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Studies in developmental cytomechanic

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ABSTRACT One of the most promising trends in modern developmental and cell biology, recently defined as «cyto», or «morphomechanics», is directed towards revealing the role of mechanical stresses, chemomechanical transduction and active stress responses of cells antissues of developing embryos. We review here the results obtained in this field by our research group and compare them with those from other labs. Our studies relate to the buds of hydroid polypes and to amphibian embryos. We describe the space-temporal patterns of mechanical stresses in these species, analyze their morphogenetical role and the tissue responses to the experimental modulations of stress patterns. In hydroid polypes we explore also the molecular events involved in mechanochemical coupling. A model, linking the passive mechanical stresses with the active stress-responses of embryonic tissues is suggested. We consider these investigations as a first approach to a developing embryo as to an «active solid body».

KEY WORDS: *cytomechanics, morphogenesis, hydroid polypes, mechanical stresses*

Introduction

In modern developmental biology two general paradigms are competing with each other, either in an open or in a hidden form. Schematically, the first implies that the development of organisms is a strictly programmed process, governed by a set of rigid instructions which are encoded within a genome, together with their exact space-temporal schedule. Without the genetical instructions, the developmental processes are assumed by this paradigm to be completely deregulated. From such a viewpoint, the main goal of investigation would be discovering these instructions one after one until a complete list of them could be obtained.

According to another point of view, there are the real and directly observable developmental processes, such as shape changes, cell movements, intercellular events etc, irrespectively of whether they are or not affected by genetical or environmental influences, which create a holistical, self-organizing set of events linked with each other by some essential feedback. Within such a framework the genetical influences may well play an important role, but only by modulating the fundamental, independently established self-organizational contours. In this case, the main research task would be exploring just these feedback contours.

For several reasons our research group joins the latter approach. Among the main arguments in its favor, there is a highly regulative character of development, hardly compatible with the assumption of its strict programming, the capacity of embryos for self-complication of their structure (spontaneous reduction in symmetry order, see Beloussov, 1993 for more details) and a wide spread of auto oscillations and autowaves during development.

Meanwhile, even this self-organizational paradigm is diverged into several trends. By one of them, the most important developmental events can be explicitly formulated in terms of chemical kinetics, such as auto- or cross-catalysis, inhibition and diffusion of certain morphogenetically active substances through a liquid medium (Meinhardt, 1982). From this viewpoint, the crucial step of development would be establishing of chemical prepatterns which are then in a one-to-one manner transformed into embryonic morphology. The visible morphogenetical processes are, according to such a viewpoint, no more than «blind end-results» of the preceding chemokinetical events and cannot play any autonomous regulatorial role.

Such a view seems to be rather unrealistical to us, or at least non-sufficient. In this presentation we would like to expose some data from our and other research groups arguing for a really active role of the visible morphogenetical processes (first of all, cell movements and rearrangements) together with the produced mechanical stresses in developmental self-organization.

It is well-known from mechanics, that stresses of tension and compression, combined with a definite geometry of a stressed body, may quite be effective tools for further complicating its shape in a regular way, without applying any precisely located forces. From the very beginning of their evolution, the living beings have

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Abbreviations used in this paper: GP, growt polsation; TMP, transmenbrane potentials; HRR, hyperrestorative reactions

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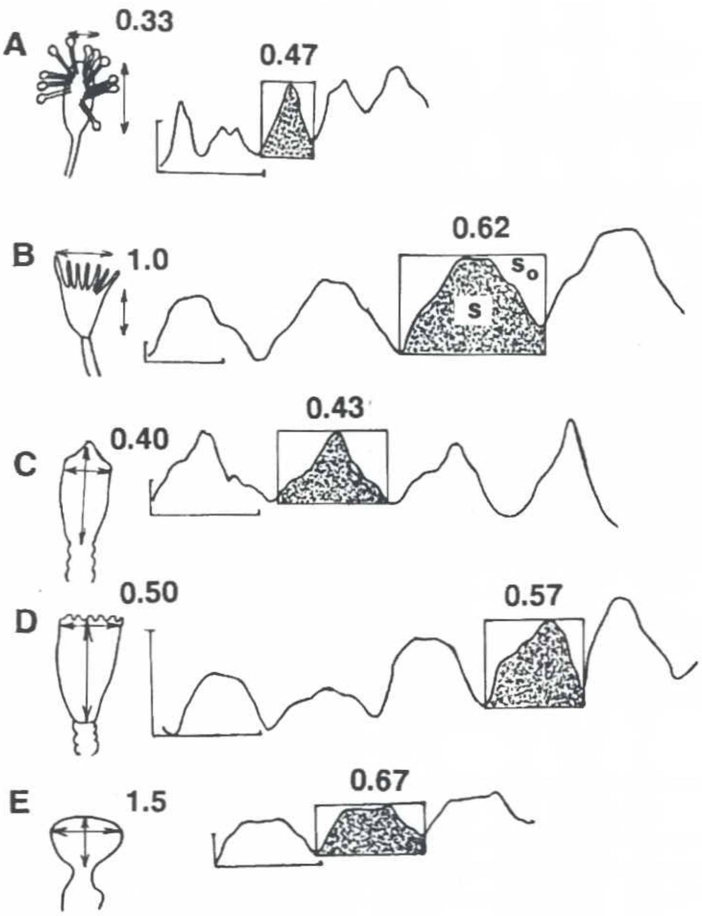

