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H1 histone synthesis by implanting pig blastocysts

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Summary. Histones were isolated from pig conceptuses on Days 8–18 of pregnancy, Acid–urea polyacrylamide gel electrophoresis (PAGE) was performed on samples of total histone and on PCA-soluble histone fractions (primarily histone H1). Conceptuses were also incubated for 24 h *in vitro* in the presence of [^{3}H]lysine, and the histones analysed by autofluorography after PAGE. A minimum of two H1 histone bands was resolved in stained gels by these techniques. The autofluorographs indicate that both H1 histones are synthesized throughout the developmental period analysed. No shift in H1 histone subtype, as encountered in sea urchin development, was observed. The results suggest that such a shift, if it occurs, may have been made before Day 8.

Introduction

H1 histones seem to be both tissue-specific proteins and regulator proteins, although direct evidence for regulatory activity is lacking. Studies of mammalian tissue culture cells reveal that various types of H1 histones characteristic of the species are expressed differentially depending on the specific genetic activity of the cells (Hohman, 1980). In embryonic systems, stage-specific changes in the synthesis of variants of H1, H2A and H2B histones of sea-urchin embryos have been observed by a number of investigators (Seale & Aronson, 1973; Ruderman & Gross, 1974; Cohen, Newrock & Zweidler, 1975; Newrock, Cohen, Hendricks, Donnelley & Weinberg, 1978; Harrison & Wilt, 1982). The changes in histone synthesis during development reflect altered histone gene expression. The histone variants, especially the H1 variants, may in turn be related to changes in chromatin structure and changes in overall transcription activity which occur as development proceeds (Arceci & Gross, 1981). There are indications that the shift in H1 histone type, from the earlier to the later variant, is not a result of the process of differentiation (Brookbank, 1978, 1980, 1982; Arceci & Gross, 1980), since the shift in H1 is made in dividing dissociated embryonic cells as well as in cleavage-arrested embryos which are synthesizing DNA.

Histone variants are represented by separate sets of tandemly repeated genes coding for early and late histones during sea-urchin development. The early histone mRNAs are transcribed from highly reiterated tandem repeat sequences (up to 1000 copies per genome). Later histones are coded by less highly reiterated sequences organized in clusters (Kunkel & Weinberg, 1978; Kedes & Maxson, 1981). The organization of histone genes in vertebrate species more closely resembles the late histone genes of sea urchins. Histone genes of human, mouse, chicken and toad cells are found as widely scattered gene clusters or in some instances as solitary genes, and are reiterated 40–80 times per genome (Kedes & Maxson, 1981; Sierra *et al.*, 1982).

Studies of histone synthesis in the preimplantation rabbit embryo indicate that overall histone synthesis is quantitatively co-ordinated with DNA synthesis (Matheson & Schultz, 1980). The present report is concerned with the synthesis of histone during the early development (Days 8–18)

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of the pig blastocyst. Placentation in the pig occurs between Days 13 and 18, and is accompanied by radical morphological changes in the blastocyst (Geisert, Brookbank, Roberts & Bazer, 1982) and by the onset of oestrogen synthesis by the preimplantation blastocyst (Flint, Burton, Gadsby, Saunders & Heap, 1979). Since a precedent exists in sea-urchin embryos for a change in type of H1 histone synthesized in advance of morphogenetic changes, pig blastocysts were examined to determine whether similar changes in histone synthesis occur between preimplantation and implanting embryos.

Materials and Methods

Conceptus material was obtained at hysterectomy (Murray, Bazer, Wallace & Warnick, 1972). Conceptuses were flushed from the uterine horns using sterile saline (Zavy, Bazer, Thatcher & Wilcox, 1980). Sexually mature crossbred gilts were used (Geisert *et al.*, 1982). The first day of oestrous behaviour in the presence of sexually mature boars was designated Day 0 of pregnancy. Gilts were mated at 07:00 and 19:00 h on Day 0. Day 8 embryos were recovered from superovulated gilts which had received 12 000 i.u. PMSG on Day 15 of the oestrous cycle (Guthrie, Henricks & Handlin, 1974). The gilts were mated at the following oestrus. Spherical conceptuses were recovered on Day 8 ($n = 40$) and Day 11 ($n = 11$); filamentous conceptuses were recovered on Days 12 ($n = 10$), 16 ($n = 11$), 17 ($n = 14$) and 18 ($n = 12$).

