Our interpretation of the primer extension analysis (Figure 5A) is supported by another MNase assay previously used to define nucleosome positions on the hsp70 promoter (Tsukiyama *et al.*, 1994). Chromatin was assembled as before in the absence of GAF or with factor added during ('early') or after the reconstitution ('late'). The MNase digestion products were resolved on an agarose gel and transferred to a nylon membrane. Hybridization with small oligonucleotide probes revealed whether the corresponding DNA was nucleosomal and part of a regular nucleosome array (Figure 5B). When the membrane was probed with an oligonucleotide specific for vector DNA ('P32'), the preponderance of 150 bp fragments upon extensive digestion demonstrated the presence of nucleosomes. Partial digestions yielded a ladder of oligonucleosome-sized fragments visualizing the regular nucleosomal repeat pattern in the vicinity. The addition of GAF, at the start or at the end of the assembly reaction, did not influence the chromatin structure on the vector ('P32') nor in bulk chromatin (not shown). Rehybridization of the membrane with probe 131, which detects distal *cis* elements (Figure 5B), detected a regular nucleosome array in the absence of GAF. This pattern was severely perturbed when GAF was included in the assembly reaction or when it was added to preassembled chromatin. In addition to a minor fraction of nucleosome-sized fragments (Figure 5, asterisks), we observed shorter fragments as well as atypical fragments of sizes between mono- and dinucleosomes (Figure 5, arrowheads) and a general perturbation of the regular nucleosome array. The membrane was subsequently probed with oligo 205 which detects sequences on the localized nucleosome. In the presence or absence of GAF the most prominent fragment species appeared nucleosome-associated (Figure 5, asterisks) but when GAF was added longer fragments also occurred (Figure 5, arrowheads) and the smear of larger fragments indicated that the nucleosome was not part of a regular array. When the proximal GAGA box was probed (Figure 5, probe 126) GAF-mediated nucleosome rearrangements were observed. The addition of GAF converted the regular nucleosome repeat unit into a pattern with only few nucleosomal fragments but prominent fragments of subnucleosomal size and of ~200 bp length. The changed MNase patterns were observed within 15 min after addition of GAF (data not shown). Purified factor expressed in either Sf9 cells or *Escherichia coli* caused the nucleosome rearrangements. These depended on intact GAGA boxes and were specifically prevented by antibodies against GAF (data not shown). Addition of GAF to histone H1-containing chromatin yielded similar results. The perturbations were confined to the promoter area; probes centered at positions −435 or +35 detected mainly nucleosome-sized fragments in the presence of GAF.

The GAF-induced changes in chromatin at GAGA boxes appear identical to the GAF-dependent nucleosome disruption previously reported in analogous experiments at the hsp70 promoter (Tsukiyama *et al.*, 1994).

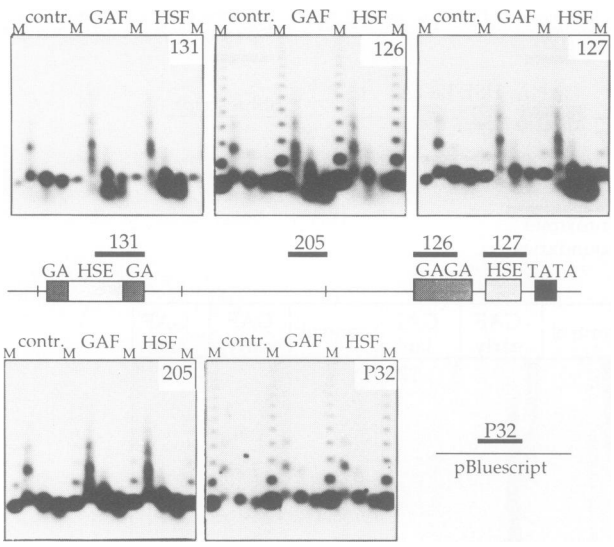

Fig. 6. Nucleosome rearrangements after GAF and HSF interaction. GAF or HSF where added to aliquots of reconstituted chromatin. Control reactions did not receive transcription factors (Contr.). MNase digestion and Southern blotting was as in Figure 5. Hybridizations were successively with probes indicated in the upper right corner of each panel, which are also shown below the schematic drawing of the promoter. M: 123 bp ladder (BRL).

