Histone Gene Expression: Progeny of Isolated Early Blastomeres in Culture Make the Same Change as in the Embryo

Abstract. *Stage-specific changes in histone synthesis during sea urchin development reflect the expression of different sets of genes. The three kinds of blastomeres formed at the 16-cell stage are the earliest "determined" cells and fall into three distinct size classes. At this stage the cells synthesize only "early" histones. Such blastomeres can survive and divide in culture after being separated from the embryo, whether or not they are permitted to aggregate. With or without reaggregation, cultured progeny cells of each type of isolated blastomere perform the same changeover of histone synthesis as takes place in the intact embryo, that is, they begin spontaneously to synthesize a new set, the "late" histone variants. Normal contact relations among cells of the embryo are, therefore, not required for this programmed change in gene expression.*

During development of the sea urchin there are stage-specific changes in the kinds of histones synthesized and incorporated into chromatin (*1*). From fertilization to the blastula stage, one set of histones, the "early" (embryonic) type, is produced. At about the mesenchyme blastula stage, synthesis of an additional set, the "late" or larval type, begins. These enter chromatin in rapidly increasing amounts, while synthesis and incorporation of the early types decline. From the early larva stage on, the bulk of histone made and associated with DNA in chromatin is of the late variety. Eventually the early variants are undetectable by ordinary chemical means. The decline follows a dilution function given by the number of nuclei at the blastula stage divided by the number present at successive later stages. The relative decrease of early variants can be accounted for quantitatively by their retention after cessation of synthesis, and by their steady dilution with accumulating late histones in the later embryo and larva (*2*, *3*).

In the 16-cell-stage embryo there are three cell types. The vegetal half of the embryo consists of four micromeres and four macromeres, and the animal half is formed by eight mesomeres, which are intermediate in size. The developmental fates of these cells are to some extent already determined at this stage (*4*). The micromeres give rise to primary mesenchyme and eventually to other mesodermal derivatives and are also inducers of gastrulation. Meso- and macromeres form ciliated epithelium, the archenteron, and the structures derived from them.

We separated the three cell types from highly synchronous populations of 16-cell embryos and cultured them under conditions that either allow or prevent reaggregation, which begins immediately (unless prevented) upon return of the isolated cells to seawater (*5*). After 40 hours, cell number in micromere cultures had increased 10- to 15-fold, whereas in meso- and macromere cultures the increase was 35- to 40-fold.

To identify the histones being synthesized at particular times, the cultures were labeled with [^{3}H]lysine and then subjected to isolation, purification, and electrophoretic procedures to separate the histones. As shown in Fig. 1, descendants of each cell type, cultured to permit reaggregation and labeled after 40 hours, synthesized only the H1, H2A, and H2B late histones, as did control embryos by that time. The reaggregated masses are not true embryos (*5*); therefore each of the blastomere types divides to yield progeny cells that make the programmed histone change without the normal embryo organization.

Still, it is possible that some nonspecific signal, generated as a simple consequence of stable cell-cell contacts, induces the observed change in gene expression. To test this possibility, blastomeres separated at the 16-cell stage were cultured in spinner flasks and stirred to prevent reaggregation. We found that such blastomere progeny do synthesize primarily the early forms of H1, H2A, and H2B within 5 hours after separation (Fig. 2). (Very small amounts of the late variants were also found labeled in these

