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HMG-D, the *Drosophila melanogaster* homologue of HMG 1 protein, is associated with early embryonic chromatin in the absence of histone H1

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We show that HMG-D, an abundant chromosomal protein, is associated with condensed chromatin structures during the first six nuclear cleavage cycles of the developing *Drosophila* embryo and that histone H1 is absent from these same structures. As H1 accumulates from nuclear division 7 onwards, the nuclei become more compact and transcriptionally active. This compaction is paralleled by a reduction in size of mitotic chromatin. In addition, we find a striking correlation between the switch in HMG-D:H1 ratios and the changes that occur between nuclear cycles 8 and 13 that are collectively termed the mid-blastula transition. This transition is characterized by an increase in the nuclear cycle times, a change in the nucleo-cytoplasmic ratio, and a 5- to 20-fold decrease in nuclear volume. We propose that this is a direct consequence of a re-organization of chromatin from a less condensed state with HMG-D to a more condensed state with H1. We argue that HMG-D, either by itself or in conjunction with other chromosomal proteins, induces a condensed state of chromatin that is distinct from, and less compact than the H1-containing 30 nm fibre and that this state of chromatin could facilitate rapid nuclear cycles.

Key words: chromatin/embryogenesis/high mobility group 1/ histone H1/mid-blastula transition

Introduction

Chromatin remodelling during the cell cycle is crucial for the correct regulation of genes (Grunstein, 1990; Fedor, 1992; Felsenfeld, 1992) and for the proper generation of higher order structures (Thomas, 1984; Bradbury, 1992; Roth and Allis, 1992). A requirement for such remodelling is particularly apparent in the early embryo of *Drosophila melanogaster*. This undergoes 13 rapid nuclear divisions in a syncytial blastoderm, of which the first seven are synchronous and take ~8 min (Foe and Alberts, 1980). During this phase of development the nuclei are larger and the chromosomes under-condensed compared with nuclei of later dividing cells. Cycles 8–13 occur at the periphery of the embryo and have progressively longer cycle times, 9.5, 12.4 and 21.1 min respectively for divisions 11, 12 and 13 (Foe and Alberts, 1980). Correlated with the increase in the cell cycle times there is a change in the nucleo-cytoplasmic ratio (Edgar *et al.*, 1986) and a 5- to 20-fold decrease in nuclear volume between cycles 8 and 14.

The paradigm for the higher-order folding of chromatin is the formation of the 30 nm fibre by the addition of histone H1 to core nucleosome particles (Finch and Klug, 1976; Thoma *et al.*, 1979; Allan *et al.*, 1980). In *Drosophila*, H1 is not detected in the early cleavage cycles (Elgin and Hood, 1973; Becker and Wu, 1992), and its first appearance has been assumed to correspond to cycle 10 coincident with the start of zygotic transcription (Anderson and Lengyel, 1980). These observations imply that H1 is not primarily involved in chromatin condensation in the earliest phases of *Drosophila* development. Thus, in the absence of H1 and in order for the DNA to undergo the condensation–decondensation process during the first nine or 10 cleavage cycles, *Drosophila* requires the presence of a histone H1-like function. However, no such protein has been identified in *Drosophila*. In this paper we address the issue of what other proteins could fulfil the role of H1 and explain the changes that take place during early phases of development.

Circumstantial evidence suggests the vertebrate high mobility group proteins of the HMG 1/2 class (Johns, 1982; van Holde, 1989; Ner, 1992) could function in a similar manner to H1 (Jackson *et al.*, 1979). They have similar properties, they stabilize and bind to bent structures (Bianchi *et al.*, 1992; Lilley, 1992) and have been suggested to interact with linker DNA sequences (Johns, 1982; Schroter and Bode, 1982). HMG-D, the *Drosophila* homologue of HMG 1/2 (Wagner *et al.*, 1992; Ner *et al.*, 1993), is an abundant chromatin-associated protein. It binds to DNA cooperatively and bends DNA but exhibits little sequence selectivity (M.E.A.Churchill and S.S.Ner, in preparation), properties also characteristic of H1.

In our ongoing series of studies to determine the biological role for HMG-D protein we have investigated, using an immunological approach, whether HMG-D could, in principle, play a similar role to H1. We have determined the spatial location of HMG-D and H1 in early embryos and show that HMG-D is detected in all nuclei from the start of embryogenesis, the most intense staining occurring in mitotic chromosomes. It is also associated with other highly condensed and transcriptionally silent chromatin structures, such as the polar bodies and polyploid yolk nuclei. In contrast, H1 is absent prior to nuclear cycle 7 and is not associated with transcriptionally inert nuclei.