HSF, like GAF, can induce nucleosome remodeling

The footprinting analysis demonstrated that HSF interacted with reconstituted chromatin as efficiently as GAF. We therefore tested whether HSF interaction would also lead to nucleosome 'remodeling' in the MNase assay (Figure 6). GAF or HSF were allowed to bind to pre-assembled chromatin before MNase treatment and hybridization analysis. When the proximal GAGA box was probed (Figure 6, panel 126) the MNase pattern changed after GAF binding but not when HSF interacted 50 bp downstream. However, probing the proximal HSEs (Figure 6, panel 127) revealed that HSF also caused changes. The appearance of short fragments of subnucleosomal size upon factor interaction was confined to a narrow area around the respective binding sites (Figure 6, control hybridizations with probes 205 and P32). As before (Figure 5B) hybridization with probe 205 revealed the perturbation of the regular nucleosome repeat pattern. Importantly, HSF apparently did not *convert* nucleosomal DNA into shorter fragments, but *recruited* additional DNA onto the membrane as judged by the increase in total hybridization signal per lane. These short fragments were only observed within a short distance from the bound transcription factor. If they were derived from disrupted nucleosomes, the perturbance of the MNase pattern would be observable over ~150 bp of DNA. We therefore conclude that these small fragments are not derived from subnucleosomal particles after nucleosome disruption, but represent the DNA protected by the corresponding factor from MNase attack (see Discussion and Figure 8). The footprint analysis (Figure 4) also supports this conclusion.

ATP-dependent nucleosome rearrangements are a prerequisite for chromatin remodeling by GAF and HSF

As will be discussed in detail below, the simplest interpretation of the observed MNase digestion patterns is that GAF bound adjacent to a nucleosome. This means that at least those nucleosomes that originally reached over the GAGA box must have moved. Since the GAF-dependent 'nucleosome disruption' described on the hsp70 promoter (Tsukiyama *et al.*, 1994) required ATP hydrolysis, we tested whether the ability of GAF or HSF to interact with hsp26 chromatin also depended on ATP. The experimental design is schematized in Figure 7A. Reconstituted chromatin was subjected to gel filtration on a Sephacryl S300 spin column, which efficiently removed free ATP. Purified chromatin was incubated with or without ATP and in the presence or absence of transcription factors, followed by MNase digestion. An aliquot of the purified DNA fragments was subjected to footprint analysis while the remaining sample was analyzed by Southern blotting to reveal nucleosome rearrangements. The footprint analysis showed that both HSF and GAF were able to bind their sites efficiently in the absence of ATP (Figure 7B, left panel). HSF binding appeared to be slightly stimulated in the presence of ATP. More strikingly, however, when ATP was added to the binding reactions both GAF and HSF footprints were now flanked by pronounced hypersensitivity indicating that the neighboring sequences had become more accessible to MNase. Analysis of the same DNA samples by hybridization was revealing (Figure 7C): the binding of GAF in the absence of ATP changed the MNase pattern around the GAGA box (Figure 7C, probe 126); in addition to unperturbed nucleosomal DNA longer fragments were abundant but only relatively few shorter fragments were observed. The addition of ATP resulted in a dramatic conversion of nucleosomal fragments to those short and long fragments. The presence of ATP also had a pronounced effect on the MNase pattern after HSF binding. In the absence of ATP, HSF did not influence the extent of nucleosome association, but protected short DNA fragments from MNase digestion (Figure 7C, probe 127). In the presence of ATP the nucleosomal DNA disappeared. Probe 127 detected only little DNA from the reaction that contained GAF and ATP, presumably because under those conditions the HSEs are rendered MNase hypersensitive (Figure 7B) and are therefore degraded. In the absence of factors, HSEs were nucleosomal to a certain extent, but were rendered entirely nucleosome free upon interaction of GAF. In this experiment, transcription factor binding and ATP-dependent nucleosome rearrangements were more pronounced than in the previous experiments (Figures 4 and 6). We attribute this to the purification of the chromatin on a gel filtration column which removes the bulk of soluble components and results in a higher factor/chromatin ratio due to chromatin losses.

