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Developmental switch in chromatin structure associated with alternate promoter usage in the *Drosophila melanogaster* alcohol dehydrogenase gene

Iain L.Cartwright

Department of Biochemistry and Molecular Biology, University of Cincinnati College of Medicine, Cincinnati, OH 45267, USA

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During the development of *Drosophila melanogaster* a switch in alcohol dehydrogenase gene promoter usage occurs, such that proximally initiated mRNA is replaced by mRNA initiated from a more distal location. Investigation of the nucleoprotein organization at this gene in cells inactive for *Adh* expression, or derived from tissues active at either the proximal or distal promoter, reveals distinct changes in patterns of nucleosome organization and regions of nuclease sensitivity that are strongly correlated with the activity of the gene and its promoter usage. A positioned array of nucleosomes covers the coding region of the inactive gene, but is partially disassembled on gene activation. A series of proximally located hypersensitive sites, detected in early third instar larval fat body cells, are replaced by new, distally located regions of hypersensitivity in late third instar larval fat body, the change apparently coinciding with the promoter switch. Further developmental stage differences are detected in regions over 1 kb upstream of the distal start site. In addition, for both proximally and distally expressing cells, separate and different regions of apparent resistance to DNase I cleavage in chromatin are detected in locations that, in some instances, were previously demonstrated to bind specific factors *in vitro*.

Key words: DNase I hypersensitivity/methidiumpropyl-EDTA.iron(II)/nucleosome positioning/nuclear footprint

Introduction

The single gene encoding the enzyme alcohol dehydrogenase (*Adh*) in *Drosophila melanogaster* is expressed in both a stage- and tissue-specific manner. In addition, the *Adh* gene displays a rather unusual switch in promoter usage during development. As initially characterized (Benyajati *et al.*, 1983) a gene proxmal site of message initiation is utilized in the larval stage, but in the adult fly the site of transcription initiation has moved distally some 707 bp. The mature, spliced messages from these stages differ in their 5′ sequences, but apparently encode identical protein products since the differences are confined to untranslated regions of the respective messages only. The significance of the developmental switch in promoter utilization is unknown at this time, but it is found as a conserved feature in at least one distantly related *Drosophila* species (Rowan *et al.*, 1986) as well as in a number of sibling species (Bodmer and Ashburner, 1984). Interestingly, other *Drosophila* species have been shown to possess duplicate *Adh* genes, one of which is utilized primarily in larval stages of development, the other primarily in adult life (Batterham *et al.*, 1983; Fischer and Maniatis, 1985). Closer examination of the switch in promoter usage through different developmental stages in *D. melanogaster* revealed a somewhat more complex story (Savakis *et al.*,1986). In mid- to late-embryogenesis some accumulation of message derived from the proximal initiation site is observed, accompanied by a transient peak derived from the distal site. By mid-third larval instar, message derived from the proximal site alone has accumulated to high levels. However, an abrupt switch occurs, so that by late third instar a small amount of message accumulates that is initiated exclusively from the distal site. Coincidentally there is a complete disappearance of message (and presumably transcription) derived from the proximal initiation site. During pupariation message accumulation is again minimal, but increases slowly during pharate adult development and then dramatically at eclosion. Such transcription is derived exclusively from the distal promoter (Savakis *et al.*, 1986).

By taking advantage of the fact that it is possible to isolate specifically expressing fat body tissue from third instar larvae in usable biochemical quantities, I have investigated whether this promoter switch is accompanied by changes in nucleoprotein organization in and around the *Adh* gene. The rationale for such experiments lies in the observation that strong discontinuities and/or rearrangements in the nucleoprotein organization around eukaryotic genes will, with high probability, be located at (or directly flank) sequences important for regulation of expression of that gene (Shermoen and Beckendorf, 1982; Cartwright and Elgin, 1986). The results of such an analysis using the chemical probe, methidiumpropyl-EDTA.iron(II) [MPE.Fe(II)], demonstrate that the nucleosome organization of the active *Adh* gene is perturbed throughout the coding region compared to that of the inactive gene, where a specifically positioned array is detected. Moreover, sharply defined regions of DNase I sensitivity and protection are located in and around both proximal and distal sites of transcription initiation as well as in regions much further upstream. However, these patterns differ distinctly from one another depending on which developmental stage is examined. Such changes in nucleoprotein organization occur in a tissue in which cell division has already terminated, and can probably occur within a single nucleus.

