

IMAGE ANALYSIS OF CHROMATIN IN CELLS OF PREIMPLANTATION MOUSE EMBRYOS

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ABSTRACT

Mouse two-celled embryos and blastulae were Feulgen stained and the DNA content of their nuclei was measured with an integrating microdensitometer. The cells considered on the basis of their nuclear DNA content to be in G_1 , S, and G_2 phases of the cell cycle were selected and their total chromatin area and chromatin areas at different gray levels were measured by the image analyzing computer, Quantimet. The measurements were aimed at quantitation of several features of the chromatin morphology of cells in different functional states. The total area of chromatin was found to increase, and the mean density of chromatin to decrease, from the G_1 to the G_2 phase of the cell cycle in both two-celled embryos and blastulae. The area of chromatin decreased, and the mean density of chromatin increased, as embryos developed from two-celled to blastula stage. It was concluded that nuclear morphology in preimplantation mouse embryos depends on both the phase of the cell cycle and the stage of development. The method of image analysis described was found to be useful for quantitation of changes in chromatin morphology.

The relationship between the chromatin morphology of the interphase nucleus and various functions of the cell continues to be an enigma. Among the difficulties encountered in approaching this problem has been a lack of well-established methods for the quantitation of chromatin morphology. Chromatin morphology includes a complex of features such as overall size, shape, and staining intensity of chromatin, the spatial distribution of chromatin lumps within the nucleus, and the relative amounts of chromatin with different staining intensities. Quantitation of some of these chromatin features was recently made possible through the use of image analysis (Sandritter et al., 1967; Bartels et al., 1968; Mertz, 1971; Rowiński et al., 1972; Sprenger et al., 1973; Bartels et al., 1973; Klawe and Rowiński, 1974). Although it is not yet

possible to relate such quantitative evaluation of chromatin to nucleus and/or cell function, it may be a useful tool in the search for the morphological expression of nuclear function.

This study evaluates the use of image analysis to answer the following questions: (a) Does chromatin morphology change in relation to the cell cycle? and (b) Does chromatin morphology depend on the stage of preimplantation development? Mouse two-celled embryos and blastulae obtained after superovulation were used.

MATERIALS AND METHODS

Supply of Embryos

Embryos were obtained by the superovulation (Fowler and Edwards, 1957) of randomly bred female Swiss mice

weighing 20–25 g. Each female mouse received 7.5 international units (IU) of pregnant mare serum (Equinex, E. R. Squibb & Sons, Princeton, N. J.) in 0.9% NaCl, injected intraperitoneally, followed 48 h later by 7.5 IU of human chorionic gonadotropin (HCG, Pregnyl, Organon Inc., West Orange, N. J.) in 0.9% NaCl, injected intraperitoneally. At the time of HCG injection, the females were placed with male Swiss mice and examined the following morning for vaginal plugs. Those with plugs were killed by cervical dislocation at the following time intervals after HCG: 30 h (supplying one-celled embryos); 34, 44, and 54 h (supplying two-celled embryos); and 116 h (supplying blastulae). One-celled and two-celled embryos were flushed from oviducts and the blastulae were flushed from uteri and placed into Whitten's medium (Whitten, 1971). The kidneys were excised from the same mice, cut perpendicular to the long axis, and surfaces of sections were gently touched to the glass slides, delivering kidney cells. Epithelial kidney cells and pronuclei of 30-h one-celled embryos were used as a reference for 2C DNA content.

Fixation and Staining

The one- and two-celled embryos, blastulae, and the kidney cells were fixed in 10% Formalin buffered with phosphate buffer, pH 7.4, for 2–4 h and then washed three times in the buffer alone. The embryos were placed on glass slides in a thin layer of albumen (Sawicki and Lipetz, 1971); the albumen coagulated in ethanol vapor and the slides were placed in 70% ethanol. The embryos and kidney cells were hydrolyzed in 5 N HCl at 20°C for either 10 min, 30 min, 1, 2, or 6 h, stained with Schiff reagent, and mounted in Canada balsam.