We conclude that the interaction of both transcription factors with hsp26 chromatin resulted in ATP-dependent nucleosome rearrangements. Rearrangements were observed for those nucleosomes that reached into the DH site, but the neighboring nucleosomes were also realigned. The newly created boundaries already described in Figure 4 and interpreted as phased linkers of neighboring nucleosomes were only observed when ATP was present in the reaction (arrows in Figures 4 and 7B). Importantly, this experiment allowed the dissection of the process of nucleosome rearrangements at the hsp26 promoter into two phases: the interaction of the transcription factor and the energy-dependent nucleosome movement.

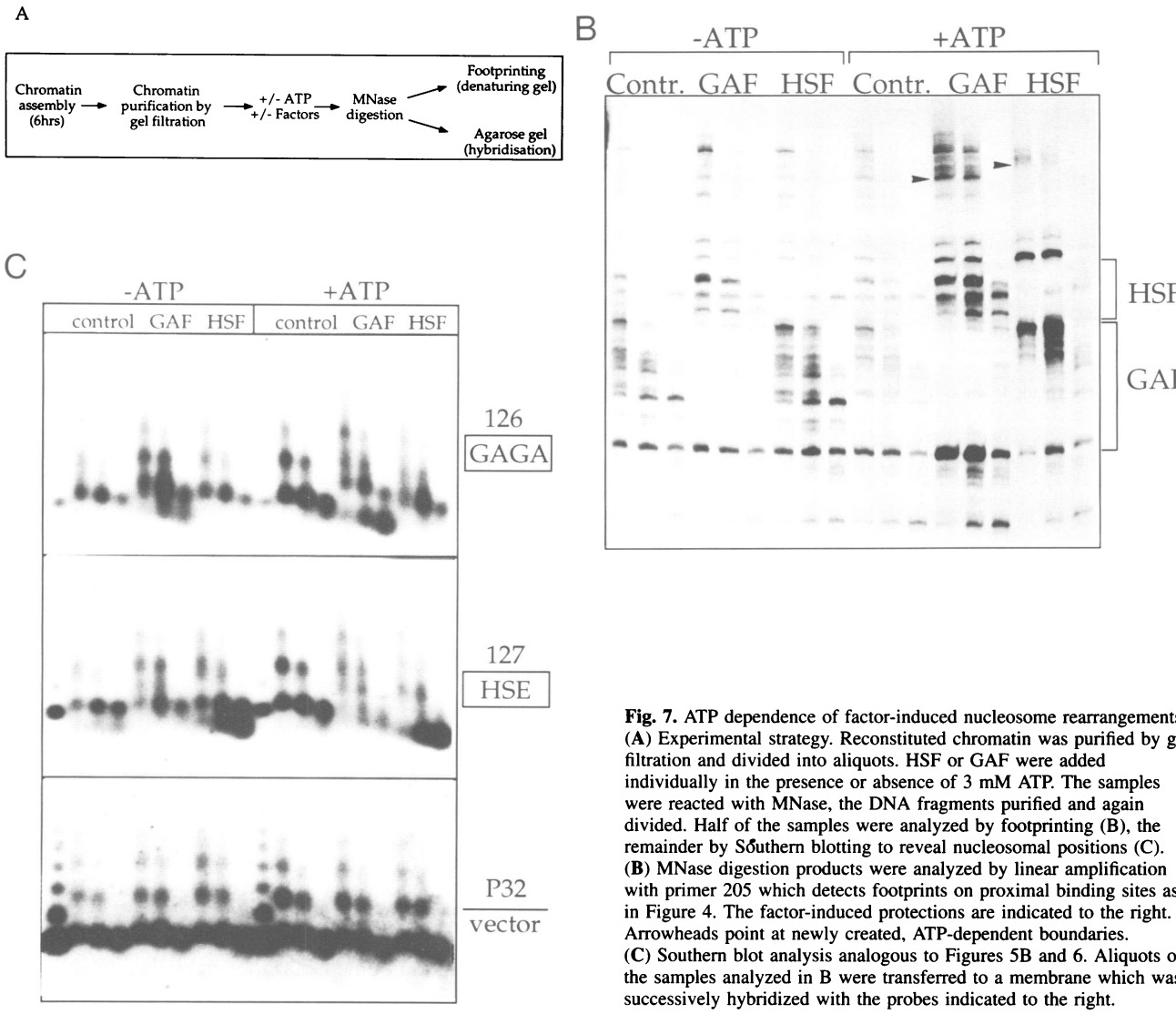

Fig. 7. ATP dependence of factor-induced nucleosome rearrangements. (**A**) Experimental strategy. Reconstituted chromatin was purified by gel filtration and divided into aliquots. HSF or GAF were added individually in the presence or absence of 3 mM ATP. The samples were reacted with MNase, the DNA fragments purified and again divided. Half of the samples were analyzed by footprinting (B), the remainder by Southern blotting to reveal nucleosomal positions (C). (**B**) MNase digestion products were analyzed by linear amplification with primer 205 which detects footprints on proximal binding sites as in Figure 4. The factor-induced protections are indicated to the right. Arrowheads point at newly created, ATP-dependent boundaries. (**C**) Southern blot analysis analogous to Figures 5B and 6. Aliquots of the samples analyzed in B were transferred to a membrane which was successively hybridized with the probes indicated to the right.

Discussion

Two modes of transcription factor interaction with chromatin

Our analysis allowed a definition of two modes of transcription factor interaction with chromatin. Their interaction with DNA within the DH sites did not require ATP and had only minimal effects on nucleosome positions. In the absence of ATP the extent of nucleosomal association of a particular sequence did not change. By contrast, binding of the transcription factors in the presence of ATP was accompanied by nucleosome rearrangements ('nucleosome remodeling'). In the presence of GAF or HSF those nucleosomes that had occupied the respective binding sites moved to a new location adjacent to the bound factor (see below). It is not known whether GAF or HSF can stably interact with nucleosomal DNA in the absence of ATP. In an earlier study with less physiological chromatin, yeast HSF was unable to bind nucleosomal DNA (Taylor *et al.