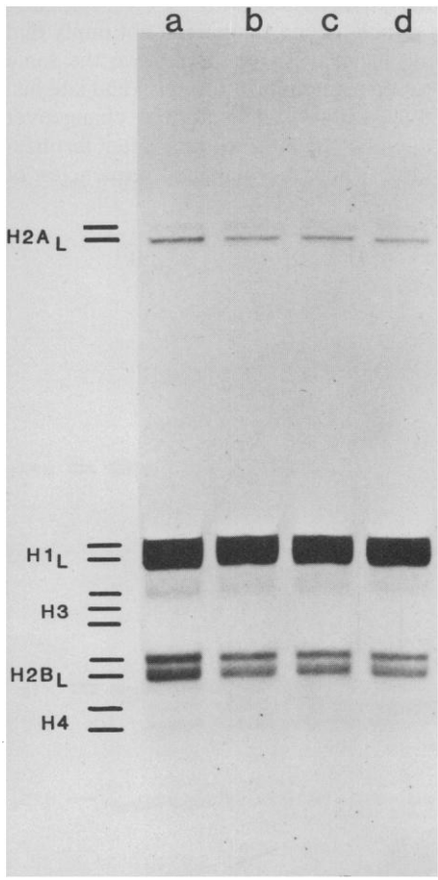

Fig. 1. Histones synthesized in cultures of separated 16-cell-stage blastomeres after 40 hours, with reaggregation permitted. Bands show histones synthesized in (a) intact control embryos, (b) the macromere culture, (c) the mesomere culture, and (d) the micromere culture. Migration in the electric field is from top to bottom. Eggs of the sea urchin *Lytechinus pictus* (Pacific Biomarine) were fertilized at a density of 10^4 per milliliter and demembranated by passing them through bolting silk as soon as the fertilization membranes had appeared. The embryos were cultured until the 16-cell stage (*15*), at which time they were chilled on ice and harvested by centrifugation at 250*g* for 1 minute. The cells were then separated by velocity sedimentation in Ficoll gradients (*7*). To maximize purity, the three bands of cells were collected separately, with only the top half of the mesomere band and the bottom half of the macromere band being retained. These were recentrifuged on separate gradients. The purity of the resulting populations was checked microscopically and by observation of reaggregation behavior. Micromeres, mesomeres, and macromeres were at least 98, 98, and 95 percent free of contaminant cell types, respectively. The cells were washed three times with artificial seawater and suspended in seawater in plastic petri dishes at a density of 1×10^4 to 2×10^4 cells per milliliter. In some cases fetal calf serum was added (2 percent), which increased cell viability but had no effect on the pattern of histone synthesis (*16*). The cells were left to divide and reaggregate for 40 hours at 21°C, and then were labeled with [^{3}H]lysine (50 Ci/ml) (specific activity 80 Ci/ml, New England Nuclear) for 4 hours while being shaken gently and intermittently. Controls (intact embryos) were labeled with [^{3}H]lysine at 5 Ci/ml. Histones were then purified as described in (*17*) but with several modifications: (i) 2 ml of packed eggs, first incubated for 30 minutes with 1 m*M* emetine to inhibit protein synthesis, were used as a carrier during chromatin isolation and (ii) histone from calf thymus was added (50 μg/ml), again as a carrier, just before ethanol precipitation of the acid-extracted histones. Histones were separated at 30 mA for 3½ hours on 12 percent polyacrylamide slab gel (which had been electrophoresed overnight at 10 mA) containing Triton X-100 (*10*). The gels were then fluorographed (*18*) to detect labeled histones. (Bands for H3 and H4 are less dense than for the others. This is due to their lower lysine content and to the lower efficiency of their extraction in the 20-minute procedure.) *L*, late histone.

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cultures. This was probably due to overlap of the labeling interval with the time at which late histone synthesis began.) However, when the cultures were labeled after 40 hours, only late H1, H2A, and H2B were detectable among the radioactive histones (Fig. 2). Stable cell-cell contacts are, therefore, not needed to elicit the normal change in histone gene expression. This statement is true at least from the 16-cell stage on.

Similar observations have been reported for nonhistone genes in cultured 16-cell-stage blastomeres and their progeny cells. The relative rates of synthesis of one class of nonhistone proteins (tubulins) and of two kinds of cytoplasmic RNA (ribosomal and transfer) change in such cultures, much as they do in the intact embryo (*5*). Insofar as histones are concerned, Brookbank (*6*) showed that late H1 can appear even in "embryos" in which cleavage is arrested but synthesis of DNA is continued.

More traditionally, new patterns of gene expression (those, at least, for which there is good evidence of change) are thought to be associated with specific inductions by external signals, such as new cell contacts or hormones. The situation described in this report, and apparently underlying the related cases mentioned, is very different. It implies the presence in early cleavage cells of information sufficient to control a future change of gene expression, independently of specific external signals. It is a situation that recalls, in fact, what is sometimes regarded as a form of determination.

The switch to synthesis of late histone must occur in most progeny cells of each of the three cell types from the 16-cell embryo. Because of the high purity of the isolated cell populations and their behavior in culture when permitted to reaggregate (*5*), we are confident that the cells responsible for the observed switch are indeed descendants of the particular cell types isolated (*7*). Of course, we cannot be certain that the switches in culture are made by the same cells and at precisely the same time as in the intact embryo. It is, however, nearly certain that the switch from early to late histone production occurs in parts of the embryo derived from both the animal and vegetal halves of the egg.

Such a conclusion need not imply that all nuclei of the embryo have the same relative amounts of the early and late histone variants. The normal changeover does not occur until long after fertilization, when the division frequencies of clones derived from early blastomeres have changed and diverged. Modulation of histone synthesis is not likely to be more synchronous than cell division after the blastula stage. Thus the histone content of embryonic nuclei may be spatially inhomogeneous.

Nuclear DNA is organized as repeating units (nucleosomes) consisting of about 200 base pairs of DNA coiled around the outside of an octomer made up of pairs of H3, H2A, H2B, and H4 (*8*). The nucleosome consists of a core particle with an invariant length of DNA (about 145 base pairs) complexed with the octomeric histones, plus a variable stretch of internucleosomal DNA termed the linker region; H1 appears to interact mainly with the linker DNA (*9*).