Cytophotometry

The nuclear DNA content of one-celled embryos, two-celled embryos, and of blastula trophoblast cells was measured using the GN2 integrating microdensitometer (Barr and Stroud, Ltd., Glasgow, Scotland). The trophoblast nuclei were used because they are not superimposed over each other as are the nuclei of the cells from the inner cell mass of blastula. The maximal intensity of Feulgen staining of one- and two-celled embryos occurs after 30 min of hydrolysis and of blastulae and kidney cells after 1 h of hydrolysis; therefore, cells hydrolyzed for these times were used for further analysis. The intensity of Feulgen staining at the maximum of hydrolysis curve for kidney cells, one-celled embryos, and 2C blastula cells was the same, while the absorption for 54-h two-celled embryos was two times higher (majority of cells in embryos of this stage have 4C DNA content, see Fig. 1). Kidney cells of adult animals were shown to have 2C DNA content (Viola-Magni, 1965; Inui and Takayama, 1970). Pronuclei of one-celled embryos at 30 h after HCG injection have completed DNA replication and become diploid (Alfert, 1950; Luthardt and Donahue, 1973). Therefore, the relative amount of DNA mea-

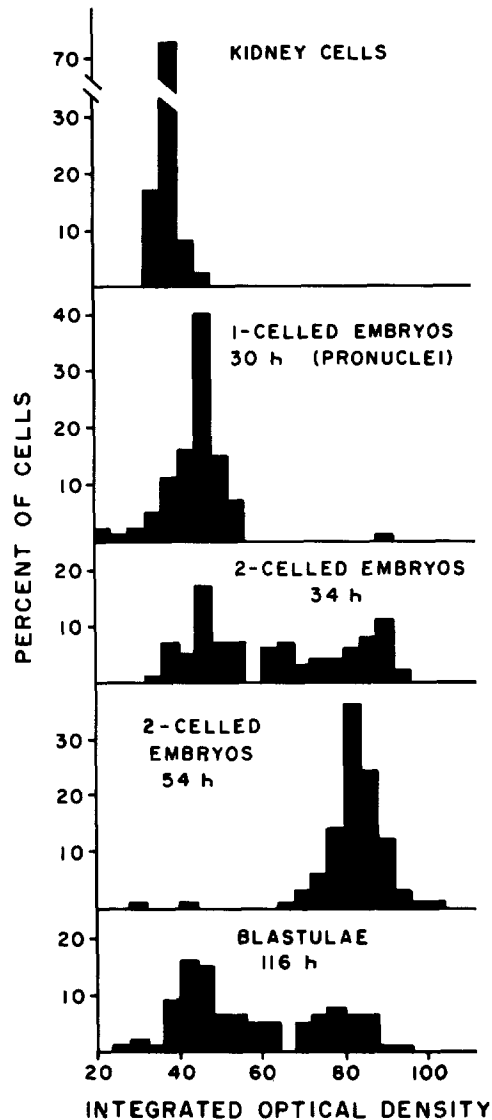


FIGURE 1 Histograms of integrated optical density of Feulgen-stained nuclei of kidney cells, one- and two-celled embryos, and blastula trophoblast cells at indicated hours after HCG injection.

asured in these two types of nuclei was considered as 2C. Those nuclei of two-celled embryos and blastula trophoblast which had the same integrated optical density as the nuclei of kidney cells and pronuclei of 30-h one-celled embryos, i.e., 36–44 relative units, were considered as 2C, those with integrated optical density of 54–66 units were considered as 3C, and those with 72–88 units of integrated optical density as 4C cells.

The integrated optical densities were recorded next to drawings made of the embryos; these served for repositi-

tioning the nuclei measured by the microdensitometer on Quantimet.

Measurements of Chromatin Area

Vs. Gray Level

The image analyzing computer Quantimet B (Metals Research, Cambridge, England) was used for chromatin measurements (Fisher and Cole, 1968; Mawdesley-Thomas and Healey, 1968; Prensky, 1971). In this instrument, the microscopic image is projected to a television camera, which passes the detected signals to a computer, and records the parameter being measured (e.g., area). A measuring frame with variable dimensions is used to restrict the measurements to a chosen part of a field of view. A threshold control of Quantimet makes it possible to display the image on the television monitor and to measure the area of a stained portion of the specimen separate from an unstained background portion. By varying the setting of threshold control, one can also "slice" the stained portion of a specimen into several levels of staining intensity which we have designated as "gray levels." Since the threshold control of Quantimet B is not calibrated in units of optical density, gray levels are expressed in terms of relative values.

Analysis of the image of a given nucleus consists of a series of measurements of chromatin areas at various settings of the threshold control, i.e., at different gray levels. The image of a single nucleus examined with $\times 100$ immersion objective was projected inside the measuring frame of Quantimet. The measurements of areas were begun from the gray level at which the overall chromatin (but not background) was displayed on the television monitor. The next measurements were made at consecutive intervals of five divisions of the threshold control scale. This interval (five divisions of the threshold control scale) was arbitrarily denoted as one gray value interval. As the gray levels increased, the increasingly smaller area of chromatin was displayed and measured. The measurements were stopped at the gray level at which no part of chromatin was detected. The gray value of background (i.e., the highest threshold level setting at which the whole area of background within the measuring frame was displayed) was used as reference and designated as 0. The method of measurements has been previously described (Mertz, 1971; Rowiński et al., 1972).