*, 1991). We have also analyzed the ability of HSF to bind to a minimal promoter containing only HSEs in front of a TATA box (Varga-Weisz *et al.*, 1995). This promoter does not display DH sites in reconstituted chromatin. Under those circumstances HSF binding to chromatin is considerably stimulated by ATP. The slight ATP stimulation of HSF binding within the DH region (Figure 7B) may therefore be due to a minor population of nucleosomal sites. Our data are consistent with the idea that, in the absence of energy, GAF and HSF can bind only to nucleosome-free regions such as nucleosomal linkers and DH sites and that ATP is required to interact with a nucleosomal binding site.

HSF, like GAF, causes nucleosome rearrangements

The ability to rearrange nucleosomes in an ATP-dependent manner was first described for GAF on the hsp70 promoter (Tsukiyama *et al.*, 1994). This observation fits well with other previously described features of GAF: its ability to act as a transcriptional antirepressor (Kerrigan *et al.*, 1991), its importance for the establishment of the correct chromatin configuration of the hsp26 promoter (Lu *et al.*, 1993) and its function as an enhancer of position effect variegation in the fly (Farkas *et al.*, 1994). It has been proposed that GAF, with its ability to create nucleosome-free regions at promoters, prepares the heat shock promoters to allow efficient interaction of HSF (Tsukiyama *et al.*, 1994; Wallrath *et al.*, 1994). The underlying

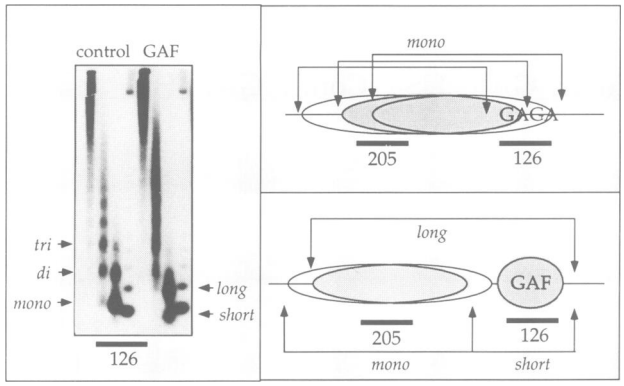

Fig. 8. Model explaining the GAF-induced nucleosome rearrangements. To the left the GAF-induced long and short fragments detected by probe 126 on the proximal GAGA box are shown. The regular oligonucleosome ladder in the absence of GAF is rearranged when GAF interacts with chromatin. The interpretation of the newly arising fragments is schematized in the right panels. For a detailed explanation see Discussion. Vertical arrows, MNase cleavages; horizontal lines, protected fragments; ellipsoids, nucleosomes.

assumption is that GAF and HSF belong to different categories of transcription factors.