The conservation of the early histone variants, with subsequent addition of late ones, implies that different kinds of nucleosomes appear at different stages of development (*10*). Micrococcal nuclease digestions of embryonic nuclei have shown that coincident with the increase of late histones is a reduction in the release of acid-soluble DNA from chromatin (*11*). Furthermore, we have observed that during the interval of steady increase of the late histones in chromatin, the mean length of the repeating nucleosomes increases 20 percent (*12*). During the same interval there is a significant reduction of the per-cell synthesis rate of heterogeneous nuclear RNA (*13*) and a similar decline in the rate of cell division (*14*).

Although it has not yet been demonstrated, it would not be surprising if the changing pattern of histone synthesis were found to be related, through chromatin architecture, to such physiological processes as transcription and cell division. Whether or not this is the case, it is increasingly important to define the events that regulate differential histone gene expression.

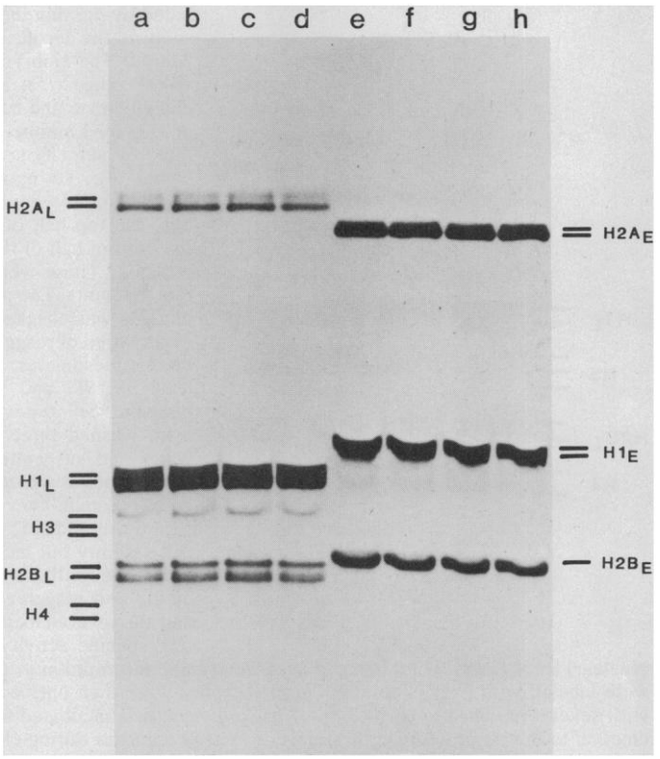

Fig. 2. Histones synthesized in cultures of separated 16-cell-stage blastomeres after 40 hours, with reaggregation prevented. Bands show histones synthesized in (a) 40-hour control embryos, (b) 40-hour macromeres, (c) 40-hour mesomeres, (d) 40-hour micromeres, (e) early micromeres, (f) early mesomeres, (g) early macromeres, and (h) early intact embryos. Migration is from top to bottom. The embryos were cultured and the blastomeres separated as described in the legend to Fig. 1. The separated cells were stirred in Bellco spinner flasks at 21°C at a rate sufficient to stop formation of any visible clumps. Cells and intact control embryos were labeled in the flasks with 50 and 5 Ci of [^{3}H]lysine per milliliter, respectively. Isolation, separation, and fluorography of histones were performed as described in the legend to Fig. 1. Slots a through d represent histones synthesized by the different cell populations during a labeling period begun 40 hours after their separation. Slots e through h show histones synthesized during a 5-hour labeling period begun immediately after cell separation. *L*, late histone; *E*, early histone.

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Effective Pulmonary Ventilation with Small-Volume Oscillations at High Frequency

Abstract. *At high oscillation frequencies (4 to 30 hertz), effective alveolar ventilation can be achieved with tidal volumes much smaller than the anatomic dead space. An explanation of this phenomenon is given in terms of the combined effects of diffusion and convection and in terms of data consistent with the hypothesis. Theory and experimental results both show that the significant variable determining the effectiveness of gas exchange is the amplitude of the oscillatory flow rate independent of the individual values of frequency and stroke volume.*

Gas exchange during normal tidal ventilation is thought to involve two distinct regions of the lung: (i) the dead space volume, comprised of the conducting airways, where gas transport is primarily convective; and (ii) the alveoli, where molecular diffusion is the predominant mechanism of gas transport. In normal ventilation, therefore, effective gas exchange is possible only if the tidal volume exceeds the dead space volume. Recently, however, it was shown that effective alveolar ventilation can occur with tidal volumes considerably less than the dead space volume if the respiratory frequencies are sufficiently high (4 to 20 Hz) (*1*).