Fig. 1. Correlations between shapes of hydranths and time-extension patterns of growth pulsations in several species of hydroid polypes. (**A**) *Coryne loveni;* (**B**) *Eudendrium rameum;* (**C**) *Campanulina lacerata;* (**D**) *Obelia loveni;* (***E***) *Dynamena pumila. In all the species the width/length hydranth relations (expressed by numbers at the left row of figures) correlate with the IPR of growth pulsations (relations of hatched zones to rectangles, S/S_o, shown by figures under pulsations records) (from Beloussov et al., 1993, modified).*

been inevitably confronted with mechanical stresses (and, first of all, with those associated to osmotic pressure). Even *a priori* it would look quite improbable that organisms could not find any use from mechanical stresses in moulding their shapes. A crucial role of osmotically-produced mechanical stresses in plants growth and development is well established (Harold, 1990). What we would like to demonstrate in this paper is that the role of mechanical stresses in animals' development is no less important, while the patterns and feedback involving mechanical stresses are much more complicated.

In our research group we have been studying, for more than two decades, two quite different groups of developing organisms: budding specimen of marine hydroid polyps (*Obelia loveni, Dynamena pumila, Podocoryne carnea*) and amphibian embryos (*Rana temporaria, Xenopus laevis*). In spite of enormous differences in their developmental patterns, we have been able to reveal, in both cases, the important morphogenetical role of mechanical stresses.

Hydroid polyps: periodically exerted mechanical stresses (growth pulsations), their morphogenetical role and chemomechanical coupling

A longitudinal growth and shaping in hydroid polyps was found to be mediated by a succession of the so-called growth pulsations (GP), that is, highly regular cooperative changes in the orientation and the vacuolar swelling of several dozens of ecto and endodermal tip cells taking place each next several minutes (Beloussov, 1961; Beloussov *et al.*, 1972). During each next extension GP phase (coincided with a transitorial increase in the local curvatures of the rudiments surfaces) the cell vacuoles became filled with osmotically-driven water, cells rotated towards transversal orientation and the tangential pressure within cell layer increased. On the contrary, during each next retraction GP phase the vacuoles were fused into prolonged channels opened to external environment, water pumped out, cells returned to oblique orientation and pressure stresses decreased (Beloussov *et al.*, 1989, 1993).

Direct correlation could be traced between both normal and experimentally modified GP patterns, as estimated by their so-called impulse-period relations (IPR) on one hand, and the resulted shapes of the rudiments, on the other. Namely, the greater being IPR, the greater also were the transversal/longitudinal ratios of the arisen hydrants (Fig. 1) (Beloussov *et al.*, 1985, 1993).

Same correlations could be imitated by modelling the rudiments shaping under the assumption that tangential pressure within a cell