Results

HMG-D is present throughout embryogenesis and is associated with all condensed chromatin structures

We have determined the temporal distribution of HMG-D by Western blot analysis on single staged embryos. This indicates that the protein is present at a constant level at all stages of development (Figure 1, data not shown for stages 9–16). Antibody staining of embryos shows the protein can be detected in nuclei from the start of embryogenesis to the blastoderm stage. Initially the most intense staining is

observed in mitotic chromosomes (Figure 3m) and the polar bodies (Rabinowitz, 1941; Campos-Ortega and Hartenstein, 1985) (Figure 3k). Subsequently, both the polyploid yolk nuclei and the pole cell nuclei (Figure 3j) stain strongly. The protein persists in the latter beyond cellularization and gastrulation. In addition, the early mitotic domains of the postblastoderm embryo are also stained by the antibody (S.S.Ner, unpublished data).

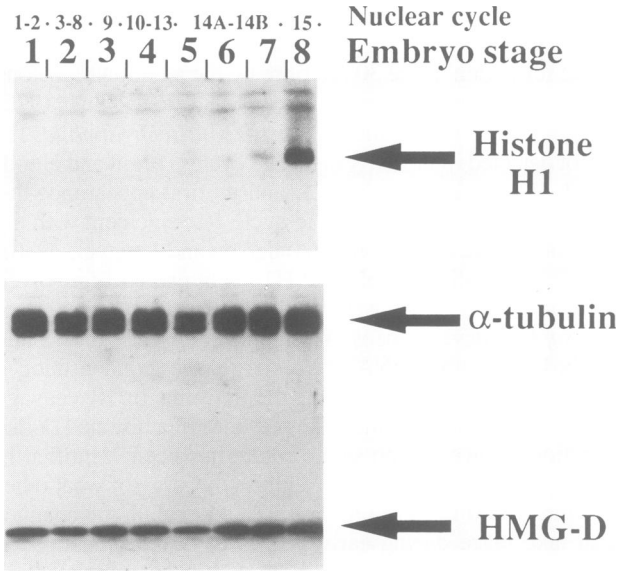

Fig. 1. Western analysis of histone H1 and HMG-D proteins in single staged embryos. Total protein from single embryos (lanes 1–8, stages 1–8, respectively) were separated electrophoretically and detected after Western transfer onto nitrocellulose with antibodies raised against purified H1. In lanes 1–3, no protein is detected; stage 4 is the first faint appearance of a band migrating at ~33 kDa (H1 has a calculated mol. wt of 29 kDa but runs anomalously on SDS–polyacrylamide gels). This approximately corresponds to cycles 9 and 10 when H1 is first detected in whole embryos. Stages 5–8 (lanes 5–8) show the rapid rise in levels of H1 detected. We estimate that 0.5–1 ng is present in stage 8 embryos. Similarly, the presence of HMG-D protein was assayed using antibodies raised against FPLC-purified protein expressed in *E.coli*. The level of protein was quantified by comparing it with known amounts of purified protein. The amount detected remains approximately constant (~0.2–1 ng) during stages 1–8. It approximately doubles during stages 10–12 (data not shown). The filter was also probed with antibodies against α-tubulin to show that equivalent amounts of protein had been loaded and transferred.

Histone H1 is first detected in nuclear cycle 7

In contrast to HMG-D, histone H1 is first detected by Western analysis (Figure 1) on staged single embryos at stage 4 corresponding to cleavage cycles 9 and 10. This timing was refined by immunofluorescence-based antibody staining which precisely localizes the first appearance of H1 to the interphase and mitotic nuclei of some nuclear division cycle 7 embryos where weak staining is observed (Figure 2b). In other cycle 7 embryos H1 is essentially absent (Figure 2a). Subsequently, during nuclear division cycles 8 and 9, this staining becomes more intense (Figure 2d) but, again in contrast to HMG-D, the polyploid yolk nuclei contain no detectable H1 (Figure 3e and f). At cycle 1 the polar bodies also lack H1 (Figure 3a and b) but later become associated with this histone (Figure 2d). After stage 7 (cycle 14), the amount of H1 protein increases dramatically due to zygotic transcription (Figure 1). We estimate stage 8 embryos contain 0.5–1 ng of H1 protein ($>10^{10}$ molecules).

