Histone modification in early and late *Drosophila* embryos

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(Received 28 June 1983/Accepted 26 October 1983)

Levels of histone acetylation and phosphorylation have been contrasted in two developmental states of *Drosophila melanogaster*. The 0–2 h nuclei of the syncytial blastula are characterized by rapid mitoses and DNA replication, but there is very little transcription. In the 18 h embryo there is considerable transcription and the mitotic rate is much slower. It has been found that (1) histone H1 from 2 h nuclei is not highly phosphorylated. This observation is not in accord with the view that H1 hyperphosphorylation is essential to mitosis, but is compatible with the hypothesis that H1 phosphorylation in *Drosophila* species is related to heterochromatization. (2) Histone H4 from 2 h embryos shows high levels of the diacetyl form (H4-Ac₂), which is principally outside the nucleus. This accords with the hypothesis that H4-Ac₂ is the form in which H4 is deposited on to newly replicated DNA and shows that H4 acetylation is linked not only to transcription. (3) Histone H3 acetylation is similar in 2 h and in 18 h embryos. As with H4, this acetylation probably correlates with chromatin assembly and is not transcription-related. (4) Histone H2B carries no modification in 2 h or in 18 h embryos, and H2A shows a single modification in 2 h embryos and two in 18 h embryos. H2B modification is thus not essential either in mitosis or replication, whereas H2A modification is important in one or both processes. (5) The nucleosomal protein D₂ is equally present in 2 h and 18 h embryos.

In the development of *Drosophila melanogaster* embryos the nuclear segmentation stage lasts for about 2½ h from the moment of fertilization and the embryo is syncytial (Rabinowitz, 1941; Sonnenblick, 1950). In this period there are 13 synchronous nuclear divisions spaced by about 10 min, although 20 min elapses between fertilization and the first division. At the end of nuclear segmentation there is a pause in mitotic activity for about 1 h for formation of the cellular blastoderm. In subsequent development all cells have individual walls, divide asynchronously and much more slowly. The 10 min nuclear-division cycle of the blastula is divided into about 6 min for the sum total of the mitotic steps, leaving 4 min for interphase, which must be regarded as S-phase, since it is the period of DNA replication. There is

very little transcriptional activity in this first 2 h of life of the embryo, but a great deal in development beyond the blastoderm (Zalokar, 1976; McKnight & Miller, 1976; Anderson & Lengyel, 1979). In particular, electron-micrograph estimates from individual timed syncytial embryos showed that only 1% of the chromatin fibres displayed ribonucleoprotein (RNP) fibrils in interphase (as compared with 5.5% at cellular blastoderm). The RNP fibril density in this 1% was also low, about one fibril/100 µm (McKnight & Miller, 1976). There is thus a sharp contrast between the syncytial blastula and subsequent developmental stages as regards nucleic acid synthesis, i.e. rapid DNA and very low RNA synthesis up to 2 h after fertilization, and the reverse later. The clear-cut temporal separation of transcriptionally inactive and active states prompted us to compare chromosomal proteins in 2 h embryos with those in 18 h embryos to look for changes correlated either with transcription on the one hand or replication/mitosis on the other. Transcriptional activity of chromatin has been

Abbreviations used: H1, histone H1 (etc.); RNP, ribonucleoprotein; HMG proteins, high-mobility-group proteins; PMSF, phenylmethanesulphonyl fluoride; SDS, sodium dodecyl sulphate; Ac₂-H₄, diacetyl-histone H4.

correlated with histone acetylation (Allfrey *et al.*, 1964) and the presence of HMG proteins (Weisbrod & Weintraub, 1979), although histone acetylation has also been implicated in the process of histone removal and deposition on to DNA (Dixon *et al.*, 1975; Woodland, 1979). Differing levels of histone acetylation might therefore be observed. Mitosis is associated with very low levels of histone acetylation, but with high levels of histone phosphorylation, particularly of H1 (Lake & Salzman, 1972; Bradbury *et al.*, 1973; Balhorn *et al.*, 1975). Levels of H1 phosphorylation are therefore of interest. The presence of histone variants or other specific proteins might also characterize one of the two developmental stages contrasted here.

