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CELL DIFFERENTIATION AND PROLIFERATION

Influence of the Activator of Transcription *gal4* on Growth and Development of Embryos and Embryonic Cells in Primary Cultures of Sand Dollar

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Abstract—In order to solve many tasks of biotechnology, constant lines of the cells of marine invertebrates with a high growth potential are required, which are absent at present. We used the universal activator of transcription *gal4* to change the degree of expression of genes of growth factors in embryonic sea urchin cells and, thereby, increase their proliferative activity. The fertilized sea urchin eggs and dissociated embryonic cells at the blastula stage were treated with plasmids containing both the functional gene *gal4* and the gene devoid of the regions encoding the activator domain. The transfection of embryonic sea urchin eggs with the functional gene led to cell dedifferentiation and formation of tumor-like structures in the embryos or increased number of embryonic cells in culture. In the cells obtained from the transfected embryos, the pigments were found within two months of cultivation, whose absorption spectrum coincided with that of echinochrome.

Key words: sea urchin, embryos, activator of transcription, cell culture, echinochrome.

More than 5000 chemical compounds from marine organisms were described during the past 10 years and many of these natural bioactive metabolites have a high pharmacological potential. Sea urchins are also a source of valuable biologically active substances, specifically the sand dollar *Scaphechinus mirabilis* contains naphtochinoid pigments, among which echinochrome is the most essential (Nishibori, 1957). The drug Histochrome developed on its basis has unique therapeutic properties (Elyakov *et al.*, 1999a, 1999b; Fedoreyev *et al.*, 2000). However, the large scale procurement may lead the organisms-producents to the brink of disappearance. The *in vitro* production of biologically active substances may become an alternative to chemical synthesis or aquaculture, provided that the cultured cells have a high growth potential.

One of the promising ways to solve this problem is to lead the cells out of the state of differentiation by changing the expression of the genes of growth factors. For this purpose we used the transcription factor encoded by the yeast gene *gal4*. This protein is an activator of transcription in heterologous eukaryotic systems (Ma *et al.*, 1988): it binds to the sequence TGACA in the promoter regions of genes and activates their expression. Such a motif was found in the promoter regions of the genes of growth factors of marine invertebrates (Delgadillo-Reynoso *et al.*, 1989; Yuh *et al.*, 1998).

The proteins–activators of transcription fulfill two functions in the same molecules, i.e., contain a DNA binding domain and a region activating transcription of the eukaryotic genes (Ptashne and Gann, 1990). We used the gene engineering construct, plasmid MA563 containing the functional gene *gal4* (Ma and Ptashne, 1987) and, as the control, plasmid MA564 containing the gene devoid of the region that encodes the activator domain (Fig. 1).

The incorporation of the plasmid DNA and its expression were earlier demonstrated by gene-specific PCR and reverse transcriptase PCR (RT-PCR) (Bulgakov *et al.*, 2002). The aim of the present work was to study the properties of embryonic sand dollar cells after their transfection with the gene *gal4*.

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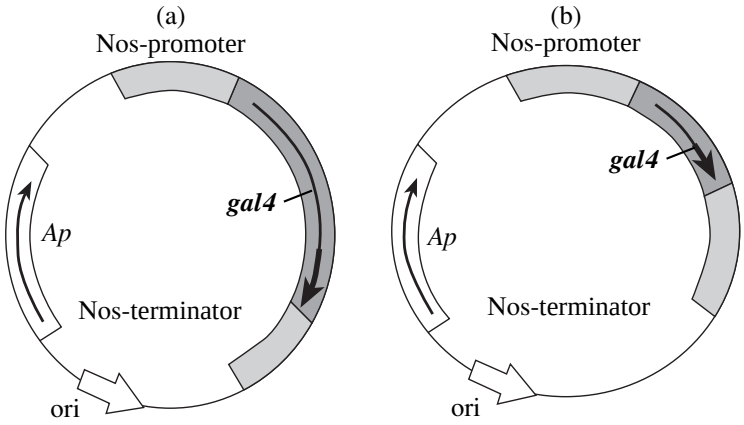

Fig. 1. Plasmid constructs used in the experiment: (a) pMA563 containing the functional gene *gal4*, (b) pMA564 containing the nonfunctional gene *gal4*. *Ap*, gene of resistance to ampicillin; ori, site of replication origin.

MATERIALS AND METHODS

Sea urchins (*S. mirabilis*) were collected in the Vostok Bay, Sea of Japan, and, during the entire experiment, kept in bath-tubs with running aerated sea water. Before the experiments, the animals were washed two–three times with UV-irradiated filtered sea water. The spawning of sea urchins was stimulated by injections of 0.5 M KCl. The embryos were reared to the blastula stage (12 h after fertilization) and then dissociated in 0.25% collagenase at 19°C for 20 min, as described earlier (Odintsova *et al.*, 1994).