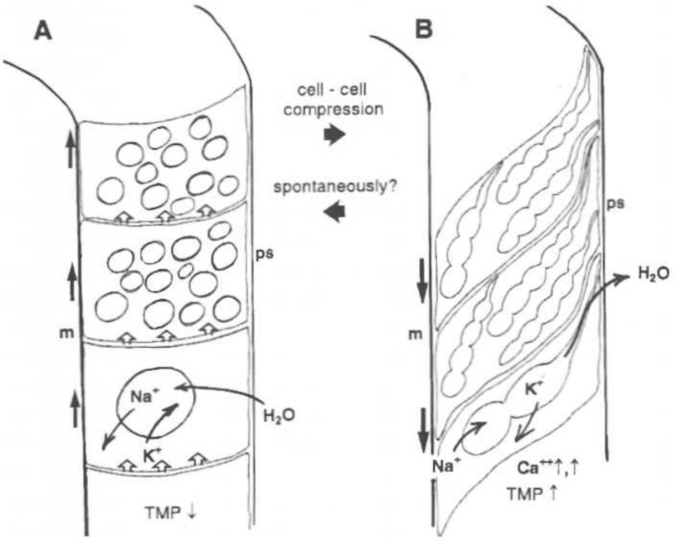

Fig. 2. A generalized dynamical scheme of extension-retraction transitions during growth pulsations in hydroid polypes. *Right halves of an apical dome are sketched, cells unproportionally enlarged. At the extension phase* (**A**), *cells are filled of isolated turgorly pressurized spherical vacuoles and compress each other (empty arrows). Mesoglea (m) is shifted upwards (solid vertical arrows) by transversely rotated cells, fixed to perisarc (ps). Ionic currents, leading to TMP decrease, as shown. At the retraction phase* (**B**) *mesoglea, due to elastical reaction, is shifted downwards (solid arrows), ionic currents reversed as related to (A). Two brief pulses of the cytoplasmic Ca^{++} uprise are registered at this phase. We suggest that A-B shift is, maybe, switched by cell-cell compression, while the reverse shift can be spontaneous to a great extent. For details see Beloussov et al., 1989, 1993, Kazakova et al., 1994, 1995.*

layer is the main morphogenetical factor (Beloussov and Lakirev, 1991). The model's capacity to reproduce rather realistically the rudiments shapes confirms a suggestion, that the latter are moulded by the finely regulated temporal patterns of pressure stresses in the growing tips. The next question would be how these patterns are produced, that is, what are the main links of a chemomechanical coupling, transforming metabolic energy into periodical mechanical stresses?

After proving the osmotic nature of GP stresses (Beloussov *et al.*, 1989), we started to detect the elemental content within the vacuolar compartment at the different GP phases with the use of X-ray analysis (Kazakova *et al.*, 1994, 1995 and in preparation). It was shown that the intravacuolar potassium concentration at the extension GP phase was considerably higher than in sea water, while for the sodium the reverse was true. Meanwhile, the retraction phase have been characterized by reverse relations. At the same time, no significant changes in chloride concentration have ever been traced. In another series of experiments, made in collaboration with Dr D. Jones from Munster University, Germany, we have measured transmembrane potentials (TMP) during GP with the use of a dye, di-8-ANEPPS (Kazakova *et al.*, 1995 and in preparation). This showed, that at the end of the extension GP phase TMP was shifted towards positive, while at the retraction phase the cells became hyperpolarized. By comparing these data with those obtained earlier, namely: (1) prolongation of an extension GP phase by adding ouabain, the inhibitor of Na^+,K^+ATPase; (2) same effects obtained by increasing external sodium under isotonicity conditions; (3) prolongation of a retraction GP phase by adding the blockator of the membrane potassium channels, tetraethylammonium (Beloussov *et al.*, 1985, 1989), we can conclude the following (Fig. 2): during extension phase the sodium-potassium pumps located within vacuolar membranes are, to a large extent, blocked promoting thus an ionic leakage between the vacuoles and the cytoplasm towards a more equilibrated ions distribution. In other words, potassium comes from the cytoplasm to the vacuoles while sodium moves in the opposite direction. Potassium, accompanied by unknown anion other than chloride (most probably, bicarbonate) seems to be the main osmogenic ion within the vacuoles, attracting water from outside and creating vacuolar swelling. The retraction phase is characterized by the opposite trend: sodium-potassium pump is restoring its activity, now driving potassium to the cytoplasm and sodium in a reverse direction, to the vacuolar compartment, which is now at least partly opened to an extercellular space. In such a way a non-equilibrium ionic ratio within a cytoplasm and, hence, TMP is restored. It is the energy, stored in TMP during retraction GP phase, which is transformed at the beginning of extension phase into the intravacuolar osmotic potential, while this one, in its turn, to the mechanical work of rotations and mutual compressing of the swelled cells, which provide the rudiments extension and its shape changes.