Relative levels of HMG-D and H1 change dramatically at onset of zygotic transcription.

The average number of molecules of HMG-D per nucleus declines with each cleavage cycle (Figure 5) although the level of HMG-D protein per embryo remains approximately constant during embryogenesis. Using pure HMG-D protein expressed in *Escherichia coli* as a standard, we have calculated the relative amounts of protein present in the *Drosophila* embryo. From our Western blots we estimate that each embryo contains ~0.2–1 ng of HMG-D protein (~$1-5 \times 10^{10}$ molecules of HMG-D which is equivalent in earliest embryos to ~10^4 molecules per nucleosome assuming ~2×10^6 nucleosomes per nucleus). However, by the cellularization stage (~5000 nuclei, cycle 14), there are ~2–5 molecules of HMG-D per nucleosome and later in embryogenesis (>50 000 nuclei) fewer than 0.2 molecules/nucleosome. By contrast H1 levels approach 1 molecule per nucleosome at approximately cycles 14 and 15 and remain at this level throughout embryogenesis.

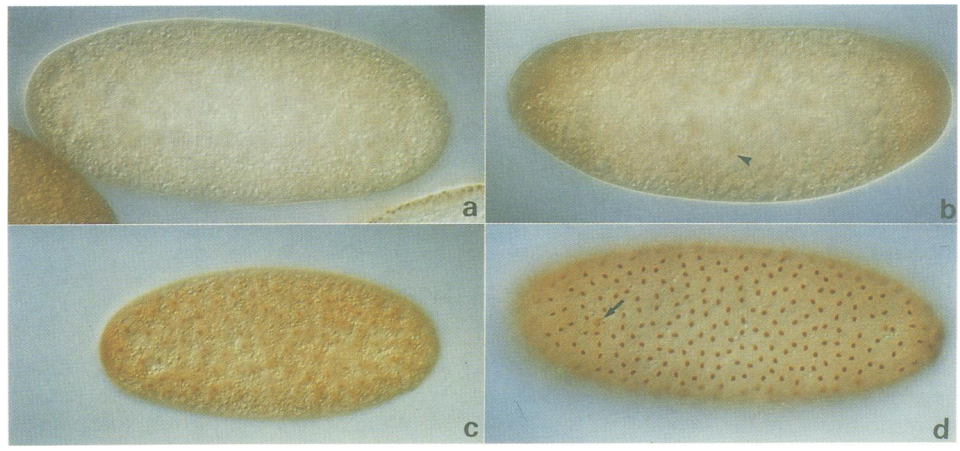

Fig. 2. Detection of histone H1 before the start of zygotic transcription. H1 is first detected weakly in some embryos during nuclear division cycle 7 (arrowhead in panel **b**). A similar cycle 7 embryo (**a**) shows no staining for H1. In cycle 9 (**c**) and early cycle 10 (**d**, the nuclei are in pairs and have just completed telophase of cycle 9) the embryos begin to stain intensely for H1. Zygotic transcription starts during interphase of cycle 10 (Edgar and Schubiger, 1986). A polar body (arrow in d) which does not stain in earlier embryos (cf. Figure 3) also has histone H1 associated with it.

Thus, during the early phases of development, there is a dramatic shift in the relative amounts of H1 and HMG-D, with H1 eventually becoming the more abundant protein (Figure 5). This absolute increase in the level of H1 and relative decrease in HMG-D correlates with the stages during which nuclei start to become competent for transcription, i.e. cycle 10 (Edgar *et al.*, 1986; Edgar and Schubiger, 1986). Full transcriptional competence is not achieved until cellularization, at which stage HMG-D protein is only weakly detected. Exceptionally the pole cell nuclei stain intensely until gastrulation (Figure 3j), correlating with the late start of transcription in these nuclei (Edgar and Schubiger, 1986). In addition, HMG-D, but not H1, is present in transcriptionally silent yolk nuclei (Figure 3j).

