DNA – histone interactions are sufficient to position a single nucleosome juxtaposing *Drosophila Adh* adult enhancer and distal promoter

Jeffrey R. Jackson⁺ and Cheeptip Benyajati*

Department of Biology, University of Rochester, Rochester, NY 14627, USA

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ABSTRACT

The alcohol dehydrogenase gene (*Adh*) of *Drosophila melanogaster* is transcribed from two tandem promoters in distinct developmental and tissue-specific patterns. Both promoters are regulated by separate upstream enhancer regions. In its wild-type context the adult enhancer specifically stimulates only the distal promoter, ~400 bp downstream, and not the proximal promoter, which is ~700 bp further downstream. Genomic footprinting and micrococcal nuclease analyses have revealed a specifically positioned nucleosome between the distal promoter and adult enhancer. *In vitro* reconstitution of this nucleosome demonstrated that DNA – core histone interactions alone are sufficient to position the nucleosome. Based on this observation and sequence periodicities in the underlying DNA, the mechanism of positioning appears to involve specific DNA structural features (ie flexibility or curvature). We have observed this nucleosome positioned early during development, before tissue differentiation, and before non-histone protein – DNA interactions are established at the distal promoter or adult enhancer. This nucleosome positioning element in the *Adh* regulatory region could be involved in establishing a specific tertiary nucleoprotein structure that facilitates specific *cis*-element accessibility and/or distal promoter – adult enhancer interactions.

INTRODUCTION

Recent genetic and biochemical studies on eukaryotic transcriptional regulation have defined the importance of *cis*-acting sequences and trans-acting factors (reviewed in 1, 2). However, there is another level of transcriptional control *in vivo* where these nucleoprotein complexes must function in the context of chromatin, in which nucleosomes can control the accessibility of regulatory elements (reviewed in 3).

The distal and proximal promoters of the *Adh* gene in *Drosophila melanogaster* are ~700 bp apart, and each has a

distinct enhancer region (see Fig. 1B) identified by P-element mediated transformation studies, and transient transfection analyses in cultured *Drosophila* cells (4–9). Transcription initiation from the proximal promoter occurs primarily in fat body tissue during embryogenesis and larval development (10, 11), and is enhanced by the *Adh* larval enhancer (ALE) (8). The distal promoter is highly expressed in the fat body of adult flies, and there are smaller peaks of expression in embryos and mid-third instar larval fat body (10, 11). Normal levels of distal promoter expression in both adults and third instar larvae require the stage-specific activity of the *Adh* adult enhancer (AAE) (4, 7). Many *cis*-acting elements required for distal promoter transcription have been defined (4–7, 12, 13); several non-histone protein binding sites have been identified, and some of the proteins have been characterized (5, 12–17). These binding sites become occupied in mid-third instar fat body following a cooperative assembly of nucleoprotein complex(es) (16). In its native context as shown (Fig. 1B), the AAE stimulates only distal transcription and has no effect on proximal transcription even though the promoters are only ~700 bp apart (6, 7, 9). One explanation for this observed specificity is that proteins bound at the AAE cannot interact functionally with proteins bound at the proximal promoter. This is unlikely however, because the AAE activates proximal transcription when the distal promoter is deleted (9).

In this study we have analyzed the chromatin structure of the *Adh* gene in larval fat body from several developmental stages and early embryos to identify chromatin structural features that may be involved in transcriptional regulation. This has revealed accessible chromatin structures in fat body in the proximal promoter, distal promoter, and adult enhancer regions at stages just prior to and during *Adh* transcription. In addition, we have revealed a single positioned nucleosome between the distal promoter and AAE before, during, and after distal promoter transcription. *In vitro* reconstitution experiments demonstrated that the region between the distal promoter and AAE contains a nucleosome positioning element (NPE) that can function via DNA – core histone interactions alone. The implications of this NPE in *Adh* transcriptional regulation are discussed.

* To whom correspondence should be addressed

⁺ Present address: Department of Molecular Genetics, SmithKline Beecham Pharmaceutical Co., 709 Swedeland Road, PO Box 1539, King of Prussia, PA 19406, USA

MATERIALS AND METHODS

Drosophila strains

Staged Oregon R P-2 larvae were reared from ~20 mls of 0–4 hr embryos on rich medium (10% glucose, 5% inactive brewers yeast (ICN), 2.5% yeast extract (ICN), 1.5% agar, sprinkled with bakers yeast) under minimally crowded conditions at 25°C.

Fat body isolation

This procedure was done essentially as in (18). Staged populations of larvae were harvested and disrupted by washing through a grinding mill with *Drosophila* Ringer solution. Tissues were filtered through 670 μ m Nitex, collected on 100 μ m Nitex, and separated by centrifugation through 2% ficoll/Ringer solution (5 min, 2000 rpm, HB-4 rotor)

Isolation of nuclei

Nuclei were released from fat body by Dounce homogenization in the presence of 0.3% NP-40 and purified by centrifugation through sucrose as previously described (19). Nuclei were digested with DNase I (Worthington) at a range of concentrations from 0 to 30 U/ml, or with micrococcal nuclease (MNase) (Sigma) at a range of concentrations from 0 to 200 U/ml, for 2.5 min at 25°C. Digestions were stopped by adding EDTA and EGTA to 12 mM each.

Isolation of nuclei from 0 to 10.5 hour embryos (20) was similar to that of fat body and these nuclei were digested with DNase I or MNase as described above for fat body nuclei. Genomic DNA was purified from nuclei as previously described (21).

Indirect end labeling

Genomic DNA samples (5 μ g/lane) were restricted to completion with Eco RI and resolved on 25 cm, 1.2% agarose, 1 $\times$ TBE gels. The DNA was then transferred to MSI magna NT nylon membrane (used to be Nytran) by standard capillary blotting as previously described (21). After blotting the membrane was dried thoroughly, and the DNA was crosslinked to the membrane by 140 Joules/meter² of UV irradiation. Blots were then hybridized with ³²P-labeled nick translated DNA (>1 $\times$ 10⁹ dpm/ μ g) whose sequence abuts the upstream Eco RI site. The probe was from –1319 to –662 of the distal transcription initiation site. Hybridization solutions were as described in (22), and modified in (23), and hybridizations were incubated for 18 hours at 62°C. Membranes were washed at 60°C as described in (22), wrapped in cellophane while still damp, and exposed with an intensifying screen.

The analysis of MNase digested DNA shown in Fig. 2 was performed as described above for indirect end labeling, except the DNA samples were not restriction digested prior to gel electrophoresis. Membranes were hybridized with ³²P labeled random primed DNA (>1 $\times$ 10⁹ dpm/ μ g) as above. The probes were: **B**, –1319 to ~+3480 (4.8 kb Eco RI fragment), **C**, –321 to –184, **D**, –662 to –321 and –181 to +53 from distal transcription initiation site. Hybridization probes were stripped off membranes by washing in 5 mM Na-Phosphate, 0.5% SDS for 10 min at 95°C.