Results

In Figures 1, 2 and 3, patterns of nucleoprotein organization in and around the *Adh* gene in various cell types, as revealed by probing of nuclear chromatin with either MPE.Fe(II) or DNase I, were visualized by employment of the indirect end-labelling technique and the use of DNA probes derived from various parts of the *Adh* region.

Previous investigations have shown that Schneider line 2 (S2) tissue culture cells do not transcribe their endogenous *Adh* gene, although copies of *Adh* transfected into these cells are accurately transcribed from both promoters (Benyajati and Dray, 1984). This implies that *trans* acting factors necessary for *Adh* transcription are present in these cells, but that for some unknown reason they are not productively utilized by the transcriptionional machinery. In Figure 1, digestion of such transcriptionally silent S2 nuclei with the chemical reagent MPE.Fe(II) (Cartwright *et al.*, 1983) and probing from downstream of the gene reveals a

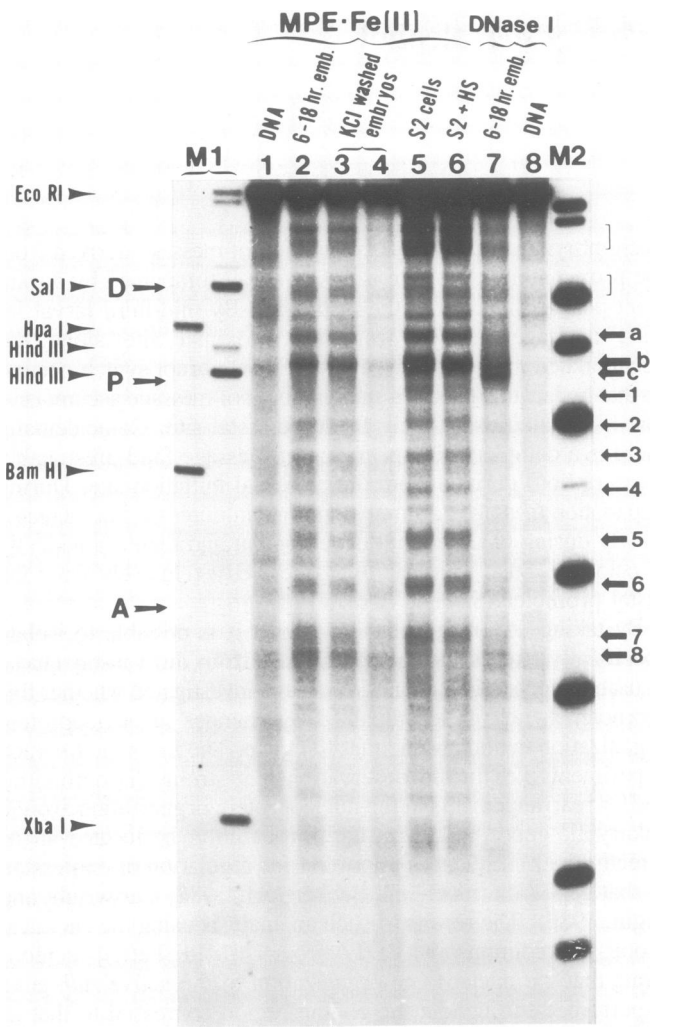

Fig. 1. Analysis of *Adh* chromatin organization in S2 cells and 6−18-h embryos. Nuclei were briefly digested with MPE.Fe(II) or DNase I, purified DNA was restricted to completion by *Eco*RI, and DNA fragments were resolved by electrophoresis on a 45-cm 1.5% agarose gel followed by indirect end label analysis of a nitrocellulose