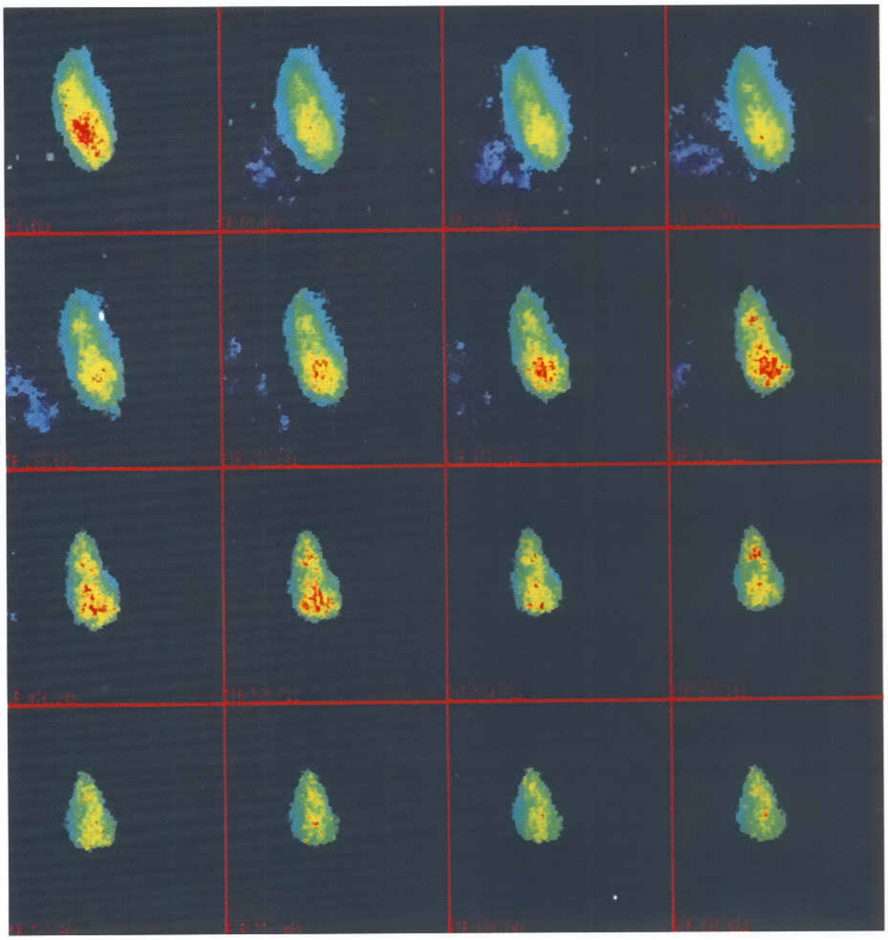

Fig. 3. A computer image record of free Ca^{++} dynamics during growth pulsations in a piece of a *Podocoryne carnea* stolon. *Ca^{++} concentration is shown in red. Note, that at the beginning of a retraction GP phase (visualized by diminishing of a sample area) there are two distinct uprises of Ca^{++} concentration (frames 1 and frame 6-10). Frames intervals are 4 sec (by courtesy of Dr D. Jones).*

What might be the triggers, shifting a cell from one GP phase to another and maintaining thus the regime of auto oscillations? In this respect it should be noted, first of all, that at the end of the extension phase the cells, due to their transversed rotations, extensively compress each other tangentially (diminishing their transversal diameters up to 30%; data taken from Beloussov *et al.*, 1989). Can it be the compression, which switches-on the cells passage towards retraction phase? This suggestion has been proved by the experiments on the artificial bending of stems compressing thus their concave side cells (Kazakova and Beloussov, in preparation). Within 1-3 min (which is just a characteristic GP time in the given species) the vacuoles within the cells have been fused and the cells took the arrangement typical for the retraction phase. Meanwhile, the molecular trigger (or one of the first molecular traces) of this transition may be the increase in the intracellular free Ca^{++}. As was shown by fluorimetric observations made in collaboration with Dr D. Jones on *Podocoryne carnea* samples loaded with FURA 2AM, excited at 340 nm and 380 nm (ratio imaging 510 nm) two brief (each one of several seconds