Genomic footprinting

The ligation mediated polymerase chain reaction (LMPCR) of Mueller and Wold (24) was used for high resolution cleavage analyses as previously described (16). For each lane 200 ng

of DNA were subjected to LMPCR amplification including 2 final cycles with ³²P end-labeled primer 3 (see below). Samples were then phenol/chloroform extracted, ether extracted, ethanol precipitated, and resolved on 60 cm denaturing 6% polyacrylamide gels.

Primer 1: 5' cctgctatgcttgac 3'

Primer 2: 5' aatattctgctcgtggccctacac 3'

Primer 3: 5' ctggctcgtggccctacactgaag 3'

DNA fragments for *in vitro* nucleosome assembly

The 200 bp *Adh* NPE DNA (–146 to –345 of the distal initiation site) was prepared by the polymerase chain reaction (PCR) using the following primers 5'tgttagaaaaccggtgttt3', and 5'aacaaatgc-ggggtaaagt3'. The fragment was either uniformly labeled with α -³²P dATP (3000 Ci/mmol, NEN), or uniquely end-labeled by using one ³²P end-labeled primer in the PCR reaction. DNA fragments were purified by electrophoresing through 5% non-denaturing polyacrylamide, excising the appropriate band from the gel and electroeluting the DNA.

Preparation of chromatin and histones

Nuclei were prepared from 1 liter of K₅₆₂ cells as previously described (17). Chromatin was prepared as outlined by Neubauer and Horz (25). Nuclei were digested with 800 U/ml MNase at 37°C for 7 min and digestion was stopped with 10 mM EDTA. Chromatin was extracted from the nuclei in 10 mM Tris–HCl pH 8.0, 0.2 mM EDTA, 0.2 mM PMSF. Histone H1 was stripped from the chromatin by adding NaCl to a concentration of 600 mM. The chromatin was then loaded onto a 5–30% sucrose gradient (also containing 600 mM NaCl and 0.1 mM PMSF) and centrifuged for 16 hours at 35,000 rpm, 4°C in an SW41 rotor (Beckman). Fractions containing mononucleosomal chromatin fragments were pooled, concentrated with Amicon 30 microconcentrators to ~5 mg/ml (DNA+protein), aliquoted, and stored at –80°C.

For purification of core histones the procedure of Stein (26) was followed. Briefly, nuclei were prepared as above and lysed by sonication. Chromatin was then bound to hydroxyapatite (BioRad) and washed with 0.6 M NaCl, 50 mM sodium phosphate, 0.1 mM PMSF. Core histones were eluted with 2 column volumes of 2.5 M NaCl, 50 mM sodium phosphate, 0.1 mM PMSF. Protein containing fractions were pooled, concentrated in Amicon 10 microconcentrators to ~1 mg/ml, aliquoted, and stored at –80°C. The purity and relative stoichiometries of the histones prepared by either method were monitored by SDS-PAGE and Coomassie staining.

In vitro nucleosome assembly

The salt dialysis method was performed as described by Neubauer and Horz (25). Less than 0.1 pmols DNA fragment, 10 μ g bovine serum albumin (BSA), and 2 μ g of nucleosome core particles were combined in a 50 μ l volume and dialyzed against 600 mls of 2 M NaCl, 10 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM β -mercaptoethanol at 4°C for 2 hrs. Dialysis was continued for ~16 hrs while the salt was gradually lowered to <10 mM by pumping 2.5 l of no-salt solution (10 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM β -mercaptoethanol) into the dialysis chamber while keeping the fluid level constant.

The salt dilution assembly (SD) was performed as described by Drew and Calladine (27). Less than 0.1 pmols of DNA fragment, were combined with ~2 μ g of nucleosome core

particles in 20 μ l of 1 M NaCl, 20 mM Tris-HCl pH 7.5, 0.2 mM EDTA, 0.1% NP40, 0.2 mM PMSF, and incubated at 24°C for 15 min. Diluent (10 mM Tris-HCl pH 7.5, 0.2 mM EDTA, 0.1% NP40) was then added stepwise so as to lower the NaCl concentration by $\sim$ 100 mM every 10 min. until the concentration reached 100 mM and the volume=200 μ l.

The poly-glutamic acid assembly (PGA) was performed as described by Stein (26). The histone-poly-glutamic acid complex (H+PGA) was prepared by combining 1 volume of purified core histones ($\sim$ 1 mg/ml in 2.5 M NaCl) with 0.5 volumes of PGA stock solution (10 mg/ml poly-L-glutamic acid (ICN, M_r $\sim$ 100K) in 20 mM Tris-HCl pH 7.5, 0.2 mM EDTA, pH neutralized with NaOH), and dialyzing this mixture against 1 liter of 100 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA for $\sim$ 15 hrs at 4°C. Aggregates were removed by centrifugation in a microcentrifuge for 2 min. at 4°C, and the supernatant ($\sim$ 0.5 mg/ml histones and 3 mg/ml PGA) was aliquoted and stored at -0° C. Nucleosomes were assembled by combining <0.1 pmols of specific DNA fragment with 1 μ g calf thymus DNA (average size of $\sim$ 1 kb) in 100 μ l of buffer containing 0.2 mg/ml PGA, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl, and 1 mM β -mercaptoethanol. 1–2 μ ls of H+PGA (0.5–1 μ gs of histones) were added to the mixture which was then incubated for 60 min. at 24°C.

Reconstituted nucleosomes were gel purified for DNase I analyses (Fig. 6) by electrophoresing the reconstitution mixture through 5% non-denaturing polyacrylamide gels in 0.5 $\times$ TBE buffer at $\sim$ 10 volts/cm (<25 mAmps), and excising the appropriate bands from the gel. Purified nucleosomes were recovered from the gel slice by crushing and soaking in 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 mM PMSF, 0.1 mg/ml BSA for 1–2 hrs at 4°C, centrifuging in a microcentrifuge for 3 min to pellet the gel fragments, and recovering the supernatant.