filter replica. The downstream *Eco*RI−*Xba*I probe used abuts an *Eco*RI site some 3.3 kb 3′ of the distal initiation site (not shown in Figure 4). Numbered or lettered arrows align with sites of enhanced cleavage and are referred to in the text. D, P and A refer to distal initiation, proximal initiation and poly(A) addition sites respectively. Genomic restriction site markers were loaded in lane M1 and their identity is noted on the figure. Lane M2 is a series of other DNA mol. wt markers.

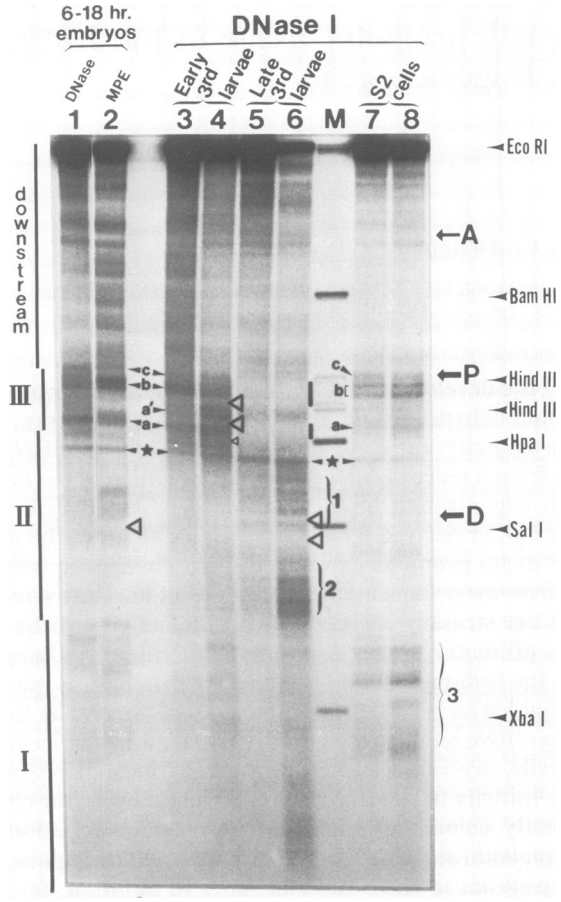

Fig. 2. High-resolution analysis of chromatin organization in and around the two promoter regions of *Adh* in different cell types. Sites of DNase I or MPE.Fe(II) digestion in each case were analyzed as described in Figure 1, except that the probe was the upstream *Eco*RI−*Xba*I fragment shown in Figure 4. D, P and A have the same connotation as in Figure 1. Arrows or brackets signify regions of DNase I sensitivity as described in the text. The open triangles denote regions of relative insensitivity to cleavage, and are also referred to in the text. Lane M shows size and position markers derived by cleavage of genomic DNA with the designated enzyme (see adjacent notation) and subsequently with *Eco*RI. The division of the locus into distinct regions is depicted at the left and shown more fully in Figure 4.