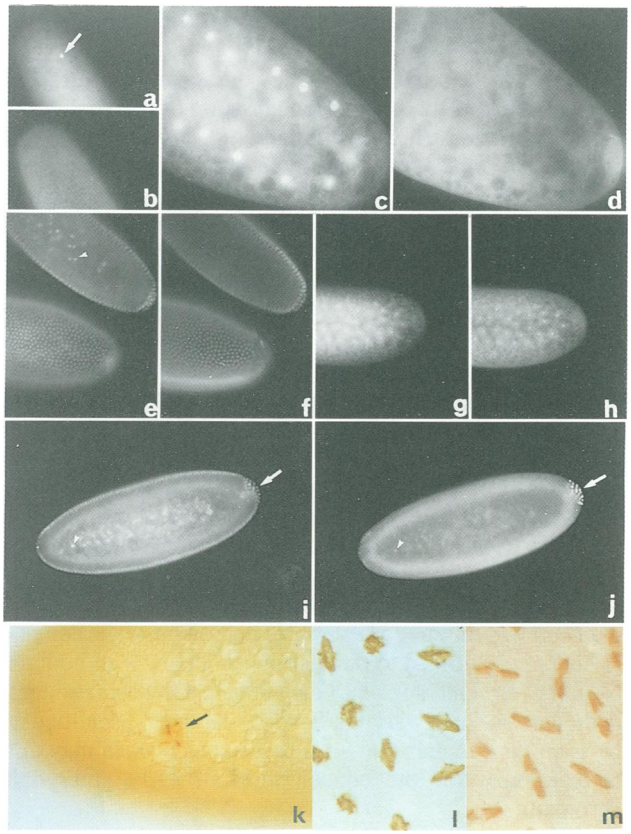

Fig. 3. Antibody staining of staged embryo to show the presence of HMG-D and histone H1 proteins in condensed chromatin. Whole mount immunofluorescence of nuclear division cycles 1 (**a** and **b**), 6 (**c** and **d**) and 12 (**e** and **f**) embryos stained with DAPI (a, c and e) or anti-H1 antibody (b, d and f) to show the absence of H1 in polar body (arrow in a) and nuclei of cycle 6 embryo (d). H1 begins to appear and localize to nuclei during cycle 8 and is prominent by cycle 9 (cf. Figure 2). At cycle 12 all nuclei stain strongly except for the yolk nuclei (arrowhead in e). In contrast immunofluorescence staining of embryos with anti-HMG-D antibody (**h** and **j**) shows localization of protein in cycle 6 embryos (h), weak diffuse staining of nuclei of a cellularizing embryo (j), weak staining of polyploid yolk nuclei (arrowhead in j) and intense staining of the pole cells (arrow in j). Panels **g** and **i**: the same embryos co-stained with DAPI; note how the yolk nuclei are less intensely stained with the HMG-D antibody than with the DAPI-stained nuclei (arrowheads in i and j), and conversely how the pole cell nuclei are more intensely stained with the HMG-D antibody (arrows in i and j). Panels **k**, **l** and **m**: higher magnification of embryos detected for HMG-D (k and m) and histone H1 using the DAB reagent. HMG-D is present in polar body in nuclear cycle 1 embryo (k) and anaphase chromosomes of cycle 11 (m). Panel l: H1 in a comparable stage embryo showing essentially identical anaphase staining pattern to HMG-D.

Nuclear volume and size of mitotic chromosomes decrease after seventh nuclear division.

The nuclei in the *Drosophila* syncytial blastoderm change in size during the course of nuclear division (Foe and Alberts, 1985). We estimate, by measurement of DAPI-stained nuclei (data not shown), that the diameter of the interphase nuclei decreases from 4.5−5.5 μm in cycles 2−8 to ~2 μm by nuclear division 13. This 2- to 3-fold decrease in diameter represents nearly a 20-fold decrease in nuclear volume. We note that there is a parallel change in size of the mitotic chromatids. We have attempted to quantify this change by calculating the length of the chromosome on the metaphase plate. In embryos undergoing the eighth nuclear cleavage the chromosomes on the metaphase plate are ~5 μm in length and in cycle 12 this value is 3.2 μm (Figure 4). We are unable to calculate how exactly this change relates to decreases in the volume of the chromosome. However, we feel confident that the change in nuclear volume is paralleled by a similar reduction in the size of mitotic chromosomes (Figure 4). We conclude that this reduction in chromosome size reflects a greater degree of chromatin condensation.