Nucleosome positioning analyses

Restriction endonuclease mapping (Fig. 5): nucleosomes were reconstituted on $\sim 2 \times 10^5$ cpm of uniformly 32 P labeled 200 bp *Adh* NPE DNA by both the salt dilution and poly-glutamic acid methods as described above. Nucleosome assembly on longer DNA fragments results in multiple high molecular weight complexes, some of which may represent association of more than 1 octamer of histones (25, 28). For that reason, fragments longer than 200 bp were not used in this study. The buffer conditions of the reconstitution mixtures were adjusted to 15 mM Tris-HCl pH 7.4, 60 mM KCl, 15 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂ (digestion buffer), the volume was adjusted to 250 μ l, and the mixtures were digested with MNase (100 units for SD assembly, 175 units for PGA assembly) for 0–30 min at 24°C. Aliquots were removed during incubation and digestion was stopped with EDTA and EGTA at a final concentration of 10 mM each. Samples were analyzed by 5% polyacrylamide nucleoprotein gel electrophoresis and DNA in the trimmed samples was phenol/chloroform extracted, ethanol precipitated and gel purified as described for DNA above. This $\sim$ 146 bp DNA was then digested with restriction endonucleases (see Fig. 5) and the fragments were analyzed on 5% non-denaturing polyacrylamide gels. The dried gels were exposed to pre-flashed Kodak XAR-5 film with no intensifying screens. Densitometry and quantitation was performed by an LKB Ultrosan XL laser densitometer.

DNase I digestion analysis: nucleosome cores were reconstituted on $\sim 2 \times 10^5$ cpm of uniquely 32 P end-labeled 200

bp *Adh* NPE DNA by both the salt dialysis and poly-glutamic acid methods, and gel purified as described above. These nucleosomes were digested side by side with free DNA, with a range of DNase I (Worthington) concentrations (0–15 U/ml for nuc., 0–1.5 U/ml for free DNA) in digestion buffer (as above, but supplemented with poly dI-dC to a concentration of 0.03 μ g/ μ l (carrier)) for 2 min. at 24°C. The digestions were stopped by adding EDTA to 10 mM and SDS to 0.1%, and the digested DNAs were phenol/chloroform extracted, ethanol precipitated, and analyzed on 60 cm 6% denaturing polyacrylamide gels.

RESULTS

Adh regulatory regions possess an accessible chromatin structure in differentiated fat body

Regulatory regions of potentially active or actively transcribed genes are often characterized by DNase I hypersensitive sites, which represent an altered chromatin conformation lacking canonical nucleosomes, and accessible to non-histone proteins (reviewed in 29). We used the indirect end labeling technique to analyze DNase I digestion patterns in the *Adh* region of early embryos (0–10.5 hrs) and larval fat body tissue from several developmental stages. Figure 1A, lanes 2–14 show that chromatin specific DNase I hypersensitive sites were present at the distal promoter, proximal promoter, and AAE in nuclei of

Figure 1. Chromatin analysis of the *Adh* gene during embryogenesis and fat body differentiation. (A) Indirect end labeling analysis of early embryo and larval fat body chromatin. Nuclei were digested with a range of concentrations (–, +, ++) of either DNase I (lanes 3–14) or MNase (lanes 16–21), purified DNAs were cleaved with Eco RI, separated by agarose gel electrophoresis, blotted to nylon membrane and hybridized with a uniformly 32 P labeled double stranded Eco RI (–1319 of the distal RNA start site) to Xba I (–662) clone. DNA: naked genomic DNA control, E-E: Early embryos (0–10.5 hours from oviposition), M-2 Fb: Mid-second instar fat body (66–70 hours), E-3 Fb: Early-third instar fat body (90–94 hours), M-3 Fb: Mid-third instar fat body (96–100 hours). Lane 1, restriction markers created by double digestions of genomic DNA with Eco RI and either Bam HI (+1262), Sal I (–63/–69), or Xba I (–662). (B) Map of *Adh* gene coding region and regulatory elements, with arrows indicating specific interactions between pairs of enhancers and promoters (6–9, 59). D: distal transcription initiation site, P: proximal transcription initiation site (+713), ALE: *Adh* larval enhancer ($\sim$ –5000 to $\sim$ –700), AAE: *Adh* adult enhancer ($\sim$ –600 to $\sim$ –450), hatched boxes indicate protein coding regions. All positions are given relative to the distal transcription initiation site.

Figure 2. Micrococcal nuclease mapping of a positioned nucleosome. DNA from MNase digested nuclei of 0 to 10.5 hr embryo or mid-third instar fat body was separated by agarose gel electrophoresis, blotted to nylon membrane, and consecutively hybridized with 3 different probes as shown. (A) Ethidium bromide stained gel showing the MNase nucleosomal DNA ladder of bulk chromatin. Mononucleosomes (1) and other oligomers are indicated. M: λ Hin D III, ϕ X 174 Hae III, and ϕ X 174 Hpa II molecular weight markers. (B) The gel from panel A, blotted and hybridized with uniformly 32 P labeled double stranded 4.8 kb Eco RI probe which spans the adult enhancer, both promoters and the coding region. The signals representing mono-nucleosomal sized DNA were weak in comparison to the ethidium bromide staining because of incomplete retention of small DNA fragments on the hybridization membrane. (C) The same blot stripped and rehybridized with uniformly 32 P labeled double stranded -321 to -184 DNA fragment which is entirely within the region occupied by the putative nucleosome. The arrow points to a 285 bp band, unique to mid-third instar fat body, visible in lanes 5 and 6. This band most likely results from cleavage in the hypersensitive sites at -135 and -430 (Fig. 1A, lanes 20, 21) and may be protected from further cleavage to the size of a single nucleosome by additional proteins stably bound to the *cis*-elements flanking the nucleosome (16). (D) The same blot stripped and rehybridized with uniformly 32 P labeled double stranded -661 to -321, and -181 to +53 DNA fragments which immediately flank the region occupied by the putative nucleosome. Probing such blots in a different order gave the same results. Positions are given relative to distal transcription initiation site. Abbreviations: see Fig. 1.

fat body tissue, however they were absent in the nuclei of early embryos. Thus, each of these *Adh* regulatory regions has an accessible chromatin conformation in fat body tissue prior to and during transcription, but not in majority of early embryonic cells, which are still undergoing differentiation and cell division. It is interesting that the relatively short region between the DNase I hypersensitive sites in the distal promoter and AAE was quite resistant to DNase I cleavage (Fig. 1A, lanes 7, 10, 13).