Surprisingly, our study reveals that the potential to rearrange nucleosome positions in our assays is not a unique feature of GAF and, indeed, in our hands HSF affects chromatin similarily. This finding does not depend on the location of HSE within DH sites. The interaction of HSF with HSEs in a different context and in the absence of pronounced DH sites leads to a similar, efficient energy-dependent nucleosome rearrangement (Varga-Weisz *et al.*, 1995). This result could mean that HSF shares with GAF the ability to rearrange nucleosome positions, a factor which distinguishes them from other proteins. Alternatively, the nucleosome rearrangement activity is intrinsic to chromatin and not associated with specific transcription factors. Then many DNA binding proteins, if not all, might be able to interact with chromatin as described. Further screening of the properties of other factors in our system will help to decide between the two possibilities.

Subnucleosomal fragments after remodeling are due to DNA protected by transcription factors

Nucleosome rearrangements of the kind we observe for both GAF and HSF have previously been attributed to nucleosome disruption because of the appearance of fragments of subnucleosomal size at the expense of nucleosomal fragments. Our data suggest that those short fragments are due to DNA protected from MNase digestion by the interacting transcription factor in analogy to the protection brought about by nucleosomes. This notion is supported by the footprint analysis (Figure 7B) that demonstrates protection of factor-bound DNA from MNase digestion and an enhanced cutting at either side effectively releasing short fragments.

Our data are most easily explained by the model given in Figure 8: nucleosomes are reconstituted in different phases over the hsp26 promoter. Major phases establish the 'localized nucleosome' with flanking hypersensitivity, but minor nucleosome phases also reach over GAGA boxes and HSEs. In the absence of ATP nucleosomal DNA will remain unaltered but GAF and HSF can interact with accessible sequences within the DH site. Previously accessible sequences—and therefore degraded by MNase—are now protected by the bound factors and give rise to small MNase resistant fragments. This is particularly obvious over the HSEs which reside well in the DH area. In those cases where a factor binds so close to the nucleosome that MNase will not cut between them, long protected fragments of ~200 bp are generated. In the presence of ATP real remodeling occurs and in addition nucleosomal DNA is quantitatively converted into long and short fragments (Figure 8).

In addition to the nucleosome rearrangement around the transcription factor binding sites, an ATP-dependent decay of the regularity of the nucleosomal array was observed upon interaction of factors (Figures 5B and 8). At present we can only speculate about the influence of local nucleosome rearrangements on the regularity of nucleosomal arrays. We propose elsewhere (T.Blank and P.Becker, in preparation) that nucleosome spacing may be a function of the folding of the nucleosomal fiber into higher order structures. Since factor-induced rearrangements will almost certainly lead to discontinuities in the folding of the fiber, changes in the regularity of nucleosomal arrays may be expected.

Chromatin remodeling may involve nucleosome sliding

The mechanism of chromatin remodeling is unclear at present, but it may not necessarily involve nucleosome disruption. In light of the dynamic features of nucleosomes in our system (see below) we suggest that the observed nucleosome rearrangements may be most easily described by the term 'sliding'. Nucleosomes that occupy factor binding sites are relocated by the interacting factor to an adjacent position, sometimes close enough that the MNase cannot cleave in between the two. The footprint analysis demonstrated an increased access of MNase on either side of the bound factor in the presence of ATP. This can be explained by a movement of nearby nucleosomes away from the new boundary created by the bound factor. It has been demonstrated that nucleosomes will be statistically positioned at a certain distance from such a boundary (Fedor *et al.*, 1988). A realignment of nucleosomes is also observed at a considerable distance from the factor binding sites (Figures 4 and 7B), which suggests that not only does the particular nucleosome that occupies the factor binding site move, but that neighboring nucleosomes move to align in concordance.

DH sites, reminiscent of native structures, are reconstituted in vitro

The reconstitution of DH sites in the *Drosophila* embryo extract in the absence of zygotic transcription factors is in surprising contrast to analogous experiments on the hsp70 promoter where no hypersensitivity was observed under these conditions (Tsukiyama *et al.*, 1994). The origin of this hypersensitivity is presently unclear but it is likely related to the mechanism(s) that define the non-random nucleosome positions that we termed the 'localized' nucleosome. Further studies will address the relative contributions of DNA sequence, secondary

structure and putative, yet unrecognized, DNA binding proteins to DH site formation.