Discussion

HMG-D is a chromatin organizer

Our data show that, during the earliest phases of *Drosophila* embryogenesis, condensed chromatin structures are associated with HMG-D but lack histone H1. These include the early polar bodies, metaphase chromosomes of the syncytial blastoderm and also the inert polyploid yolk nuclei. With the appearance of histone H1 such chromatin, as

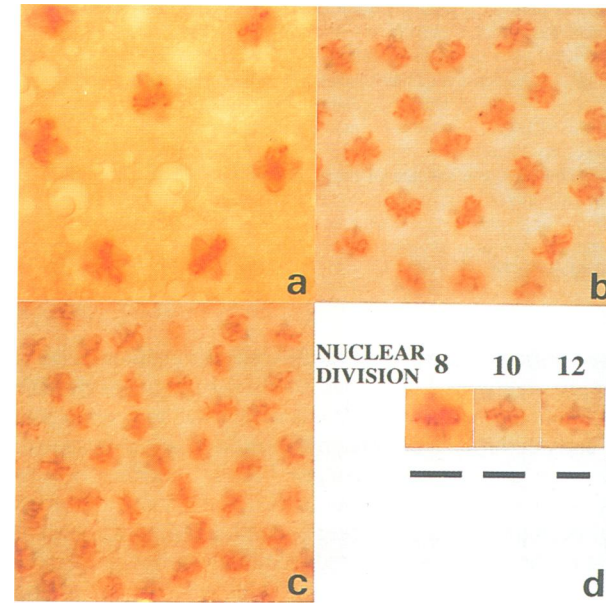

Fig. 4. Size of metaphase chromosomes decrease during mid-blastula transition. Embryos were fixed and stained with antibodies against HMG-D (brown) and α-tubulin (light brown). Embryos with the chromatids aligned on the metaphase plate were isolated and photographed at the same magnification. Embryos undergoing eighth (**a**), 10th (**b**) and 12th (**c**) nuclear divisions. The space occupied by the chromosome and the microtubule arrays clearly decreases with nuclear cycle. This is illustrated in (**d**). Individual metaphase nuclei are arranged adjacent to one another. The bar indicates the length of the chromosomes on the metaphase plate. These are ~5, 4.1 and 3.2 μm respectively for the eighth, 10th and 12th nuclear cycle embryos.

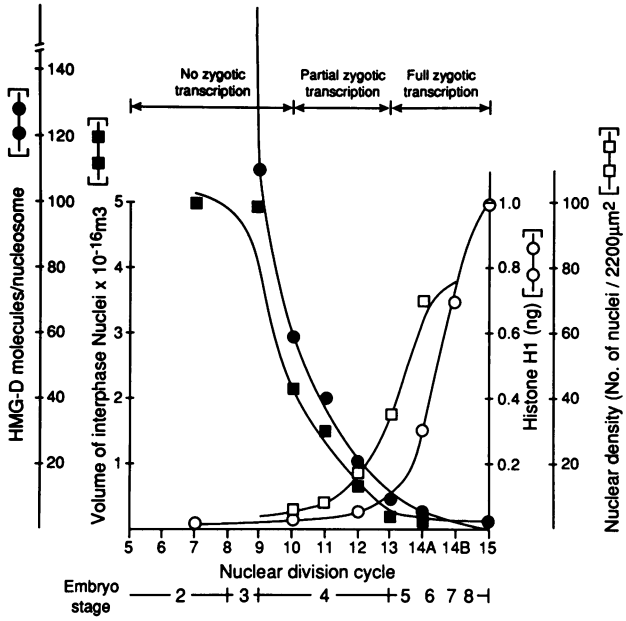

Fig. 5. Summary of changes in H1 and HMG-D levels during progressive nuclear divisions and relationship to nuclear density and volume of nuclei. The concentration of HMG-D protein remains constant during embryogenesis but the average number of molecules per nucleosome (1 nucleosome = 200 bp, Becker and Wu, 1992) during nuclear divisions falls rapidly (closed circles). This value is ~1000 at cycle 6, ~60 at cycle 10 and ~1–5 by cycle 14. The postblastoderm mitoses would bring this number to <0.2–0.5 molecules per nucleosome. These numbers are based on our estimates of HMG-D proteins to be in the range 0.2–1 ng per embryo. The data for appearance of H1 are taken from our Western analysis (open circles). Our estimates for the level of protein in stage 4 and 8 embryos indicate a rapid increase in H1, and show that the value approximates to 1 molecule per nucleosome by late cycle 14. Correlated with the changes in HMG-D and H1 levels we depict the changes in nucleo-cytoplasmic ratios (open squares) during division cycles 9–13 (taken from Foe and Alberts, 1980), and the decrease in volume of nuclei (closed squares). These values are calculated from measuring diameters of interphase nuclei of the appropriate cleavage cycle embryos; the volume of the nuclei decreases substantially following migration to surface. The diameter of nuclei decreases by half during cycles 8–13. The correlation of these changes with zygotic transcription is also indicated. Full zygotic transcription is attained during cycle 14.