Experimental

Drosophila melanogaster, wild-type Oregon R, were kept in cages containing about 5000 flies at 25°C and eggs were deposited on Petri dishes of nutrient (sucrose, yeast, agar, anti-fungal agent). For collection of '18h embryos' (i.e. 0–18h), nutrient dishes were left in the cages overnight. For '2h embryos' fresh plates were put in the cages and left for 1h and then removed and kept for 1h at 25°C; '2h embryos' therefore refer to embryos of age 1–2h. Such young embryos were collected throughout the day (light cycle 03:00–15:00h) after rejection of the first collection in the morning, which typically contains a number of older embryos.

Embryos of both ages were first washed in 50mM-phosphate buffer (pH 7.2)/0.15M-NaCl + 0.1% Triton X-100 and then treated for 2min with 5% (v/v) sodium hypochlorite for dechoriation. After further washing in the above buffer and then in 95% ethanol (Shen & Hearst, 1977), embryos were stored at –80°C until sufficient had been collected for chromatin preparation. All subsequent operations were carried out between 0 and 4°C, where possible on ice, and always in the cold-room. Embryos were initially suspended in 1–2vol. of the following medium (A): 250mM-sucrose/10mM-Tris (pH 8.0)/10mM-MgCl₂/50mM-sodium bisulphite/20mM-sodium butyrate/0.5mM-PMSF and nuclei then obtained with a Dounce homogenizer. Butyrate was present in the medium to inhibit deacetylase activity (Vidali *et al.*, 1978). The suspension was filtered through a fine plankton net and nuclei pelleted at 3000g for 10min. Nuclei were then washed three times in the above extraction medium with 0.2% Nonidet P40 added and collected by centrifugation. In earlier experiments nuclei were lysed in 0.7mM-EDTA/2mM-butyrate, pH 7.0, to give a chromatin gel which was extracted with 0.2M-H₂SO₄. For the bulk of the experiments, however,

washed nuclei were suspended directly in 0.2M-H₂SO₄ and left for about 1h at 4°C, after which DNA and other insoluble material was removed by centrifugation at 30000g for 10min. The supernatant was then dialysed three times against 1mM-HCl/5mM-butyric acid for about 4h and freeze-dried. These protein preparations were loaded directly on to gels. Partially purified nuclei were the product of an accelerated procedure in which nuclei were washed only once in the Nonidet-containing medium before addition of H₂SO₄.

Basic proteins from total embryos were obtained by suspension of frozen, dechorionated embryos in 0.2M-H₂SO₄ for 1h with stirring. After centrifugation at 50000g for 20min, the supernatant was dialysed against 1mM-HCl and freeze-dried.

Basic proteins from whole adult flies were obtained by homogenizing diethyl ether-treated flies in a Waring blender for 2min in medium A. The suspension was filtered through surgical gauze to remove connective tissue and then through a plankton net. A largely nuclear pellet was collected by centrifugation at 3000g for 10min. The pellet was washed with medium A + 0.2% Nonidet P40, and basic proteins were extracted as described above.

Electrophoresis

Acetic acid (0.9M)/4M-urea/polyacrylamide gels (20cm; hereafter called simply 'acid/urea gels') were run as described by Oliver & Chalkley (1972) with a stacking gel (Spiker, 1980) and pre-electrophoresed for 1h at 100V with 1M-cysteamine (50μl/slot). The upper reservoir was then renewed, the sample introduced [dissolved in stacking solvent + 15% (w/v) sucrose + 5% (v/v) β-mercaptoethanol] and accumulated at the bottom of the stacking gel by using 30V for 2h. Separation was obtained by using 110V for 20–24h. Gels were fixed and stained in methanol/water/acetic acid (5:4:1, by vol.) plus 1.5% (w/v) Coomassie Brilliant Blue R250 for about 5h at room temperature and destained in ethanol/water/acetic acid (25:65:8, by vol.). Gels to be used in a second dimension were finally soaked in destaining solutions containing 5% (v/v) glycerol and kept sealed at 4°C.

Second-dimension SDS/15% (w/v) polyacrylamide gels 20cm long were used as described by Laemmli (1970) as modified by Thomas & Kornberg (1975). Bands excised from the acetic acid/urea gels were incubated for 30min in gel solvent containing 1% SDS, placed on top of the 0.1% SDS gel and set in fresh 15% polyacrylamide. Electrophoresis was for about 15h at 100V. Fixing and staining was as described above for acetic/urea gels. For silver staining the procedure of Wray *et al.* (1981) was used.