very distinct and precisely positioned array of nucleosomes covering the coding region of the *Adh* gene downstream of the proximal cap site, P (lane 5, bands 1−7). As expected, this pattern is not affected by a 45-min heat shock (lane 6). In weakly transcriptionally active 6−18 h embryos, however (lane 2), the array appears substantially less distinct and some of the bands are probably due to DNA specific background cleavage. There is also a particularly noticeable difference in the pattern of cleavages in the vicinity of the poly A addition site, A (compare lanes 2 and 5). The extremely strong cuts produced in S2 cells give way to a weaker pattern in embryos. However, the embryonic pattern is terminated by a strong cleavage about 160 bp 3′ of the poly(A) site that is not present in the inactive S2 chromatin (band 8, lane 2). Both S2 and embryonic nuclei display broadly similar patterns of hypersensitivity upstream of the proximal cap site, P, in Region III (see Figure 4 for an explanation of this terminology) revealed both by MPE.Fe(II) (bands a and b in lanes 2 and 5 of Figure 1; see also bands a and b in lane 2 of Figure 2) and by DNase I (bands a, b and c in lane 7 of Figure 1; bands a, b and c in lanes 1, 7 and 8 of Figure 2, but note band a is very weak in S2 cells). These cleavages are of rather stronger intensity in the active embryos. A broad area of cleavage is seen in Region II around the distal cap site, D, in both S2 and embryo nuclei (bracketed in Figure 1; see summary in Figure 4). However, a noticeably strong site of DNase I cleavage at −150 to −190 bp upstream of D in S2 cells is weak or absent in embryos (compare band 1 in lanes 1 and 2 of Figure 3). In Region I a series of strong cleavages around the *Xba*I site (at −660 bp relative to D) are seen in S2 nuclei (Figure 2, lanes 7 and 8, bracketed region 3; Figure 3, lane 2, band 3). These are relatively weak in embryos (Figure 2, lanes 1 and 2; Figure 3, lane 1). More differences can be seen even further upstream (compare particularly bands 5, 9, 10 and 12 in lanes 1 and 2 of Figure 3). Quite clearly then, the S2 cell *Adh* gene shows a series of distinct nucleoprotein organizational differences compared to the active embryos (and to active fat body nuclei — see below) that are probably causally related to its inactivity. These are most notable within the gene, at the 3′ end of the gene, and in regions

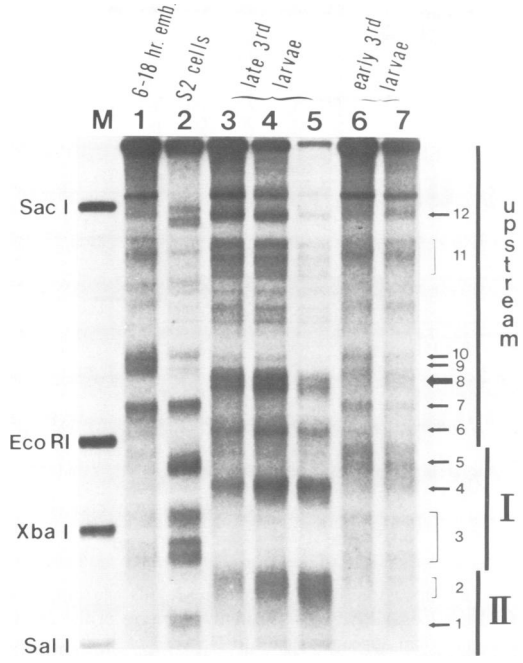

Fig. 3. Analysis of chromatin organization upstream of the distal initiation site in a variety of cell types. Indirect end-labelling of *Bam*HI-digested DNA samples was facilitated by use of the *Bam*HI−*Hin*dIII probe shown in Figure 4. Arrows or brackets signify regions of DNase I sensitivity as discussed in the text. Lane M is a set of genomic DNA markers as described in Figure 1.

upstream of the gene i.e. from 150 bp to > 2 kb away from the distal cap site.