exemplified by the size of the mitotic chromatids, becomes progressively more compact until eventually HMG-D is supplanted by the histone. We conclude that the organization of early embryonic chromatin differs from that characteristic of later developmental stages. Other evidence for the existence of a different state of early embryonic chromatin has been presented by Becker and Wu (1992) who observed that in chromatin reconstituted from early embryonic extracts the nucleosome spacing is ~180 bp whereas on addition of H1 this value increases to ~200 bp.

In the early embryo HMG-D is highly abundant. It is deposited in the egg by the mother but thereafter is maintained at an approximately constant level per embryo. Consequently, with each nuclear division the average number of HMG-D molecules per nucleus falls while during nuclear cycles 7–14 the amount of H1 rapidly increases. These observations are compatible with HMG-D, either by itself or in conjunction with other chromosomal components, providing a role in the condensation of nucleosomes into higher order structures in the early embryo and thus may perform a function analogous to that of histone H1.

A structural role for HMG-D?

The abundance of HMG-D and its properties are consistent with a structural role for this protein. The higher-order structures produced in the presence of HMG-D would be distinct from the chromatin containing H1. Such a role for HMG-D is also consistent with the observed properties of the vertebrate homologues HMG 1 and HMG 2. These proteins bend DNA (Paull *et al.*, 1993; Pil *et al.*, 1993), can interact directly with H1 *in vitro* (Carballo *et al.*, 1983; Kohlstaedt *et al.*, 1987) and, under physiological conditions, facilitate the *in vitro* formation of complex nucleoprotein structures, including both nucleosomes (Bonne-Andrea *et al.*, 1984) and invertasomes functional for site-specific recombination (Paull *et al.*, 1993).

Histone H1 is required both for the formation of the 30 nm fibre and for the further condensation of chromatin into more compacted structures (Allan *et al.*, 1980; Hill *et al.*, 1991). The former involves both the sealing of the two gyres of nucleosomal DNA by the bivalent globular domain of H1 and also cooperative interactions between adjacent H1 molecules (Clark and Thomas, 1988), while the N-terminal domain is necessary for the latter process (Hill *et al.*, 1991). Although the precise disposition of H1 in the 30 nm fibre is not established it seems plausible, from the known properties of HMG-D, that the latter protein could fulfil one or more functions of H1. In particular, HMG-D possesses a region rich in alanine and lysine residues which is similar to the C-terminal domain of H1 (Ner *et al.*, 1993). In certain other organisms (sea urchin and *Xenopus*) the early embryo contains a homologue of H1 termed 'cleavage cycle' histone H1 (Levy *et al.*, 1982; Smith *et al.*, 1988; van Holde, 1989) that replaces the normal somatic H1. In *Xenopus* this is termed B4 and is restricted to early development (Dimitrov *et al.*, 1993). In these H1 variants homology to the somatic H1 is largely limited to the globular domain, with both the N- and C-terminal domains being deficient in basic residues. Although no such variants have so far been identified in *Drosophila* it remains possible that such a protein may also be necessary for the condensation of early embryonic chromatin.

Physiological significance

What could be the physiological significance of different forms of condensed chromatin? In principle the structures containing HMG-D and H1 could represent alternative modes of compaction. A second possibility is that the HMG-D-containing structure is a less compacted intermediate on the pathway to solenoid formation. In either view, a looser structure could form in the absence of H1 and could facilitate the rapid condensation and decondensation required during the very short early cleavage cycles.