To further assess the chromatin structure in *Adh* regulatory regions we used micrococcal nuclease (MNase), a chromatin structural probe that preferentially cleaves the linker regions between nucleosomes (30) (Fig. 1A lanes 15–21). This revealed structural features similar to those detected by DNase I. In mid-late third instar fat body tissue MNase hypersensitivity was seen at the distal promoter, proximal promoter, and AAE coinciding with the DNase I hypersensitive sites (Fig. 1A, lanes 20, 21). Thus both MNase and DNase I have revealed accessible chromatin structures in the *Adh* regulatory regions of fat body tissue which is expressing *Adh*. In addition, between the nuclease hypersensitive sites in the adult enhancer and distal promoter there was ~160 bp of DNA (from ~-190 to ~-350) that was highly resistant to MNase at all developmental stages studied

Figure 3. High resolution analysis of chromatin structure within the MNase resistant region between the adult enhancer and the distal promoter. DNase I digestions were performed on nuclei from the various developmental stages. Digestion patterns were visualized by the ligation mediated polymerase chain reaction (LMPCR) footprinting technique of Mueller and Wold (24). Shown is the antisense strand. Lane 1: restriction markers, 15 ng of genomic DNA digested with Dra I (-321), and 15 ng digested with Asp 700 (-243). Lane 2: G ladder, 200 ng genomic DNA treated with dimethyl sulfate and then piperidine *in vitro*. Note some positions in the marker lanes have more than 1 band. This could be attributed to inappropriate cleavage reactions or the LMPCR detection method. Lanes 3–14: DNase I digested nuclei (200 ng DNA per lane), for each developmental stage there are 3 lanes with increasing DNase I digestion from left to right. The first lane of each stage is a no DNase I control; low levels of endogenous nuclease(s) digestion were evident. Lanes 15–18: naked DNA control, 200 ng per lane naked genomic DNA digested with increasing DNase I *in vitro*. The MNase protected region is from Fig. 1A. ← denotes the periodic ~10 base cleavage pattern on the nucleosome present at all developmental stages. Cleavage sites are on the upstream side of nucleotide position listed, i.e. the band labeled -175 represents cleavage between positions -175 and -176. * Cleavage at -212 is an anomaly in the 10 base ladder because it is located between positions -205 and -219 away from the exposed face of the helix (see Fig. 4). E-P Fb: Early pupae fat body (120–124 hours from oviposition), all other abbreviations as in Fig. 1A.

(Fig. 1A, lanes 16–21). This same region was protected from DNase I cleavage in fat body of all stages (Fig. 1A, lanes 6–14), as well as in chromatin of all tissue culture cell lines tested regardless of *Adh* expression patterns (31, 32). MNase resistant regions of this size (~160 bp) often represent a nucleosome. On either side of this region in fat body tissue, there are broad DNase I and MNase hypersensitive domains. This suggests that a single nucleosome, which is not part of a nucleosomal array, is located

Figure 4. Summary of DNase I cleavages on the nucleosome. **A) *in vivo*.** The sequence of the antisense strand (from -350 to -151) is shown below a helix map, which represents the DNA double helix having been imaginarily cut along 1 side and laid flat. The diagonal lines represent the phosphate backbones of each strand and the major and minor grooves are illustrated by the distance between the backbones. The helix map is drawn with a pitch of 10.2 bp/helical turn and a minor groove stagger of 3 bp between strands because these are the average figures observed for nucleosomal DNA (27, 34, 47, 51). open arrows: major DNase I cleavages in naked DNA (Fig. 3), solid arrows: major DNase I cleavages in chromatin (Fig. 3), the height of the arrows reflects the extent of cleavage, solid circles: average positions of antisense strand ~10 base periodic DNase I cleavage on the nucleosome (from cleavage summary below the map), solid triangles: average positions of sense strand DNase I cleavage on the nucleosome (gel not shown), open circles: the only cleavage positions that lie significantly away from the exposed face of the helix. **B) Summary of the nucleosome positioning analyses *in vitro*.** The results of the MNase and DNase I studies from Figs. 5 and 6 are shown with respect to a helix map as in A. The shaded rectangles represent the ~146 bp of DNA protected from MNase digestion in nucleosome positions a, b, and c (Fig. 5). Solid lines extending vertically from the center of each ~146 bp rectangle to the minor groove on the helix map indicate the location of the dyad for each nucleosome position. Solid circles represent the positions of prominent ~10 base periodic DNase I cleavages on the nucleosome, and demonstrate which face of the helix is oriented away from the histone octamer (Fig. 6). Dra I cuts between -321 and -322 on both strands. Asp 700 cuts between -243 and -244 on both strands. Dde I cuts between -200 and -201 on the sense strand, and -197 and -198 on the antisense strand.

between the *Adh* distal promoter and adult enhancer. The coding region was also largely resistant to MNase, and is known to exist as a regularly spaced nucleosomal array (18).

A single nucleosome is located between the distal promoter and adult enhancer

We performed additional MNase analyses to confirm the nucleosomal structure of the *Adh* gene. Nuclei from early embryos or third instar fat body were digested with MNase, and the resulting nucleosomal DNA ladders were resolved by agarose gel electrophoresis. Southern analysis was then performed using a variety of DNA probes which span (B), flank (D), or are within (C) the nuclease resistant region between the distal promoter and adult enhancer (Fig. 2). Ethidium bromide staining revealed a typical bulk chromatin nucleosomal ladder extending for at least 6 nucleosomal repeats in both embryos and fat body (Fig. 2A). After blotting this gel, hybridization with probe B (4.8 kb of DNA which spans the *Adh* locus) also revealed a clear nucleosomal ladder in embryos and fat body. This indicates a regularly spaced nucleosomal array(s) exists within the *Adh* locus (Fig. 2B).

Stripping the blot and rehybridizing with probe C (within the 160 bp MNase resistant region) demonstrated a distinct difference between fat body and embryo nucleosomal structures in the distal promoter region. In early embryos probe C detected a nucleosomal ladder which was indistinguishable from that detected by probe B (Fig. 2C, lanes 9,10) suggesting that the distal promoter or AAE are incorporated into a nucleosomal array at this stage. In contrast, probe C did not detect a nucleosomal ladder in the fat body DNA. It revealed a relatively strong broad band, similar in size to mononucleosomal DNA, that was protected from heavy MNase digestion (Fig. 2C, lane 6, and also see legend), but there are no protected bands representing dinucleosomes or longer arrays in this lane. When this blot was stripped and rehybridized with probe D, which flanks the putative nucleosome and is within the nuclease hypersensitive regions, a very weak smear of DNA fragments <500 bp long was all that was detected in fat body DNA (Fig. 2D, lanes 5, 6). This indicates the sequences recognized by this probe were more heavily digested by MNase and not occupied by nucleosomes in fat body tissue. In the embryonic DNA probe D revealed a

MNase ladder indistinguishable from those detected by the other probes (Fig. 2D, lanes 9, 10), suggesting that unlike fat body tissue the distal promoter or AAE or both are protected by nucleosomal arrays in embryos. Taken together, these results are consistent with an altered chromatin structure in *Adh* expressing fat body tissue where a single nucleosome is located between the adult enhancer and distal promoter, which are highly accessible and stably bound by non-histone proteins when the distal promoter is actively transcribed (16).