Isolation of nuclei utilized a modified procedure of Bentinnen & Comb (1971). Blastocysts were collected by centrifugation and rinsed in cold 0·85% (w/v) NaCl to remove uterine cells and mucus. The final rinse was replaced with five volumes of Buffer A (0·045 M-NaCl, 0·01M-EDTA, 0·05 M-$NaHSO_3$, 0·01M-Tris, pH 7·4 containing 20 µl Triton X-100 per 100 ml). Conceptuses were homogenized for 15 sec using a micro-cup and Waring blender or by repeated passage through an 18-gauge needle fitted to a 5-ml syringe. Homogenates were inspected for nuclei and unbroken cells under phase-contrast optics, and then centrifuged at 800 *g* for 10 min to sediment nuclei. The pellet was suspended in a small volume (< 0·5 ml) of Buffer A layered over 2 volumes of 1 M-sucrose in Buffer A, and centrifuged at 5000 *g* in a Fisher microcentrifuge for 10 min. Since the samples were small, and since repeated centrifugations tended to result in losses of nuclei, the nuclei were

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PLATE 1

Migration towards cathode, at bottom of gel; 13·5% acid–urea gel, 4% spacer gel in Fig. 1, Lanes 2–6.

Fig. 1. Lane 1 (silver stain): 20 µl from Day-12 histone sample. Lane 2: 20 µl of Day-17 total histone. Lanes 3 and 4: 15 and 25 µl 5% PCA-soluble histone. Lanes 5 and 6: 10 and 15 µl 5% PCA-insoluble histone. Bands numbered 1–4 represent histones H1 (one 'slow' component = H1s, and three 'fast' components = H1f, resolved into three closely spaced bands), H2 (a and b), H3 and H4. Histones H1 and H2 are soluble in 5% PCA; histones H3 and H4 are insoluble in 5% PCA. Lanes 2–6, Coomassie stain.

Fig. 2. Coomassie-stained gel. Lanes 1, 2 and 3 are histone patterns from Day-17 blastocysts (5, 10 and 20 µl; 6, 12 and 24 × 10^3 c.p.m. [^{3}H]lysine). Lanes 4, 5, and 6 are patterns from Day-11 blastocysts (10, 20 and 40 µl; 3, 6, and 12 × 10^3 c.p.m.).

Fig. 3. Autofluorographs of gel in Fig. 2. Lanes 1–3, Day-17 embryo histones. Lanes 4–6, Day-11 embryo histones. Kodak XRP-5 X-ray film at − 70°C, 18-day exposure. Bands numbered 1–4 represent histones H1f and H1s, H2 (a and b), H3 and H4. Histone H4 is not represented in the autofluorograph.

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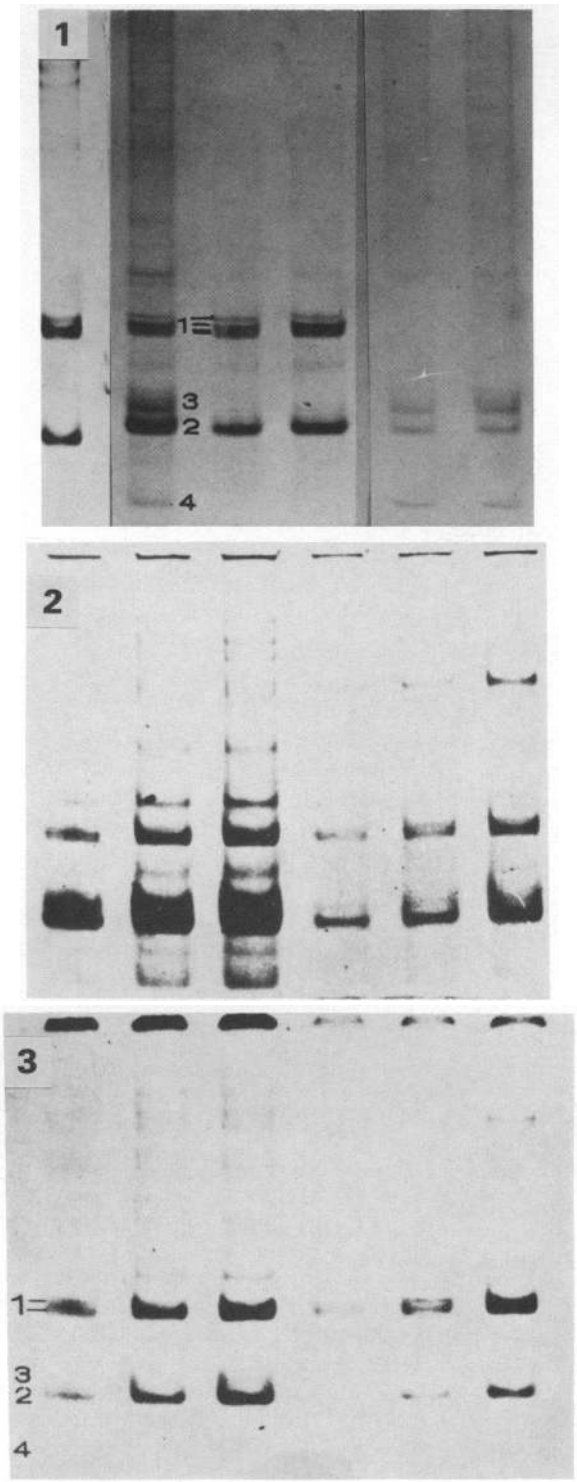
PLATE 1