Six classes of nuclei were selected for measurements on Quantimet: 2C, 3C, and 4C nuclei of two-celled embryos and 2C, 3C, and 4C nuclei of blastula trophoblasts. The nuclei with 2C, 3C, and 4C DNA content were considered as being in G_1 , S, and G_2 phase of the cell cycle, respectively. Since the presence of G_1 phase in two-celled mouse embryos is controversial (Samoshkina, 1968; Gamow and Prescott, 1970), the 2C cells of this stage of development represent either G_1 or early S phase. Each nucleus was measured three times at each gray level. 25 nuclei of each class was measured with the

Quantimet. The area of chromatin at each gray level was averaged for nuclei of each class, the standard errors of means were calculated, and the resulting data plotted.

This plot of area of chromatin vs. gray level describes the distribution of chromatin into areas of different staining intensity. This distribution is one of the main features of the nucleus, contributing to what can be recognized by the human eye and brain as "chromatin pattern."

RESULTS

Feulgen-DNA value distributions for nuclei of one- and two-celled embryos and blastula trophoblast cells, obtained at various times after HCG injection, are presented in Fig. 1. Most of the pronuclei of one-celled embryos obtained 30 h after HCG injection contain 2C amount of DNA. Two-celled embryos, 34 h after HCG injection, and blastula trophoblast cells, 116 h after HCG injection, contain the nuclei with broad distribution of Feulgen-DNA values ranging from 2C to 4C. Two-celled embryos obtained 54 h after HCG injection contain almost exclusively nuclei with 4C DNA content.

Fig. 2 presents the curves of the chromatin area vs. gray level for six classes of nuclei, measured with the Quantimet. The areas of chromatin in blastula trophoblast cells are significantly smaller than the area of chromatin of two-celled embryos, at each of the gray levels, and for each of the stages of the cell cycle. It should be noted that the standard errors of the mean area of chromatin at a given gray level are much higher for S cells than for G_1 and G_2 cells. This indicates the greater variability among individual curves for S nuclei than for G_1 and G_2 nuclei.

The maximal value of the curve shows the mean total area of chromatin for a given class of nuclei. These values are listed in Table I for G_1 , S, and G_2 nuclei of both two-celled embryos and blastulae. The total chromatin area of smeared blastomeres of two-celled embryos depends on the cell cycle phase: G_2 nuclei average about $160 \mu\text{m}^2$ and G_1 nuclei average about $58 \mu\text{m}^2$. Similar differences in the total chromatin area have been found between G_2 and G_1 cells of blastulae (65 and $24 \mu\text{m}^2$, respectively). The total area of chromatin in S cells was found to be intermediate between the values for G_1 and G_2 nuclei of the respective embryo stage. Since DNA content per nucleus doubles between the G_1 and the G_2 phase of the cell cycle, and since at the same time the area of chromatin nearly triples, it can be inferred that the mean