The nucleosome between the distal promoter and adult enhancer is specifically positioned with respect to the DNA sequence

We performed high resolution analyses of the DNase I digestion pattern within the nucleosome occupied region using the ligation mediated polymerase chain reaction (24). This confirmed the presence of the nucleosome at all developmental stages and also demonstrated specific positioning by revealing a ~ 10 base periodic DNase I cleavage pattern on the antisense strand, that extended for ~ 145 bases (from ~ -186 to ~ -331 within the MNase resistant region), and was distinct from the DNase I cleavages on naked DNA (Fig. 3). DNase I cleavages on the sense strand (data not shown) also occurred with a ~ 10 base periodicity and were staggered ~ 3 bases (in the 3'OH direction) from the antisense strand cleavages (summarized in Fig. 4A). Such cleavage patterns, with periods approximately equal to the helical repeat length of B-form DNA, and ~ 3 bp staggers between strands, represent the periodic accessibility of DNA when it is constrained on a surface such that the same face of the helix is always oriented away from the surface. In the case of a nucleosome this indicates that the DNA wraps around a histone octamer in a specific rotational orientation with respect to the surface of the octamer (20, 33, 34). Figure 4A shows a summary of the DNase I cleavages on the nucleosome and naked DNA, and displays the periodic pattern on a helix map. The horizontal line running through the helix map approximates the outward oriented face of the helix and demonstrates that the observed periodic DNase I cleavages occur very close to that face.

It is difficult to precisely determine the upstream and downstream limits (the translational position) of the nucleosome from the MNase digestion data because the resolution of the analysis in Fig. 1 was no better than ± 10 bp. However the specific rotational orientation adopted by the DNA on this nucleosome restricts the possible translational positions, because the center of pseudo-dyad symmetry (dyad) will be located at a position where the minor groove of the DNA is oriented away from the nucleosome (35). We know the minor groove is oriented away from the nucleosome at or near the positions of DNase I cleavage because the enzyme binds to and cleaves across the minor groove (36). Based on the limits of the ~ 10 base ladder and MNase protection, and the DNA rotational orientation suggested by the ~ 10 base ladder, we estimate the dyad of the nucleosome is located at -258 . Therefore the upstream and downstream limits would be -330 and -186 , each 72 bp from the dyad because the histone octamer is known to occupy ~ 145 bp of DNA (30).

During development the positions of DNase I cleavage on the nucleosome surface did not change (Fig. 3), but the sensitivity to DNase I increased, beginning at mid-second instar, and peaking at mid-third instar, when distal promoter transcription peaked (28). These reproducible increases in cleavage could be seen at

Figure 5. Nucleosome position analyses by MNase trimming and restriction endonuclease digestion. **A)** Nucleosomes (Nuc.) reconstituted on uniformly ^{32}P labeled 200 bp *Adh* NPE DNA as described in Materials and Methods were digested with MNase for 0 to 30 minutes to trim them to 146 bp nucleosome core particles (Trimmed). Lanes 1–5; nucleosomes assembled by salt dialysis. Lanes 6–10; nucleosomes assembled with poly-glutamic acid (PGA). The trimmed nucleosome core particles were phenol/chloroform extracted to remove the histones and ethanol precipitated. Electrophoresis through 5% polyacrylamide demonstrates that the predominant band is ~ 146 bp (shown for lanes 4, 5, 9, and 10). The larger (~ 180 bp) fragment may have resulted from incomplete trimming of the nucleosomes, consistent with its reduction upon longer digestion (compare lane 4 to lane 5, or lane 9 to lane 10). Similarly the shorter fragments represented incompletely digested free DNA. These small fragments were more prominent in the PGA assembled nucleosomes due to the inhibitory effect of PGA on MNase (60). M: molecular weight markers derived from restriction digestion of the 200 bp *Adh* DNA fragment, 200 bp, 176 bp, 146 bp, 98 bp, 78 bp, 53 bp, and 24 bp. **B)** Gel isolated 146 bp DNA from MNase trimmed core particles in A was incubated with no enzyme (Uncut), Dra I, Asp 700 (Xmn I), or Dde I and the resulting fragments were resolved by electrophoresis through 5% polyacrylamide. M: markers as in A. **C)** Densitometer scans of lanes 10 and 4 from B. Relative quantities of each fragment were compared after adjusting the quantitation for DNA length (fragments are uniformly labeled). Pairs of fragments whose lengths added to ~ 146 bp and whose relative quantities matched were identified. Each such pair was interpreted to represent a single nucleosome position. Note, in some cases a doublet was interpreted as representing a single nucleosomal border that was not trimmed completely by MNase. For example, the 58 and 62 bp fragments from the Asp 700 digestions must represent a single border and be paired with the 88 bp fragment (position a) because there is no 84 bp fragment to pair with the 62 bp fragment. In addition, the combined quantities of the 58 and 62 bp fragments match that of the 88 bp fragment. The 46 and 51 bp Dde I fragments were interpreted in a similar manner. These discrete sites of incomplete trimming may have been due to DNA structural features which paused the exonucleolytic activity of MNase. The distribution of the positions was determined from the corrected relative quantities of the matched pairs of fragments. These distributions were reproducible between the 2 assembly methods. The accuracy of the DNA length measurements was ± 2 bp.

most positions of the ~10 base ladder, however they were most pronounced at positions -229, -255, -266, -276, and -287 flanking the putative dyad of the nucleosome. In another high-resolution study, a similar increase in DNase I sensitivity was observed on nucleosome B of the MMTV promoter after steroid hormone induction, and this was attributed to depletion of histone H1 (37). While it is possible that an equivalent mechanism may be at work on the *Adh* positioned nucleosome, it is not known whether H1 is associated with this nucleosome, or what its role might be since the nucleosome is not part of a nucleosomal array in fat body tissue.

Nucleosome reconstitution on the *Adh* nucleosome positioning element

In the digestion pattern of the naked DNA (Fig. 3, lanes 15–18, Fig. 4A) it is evident that the DNase I cleavages in this region (-330 to -186) were somewhat periodic. Although the patterns of cleavage in naked DNA were distinct from chromatin, many of the cleavages were clustered near the positions of ~10 base periodic cleavage on the nucleosome (see Fig. 4A). Examination of the DNA sequence between these clustered cleavages revealed stretches of A's and T's that occur with a ~10 base periodicity and are ~5 bases out of phase with the cleavages. This periodic sequence arrangement and DNase I digestion pattern suggests there are local structural variations in this DNA which could result in curvature or non-uniform (anisotropic) flexibility (38, 39). Such sequence-specific DNA structure could provide a nucleosome positioning signal. We tested this putative nucleosome positioning element (NPE) for the ability to position a nucleosome based on core histone-DNA interactions alone as implied by its potential anisotropic flexibility. This was done by assembling nucleosomes on a 200 bp fragment (-345 to -146 from the distal RNA start site) containing the putative *Adh* NPE *in vitro* with *Drosophila* histones. We compared both salt dilution (SD) and poly-glutamic acid (PGA) mediated assembly methods (see Materials and Methods) to address concerns regarding the conditions under which histone-DNA interactions reach equilibrium (40).