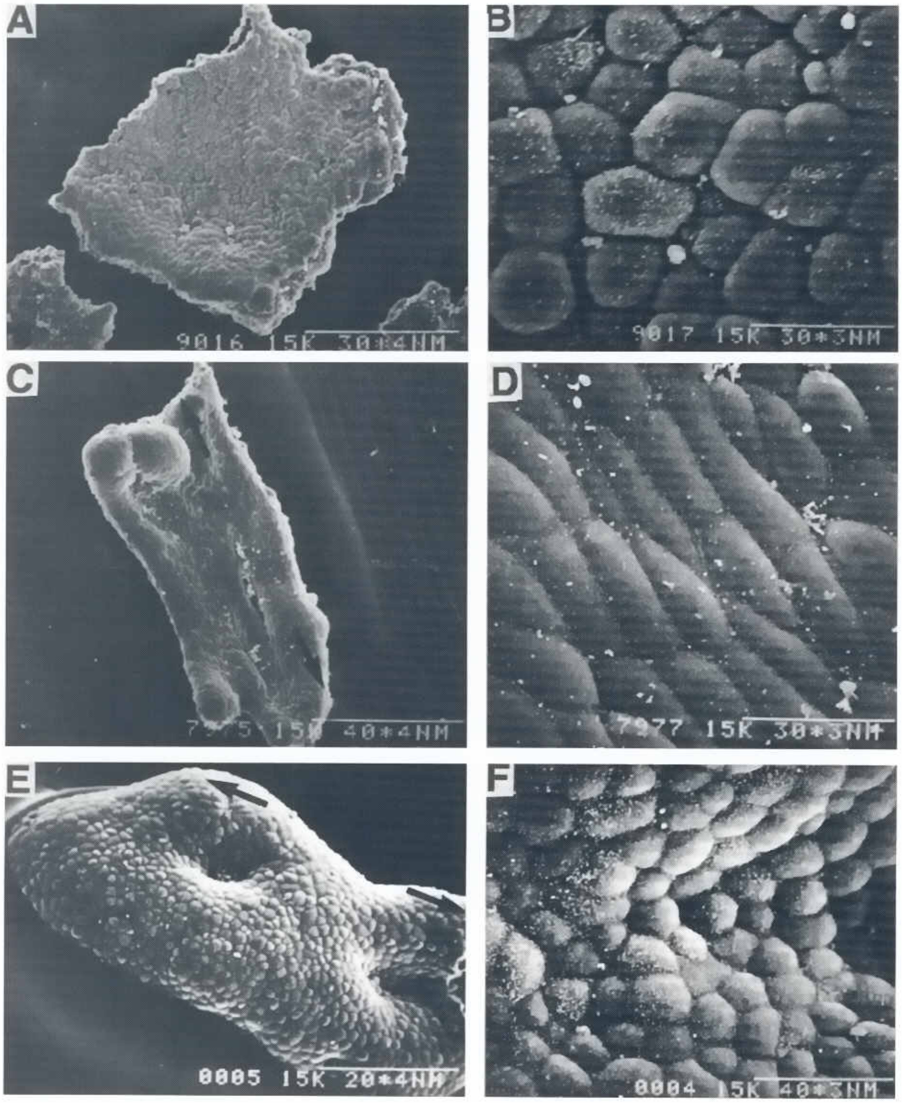

Fig. 4. Explants of ventral ectoderm of Xenopus laevis early gastrula embryos stretched onto the latex substrate. (**A,B**) *prior to stretching.* (**C,D**) *immediately after the cessation of 0.5 h stretching.* (**E,F**) *4 h later, without additional stretching. Left column: general views from the top. Right columns: details of an outer ectoderm structure. Immediately after stretching (D) many cells (although not all of them) become elongated in the stretching direction to a much greater extent that an entire explant. Later on (F), they return towards isodiametrical shapes and relax (as shown by dissection tests) the imposed stresses. Bars: A, 300 mkm; B and D, 30 mkm; C, 400 mkm; E, 200 mkm; F, 40mkm. For more details see Beloussov and Luchinskaia, 1995b.*

duration) successive pulses of Ca^{++} increase have been registered within the the first half of the retraction phase (Fig. 3) (Kazakova *et al.*, 1995 and in preparation). Obviously, Ca^{++} increase (triggered itself probably by the preceding mechanical signal) promotes the vacuoles fusion and activates the contractile processes, required for pumping water out of the vacuoles (see Fig. 2).

Amphibian embryos: patterns of mechanical stresses and results of their experimental modulations

Contrary to hydroids, we could not detect in amphibian embryos any signs of several minutes periodical dynamics. Instead, by tracing fast tissue deformations after localized dissections (Beloussov *et al., 1975*, 1994) and measuring cells surface geometry (Beloussov and Lakirev, 1988), we discovered several spatially extended patterns of mechanical stresses which remained topologically invariable during the substantial time periods, roughly coincided with blastulation, gastrulation and tail bud formation (Beloussov *et al.*, 1994, Fig. 1). A transition towards each next developmental period was associated with a relatively rapid transformation of a stress pattern, namely with the formation of new bundle(s) of the stretched cell files which cross cell sheets (so-called cross-lines) and the domains of columnarized cells (Belintzev *et al.*, 1987). These stress patterns, while being established, predict unambiguously the shape to be taken by the rudiment at the end of the corresponding topologically invariable period. This is because its immediate shape changes can be interpreted as a drive towards relaxation of the pre-established mechanical stresses (although if the shape changes are energy consuming). In such a framework, we consider normal morphogenesis as a retarded quasi-relaxational process, determined by the pre-established stress patterns. Their morphogenetical importance can also be demonstrated by the following experiments:

1. Isolation of an embryonic tissue piece with already well-established stress field leads to its extensively accelerated development towards a normal shape. This is obviously due to the removal of mechanical resistance of the surrounding tissues. On the other hand, isolation of a piece without as yet established field leads to the formation of highly variable morphogenetical patterns (Beloussov, 1979).

2. A relaxation of an overall tangential tension of an embryo surface, performed at the early gastrula stage, leads to an increase of an area, occupied by extensively columnarized cells (related mostly to a presumptive neural plate) and, as a consequence, to substantial abnormalities in the development of axial rudiments (Beloussov *et al.*, 1990; Beloussov and Luchinskaia, 1995a). Worth mentioning is the fact that such a relaxation can be only rather short-termed, since embryonic tissue tends to restore actively the relaxed stresses (see below).