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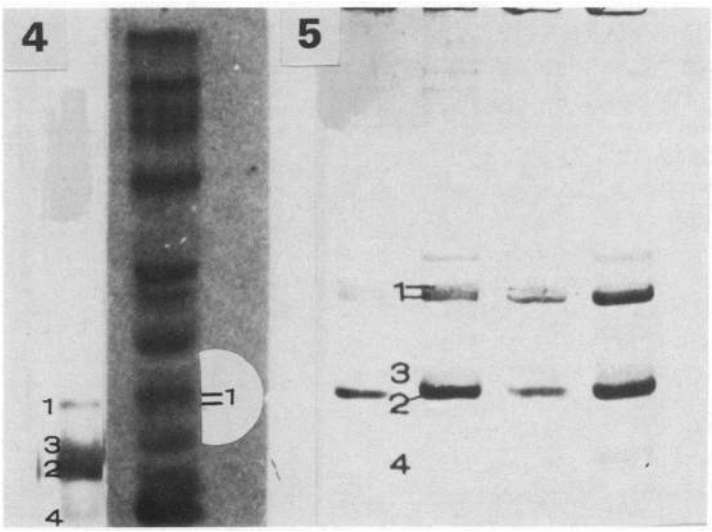

Migration towards cathode, at bottom; 13·5% acid–urea gels.

Fig. 4. Lane 1 = stained gel, Day-8 embryo basic proteins plus calf thymus histone carrier. Position of H1 band marked on filter paper supporting gel before drying the gel for fluorography. Lane 2 = autofluorograph of basic proteins of 8-day embryos (gel pictured in Lane 1). Lines at '1' indicate position of H1 histone bands. H1f (towards the cathode) is distinct from H1s by virtue of lighter deposition of silver grains in H1f. Total c.p.m. = 7×10^3; 4-month exposure in conditions as in Fig. 3.

Fig. 5. Stained gel, pig thymus histone. Lane 1: 2 µl total unfractionated histone. Lane 2: 10 µl. Lane 3: 5 µl. Lane 4: 15 µl.

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recovered from beneath the sucrose, rinsed with deionized water and extracted without further purification for 2 h at 0°C in 0·5 N-H_2SO_4. The acid extract was then centrifuged, and the supernatant containing histones precipitated overnight at −20°C with 2·5 volumes of cold absolute ethanol. Histone precipitates were recovered by centrifugation, and dissolved in appropriate volumes of a mixture of 0·5% glacial acetic acid, 4 M-urea and 0·01 M-2-mercaptoethanol (Buffer B). Thymus tissue was obtained from newborn pigs and the histones extracted by the above procedure. Portions of the histone preparation from Day-17 conceptuses were fractionated with 5% perchloric acid (PCA) (Johns, 1978) to help identify the stained bands. H1 histone can be precipitated from PCA solutions using 8 volumes of ethanol after making the solution 0·5 N with H_2SO_4. The precipitated histones are redissolved in Buffer B after twice rinsing the precipitate with cold 5% PCA. Due to the small volume of material, the Day 8 embryos were processed as whole homogenate without isolation of nuclei. Histones were labelled with [^{3}H]lysine (below) during embryo culture, and the histone fraction was carried through with added unlabelled calf thymus histone.

Day 8 and some Day 11 and Day 17 blastocysts were separately cultured for 24 h under sterile conditions in Hank's medium at a temperature of 37°C in an atmosphere of 50% N_2, 45% O_2, CO_2 (Godkin, Bazer, Lewis, Geisert & Roberts, 1982) with 10 μCi [^{3}H]lysine/ml (sp. act. = 75 Ci/mmol). Conceptuses so treated continue synthesis of a number of extracellular and intracellular proteins (Godkin *et al.*, 1982). The labelled embryonic material was processed as above, and the histones electrophoresed. Radioactivity in dried gels was detected using salicylate fluorography (Chamberlin, 1979).

Electrophoresis was performed on 13·5% (75:1 (v/v) acrylamide:bis-acrylamide) polyacrylamide slab gels containing 5% acetic acid and 4 M-urea for 5 h at a constant voltage of 160–180 V with 5% acetic acid in the buffer wells. Best resolution was finally obtained when a spacer gel of 4% acrylamide (75:1) and occupying 20% of the total gel volume was interposed between the bottom of the wells and the main gel. Slabs were stained with Coomassie Brilliant blue R, and, if necessary, silver nitrate (Merril, Goldman, Sedman & Evert, 1980). Extraction procedures did not quantitatively remove H3 and H4 from the nuclei, especially at Days 11 and 17. The relative amounts of H1 histones in a given sample well can be visually compared following electrophoresis and staining; otherwise the gels are not quantitative.