The interaction of GAF and HSF refine nucleosome positions

The genomic footprinting analysis of Thomas and Elgin (1988) suggests a precise rotational and translational position for the nucleosome between the proximal and distal DH sites. By contrast the *Drosophila* embryo extract assembles a series of non-random nucleosome phases, with a nucleosome localized around the genomic site. The binding of GAF and the associated nucleosome movements refine the nucleosome position such that the genomic position is approximated. This refinement is best illustrated in the DH site analysis (Figure 2E) where the gap between the DNase I cutting sites in the presence of both HSF and GAF is reduced to ~150 bp. Since we have not detected a rotational phasing of the nucleosomal DNA even with GAF and HSF bound, we envisage constraints on nucleosome positions in nuclei that we do not reconstitute in our system. One such constraint could come from the unpredictable effect of a higher order folding. On the other hand it is also conceivable that native GAF, perhaps in association with other proteins, influences the hsp26 promoter structure more profoundly than recombinant GAF.

Energy-dependent nucleosome movements may be a general principle contributing to chromatin dynamics

Bradbury and colleagues (Meersemann *et al.*, 1992) have demonstrated that nucleosomes in principle can move on DNA. We have recently demonstrated nucleosome sliding in the absence of any interacting protein (Varga-Weisz *et al.*, 1995). The fact that those nucleosome movements in our system require ATP hydrolysis suggests that nuclei are furnished with activities that facilitate nucleosome movements, thereby implementing a dynamic state. We propose that transcription factors such as GAF and HSF, take advantage of nucleosome movements to integrate themselves into chromatin. Once integrated these proteins function as boundaries that redirect nucleosome positions. Realignment of nucleosomes with respect to the newly created boundaries again relies on energy-consuming nucleosome movements. Importantly, the mobility of nucleosomes in this model does not depend on the interacting transcription factor.

The nature of the activity that hydrolyses ATP to render nucleosomes mobile is presently unclear. It appears to be chromatin-associated, since we have reproduced essential features using immobilized chromatin on paramagnetic beads (Sandaltzopoulos *et al.*, 1994) and purification in a magnetic field without contribution of soluble proteins (data not shown). Future studies will reveal whether the nucleosome mobility observed in chromatin reconstituted in extracts from fly embryos is characteristic for preblastoderm chromatin or a property of chromatin in general.

Materials and methods

Plasmids and probes

The construction of the relevant mutant hsp26 templates is described elsewhere (C.Mitchelmore, R.Sandaltzopoulos, E.Bonte and P.Becker, in preparation). Details are available upon request. The wild-type plasmid, phsp26HH4.8, contains the *hsp26* gene with flanking sequences as a 4.8 kb *Hin*dII fragment from pJ1 (Craig and McCarthy, 1980), cloned into the *Hin*dII site of pBluescriptII SK$^-$ (Stratagene). Mutant promoters were analyzed in a minigene context which was derived by deleting sequences betwen +70 and +90 (all numbers relative to the transcription start site, +1). Wild-type and minigene promoter yielded comparable results. HSEs were destroyed by replacing the central three consensus bases by non-complementary transversions. The oligonucleotides used in various assays where as follows (u = upper strand, l = lower strand): 131: u −392/−356 ; 205: u−217/−193; 601: l −193/−215; 126: u −117/−90; 127: u −69/−41. The probe used for indirect endlabeling was an *Ava*II−*Cla*I fragment corresponding to hsp26 sequences between +727 and +1014.