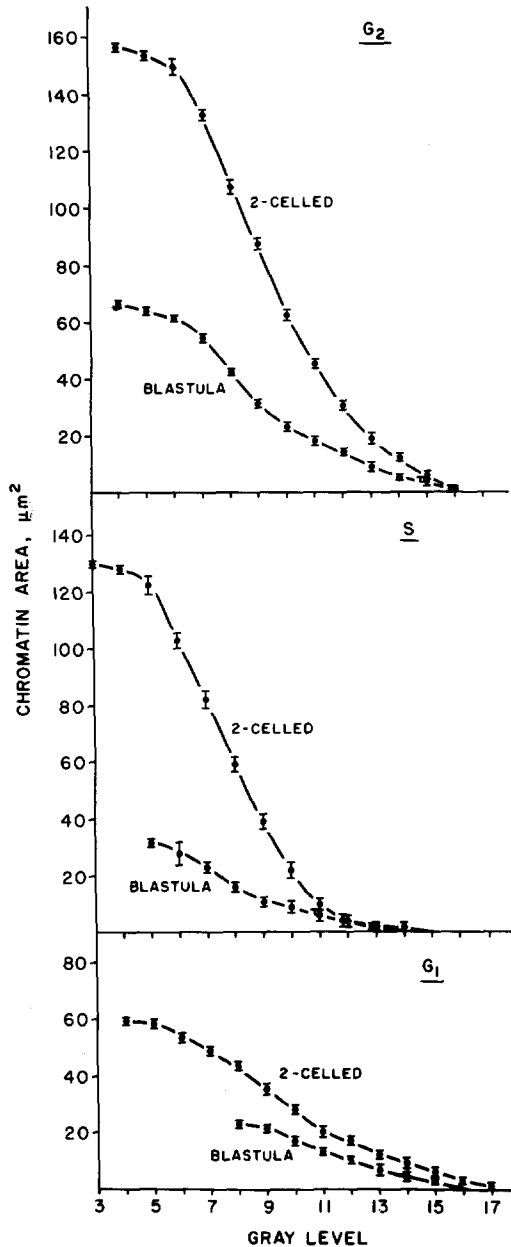


FIGURE 2 Curves of chromatin area vs. gray level, measured on Quantimet. The vertical bars present standard errors of means.

density of chromatin decreases during the cell cycle in both two-celled embryos and trophoblast cells of blastulae.

The overall area of chromatin differs significantly between two-celled embryos and blastulae. The area of chromatin for both G_1 and G_2 nuclei of

two-celled embryos is about two times as large as the respective chromatin areas of blastulae; the difference is even higher (about four times) for chromatin of S cells (Table I). This indicates that the mean density of chromatin increases during development from two-celled embryo to blastula by a factor of 2 or more.

To compare the pattern of curves of chromatin area vs. gray level for G_1 , S, and G_2 cells of both two-celled embryos and blastulae, the curves presented in Fig. 2 were normalized to a total area of chromatin, considered as 1. They show the fraction of total area detected at a given gray level (Fig. 3). Fig. 3 reveals the pronounced similarity between the curves for nuclei of two-celled embryos and blastulae in respective cell cycle phase. The two curves for G_2 nuclei occupy the middle position between curves for S and G_1 nuclei: both curves for S nuclei are shifted to lower gray levels while G_1 curves are shifted to higher gray levels. The curves for G_2 nuclei of both two-celled embryo and blastulae are nearly identical. The two curves for S nuclei are very close at 4–9 gray levels, and they have a different course at 10–16 gray levels. The curves for G_1 nuclei are different for two-celled embryos and blastulae: the curve for two-celled embryos is spread over the wide range of gray levels (from 5 to 18), while the respective curve for blastula cells is confined to 8–16 gray levels. The narrower range of gray levels for the latter nuclei indicates that they have much more homogeneously distributed chromatin than the former ones.

DISCUSSION

The measurements described in the present paper were aimed at quantitative description of chromatin morphology. They included: (a) measurement of the total area of chromatin, (b) estimation of the mean density of chromatin, and (c) measurements of chromatin areas at different gray levels. Although this is far from a complete description of

TABLE I
Mean Total Area of Nuclear Chromatin of
Two-Celled Embryos and Blastulae

| DNA amount (cell cycle phase) | Mean area ($\mu\text{m}^2 \pm \text{SE}$) | |
|----------------------------------|---|-----------------|
| | Two-celled embryo | Blastula |
| 2C (G_1 , early S phase) | 58.0 ± 2.89 | 23.8 ± 1.46 |
| 3C (S phase) | 130.3 ± 4.22 | 32.1 ± 4.12 |
| 4C (G_2 phase) | 160.8 ± 8.94 | 65.6 ± 3.09 |