Gels containing Triton X-100

Second-dimension Triton X-100/15%-polyacrylamide gels 20 cm long were run in 0.9M-acetic acid/7.5M-urea/6mM-Triton buffer for 30h at 100V (Zweidler, 1978). Gels were pre-electrophoresed for 1h at 100V with 1M-cysteamine.

Results

Characterization of proteins from 18h embryos

Core histone region. From published gels (Oliver & Chalkley, 1972; Elgin & Hood, 1973; Alfageme *et al.*, 1974; Levy-Wilson *et al.*, 1977; Palmer *et al.*, 1980), bands a and b in Fig. 1 represent unmodified and monoacetyl-histone H4, band c represents H2B, bands d and e represent unmodified and modified H2A and the broad band f represents species of histone H3. To verify assignments, bands a-f, and also calf thymus and adult histones, were separately cut from the Fig. 1 gel and run in a 20cm-long SDS/polyacrylamide gel (Fig. 2). Bands a and b run exactly as calf H4. Band c runs slightly faster than calf H2B in the presence of SDS and has been positively identified as H2B by sequencing (Elgin *et al.*, 1979). Bands d and e both run identically in SDS and are probably both H2A species (Oliver & Chalkley, 1972). Alfageme *et al.* (1974) did not observe band e in their preparation and nor did we when butyrate was omitted. Band e is probably an acetylated form of H2A and the internal acetylation is readily lost from this histone. The strongest component of band f is presumably H3, but in addition there is an H2A species that is not overlap from band e, since both bands were cut conservatively from the first-dimension gel. Embryos at 18h clearly contain low levels of a doubly modified H2A species. The third component of band f is identified as protein D₂ (Alfageme *et al.*, 1974; Palmer *et al.*, 1980), since it runs with H3 in acid/urea and between H3 and H2B in SDS. Since band f in Fig. 1, lane 3, is rather broad, a longer acid/urea gel was obtained and this showed three separate bands. These three bands were separately excised and run in a second SDS/polyacrylamide-gel dimension together with band e (Fig. 2, inset). It can be seen that the minor H2A species runs together with the fastest H3 component, and D₂ with the slowest. In comparison with published gels (Oliver & Chalkley, 1972; Alfageme *et al.*, 1974; Palmer *et al.*, 1980) we observed: (1) somewhat enhanced amounts of the slowest H3 component; (2) a D₂ mobility in acetic acid/urea significantly less than that of unmodified H3.

H1 region of the gel. Band i in lanes 2 and 3 of Fig. 1 has a mobility about 0.53 that of H4. Comparison with the acid/urea gels described by Oliver & Chalkley (1972) and Alfageme *et al.* (1974) shows that this is clearly histone H1. The

Fig. 1. *Acetic acid/urea/polyacrylamide-gel electrophoresis*

Lane 1, calf thymus (CT) histone; lanes 2 and 3, total histone extracted from 18h embryos without and with butyrate in the medium; lanes 4 and 5, total histone extracted from adult flies with medium containing butyrate. Loadings $\times 3$ (lane 4) and $\times 1$ (lane 5).

band labelled i from the chromatin of adult flies appears to be the same protein, in that it has the same mobility as 18h-embryo H1 also in a second SDS/polyacrylamide-gel dimension (results not shown). Band i is very sharp and shows no evidence of the microheterogeneity or phosphorylation in acid/urea gels (Fig. 1) that is seen in other organisms (Balhorn *et al.*, 1972; Bradbury *et al.*, 1973; Hohmann *et al.*, 1976). Bands g, h and j from adult flies do not run like H1 in the presence of SDS and must be other protein types.

Analysis in 6mM-Triton X-100-containing gels. Fig. 3 shows a second-dimension Triton-containing gel of the 18h-embryo histones from lane 3 of Fig. 1. Band a runs as calf H4 and band b slightly

18h chromatin

Fig. 2. Second-dimension SDS/polyacrylamide-gel electrophoresis of lane 3 of Fig. 1 (18h-embryo chromatin). Bands a-f were separately excised and blank gel intercalated at the top of the SDS/polyacrylamide gel. Bands of calf thymus (CT) histones were also cut from the Fig. 1 gel as were the two H4 bands from adult flies (a and b taken from Fig. 1, lane 4). The inset shows modified H2A and H3 (bands e and f) from a long (30cm) first-dimension acetic acid/urea/polyacrylamide gel separated into four slices, intercalated with blank gel and run in the second dimension in the presence of SDS.