To explain the gas exchange effected by high-frequency, low tidal volume ventilation, we present a theoretical model based on the concept of augmented transport, or diffusion coupled to convection (*2*). We also present experimental data that confirm the observations of others concerning the efficacy of this technique and that are in general agreement with the predictions of the theory.

Augmented mass transport in both laminar and turbulent flow through a long straight tube was originally analyzed by Taylor (*2*). Subsequently, Chatwin, using concepts similar to those of Taylor, provided a solution for oscillatory laminar flow (*3*). The augmented mass transport can be represented in a way analogous to molecular diffusion by replacing molecular diffusivity D_{mol} by effective diffusivity D_{eff}. The effective resistance to diffusion R (in units of inverse volume flow) for a tube of length L and cross-sectional area A may then be expressed as

$$R = L/(AD_{eff}) \quad (1)$$

The appropriate formula for D_{eff} depends mainly on two parmeters: (i) the Womersley parameter, $\alpha = d\sqrt{(\pi/2)f/\nu}$, where d is the tube diameter, f the oscillation frequency, and ν the kinematic viscosity; and (ii) the Reynolds number, $Re = ud/\nu$, where u is the root-mean-square velocity.

For this simple analysis we assume that the stroke volume of oscillation is less than the dead space volume and that there exists a critical Reynolds number, Re_c, such that the flow is laminar when $Re < Re_c$ and well mixed when $Re \geq Re_c$ (*4*). Given the theoretical results of Chatwin (*3*) for laminar flow and the theory of Taylor (*2*) and experiments of Scherer *et al.* (*5*) for well-mixed flow, we employ the following expressions for D_{eff}:

$$D_{eff}/D_{mol} = 1 + K_1 (ud/D_{mol}), \quad \text{if } Re \geq Re_c \quad (2)$$

$$D_{eff}/D_{mol} = 1 + (1/192)\,(ud/D_{mol})^2, \quad \text{if } Re < Re_c \text{ and } \alpha < 1 \quad (3)$$

$$D_{eff}/D_{mol} = 1 + (K_2/192)\quad (ud/D_{mol})^2\alpha^{-7}, \quad \text{if } Re < Re_c \text{ and } \alpha \gg 1 \quad (4)$$

where K_1 and K_2 are dimensionless constants of order unity.

Now consider the lung as a network of branching tubes. As in the calculation of resistance in an electrical circuit, the effective resistance to diffusion of the entire bronchial tree, R_T, may be found by summing the individual resistances. Note that in a particular generation, the area in Eq. 1 would be the total cross-sectional area of that generation.

The rate of CO_2 removal from the lung, $\dot{V}_{CO_2}$, is proportional to the difference between the volume fraction of CO_2 present in the alveoli, F_{A,CO_2}, and at the airway opening, F_{AO,CO_2}, and can be expressed as

$$\dot{V}_{CO_2} = (F_{A,CO_2} - F_{AO,CO_2})/R_T \quad (5)$$

To obtain the results shown in Fig. 1A, we used the anatomic data from the dog lung model of Horsfield and Cumming (*6*) and $K_1 = 0.7$ [the mean value for inspiratory and expiratory flow given by Scherer *et al.* (*5*)]. Results are shown for three cases that bracket the anticipated range of Re_c and α_c, where α_c represents the critical value of α; α_c determines whether Eq. 3 (for $\alpha < \alpha_c$) or Eq. 4 (for $\alpha > \alpha_c$) is used (*7*). The value of Re_c is not known, but is thought to be on the order of 10 (*8*). The discontinuities in the curve for $Re_c = 30$ are an artifact of the model caused by the abrupt transition from laminar to mixed flow as the Reynolds number in a given generation changes through its critical value. In reality these transitions are probably not abrupt, but continuous, due to entrance effects, secondary motions, and asymmetries in the bronchial tree.

The model predicts that CO_2 output will increase as the amplitude of the oscillatory flow increases, regardless of the individual values of f and stroke volume, as long as f is low enough for α to be small in regions of laminar flow. In addition, theoretical results not shown in Fig. 1 indicate that the CO_2 output as a function of tracheal flow is relatively independent of animal size or lung volume for a single animal, provided the lung geometries are similar (*9*).

Experiments were performed on four dogs in which we varied f and the stroke volume and observed the effect on CO_2 elimination. Our experimental setup (Fig. 2) consisted of a standard animal piston ventilator with the following added features: (i) a high-frequency oscillator (HFO), which consisted of four 12-inch-diameter speakers sealed in a special chamber, acoustically coupled in series, and driven by an amplifier connected to a wave-form generator used to

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