A similar set of experiments was performed on nuclei isolated from fat body cells derived from both early third instar and late third instar larvae collected as described in Materials and methods. Due to a relative scarcity of material only DNase I digestions were performed on these nuclei. Figure 2 demonstrates the relatively high level of resolution obtained by fractionating these DNA samples on long agarose gels. Using a probe derived from the region spanning the upstream *Eco*RI and *Xba*I sites (see Figure 4) resolution equivalent to ± 10 bp could be achieved in and around D and only slightly less (± 15 bp) near P. Of immediate interest is the strong disparity between the patterns of sensitivity to DNase I cleavage close to the respectively utilized transcriptional initiation sites. Thus there are pronounced regions of hypersensitivity immediately upstream of P in Region III in the early third instar cells (bands a, a′, b and c, lanes 3 and 4) with very little cleavage around D in Region II (the starred band appears to be a region of high DNA sequence preferential cleavage). Conversely, late third instar cells show little cleavage around P in Region III, but two distinct areas of sensitivity in Region II, one located in the vicinity of D (bracketed region 1) and the other about −250 to −400 bp upstream of it (bracketed region 2, lanes 5 and 6; see also region 2 of Figure 3). The strong cuts noted around the upstream *Xba*I site (bracketed region 3 in Figure 2) in S2 cells (lanes 7 and 8) are weak in early third instars and virtually absent in late third instars (see also region 3 in Figure 3). Very noticeable, however, is a broad area of sensitivity in late third instar cells located at −800 to −1000 bp in Region I which is absent in the early third instars (Figure 3, band 4). Figure 3 in fact shows substantial differences in far upstream regions between many of the cell types. There is a series of pronounced sites of DNase I hypersensitivity in this region unique to late third instar fat bodies (bands 4, 6 and 8), and indeed further strong sites are seen out to −3.5 to −4.5 kb relative to D.

Close inspection of the sensitivity patterns detected around the cap sites in Figure 2 reveals some interesting data. Between the hypersensitive bands a, a′ and b in Figure 2, lanes 3 and 4 are regions that appear protected from (or hyposensitive to) DNase I (denoted by large open arrowheads in lane 4). Careful mapping of the extent of these regions reveals potential footprints from −122 to −167 bp and −257 to −282 bp upstream of P. A region slightly further upstream of these fairly prominent footprints also appears somewhat refractory to DNase I digestion in these cells (denoted by the smaller open arrowhead in lane 4); this region is located −360 to −395 bp upstream of P. Much of this proximal region becomes highly refractile to digestion by DNase I in late third instar fat body as denoted by the solid black bars in this region adjoining lane 6. The impression given is that these regions have been completely blocked by some form of protein association that directly correlates with the switch to distal promoter usage. Moreover, upstream of D in these cells two regions which appear refractory to DNase I cleavage can be detected between the DNase I hypersensitive (DH) sites (denoted by open arrowheads in Figure 2, lane 6). Mapping here reveals apparent footprints from −5 to −35 bp and −63 to −120 bp upstream of D. Interestingly, the more distal of these footprints appears particularly clear in MPE.Fe(II) digested embryonic nuclei (open arrowhead in lane 2). Furthermore, inspection of the hypersensitive pattern immediately downstream of D (Figure 2, lane 6) reveals further DNase I-resistant regions, located between cleavage sites, at approximately +10 to +50 bp, +65 to +80 bp, and +95 to +135 bp (not marked on the figure). Much of the information derived from Figures 1, 2 and 3 with respect to S2 cells and the proximally and distally expressing third instar fat body cells is summarized in diagrammatic form in Figure 4.

A further point of interest can be seen in Figure 2. The coding region between P and the downstream poly A addition site is noticeably sensitive to DNase I in cells from early third instar larvae (lane 3) in which the gene is apparently highly transcribed (Savakis *et al.*, 1986). This is not true of *Adh* negative S2 cells (lanes 7 and 8), nor of fat body cells from late third instar animals (lanes 5 and 6) where the gene is probably not transcribed at a high rate [a conclusion based on relative message accumulation; Savakis *et al.* (1986)].

Discussion

The present data demonstrate an extremely strong correlation between the underlying nucleoprotein organization of the *Adh* in *D. melanogaster* (as revealed by chromatin accessibility in nuclei) and the apparent stage-specific promoter usage of this gene. In particular these observations imply significantly altered associations of DNA with *trans* acting factors occurring within a given nucleus when the switch from proximal expression to distally derived expression occurs. Such reasoning follows from the arguments of M.Ashburner and colleagues, who have contended that the switch in promoter usage between early third instar and late third instar larvae occurs within individual fat body cells (Savakis *et al.*, 1986). The chromosomes of fat body cells are polytene in nature, a characteristic typical of many fully differentiated dipteran tissues. This means that during larval development fat body cells do not divide, although the chromosomes themselves are endo-reduplicated many fold. There are apparently no data that address the question of whether, during the time interval investigated here (i.e. from early to late third larval instar), DNA synthesis occurs in these fat body cells. Clearly it would be quite surprising if such major shifts in DNA−protein