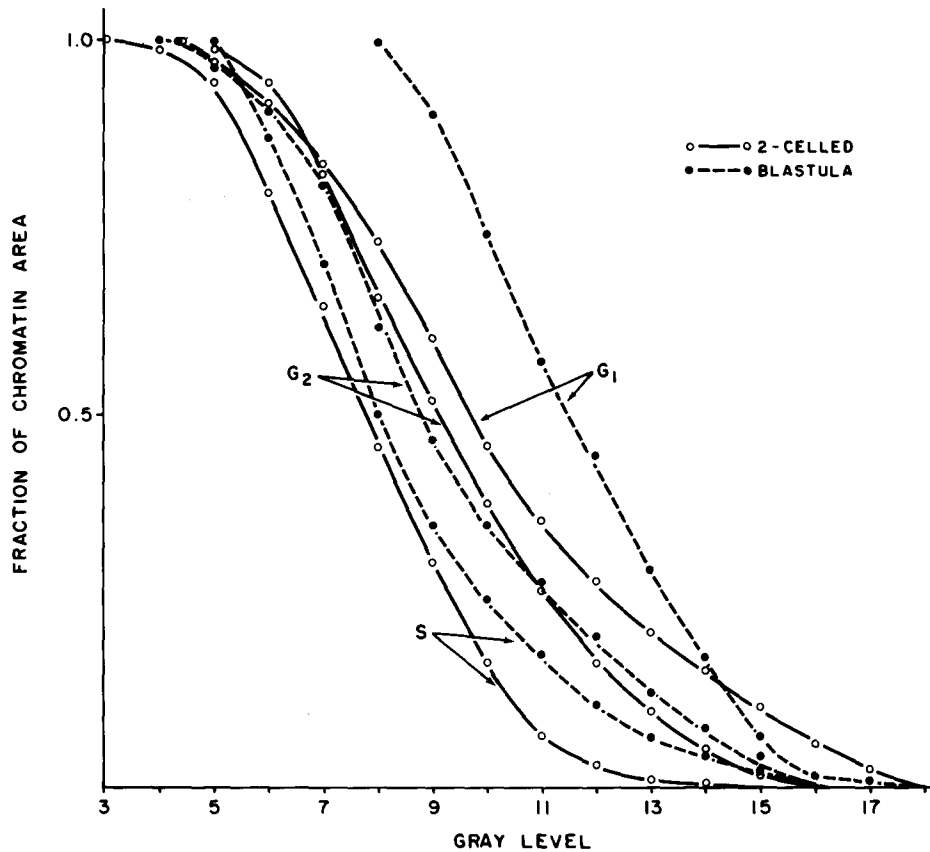


FIGURE 3 Curves of chromatin area vs. gray level. The curves were normalized to the common area of chromatin denoted as 1.

all of the features comprising chromatin morphology, we were able to demonstrate, in quantitative terms, the dependence of chromatin morphology on both the phase of the cell cycle and the stage of embryonic development.

The measurements describe properties of the image of fixed chromatin; the relationship between these properties and the properties of chromatin in living cells remains to be determined. That a close relationship between these two exists can be inferred indirectly from differences revealed between the chromatin of fixed cells in different cell cycle phases and in different stages of embryonic development at the time of fixation.

The decrease in the mean density of chromatin in cells of the early mouse embryo as they progress from the G_1 to the S phase of the cell cycle is consistent with the observations of Hay and Revel (1963) and Tokuyasu et al. (1968) who reported that in stimulated lymphocytes, lumps of chromatin progressively transform into diffuse chromatin

regions, and that both DNA and RNA syntheses are observed predominantly in such regions. It must be emphasized that despite the considerable difference in total chromatin area and mean chromatin density between nuclei of two-celled embryos and blastulae, comparison of chromatin areas vs. gray levels as measured by Quantimet revealed similarities between the chromatin distribution patterns of these two embryonic stages, for cells in the same cell cycle phase. These similarities were most clear for G_2 (4C) nuclei of two-celled embryos and blastulae. The curves for 2C nuclei are quite different for the two embryonic stages. It is uncertain whether the 2C nuclei of two-celled embryos represent G_1 or early S phase. According to Samoshkina (1968) there is a G_1 phase in two-celled mouse embryos, while data published by Gamow and Prescott (1970) indicate the absence of a G_1 phase in the cell cycle of two-celled mouse embryos. If the conclusion of the latter authors is correct, the 2C nuclei of two-celled

embryos represent an early S phase and this would explain the difference between curves of chromatin areas vs. gray level for 2C nuclei as due to different cell cycle phases (early S phase in two-celled embryos and G₁ phase in blastulae).

Cells of mouse two-celled embryos and of blastulae have been shown to differ in a number of features: The cells in the two-celled embryo have no mature nucleoli, while in blastula cells the nucleoli are morphologically and functionally mature (Alfert, 1950; Mintz, 1964; Calarco and Brown, 1969; Hillman and Tasca, 1969). In cells of blastulae there is a rough endoplasmic reticulum, and an ultrastructure rarely found in two-celled embryos (Calarco and Brown, 1969; Hillman and Tasca, 1969). The difference in chromatin area and density, reported here, is another sign of early differentiation of mouse embryonic cells.

Our results showing an increased mean density of chromatin during the early embryonic development of mice are consistent with the results of Kiliovska and Radeva (1971) who found that the mean density of nuclear chromatin, as measured by cytophotometry, increases during the development of the chorda dorsalis of *Triturus vulgaris*.

These data suggest that the changes in cell function that occur during differentiation and/or in the course of the cell cycle are expressed as a measurable alteration of chromatin morphology. The latter can be quantitated by using the microdensitometer and image analyzer.

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