Nucleosomes form in specific positions on the 200 bp *Adh* fragment *in vitro* and the major position is the same as that identified *in vivo*

Nucleosome cores were assembled on the 200 bp *Adh* DNA fragment (uniformly ³²P-labeled) and trimmed with micrococcal nuclease (MNase) to remove free 'linker' DNA, leaving ~146 bp of nucleosome core DNA that is protected by the histone octamer (30). Subsequently we cleaved the 146 bp DNA with restriction enzymes to map the limits of MNase protection with respect to the restriction cleavage site. If the histones had adopted a single position on the 200 bp DNA, then unique restriction enzyme cleavage within the 146 bp DNA should result in 2 fragments whose lengths represent the borders of MNase protection with respect to the restriction site. However if the nucleosome positioned randomly on the DNA, then no discrete restriction fragments would be possible.

When either SD or PGA nucleosome assembly reactions were digested with MNase, the uncomplexed DNA was digested very quickly, while the nucleosome core complex was more stable (Fig. 5A). After extended digestion this complex was converted to a tighter band with increased mobility, which suggests the free DNA protruding from the nucleosome cores has been trimmed

off. The DNA in these trimmed nucleosomes was then deproteinized and analyzed by polyacrylamide gel electrophoresis (Fig. 5A, bottom panel). Although this DNA consists of a mixture of sizes, the predominant band migrated at the position of the 146 bp marker, further indicating that both assembly methods produced canonical nucleosome core particles.

We isolated the 146 bp DNA from a gel and then cleaved it separately with 3 restriction enzymes (see Fig. 4B for cleavage positions). *Dra* I did not appear to cleave the 146 bp DNA suggesting that its site was either not protected from MNase digestion or lies within close proximity of the fragment end (Fig. 5B, lanes 3, 8). *Asp* 700 (*Xmn* I), and *Dde* I both yielded several discrete restriction fragments, suggesting the nucleosome occupies more than 1 non-random position on the 200 bp *Adh* fragment. Densitometer scans of *Dde* I and *Asp* 700 digestions are shown in Figure 5C. For each digestion, we identified pairs of fragments whose sizes add to ~146 bp, and whose quantities match after correcting for DNA length. Based on the data from both enzymes, which cleave 45 bp apart, 3 positions could be distinguished. Position a was -331 to -186 from the distal transcription initiation site. Position b was -298 to -154, and position c was -317 to -173. This is summarized in Figure 4B. From these scans we have also been able to quantitate the percentage of nucleosomes at each of these positions. ~50% of the nucleosomes were in position a, ~35% were in position b, and ~15% were in position c. The major position (position a, -331 to -186) is the same as the nucleosome position identified *in vivo* (Figs. 3, 4A). Note that both assembly methods gave rise to the same 3 positions in the same relative quantities. Only minor differences in the quantities of restriction fragments from the two methods were detectable and these were attributable to slight differences in the extent of trimming by MNase (see Fig. 5 legend).

DNase I digestion suggests the *Adh* DNA adopts a single rotational orientation on the nucleosome *in vitro*

We originally identified this nucleosome positioning element after observing a ~145 bp, 10 base periodic DNase I cleavage pattern in nuclei which represents unique DNA rotational positioning on a nucleosome (Fig. 3, 4A). To determine whether the same rotational positioning occurs *in vitro* we digested the assembled nucleosomes with DNase I. This revealed that the DNA adopts a single rotational orientation on the nucleosomes (Fig. 6) which was very similar to the rotational position observed *in vivo*.

Nucleosomes were assembled by both methods (SD or PGA) on uniquely ³²P end-labeled 200 bp *Adh* NPE DNA, the nucleosome complexes were gel purified and digested with DNase I, and the cleavage patterns were analyzed on DNA sequencing gels. DNase I cleavages on the antisense strand of SD assembled nucleosomes are distinctly different from the naked DNA (Fig. 6A). The prominent cleavages on the nucleosome were separated by multiples of ~10 bases, and many of the bands between these positions are clearly protected compared to the naked DNA, e.g. -275, -265, or -247. This ~10 base ladder of DNase I cleavage represented periodic accessibility of the minor groove (20, 33, 34), and suggests that the same face of the helix was always oriented away from the histone octamer.

On PGA assembled nucleosomes a similar DNase I cleavage pattern was evident on the antisense strand (Fig. 6A). Some positions between the periodic cleavages were not protected as well as on SD assembled nucleosomes, e.g. -275, -265, -247.

Figure 6. DNase I digestion of reconstituted nucleosomes. Nucleosomes assembled by either salt dialysis (SD) or the poly-glutamic acid (PGA) method on uniquely ^{32}P end-labeled 200 bp *Adh* NPE DNA were gel purified and subjected to a range of DNase I digestion (0 to 15 U/ml for nucleosomes, 0 to 1.5 U/ml for free DNA control). The cleavage patterns were resolved on denaturing 6% polyacrylamide gels. **A)** Cleavages on the antisense strand. The prominent ~ 10 base periodic cleavages on the nucleosome are indicated with arrows and labels. The labeled positions represent cleavages on the 3' side of that position, e.g. on the antisense strand -299 indicates cleavage between -299 and -300. There were positions on the nucleosomal DNA that should have been accessible, but were not cleaved strongly by DNase I, e.g. ~ -289 , ~ -260 , ~ -217 , ~ -207 . This probably resulted from DNA structural specificities required by DNase I that involve more than simple accessibility, e.g. minor groove width (14, 30). Positions -218 and -206 and several others between -228 and -196, which were protected from DNase I cleavage on SD assembled nucleosomes, were not strongly protected on PGA assembled nucleosomes. These reproducible differences may reflect subtle nucleosomal structural differences resulting from the 2 assembly methods. The bands between -299 and -279 in the free DNA (lanes 8–12) of the SD panel are due to the presence of a minor contaminant in the undigested 200 bp DNA (see lane 8, no DNase I control). **B)** Cleavages on the sense strand. The labeled positions represent cleavages on the 3' side of that position, e.g. on the sense strand -184 indicates cleavage between -184 and -183. M: restriction marker created by cleavage with Asp 700, which cuts between -243 and -244 on both strands. GA: G+A sequence markers created by chemical cleavage (61).