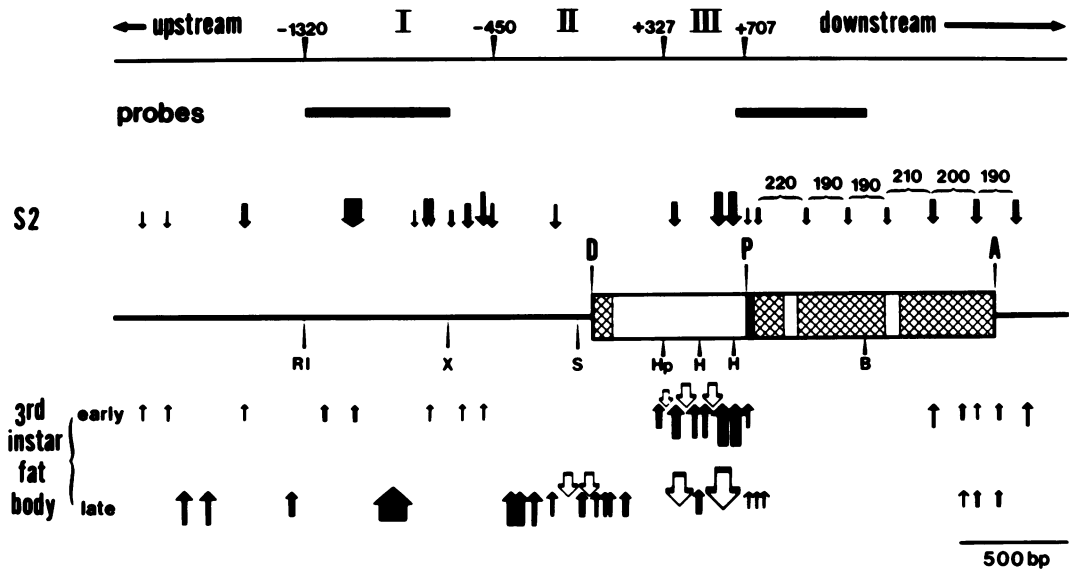

Fig. 4. Schematic representation of the comparative chromatin organization within a 4 kb region encompassing the *Adh* gene in S2 cells and in cells derived from early third and late third instar larval fat body. Vertical black arrows represent sites of DNase I or MPE.Fe(II) sensitivity; the size of the arrow denotes relative sensitivity to cleavage at a given site. The open arrows near each promoter delineate regions that appear resistant to DNase I cleavage and represent potential sites of specific *in vivo* DNA−protein binding interactions. D, P and A have the same connotation as in Figure 1. The primary transcripts initiated from either D or P are shown in the center: exons are cross-hatched boxes separated by open boxes representing intron sequences. The short black shaded region immediately downstream of P represents the 3′ end of the first intron of distally initiated mRNA: this region is also transcribed as the 5′ end of exon 1 in proximally initiated mRNA. The locus has been subdivided into five regions (upstream, I, II, III, and downstream) as shown above the map for ease of discussion in the text; the numbering scheme is in base pairs relative to D (which is +1 bp). The indirect end label probes used in Figures 2 and 3 are also shown above the map, and relevant restriction sites referred to in the text are: R1, *Eco*RI; X, *Xba*I; S, *Sal*I; Hp, *Hpa*I; B, *Bam*HI. Distances (in bp) between MPE.Fe(II) cleavage sites on the protein coding regions are shown for the S2 cell pattern.