3. Artificial stretching of a suprablastoporal region, extirpated from an early gastrula embryo, in the direction perpendicular to its normal antero-posterior stretching led to the partial rearrangement of the axial rudiments along a new stretch direction. This was due to the reorientation of the convergent intercalation movements of the cells, now going perpendicularly to their normal direction and, at the same time, perpendicularly to the artificial stretching. For triggering these activities, no more than 30 min stretch was enough. Therefore, the described responses were mostly the post-stretching ones. On the other hand, on the intracellular level the

effects of stretching or non-stretching a tissue piece onto an elastic substrate could be revealed already within few minutes after starting the experiment: stretching promoted the development of microfilament bundles and the associated lateral cell-cell contact areas, while in the absence of stretching cell-cell contacts have been immediately reduced and cells dissociated (Beloussov *et al.*, 1988).

4. Same experiments made with pieces of a blastocoel roof (presumptive ventral ectoderm) showed similar short-termed and post-stretching results. In particular, in post-stretched explants an extensive cell intercalation, never occurring in the similar non-stretched pieces, took place. As in the previous experiments, the intercalation cell movements were going on perpendicularly to the stretching direction. As a result, the cells, initially elongated in the stretching direction (Fig. 4D), returned later on to the approximately isodiametrical shapes (Fig. 4F). At the same time, a shape of the stretched pieces was more complicated, up to the formation of the variable «embryoid bodies» with one or the both poles inflated and the central or «tail» region narrowed (Fig. 5). Later on we discuss these results in more detail.

5. A rearrangement of tensile fields within early gastrula marginal zone considerably affects cell differentiation, distorting the presumptive cell fates (Beloussov and Snetkova, 1994).

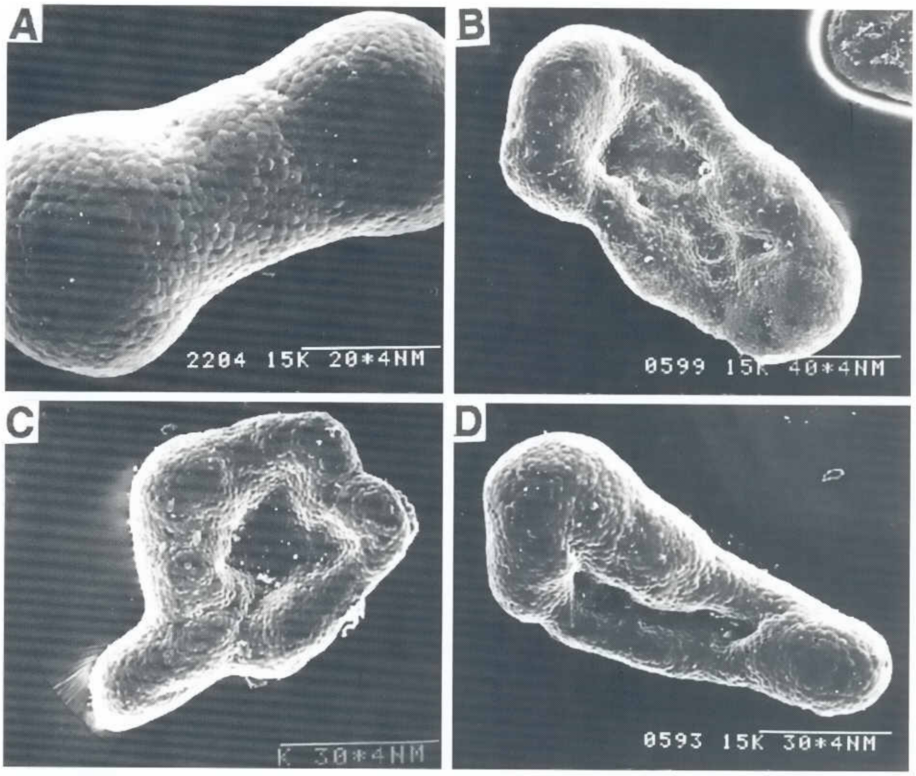

Fig. 5. Different shapes taken by explants similar to those shown in Fig. 4, 4 h after the cessation of stretching *(see also Fig. 4E).Those in C, D especially resemble «embryoid» structures. Bars: A, 200 mkm; B, 400 mkm; C and D, 300 mkm.*

Some single observations made in our lab indicated also the existence of regular patterns of mechanical stresses at the pregastrula stages of sea-urchin embryos (Beloussov and Bogdanovsky, 1980) and in the ectodermal and mesodermal tissues of 2-3 day old chick embryos (Beloussov and Naumidi, 1977 and unpublished).