Results

The patterns obtained from conceptuses between 12 and 17 days of gestation are shown in Pl. 1, Fig. 1. Plate 1, Fig. 1 also shows the pattern obtained with PCA-fractionated histone samples from 17-day conceptuses. Taken together, the figures show one slow H1 band (H1s) in all samples, preceded (towards the cathode) by a heavier band (H1f) which resolved into 3 components under ideal conditions. Other more slowly moving bands represented contamination of the sample by other basic proteins, possibly ribosomal proteins. Histone electrophoretic patterns of Day 16 and Day 18 conceptuses (not shown) were indistinguishable from those illustrated.

Plate 1, Figs 2 and 3 represent the results of autofluorography of [^{3}H]lysine labelled histones from Day 11 and Day 17 conceptuses. The first bands to appear in the fluorographs were the H1 bands, a finding consistent with these proteins being very lysine rich. Both H1 histone bands, including the minor component with slower mobility, appeared to incorporate label at 11 and 17 days.

Plate 2, Fig. 4 shows the stained gel (pig thymus carrier histone) and autofluorograph obtained from Day 8 conceptuses. The H1 histones at Day 8 appear in the autofluorograph to be resolved into H1f and H1s components, as found in samples from later embryos. The electrophoretic pattern derived from neonatal pig thymus is shown in Pl. 2, Fig. 5, The H1 patterns of neonatal and conceptus material are not readily distinguishable from one another.

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Discussion

Plate 1 (Figs 1 and 2) indicates that two major H1 histone components (H1s and H1f) are present in all samples from Day 12 to Day 17. PCA solubility indicates that these bands are in fact H1 histone. Day 8 embryos reveal similar patterns through fluorography. The Day 8 fluorography pattern shows more intense label in H1s than in H1f, just the reverse of the later stages. It is possible that these embryos are just beginning synthesis of H1f. Since H1f is the major component of later developmental stages, it is possible that shift in H1 histone from H1s to include H1f was made before Day 8, even though H1s seems to have incorporated more label during in-vitro embryo culture.

Under the present conditions (extraction from Buffer B solution with 5% PCA), it appears that histone H2 (a and/or b) is soluble in 5% PCA along with H1 histone(s). The acid–urea system employed does not permit estimates of molecular size, e.g. for H1f and H1s. The autofluorographs in Pl. 1, Fig. 3 indicate that the H1 pattern visualized on stained gels reflects the pattern of H1 histones being synthesized over the experimental period between Days 11 and 17. The multiple H1 bands which are resolved in Pl. 1, Fig. 1 might represent H1 variants, but more probably represent phosphorylated forms of H1 histones. Similar if not identical H1 histone patterns are found in the pig thymus histone sample (Pl. 2, Fig. 5).

In sea-urchin embryos a definite H1 shift is encountered at the late blastula stage, some hours before the beginning of gastrulation. Similar shifts have been described for other unrelated invertebrates (Mackay & Newrock, 1982). The earlier H1 is typically conserved in later embryonic stages. If mammalian embryos follow a similar pattern, one might expect a shift in H1 subtype during development. In terms of time, sea-urchin embryos make the H1 shift at blastulation (about 15 h after fertilization, depending on temperature and species). Mammalian embryos are likely to be composed of only 1 or 2 cells at this time. From studies of ethanol-treated sea-urchin eggs, it is clear that time after fertilization and not developmental stage is the controlling factor in the H1 histone shift in sea-urchin embryos (Brookbank, 1982). In terms of time after fertilization, one might therefore expect the shift in mammalian embryos to occur during the first day of pregnancy.

Failure to observe a histone shift in pig blastocysts might be ascribed to any or all of the following. (1) The shift may have occurred before the first sampling day (Day 8). (2) The shift may be restricted to embryonic tissue only, and be masked by the predominance of extraembryonic tissues in the early conceptus. (3) A shift may have occurred between Days 8 and 18, but the new H1 variant was not resolved from the pre-existing H1 variant by the gel system used. A number of different acrylamide concentrations (10–15%) and acrylamide : bis-acrylamide ratios (37·5 :–150 : 1) were tried, as were linear 10–15% gradient gets, but resolution was not improved. (4) The shift may be scheduled to occur later than Day 18,.

Alternative (1) seems most probable from all considerations, since H1f is apparently incorporating less radioactivity on Day 8 than on Days 11 or 17. Perhaps this is an indication that this histone has recently been switched on. A direct parallel with sea urchin development would presumably show an early H1 being conserved (and diluted out) while a later H1 is being synthesized and added to the nuclei at successive cell cycles. The present data are consistent with such an interpretation, but do not prove it.

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