18h chromatin

Fig. 3. Second-dimension acid/urea/Triton X-100/polyacrylamide-gel electrophoresis of bands a-f separately excised from a gel identical with Fig. 1, lane 3 (18h-embryo chromatin).

Calf thymus (CT) histones, also cut from a first-dimension acid/urea/polyacrylamide gel are included for comparison. Abbreviation: ^{ox}, oxidized forms.

slower, as expected for monoacetylated H4. Band c (H2B) runs as a single spot with no sign of modified species. Band d (H2A) shows three bands in Triton-containing gels. The fastest running is an overlap of H2B from band c. It cannot be due to acetylated H2B, since it runs in the presence of Triton identically with the main H2B spot. The other two components of band d are H2A species. The minor component runs like calf H2A in the presence of Triton, but the major component migrates very much faster. Published results from Triton-containing gels (Alfageme *et al.*, 1974)

indicate that the fast-migrating species are oxidized forms. Band e (monoacetylated H2A), shows two components that run somewhat slower than the H2A components of band d. These must be the monoacetylated forms of oxidized and reduced H2A. Band f (cut as a single broad slice from the acid/urea first-dimension gel) shows a major component running similarly to mammalian H3₃ (Zweidler, 1978) (below which can just be seen the doubly modified H2A) and a minor component running similarly to calf H3₁. Protein D₂ has intermediate mobility in the presence of Triton

2 h-embryo chromatin

Fig. 4. Acetic acid/urea/polyacrylamide-gel electrophoresis of total histone from 2h embryos

Lanes 1 and 2 are one preparation; lane 3 is a second preparation. Calf thymus (CT) histones are included for comparison.

(Alfageme *et al.*, 1974; Palmer *et al.*, 1980). The results obtained in the presence of Triton are thus in accord with those of the SDS-containing second-dimension gel (Fig. 2).

Chromatin from 2h embryos

Fig. 4 shows a first-dimension acid/urea gel of acid-soluble proteins from the chromatin of 2h embryos. The bands have been labelled as those in Fig. 1 (18h chromatin), since the pattern is similar. A second dimension was run in the presence of SDS, as with 18h chromatin (Fig. 5).

The core histone region. Bands a–f were cut from the acetic acid/urea gel as a whole (Fig. 4, slot 2), and the second-dimension gel run without cutting out the separate bands. Fig. 5(a) can be immedi-

ately interpreted: two forms of H4 can be seen (mono- and non-acetylated), one form of H2B and at least two of H2A. There is probably more than one form of H3, and protein D₂ is present. Fig. 5(b) shows a gel that has been stained with silver, since this gave better definition of the components. The most notable difference between the two stained gels is that H4 species appear spread over a longer region in Fig. 5(b), all the way from band a to the start of band c (H2B). This suggests that some highly acetylated H4 species are present in the 2h-embryo chromatin.

The histone H1 region. The two bands i and i' were cut from Fig. 4 (lane 3) in a single gel slice for the second dimension (Fig. 5b). It is clear that whereas i is the same protein as the H1 from 18h embryos, band i' has a different mobility from i in the second as well as the first dimension. Band i' is not protein D₁, which has much lower mobility (Alfageme *et al.*, 1974); it is probably of cytoplasmic origin. Importantly there is no evidence of multiplicity in the H1 band i of Fig. 4 that would indicate the presence of multiple phosphorylation states.

Total 2h embryos and partially purified nuclei

Nuclear purification takes time, during which the modifying groups of histones might be lost. To isolate histone as quickly as possible, total basic protein was obtained by acid extraction of complete dechorionated 2h embryos (Fig. 6, lane 2). A second SDS/polyacrylamide-gel dimension of bands 1–4 was run to unscramble the overlapping bands (Fig. 6b). Whereas band a from 2h embryos is H4 by comparison with the calf standard, band 3 also appears to contain an H4 component, in addition to two other proteins. The mobility of the H4 component in band 3 is less than that of band b, which has previously been assigned to monoacetyl-H4. It appears that the 2h embryos contain an H4 species with more than one acetyl group. The assignment of the other histones follows from the previous Figures (Fig. 6b).