Recombinant transcription factors

GAF was expressed in Sf9 cells from a baculovirus vector. The cDNA (Soeller *et al.*, 1994) was amplified by PCR with oligos that added an *Xba*I site to the 3′end and a *Bgl*II site/insect translation start/hexahistidine tag to the 5′ end and inserted into *Bgl*II/*Xba*II cut pVL1392 (Invitrogen). GAF was purified from infected Sf9 cells 2 days after infection. Cells washed with PBS were suspended in 500 µl NB (60 mM Tris−HCl, pH 8.0, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 2 mM EGTA, 0.5 mM EDTA, 0.3 M sucrose). 500 µl NB/1% NP-40 was added and the cells lysed for 5 min on ice. Nuclei were collected by centrifugation and suspended in two packed cell volumes of HEMG/400 mM KCl/0.05% NP-40 (HEMGN400; Sandaltzopoulos *et al.*, 1994), gently mixed for 1 h at 4°C and then spun for 10 min at 14 000 r.p.m. The supernatant was applied to a 200 µl DEAE Sepharose CL6B column (Pharmacia), pre-equilibrated in HEMGN400. The flow through including a 150 µl wash with HEMGN400 was added to 50 µl packed Ni-NTA resin (Qiagen), and gently mixed for 1 h at 4°C. The resin was collected by centrifugation in an Eppendorf centrifuge type 5415C, 14 000 r.p.m., washed with 200 µl HEMGN400, and 200 µl HEMGN400/20 mM imidazol (pH 8) and incubated for 5 min. 100 µl HEMGN400/200 mM imidazol were added, the resin incubated for 10 min and then spun out. GAF in the supernatant was used for the experiments shown. Expression of GAF in *E.coli* was according to a protocol from T.Tsukiyama and C.Wu (personal communication) by chromatography over heparin sepharose and Mono S resins (Pharmacia) followed by Centricon concentration (Amicon). Both proteins were ~80% pure. Histidine-tagged baculo GAF and untagged GAF expressed in *E.coli* had similar effects in our assays. HSF was expressed in *E.coli* and purified as in Clos *et al.* (1991).

Chromatin assembly and nuclease digestions

Chromatin assembly and MNase digestion were as described (Becker *et al.*, 1994; Tsukiyama *et al.*, 1994). Typically a reaction contained 100 ng pHH4.8 and 400 ng RF FX174 DNA. At the end of an assembly reaction usually enough ATP remains to allow chromatin remodeling. To avoid variations due to variable ATP levels we generally added another 4 mM ATP along with 1–3 footprint units of transcription factors. Remodeling happened during further incubation for 15–30 min. For DNase I digestion the reaction volume was increased with EX buffer to 100 µl. 20 µl aliquots were digested with 0, 1.5, 3 and 6 U of DNase I (Boehringer) in a final volume of 50 µl Ex buffer/3 mM $CaCl_2$ for 2 min at 26°C before addition of 12.5 µl stop mix and 0.5 µl glycogen (10 mg/ml, Boehringer). DNA was purified by treatment with RNase and Proteinase K as described (Becker *et al.*, 1994) followed by organic extractions and ethanol precipitation in the presence of ammonium acetate. DNA was cleaved with 3 U *Cla*I (Bsu15I, Fermentas) at 37°C for 2 h, precipitated and run along with ^{32}P-labeled 1 kb ladder (Gibco BRL) on a 1.0% agarose gel at 90 V. Southern blotting and hybridization was as described (Becker *et al.*, 1994; Tsukiyama *et al.*, 1994). For rehybridizations membranes were stripped with 0.2 M NaOH for 15 min at RT. The probe fragment for indirect endlabeling was cleaved from the hsp26 coding region with *Cla*I (Bsu15I, Fermentas) and *Eco*0109I (Biolabs), gel purified (Jetsorb kit) and labeled by random priming (Prime-A-Gene kit, Promega).

Primer extension footprint analysis

Purified MNase digestion products (10–20 ng) were analyzed by linear amplification with ^{32}P-labeled primers. The 10 µl reaction in Vent-Exo$^-$ buffer contained 0.1–0.2 pmoles of primer in 4 mM $MgSO_4$, 0.2 mM dNTPs and 0.6 U Vent-Exo$^-$ polymerase (NE Biolabs). Cycling involved 4 min at 95°C, then 20 cycles of 1 min at 95°C, 2 min at 55°C, 3 min at 72°C, and a final 10 min at 72°C. The reaction was mixed with 10 µl

formamide loading buffer (Sambrook *et al.*, 1989), heated to 75°C for 5 min, chilled on ice and 2–4 μl were resolved on an 8% sequencing gel in TBE. The gel was fixed, dried and autoradiographed.

Purification of chromatin by gel filtration

120 μl assembly reactions were spun at 1100 *g* through a 1.8 ml settled Sephacryl S-300 HR (Pharmacia) spin column pre-equilibrated with 10 mM HEPES–KOH, pH 7.6/ 120 mM KCl/10% glycerol/5 mM $MgCl_2$/ 0.5mM EGTA/10 mM β-glycerophosphate/1 mM DTT.

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