organization as seen here were to occur in the absence of DNA replication, since it has been widely held that the S phase in the cell cycle provides the opportunity to reorganize chromatin structure and, potentially, gene expression during the subsequent growth (or G2) phase. It has been known for a long time that DNA synthesis does occur during this interval in the polytene cells of *Drosophila* salivary glands (Rodman, 1967). Interestingly however, Richards (1980) has found the level of polyteny in fat body to be substantially lower than that of salivary gland cells from the same third instar stage. Whether this means that DNA synthesis terminates earlier with a lower level of polyteny in fat body, or is delayed relative to salivary glands is unknown. Clearly, switching promoter usage within a single cell implies the action of an exogenous factor(s) in the switch [perhaps involving ecdysterone, of which there is a small mid-third instar peak (Schwartz *et al.*, 1984)]. Moreover, switching the program of gene expression of a differentiated cell in the absence of DNA synthesis has been reported in at least one experimental system (Chiu and Blau, 1984). Further work is needed to resolve this point.

As detailed in the results and depicted in summary form in Figure 4, in both early and late third instar fat body cells there are (different) regions of DNase I hypersensitivity flanking rather highly resistant (or *hypo*sensitive) regions near the respective cap sites. In particular, it is noticeable that Region II (from −450 bp relative to D to the *Hpa*I site at +327 bp) reveals mainly distal promoter-specific information while Region III (from the *Hpa*I site to P) provides most information in proximally expressing cells. In addition, Region I contains a number of DH sites that differ between cells utilizing the different promoters. In early third instar cells two prominent hyposensitive regions occur at approximately −120 to −170 bp and −257 to −282 bp relative to the proximal start site. Conversely, in late third instar fat body cells such regions are especially noticeable at −5 to −35 bp and −62 to −120 bp relative to the distal initiation site. In common with studies at this resolution that were performed previously on inducible heat shock genes (Cartwright and Elgin, 1986) a likely interpretation of such highly resistant regions is that strong, specific DNA−protein associations have been established at these locations and that they probably encompass regions essential for regulated expression of the genes in the respective developmental compartments. Recently, Heberlein *et al.* (1985) analyzed *Drosophila Adh* transcriptional requirements by both *in vitro* transcription assays and *in vitro* DNase I footprinting of the *Adh* gene using partially purified Kc cell extracts. Kc cells are another line inactive for transcription of their endogenous *Adh* gene. It was found that certain protein factors from these cells conferred specific transcriptional initiation upon *Adh* genes *in vitro*. Two regions, important for proximal initiation, and specifically footprinted by protein factors, were located at −75 to −150 bp (actually two contiguous protein binding sites) and −230 to −270 bp upstream of the proximal cap site. These sites are in very close agreement with our *in vivo* data. We have detected at least one further upstream binding site at −360 to −395 bp. For distal-specific expression Heberlein *et al.* (1985) noted a region both important for transcription and strongly footprinted by a protein factor located at −45 to −85 bp relative to the distal cap site. We find that the center of this region is in fact sensitive to DNase I *in vivo* (see cut in lane 6 of Figure 2 virtually coincident with the *Sal*I marker site at −66 bp) but that regions both immediately upstream and downstream are DNase I-resistant. The downstream region covers the distal TATA homology and might be the binding site for the required general transcription factor noted by these workers. As mentioned in Results, three further regions of resistance to DNase I can be seen in the region spanning 150 bp 3′ of the distal initiation site (not marked in Figure 4). It is possible that these also represent sites of specific DNA−protein association related to distal promoter usage. A further interesting

observation is that switching to distal promoter usage is accompanied by a complete blocking of the previously hypersensitive region upstream of P. It seems conceivable that the switch is engendered, at least in part, by the binding of repressive factors (perhaps even nucleosomes) to these regions, thereby provoking the transient rise in initiation from the distal promoter that characterizes late third instar fat body cells.