The strong H4 component in band 3 from total embryos is an unexpected finding. Nuclei were therefore prepared by a rapid procedure that resulted in some syncytial cytoplasm remaining. Fig. 7 shows an acid/urea gel of this preparation and a second SDS/polyacrylamide-gel dimension of the bracketed region, taken as a single slice. In the first dimension, band a looks like unmodified H4, and this is confirmed in the second dimension. The presence of an H4 component in band 3 in addition to two other proteins is clear from the second-dimension gel, but is weaker than in total 2h embryos (Fig. 6). From the mobility of band 3 in acid/urea (4% less than that of unmodified H4) the H4 component must be the diacetyl species.

Fig. 5. Second-dimension SDS/polyacrylamide-gel electrophoresis of lane 2, Fig. 4 (Coomassie Blue-stained, a) and lane 3, Fig. 4 (silver-stained, b)

CT (calf thymus) and *D.m.* 18h (*Drosophila* 18h-embryo) histones are included for comparison.

Discussion

Histone H1

In both 18h- and 2h-embryo chromatin, band i shows no indication of the multiplicity or increased breadth in acid/urea gels that would indicate the presence of multiple phosphorylation. That from 2h-embryo chromatin in Fig. 4 has a width of about 1½% of the distance migrated, which must be compared with the known reduction in H1 mobility of about 1.0% per added phosphate group (Chalkley *et al.*, 1973; Gurley *et al.*, 1978). The 2h-embryo chromatin extracted therefore contains at most only one phosphate group per H1 molecule (Levy-Wilson *et al.*, 1977). The same is true of band i from partially purified 2h-embryo nuclei (Fig. 7). Since blastula nuclei are at some stage of mitosis for about 60% of the total time, this is unexpected, considering the strong association between H1 hyperphosphorylation and mitosis found with other organisms (Lake & Salzman, 1972; Bradbury *et al.*, 1973; Balhorn *et al.*, 1975; Gurley *et al.*, 1978). Since mitosis is immediately followed by S-phase in 2h embryos, as in the syncytial macroplas-

modium of the slime mould *Physarum polycephalum*, it might be that phosphorylation of H1 follows the same temporal pattern in both organisms and decreases from a peak in prophase to a lower value at metaphase, rather than continue at high level throughout mitosis as in mammalian cells. A lower average degree of phosphorylation would result, but a very low net level of phosphorylation as observed in 2h embryos implies that hyperphosphorylation of H1 is not a feature of mitosis in the rapidly dividing nuclei of the syncytial blastula. It has been proposed (Blumenfeld *et al.*, 1978) that H1 phosphorylation levels in *Drosophila* correlate with the level of heterochromatinization (formation of heterochromatin) rather than with cell replication (Billings *et al.*, 1979). Since blastula nuclei do not contain constitutive heterochromatin, a low degree of phosphorylation is expected on this explanation.

Histone H4

Adult flies and 18h embryos contain significant amounts of monoacetyl-histone H4, but the 2h-embryo chromatin (Fig. 5) exhibits a low level of

Fig. 6. (a) Acid/urea/polyacrylamide-gel electrophoresis of total acid soluble proteins from 2h total embryos (lane 2) compared with histones extracted from the chromatin of 18h embryos (lane 1), and (b) second-dimension SDS/polyacrylamide-gel electrophoresis of the region covering bands 1-4 of lane 2 in (a)

Abbreviations used: CT, calf thymus (histones); 2D SDS, bands taken for second-dimension electrophoresis shown in (b).

additionally acetylated H4 running between bands b and c. Gels from partially purified nuclei show a strong H4 component in band 3 at the position of Ac_2 -H4 (Figs. 6 and 7), and the increasing intensity of the spot in the series (a) extracted 2h chromatin, (b) partially purified nuclei and (c) total embryos implies that the Ac_2 -H4 is in a free pool of H4 outside the nuclei. This is similar to the early stages of *Xenopus laevis* development (Woodland, 1979) in which a large store of Ac_2 -H4 is found, largely in the nucleus rather than in the cytoplasm. Rapid nuclear division takes place in the early stages of development of both organisms and a large store of histone is required. Ac_2 -H4 may therefore be the form of its deposition on to newly replicated DNA (Ruiz-Carrillo *et al.*, 1975). A correlation of Ac_2 -H4 with chromatin assembly has also been reported for the vegetative macropod of *Physarum polycephalum* (Chahal *et al.*, 1980). It is clear that H4 can be considerably modified in the absence of transcription.