As a consequence of the different condensed chromatin structures generated and changes in HMG-D and H1 levels a model can be proposed which also explains the observed changes associated with mid-blastula transition (MBT, Newport and Kirschner, 1982). The appearance of H1 is correlated with a decrease in the size of nuclei and the metaphase chromosome as well as a lengthening of nuclear division times, and the acquisition of transcriptional competence during cycle 10 (Anderson and Lengyel, 1980;

Edgar and Schubiger, 1986). In general, nuclei which stain strongly for HMG-D are transcriptionally inactive. For example, yolk nuclei remain transcriptionally inactive and pole cells are not competent for transcription until gastrulation has begun. These observations strongly suggest that the chromatin generated in the presence of HMG-D is transcriptionally silent, and lead us to argue that transcription only begins when H1 levels have reached a particular threshold value and overcome the HMG-D effects, such as in nuclear cycle 10. Acquisition of transcriptional competence is not purely a function of the length of interphase or the nucleo-cytoplasmic ratio (Edgar *et al.*, 1986). Nuclei of cycle 8 and 9 embryos are unable to transcribe genes even when interphase is extended through the use of cell cycle inhibitors (Edgar and Schubiger, 1986). Therefore, we suggest that the ratio of H1/HMG-D and the extent of remodelled chromatin are the crucial determinants in the acquisition of transcriptional competence. Similar observations have been described in the *Xenopus* system in which B4, the H1 variant, disappears during MBT and there is a correlated change in the accessibility of embryonic chromatin to class III transcriptional machinery (Dimitrov *et al.*, 1993).

The first appearance of H1 during cycles 7 and 8 is significantly earlier than previously estimated (Becker and Wu, 1992) and implies that H1 must arise from translation of the maternally deposited mRNA (Ruddell and Jacobs-Lorena, 1985) since zygotic transcription of the H1 gene begins during cycle 10 (Anderson and Lengyel, 1980). If this implication is correct it follows that translation of this RNA is tightly regulated to start at cycles 7 and 8. It is during cycle 7 that the size of the nuclei begins to decrease. By cycles 10–12 a sufficient amount of histone H1 has accumulated to allow the reorganization of chromatin to a transcriptionally active state. Subsequently, increased zygotic transcription elevates histone H1 levels further. This exponential increase together with the increased number of nuclei rapidly deplete HMG-D protein to levels that cannot have global effects on chromatin structure. Thus, in structures where H1 is absent and HMG-D is present, such as yolk nuclei, or in structures where HMG-D protein persists for longer periods, such as in pole cells, we suggest HMG-D suppresses transcription. We note that HMG-D is present in later stages of embryogenesis as is HMG-Z, a related protein which has extensive sequence homology to HMG-D (Ner *et al.*, 1993). The combined levels of these proteins during the later stages of embryonic and larval development remain relatively constant and do not exceed the values per nucleosome for H1 (S.S.Ner, unpublished). Hence, we suggest HMG-D and HMG-Z have a second function analogous, perhaps, to that of H1 in organizing chromatin locally into active or inactive domains.

Our future efforts are directed towards binding studies of HMG-D to nucleosomes and competition experiment with H1 to provide biochemical evidence in support of the model proposed in this study.

Materials and methods

Generation of antibodies against HMG-D

Full-length HMG-D was overproduced in *E.coli* and purified to homogeneity (M.A.Searles and S.S.Ner, unpublished). Polyclonal antibodies were raised in rabbits against this protein. IgG-containing fractions were isolated by affinity purification from a protein A–Sepharose column (Harlow and Lane, 1989). These fractions were preadsorbed against a large volume of 0–20 h old fixed *Drosophila* embryos prior to use in Western analysis and for embryo staining.

Single embryo Western analysis

Embryos were staged under a dissecting microscope (Campos-Ortega and Hartenstein, 1985) and placed on the inverted lid of an Eppendorf tube which contained 2 μl of standard SDS gel loading buffer. The embryo was crushed by placing upon it a small cut piece of coverslip and pressing gently with a pair of forceps. The lid was then replaced on the tube and spun in a centrifuge for 30 s. A further 5 μl of SDS loading buffer was added, and the sample was boiled and loaded onto a 15% SDS–polyacrylamide mini-gel. Electrophoresis and Western transfer were performed using standard conditions. The protein bands were detected using secondary antibodies coupled with horseradish peroxidase and a chemiluminescence detection system (Amersham).

Immunohistochemistry

Procedures for fixation and antibody staining of embryos were carried out as described (Lawrence and Johnston, 1989). Anti-rabbit secondary antibodies (Amersham) were either conjugated with biotin and the antigens visualized with the Vectastain Elite ABC system (Vector Laboratories), or conjugated with fluorescein isothiocyanate (FITC) and the antigens detected using epifluorescence optics. The embryos that were stained with the FITC-conjugated secondary antibody were counterstained with the nuclear dye DAPI.

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