A final point concerns the inability of S2 cells (and Kc cells) to transcribe their endogenous *Adh* gene while clearly in possession of the relevant transcriptional machinery. The current analysis shows that in S2 cells the *Adh* chromatin structure is quite different from that found in embryos and both larval stages examined. In particular, the distinct and precisely positioned nucleosome array over the coding region is a characteristic (although not necessarily a cause) of many inactive genes (see Eissenberg *et al.*, 1985 for examples). Other strong differences were noted both upstream and downstream of the gene in S2 cells, an observation that certainly supports the notion that the *Adh* gene in these cells lies in an altered chromatin domain not conducive to transcriptional activity. Interestingly, sites of hypersensitivity around the proximal and (more weakly) the distal cap site in these cells may mean that some of the protein factors mentioned above are accurately bound, but that other sequences, e.g. more distantly located enhancer sequences, are not exerting (or are not able to exert) any positive regulatory control in these cells.

Materials and methods

Nuclear isolations and digestions

Nuclei from S2 tissue culture cells and 6–18-h *Drosophila* embryos were isolated as previously described (Wu *et al.*, 1979; Cartwright and Elgin, 1984). Early third instar (70-h post egg-laying) or late third instar (110-h post egg-laying) larvae were raised at 25°C in plastic food boxes seeded with synchronous (± 1 h) embryos (Miller and Elgin, 1978) and harvested free of food medium by washing with *Drosophila* Ringer solution and subsequent flotation in 20% (w/v) sucrose. Fat body was isolated by procedures quite similar to those reported recently (Jowett, 1985). The small pieces of fat body tissue released from early third instar larvae by a mechanical grinder (VWR Scientific, no. 48962-008) were fractionated from other tissues merely by low-speed centrifugal flotation (660 g) in 2% (w/v) Ficoll in *Drosophila* Ringer solution. The larger pieces of fat body released from late third instar larvae were differentially filtered through nylon mesh screen (Nitex) using an initial pre-filtration of larval debris through 600-μm mesh and subsequent collection of tissue on 100-μm mesh. Fat body was further purified by centrifugal flotation in Ficoll as above. Nuclei were released by Dounce homogenization of fat body (10 strokes with B pestle, 3 strokes with A pestle) in buffer A–0.25 M sucrose (Wu *et al.*, 1979) supplemented with 0.25% (w/v) NP40 and 2 mM EGTA. After pelleting for 5 min at 4125 *g*, crude nuclei were resuspended in buffer A–0.25 M sucrose–0.25% NP40, vortexed hard for 20 s, layered on a pad of buffer A–1.7 M sucrose and pelleted at 16 500 *g* for 20 min.

Nuclei from tissue culture cells, 6–18-h embryos or from larval fat body cells were resuspended in DNase I or MPE.Fe(II) digestion buffer as required, brief digestions performed, the reaction terminated and DNA purified, all as prevously described in detail (Wu *et al.*, 1979; Cartwright and Elgin, 1986).

Analysis of sites of chromatin accessibility

Samples of DNA derived from the nuclear digestions (or from control digestions of deproteinized genomic DNA) were restricted to completion with a suitable enzyme spanning the region of interest and then subjected to indirect end-label analysis (Nedospasov and Georgiev, 1980; Wu, 1980). This involved fractionation of samples on short (20-cm) or long (45-cm) agarose gels, transfer to nitrocellulose and hybridization to a highly active radiolabelled probe abutting one restriction cut site. All methods used in this type of analysis have been previously described in detail (Cartwright and Elgin, 1986). For preparing radioactive probes, the random priming method (Feinberg and Vogelstein, 1983) was used on both purified linear plasmid DNA or on linear fragments cut from low melting point agarose gels and used without further purification (Feinberg and Vogelstein, 1984). Probes were routinely labelled to $> 1 \times 10^9$ d.p.m./μg. Nitrocellulose filters, washed as described (Cartwright and Elgin, 1986) were dried and then exposed at −70°C to pre-flashed Kodak X-AR5 film with Dupont Lightning Plus intensifying screens.

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