A comparison with other labs data

Although only few researchers were interested in studying mechanical stresses in developing animal embryos, their scattered data, while being collected together, look impressive. To mention only two extremes: a hydra bud cannot grow without being stretched by a turgor pressure within its gastral cavity (Wanek *et al.*, 1980), and the same is true for the development of a chicken blastoderm, which starts to stretch itself immediately after the onset of incubation (Kucera and Monnet-Tschudi, 1987). A complicated regular pattern of mechanical stresses exists also within embryonic neural tube, the neuroepithelial cells after its relaxation losing their normal orientation and becoming degraded (Saveliev, cited from Beloussov *et al.*, 1994). A necessity and a sufficiency of tensile stresses exerted by individual cells for inducing a morphological organization within a huge cell collective have been demonstrated by Harris *et al.* (1984) with the use of an artificial elastic substrate.

In spite of extensive gaps in our knowledge of mechanical stresses and their dynamics (for example, nothing is known about these in the insect eggs and embryos, although if their geometry indicates the existence of quite non-trivial stress patterns) one may definitely claim, that the tissues of all the developing organisms studied in this respect are mechanically stressed, the stresses are organized according to regular patterns and clearly correlated with the subsequent morphogenetical processes; experimental modulations of stresses lead to considerable morphogenetical abnormalities. Under these circumstances, the next question will be: what might be the place of mechanical stresses in the regulatorial feedbacks which, according to a self-organizational paradigm, are driving forth development?

A possible involvement of mechanical stresses into regulatorial feedbacks: a hypothesis of a hyperrestoration of mechanical stresses.

As it have been already mentioned, in a living physiologically active embryonic tissue a state of artificially caused mechanical relaxation cannot last more than few dozens of minutes: an entire embryo or its tissue piece behaves in such a way as if it tended not only to restore, but even to hyperrestore the relaxed tensions in various manners, depending mostly upon the initial and/or border conditions. In particular, if the edges of a tissue piece are fixed, the tensions are often restored due to tangential contraction and the radial extension (columnarization) of its cells; in the case of free edges a tissue piece tends to curl; increase of a turgor pressure within a newly formed internal cavity is another way for doing the same (see for more details Beloussov, 1994; Beloussov *et al.*,

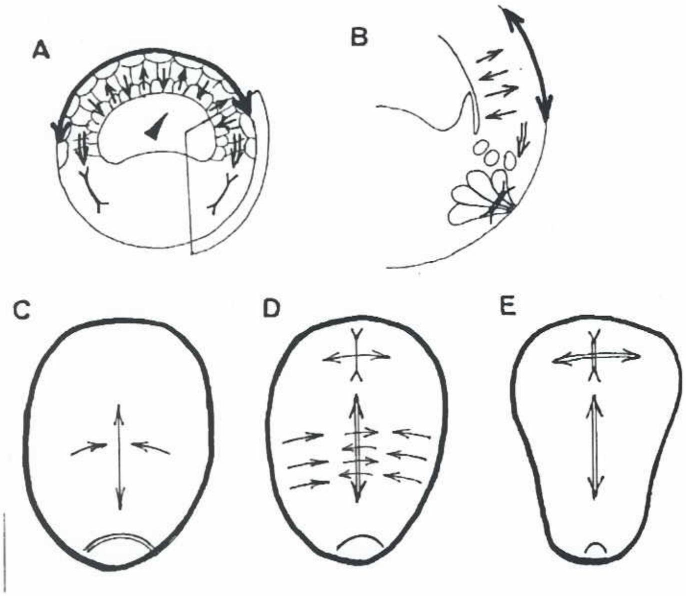

Fig. 6. A sketch of the passive and active MS which may drive the development of an amphibian embryo from blastula up to a tail-bud stage. *Passive stresses are denoted by single-line bidirectional arrows while the active ones are marked by double-line arrows. Small arrows pointing to opposite directions indicate cell intercalation movements. Other designations as in Fig. 1.* (**A**) *blastula stage.* (**B**) *a marginal zone of an earl gastrula, framed in A.* (**C-E**) *MS patterns at the successive stages of a neurulation, dorsal view, anterior pole to the top.*

1994). On the other hand, an artificially stretched tissue piece tends to diminish the tension by redistributing its cell material in such a way that the piece contracts in the direction perpendicular to the stretching. That corresponds to the so-called cell intercalation, an ubiquitous morphogenetical process (Keller, 1987). The hyperrestorative reactions (HRR) seem to be rather wide-spread (see Beloussov *et al.*, 1994 for details). That means that the changes in the stationary stress values are capable of activating some cytoskeletal and membrane-bound mechanisms which might produce new stresses, etc. In such a way closed feed-back circuits may be created, playing a role not only in the artificial situations, but in the normal development as well. Let us trace now some of these, more or less schematized feedbacks:

(1) A planal (unilayered) feedback loop. Suggests that a more or less planar piece of a (multilayered) cellular sheet with its edges fixed is stretched by an external force (applied either to the edges, or to the basal surface). That will switch-on a HRR of cell intercalation. As a result, a more or less prolonged central part of a piece will elongate itself, while its mechanically fixed polar regions relax or even compress. Within these parts the HRR will be expressed in the contraction of the apical cell surfaces, that is in the columnarization and/or immigration of cells. This will lead, in its turn, to further stretching of a central part, this one to a prolonged cell intercalation and so on. In such a way a positive feedback loop is created which may transform an initially homogeneous tissue piece into that with the elongated central part and inflated polar regions, may be bearing invagination holes. This is just what have been observed in the corresponding experiments. The resulted structures (see, in particular, Fig. 5) resemble an archetype of a vertebrate embryo.

(2) Increase of a curvature. Suggests that a tissue piece is slightly bent by an external force. As a rule, this will lead to the stretching of its convex surface and the compression of the concave one. HRR will be directed now towards further contraction of the concave surface and the extension of the convex surface, that is, to an autonomous increase of an initiated curvature. This is just what is reproduced by the model of epithelial folding (Beloussov and Lakirev, 1991).

(3) Interaction of two adjacent cell layers. Suggests that two cell layers A and B are mechanically bound to each other and one of them (A) stretched by an external force. According to (1) this will lead to its active elongation due to cell intercalation and thus to passive stretching of B layer. The latter will trigger cell intercalation in B, which starts now to stretch A, etc. In such a way a positive feedback loop is established, stimulating the coordinated elongation of both A and B. Such may be the way for the pre- and postinvoluted cell layers to interact with each other during gastrulation, promoting thus epiboly and involution at the same time.

Let us employ now this feedback for interpreting the succession of the morphogenetical events, taking place between blastula and neurula stages of an amphibian embryo (Fig. 6).

Normally blastocoel is under a substantial osmotically-driven turgor pressure of about 325 mOsm (see Beloussov *et al.*, 1994, for discussion). We suggest that it is a stretching of the blastocoel roof by this turgor pressure which initiates cell intercalation in the roof and, thus, its active extension (Fig. 6A). This view is supported, on one hand, by cessation of cell intercalation (and even by increase of a number of cell layers) in the mechanically relaxed blastulae (Beloussov and Luchinskaia, 1995a and in preparation) and, on the other hand, by stimulation of cell intercalation after artificial stretching (Beloussov and Luchinskaia, 1995b).

Meanwhile, according to (1), the active roof extension will lead to cell columnarization and/or immigration on its edges (Fig. 6B), thus outlining the marginal position of a circular blastopore. The dorso-ventral differences in the morphogenetical movements of a marginal zone are, according to an accepted view, firstly outlined during the rotation of fertilization and should be taken in our construction as initial conditions. Meanwhile, what can be obtained by applying (1) to the marginal area is that even quite slight initial regional differences in the contractile activity should be largely reinforced, making a uni-axial pattern most of all stable.

Actually, however, any axial stretching is accompanied by a transversal schrinkage. This is what initiates, in our view, the geometrical reconstructions in the dorsal part of embryo, associated with neurulation and the axial organs formation. As previously, the stretching initiates cell intercalation (which actively extends the dorsal embryo part further longitudinally) while even slight transversal compression or relaxation stimulates cell columnarization, which is self-restricted towards its flanks (Belintzev *et al.*, 1987). If relaxing, prior to this stage, the circumferential tensions of the embryonic surface, a convergent later-medial cell intercalation will be largely inhibited, while cell columnarization will spread ventrally much more than normally (Beloussov *et al.*, 1990). Further action of (1) promotes the longitudinal compression, or at least relaxation anteriorly to the stretched dorsal area, that is in the presumptive head region (while posteriorly to it the relaxation is largely hampered by a continuous involution). Hence, the head region contracts longitudinally and extends transversely, taking thus its typical configuration. A same way of reasoning can be largely prolonged. At the present moment we see our main task in continuing its experimental approval.

Concluding remarks

One may suggest that the molecular cell biology of a near future will become to a large extent, a science about "active solid bodies", that is the energized structures capable of generating and transmitting mechanical stresses and actively responding to them (see, e.g., Agutter, 1994, Forgacs, 1995). This approach can, hopefully, largely promote our knowledge of genes regulation (Opas, 1994) and the understanding of the higher levels phenomenae, such as morphogenesis. The above presented results and considerations are no more than the first tentative steps on this way.

Acknowledgments

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