Histone H3

In 18h embryos, H3 occurs in three forms (f_1 , f_2 and f_3). Since in the chromatin prepared from 18h embryos *not* using butyrate the H3 appears as a single band in the f_1 position, the variant species of H3 (two in number, see Fig. 3) run identically on acid/urea gels. Bands f_2 and f_3 are therefore modified species. The modification is probably acetylation, although phosphorylation could be involved (Whitlock *et al.*, 1980). Phosphorylation of H3 has been observed at mitosis, but not at other times in the cell cycle (Gurley *et al.*, 1978), so bearing in mind the high level of both modified species, phosphorylation is probably not involved. Histone H3 from 2h-embryo chromatin (Fig. 4) is a broad band in the first dimension. In the second SDS/polyacrylamide-gel dimension, species are seen that run in the first dimension as slowly as protein D₂, i.e. carry two modifications. An H3 species running as protein D₂ can also be seen in

Fig. 7. (a) First-dimension acid/urea/polyacrylamide-gel electrophoresis of partially purified nuclei from 2h embryos compared with calf thymus (CT) histones, and (b) second-dimension SDS/polyacrylamide-gel electrophoresis of two regions of the acid/urea gel of (a) bands 2-f and bands i and i'.

Abbreviations: 2D SDS, bands taken for second-dimension electrophoresis shown in (b); *D.m.*, *Drosophila* histones.

the extracts of 2h total embryos. Histone H3 modification in the 2h embryos is thus similar to that of the 18h embryo, and acetylation of H3 is therefore involved in processes other than transcription. As with H4, this is probably histone deposition on to newly replicated chromatin.

Histone H2A

More than half the H2A carries one modification and a small amount carries a second modification in 18h-embryo chromatin. This could be acetylation at residue 5 and phosphorylation at residue 1, as documented by Pantazis & Bonner (1981) for mouse cells. At present we cannot decide the distribution of these modifications in *D. melanogaster*, although the high degree of retardation in the acid/urea gel suggests acetylation rather than phosphorylation. In 2h-embryo chromatin, band e can be assigned to a modified H2A (Fig. 5), as in 18h chromatin. There is no evidence of doubly modified H2A in the 2h chromatin, but it might escape detection if the level were as in the 18h chromatin. Interpretation of such a high level

of H2A modification requires knowing the distribution between acetylation and phosphorylation.

Histone H2B

In 2h chromatin the second-dimension SDS/polyacrylamide gel (Fig. 5a) stained with Coomassie Blue shows no indication of H2B (band c) in band d. There is some tailing of band c in Fig. 5(b) towards band d, but this probably does not indicate the presence of modified H2B. The second-dimension SDS/polyacrylamide gel in Fig. 6 confirms the absence of modified H2B from 2h embryos, since band d shows no H2B. We conclude that H2B is unmodified in 2h embryos as well as in 18h embryos. Thus, although *D. melanogaster* H2B contains potential sites of acetylation in its *N*-terminal region (Elgin *et al.*, 1979), there is no modification in either developmental state.

Protein D₂

Palmer *et al.* (1980) have shown that protein D₂ is present in 18h embryos, in adult head chromatin and is a component of mononucleosomes. The situation in 2h-embryo chromatin is similar to that in 18h embryos (Fig. 5). D₂ is also present in the 2h-total-embryo extract (Fig. 6). Protein D₂ is thus a component of chromatin from the earliest nuclei. This extends the conclusion of Palmer *et al.* (1980) that the presence of D₂ is not restricted to a defined developmental period.

We acknowledge the support of the 'Ministero della Pubblica Istruzione', the 'Consiglio Nazionale delle Ricerche' of Italy and the University of Trieste. The work was also supported by NATO grant RG 9181. C. C.-R. acknowledges the continuing support of the Science and Engineering Research Council (U.K.).

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