This may have been due to some free DNA contamination in the PGA assembled nucleosomes based on the disappearance of these bands at high levels of digestion.

On the sense strand DNase I cleavages on the nucleosome also occurred with a ~ 10 base periodicity distinct from the naked DNA (Fig. 6B). For example cleavages between the exposed positions at -184, -202, -210, -222, and -235 were clearly protected on the nucleosomes compared to the naked DNA. Most of the prominent sense strand cleavages were ~ 2 to 4 bases downstream from the corresponding antisense strand cleavages, e.g. -299/-297 (sense/antisense), -279/-275, -270/-266, -186/-184 (Fig. 6, A and B, and Fig. 4B). This is typical of DNase I cutting across the minor groove (41, 42), indicating that indeed the prominent positions of DNase I cleavage on both

strands represent positions of minor groove accessibility on the nucleosome surface.

The positioning data for nucleosomes assembled on the *Adh* NPE *in vitro* is summarized in Figure 4B. The 3 nucleosome positions (a, b, and c) demonstrated by MNase are shown along with their relative distributions. Position a, the major position, is virtually identical to the position we observed on this NPE *in vivo* (Figs. 3, 4A). The summary of DNase I cleavages on the nucleosomes *in vitro* demonstrates that they were staggered by ~ 3 bases between strands and occurred very close to the same face of the DNA helix. The cleavages had an average periodicity of 10.27 bases and the periodic pattern was only detectable in the region where all 3 nucleosome positions overlap. This was not surprising because all other regions would have been

Figure 7. Model of facilitated promoter-enhancer interactions via a positioned nucleosome. (A) Relevant regulatory elements within the proximal and distal promoters are indicated to illustrate their positions with respect to the adult enhancer (AAE). Without a defined tertiary structure the adult enhancer could loop out and interact with either promoter. (B) Promoter–enhancer interactions in mid-third instar fat body. Early in embryogenesis (prior to 10.5 hours from oviposition) a nucleosome forms over the positioning element between the adult enhancer and distal promoter. By mid-second instar in fat body both promoters as well as the adult enhancer are DNase I hypersensitive and thus accessible to non-histone DNA binding proteins. The nucleosome could act to bring the accessible distal promoter and AAE within close proximity of one another. The adult enhancer cannot activate transcription yet, but it may be bound by non-histone protein(s) and be involved in protein–protein contact with the distal promoter. By mid-third instar in fat body the adult enhancer and distal promoter have likely attained a conformation involving protein–protein interactions among enhancer and promoter binding factors (as shown), and resulting in distal transcription (wavy arrow). This could have been facilitated by the early positioning of the nucleosome which aligns these sequence elements in close proximity to one another. This model does not address potential interactions between the proximal promoter and *Adh* larval enhancer (ALE). The ALE is large, complex, and its *cis*-elements and chromatin structure are not precisely defined, thus its possible interactions are difficult to speculate. All proteins shown are bound to known binding sites identified *in vitro* or *in vivo* (5, 14–16, 21), however it is not known whether they interact with each other directly or indirectly, and more proteins may be involved than are pictured. D: distal transcription initiation site, P: proximal transcription initiation site.

unprotected in some portion of the nucleosome population. We estimated the dyad location for each nucleosome position by locating the center of the ~146 bp MNase protected regions of positions a, b, and c. Extending lines from these centers to the helix map above demonstrated that the minor groove at each dyad position is on the exposed face of the helix where it was accessible to DNase I. This agrees with X-ray crystallographic studies of the nucleosome core, in which the minor groove was observed facing outward at the dyad (35).

DISCUSSION

The AAE, and both *Adh* promoters are DNase I hypersensitive in fat body at stages prior to and during *Adh* transcription, suggesting that these regulatory elements have an open chromatin conformation lacking canonical nucleosomes and accessible to non-histone proteins. Between the distal promoter and the AAE there was a nuclease resistant region that contains a specifically positioned nucleosome. This nucleosome is present constitutively at all developmental stages tested, before during and after distal promoter transcription. In mid-third instar fat body when the distal promoter and AAE are highly accessible and bind a cooperative complex of non-histone proteins (16), this nucleosome appears to be singular and not part of a nucleosomal array. The early developmental presence of this nucleosome between the closely spaced distal promoter and AAE suggests that its position could be important for *Adh* transcriptional regulation.

Histone–DNA interactions alone are sufficient to promote nucleosome positioning between the *Adh* adult enhancer and distal promoter

Our *in vitro* reconstitution experiments have revealed that core histones assemble with the 200 bp *Adh* NPE DNA non-randomly to form nucleosomes in 1 major and 2 minor translational positions (Figs. 5, 6). Position a, the major position (–331 to –186), agrees very closely with the nucleosome position

identified in nuclei (–330 to –186) (Figs. 3, 4A). In addition to this agreement in translational positioning, the single rotational orientation of the DNA on the nucleosome is very similar *in vitro* and in nuclei. Thus the region from –330 to –186 of the distal RNA start site contains a NPE that can position a nucleosome on the basis of histone–DNA interactions alone.

Interestingly, the DNase I cleavage patterns on the nucleosomal DNA are not identical *in vitro*, and in nuclei. Most of the prominent ~10 base periodic cleavages on the reconstituted nucleosomes are within 1–2 bases of those detected in nuclei, except near the dyad (–258), where the difference is as large as 5 bases. On average over the entire length of the ~10 base periodic cleavage pattern the same face of the helix is oriented outwards in nuclei and *in vitro*, indicating the same direction of DNA curvature. Thus the differences observed may reflect slightly different conformational properties. In nuclei this could be induced by interaction with other proteins, including but not limited to higher order chromatin folding. The larger differences near the dyad may reflect the deviation from uniform DNA curvature at that location (30, 43, 44). These severe bends or kinks would be expected to cause a disruption in the periodic ~10 base cleavage pattern within ~1.5 helical turns from the dyad, which could have been partially masked during analysis *in vitro* because there are 3 nucleosome positions and thus 3 dyad positions within the population of nucleosomes. Another possibility is that histone H1 is bound to the dyad of the nucleosomal DNA *in vivo* and results in an alteration of DNase I cutting in that location. Minor discrepancies in DNase I cleavage were also observed when the *in vivo* digestion patterns of 2 positioned nucleosomes in yeast were compared with typical nucleosomal DNase I digestion patterns *in vitro* (45), suggesting a general property that nucleosomes exhibit minor conformational differences *in vitro*.

The reconstituted nucleosomes adopted a reproducible distribution of 3 translational positions on the *Adh* NPE DNA when either the SD or PGA assembly method was used suggesting that multiple positioning is an inherent property of this NPE *in*

vitro. A similar phenomena has been reported for the sea urchin 5S NPE, which was shown to adopt 1 major (~50%) and several minor nucleosome positions upon reconstitution by salt dialysis (46). It is possible that *in vivo* there are additional interactions which shift the nucleosome distribution in favor of position a on the *Adh* NPE. These could be subtle effects of higher order chromatin structure, and/or the binding of histone H1 or other non-histone proteins on or near the nucleosome. However, we cannot rule out the possibility that the same distribution of positions exists *in vivo*, because we were unable to perform the precise MNase mapping experiments *in vivo*.

Nucleosome positioning on the *Adh* NPE is mediated by a combination of DNA structural features

Anisotropic DNA flexibility or intrinsic DNA curvature have been considered potential DNA structural features that could promote nucleosome positioning (47–50). These structures can be caused by periodic variations in minor groove width, which result from periodicities in DNA sequence (38, 39). Basically, short runs of A and/or T favor a narrowed minor groove, and short runs of G and/or C favor a widened minor groove. If for example short AT rich runs occurred every 10 bp, in phase with the helical pitch of the DNA, then the minor grooves along 1 face of that helix would favor being narrowed. Thus it would be energetically favorable to bend this DNA towards that face where the grooves must narrow to accommodate the curvature. Because DNA must be sharply bent around the histone octamer when forming a nucleosome, anisotropic flexibility is expected to promote nucleosome positioning. The *Adh* NPE (from –186 to –330 of the distal initiation site) may possess such anisotropic flexibility due to periodically spaced (~10 bp) AT rich runs which occur between the prominent DNase I cleavages on the nucleosome (Fig. 4A).

A preferred direction of DNA curvature around a nucleosome cannot completely account for nucleosome positioning, but it does limit the possible translational nucleosome positions. The crystal structure of the nucleosome has shown that the minor groove faces outward at the dyad (35). Thus nucleosomes can only be positioned where their dyad is aligned with an outward facing minor groove (approximately every 10 bp) on the curved DNA. In addition there may be other DNA structural features which cause 1 potential dyad position to be favored over the others. This is certainly the case for the *Adh* NPE where *in vitro* we observed an uneven distribution of nucleosomes in 3 positions and no nucleosomes in 1 potential dyad position (–238) (Figs. 4B, 5). Several investigations have implicated alternative helical pitch and curvature requirements near the dyad in defining nucleosome translational positioning. High resolution hydroxyl radical cleavage analyses of both mixed population, and 5S NPE nucleosomes revealed that the helical pitch of nucleosomal DNA is 10.05 for the outer regions, but 10.7 for ~30 bp over the dyad (51, 52). DNA structures that could accommodate these altered pitch and curvature requirements could serve as translational positioning signals. In another study, Satchwell et al (44) found that the preferred rotational orientations of dinucleotides and trinucleotides differed near the dyad from those found in the outer turns of the nucleosomal DNA. In 177 nucleosome core DNA sequences they observed preferential occurrence of the trinucleotide AAA or TTT at the dyad where the minor groove faces out. Elsewhere on the core DNA this trinucleotide preferred to have its minor groove facing in. Position a on the *Adh* NPE has 5 continuous As across the dyad, consistent

with the observed preferential occurrence of the trinucleotide AAA at the dyad (44, 53). The trinucleotides GTT and GAA at the dyads of nucleosome positions b and c respectively are also preferred dyad sequences, while the potential dyad position at ~–238, which was avoided by the reconstituted nucleosomes (Fig. 4B), contains the trinucleotide CAT, which is not a preferred dyad sequence. Thus the dyad sequences in the distribution of nucleosome positions we observed, agrees with the statistical sequences preferences at the dyad (44, 53).

A model for *Adh* promoter–enhancer interactions

The presence of a NPE in the *Adh* regulatory region suggests that nucleosome positioning could play a role in setting up a specific chromatin structure involved in distal promoter transcriptional activation. Figure 7 illustrates a model that could explain why the AAE specifically stimulates only the distal promoter in its native context (see Introduction). In this model, a nucleosome is positioned between the adult enhancer and distal promoter in all cells prior to differentiation by a mechanism involving sequence-specific DNA conformation. In differentiated fat body tissue at mid-second instar the nucleosome is flanked by DNase I hypersensitive sites, which are highly accessible, and lack canonical nucleosomes. Two DNA binding interactions that have been detected in mid-third instar fat body (16) could be involved in aligning the distal promoter and AAE as shown in Fig. 7B. Specifically, interactions between proteins bound at the Adf-2a site on the surface of the nucleosome (–198 to –206), and the d5 binding site immediately upstream of the nucleosome (–330 to –337) (16) could potentially affect the direction of the protruding DNA due to their proximity to the DNA entry/exit point of the nucleosome. As additional regulatory proteins bind cooperatively (16) and assemble on the accessible distal promoter and adult enhancer in mid-third instar fat body, their close proximity and alignment, as a result of the nucleosome, may facilitate protein–protein interactions that lead to an active transcription initiation complex (Fig. 7B). The interactions within this structure and the timing of its assembly would make it unlikely that the adult enhancer would adopt an alternate conformation and activate the proximal promoter.

Another possible role of this positioned nucleosome in transcription would be to facilitate the specific removal of nucleosomes over the distal promoter and AAE. This could be accomplished by setting up a nucleosomal structure in which specific binding sites or *cis*-elements remain accessible to stage- and/or tissue specific factor(s) that can remove the flanking nucleosomes and provide the transcription machinery with access to other *cis*-elements in the distal promoter and AAE. An example of such a binding site is the Adf-2a site, on the surface of the nucleosome, which we have previously shown to be occupied by additional protein in mid-third instar fat body (16). The precise rotational positioning of the DNA on this nucleosome ensures that the Adf-2a binding site is always accessible (i.e. facing away from the surface of the nucleosome).

Nucleosome positioning has been implicated in the transcriptional regulation of several other genes

The promoter of the yeast *PHO5* gene (54, 55) and the long terminal repeat of the mouse mammary tumor virus (56, 57) are occupied by arrays of positioned nucleosomes that appear to prevent transcription initiation in the absence of specific induction signals. Also, repression of several α -specific genes in yeast α cells by $\alpha 2$ protein is mediated through precise positioning of

a nucleosome over the TATA box regions (45, 58). In contrast to these negative transcriptional roles, a positioned nucleosome on the *D.melanogaster hsp26* promoter juxtaposes 2 heat shock factor binding sites near the already occupied TATA box and possibly facilitates protein-protein interactions during heat shock transcriptional activation (20). Thus, in the native context of the genome, establishing the proper local tertiary structure during development by appropriate placement of specific nucleosomes and/or other chromosomal proteins, will likely prove to be a general mechanism for gene regulation rather than an exception.

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