We used plasmid DNA (pMA563 and pMA564) purified by the method of alkaline lysis (Maniatis *et al.*, 1982) and kindly provided by Dr. M. Ptashne (Harvard University, USA). The nativity of plasmid DNAs was tested by electrophoresis in 0.8% agarose gel (Pharmacia, Sweden). Within 15–20 min after fertilization, the eggs were treated with plasmid DNA, as described earlier (Bulgakov *et al.*, 2002). Exogenous DNA was transferred in the presence of polyethylene glycol (PEG) (Loba Feinchemie, Austria) with the molecular mass 4000 Da at a final concentration of 7.5%. The control and transfected embryos developed in a climatic chamber (19°C) in Petri dishes.

The cells of primary cultures from the blastula stage were incubated with plasmid DNA (0.01–2 μg) for 1 min and then, after addition of PEG, in the climatic chamber (18–19°C) for another 15–20 min. The cells were washed in 10-fold volume of sterile sea water, resuspended in a modified Leibovitz medium (Flow Laboratories, USA) (Odintsova and Khomenko, 1991), and cultivated in 24-well plates (Corning, USA) at 19°C. The initial concentration of cells at plating was $2–6 \times 10^6$/ml.

The proliferative activity of cells was estimated by direct counting during the entire period of cultivation. For registration of DNA synthesis in the cells, ^{3}H-thymidine (18.5×10^4 Bq/ml, specific activity 307.1×10^{10} Bq/mmol, Amersham, UK) was introduced in the wells. In order to arrest incorporation, an equal volume of cold 10%trichloroacetic acid was added to the wells. The mixture was kept at 0°C for 30–60 min and the contents of wells was then transferred onto membrane filters (0.45 μm), as described earlier (Odintsova *et al.*, 1999), Dry filters were placed in vials containing 5 ml toluene scintillator each. Radioactivity was measured on a LC-30 scintilation counter (Intertechnique, France) for 100 s. No less than three parallel experiments were carried in each case.

For preparation of semithin sections, the larvae were fixed by 2.5% glutaraldehyde (Sigma, USA) on 0.05 M cacodylate buffer with 2.14 mg/ml NaCl for 2 h, washed by cacodylate buffer (20 min), postfixed by 1% OsO_4 on the same buffer for 75 min, again washed by cacodylate buffer (20 min), and passed through ascending alcohols. The materials were embedded in Araldite (Sigma). Semithin sections (0.75 and 0.5 μm) were made on an HM360 Microm rotation microtome (Carl Zeiss, Germany). The sections were stained by methylene blue.

Chinoid pigments were extracted from embryonic cells as described by Koltsova *et al.* (1981). The pellet of cells was homogenized in 96° ethanol, an equal volume of 3N HCl was added to the homogenate, and the resulting mixture was left for infusion for 20 min. The extract was then poured off, the residue was quantitatively washed by ethanol and united with the extract. The resultant mixture was then clarified by centrifugation at 3000 rpm for 15 min. The absorption spectrum of the obtained extract and echinochrome solution of known concentration in acidified ethanol was recorded on a Specord M-40 spectrophotometer (Carl Zeiss).

The data we obtained were statistically processed using the FraphPadPrism software and presented as mean ± standard error. The data were checked by paired Student's *t*-test. The level of 0.05 was chosen as the minimal value of statistical difference in all experiments.

RESULTS

Transfection of the embryonic cells of primary cultures obtained from the blastula stage led to significant changes in their proliferative activity. The total number of cells in primary cultures treated with pMA563 and pMA564 increased 4.4–4.6- and 2.7–2.9-fold, respectively, during two months of cultivation (Fig. 2). In the control cultures, the growth of cells was insignificant (within the limits of experimental error). Note that the control culture died already in the beginning of the second week of cultivation. On the contrary, the cells treated with the functional gene *gal4* survived during two–three months. In addition, the level of DNA synthesis (Fig. 3) in these cultures increased 2.2–2.3-fold, as compared to the control cells.

The treatment of fertilized sea urchin eggs (until the two-cell stage) with a plasmid containing the functional gene *gal4* (pMA563) led to the appearance of abnormally developing larvae already within 48–52 h of cultivation. The transfected larvae had a rounded shape and their developed was slowed down, while in the control the development was normal: plutei were formed by three days of cultivation (Fig. 4a). In 15–20% of transfected embryos, cell aggregates were formed on the surface as tumor-like structures (Fig. 4b). These aggregates varied in morphology, number, and position on the larva and many of them were pigmented. After transfection of the nonfunctional gene *gal4* (pMA564), tumor-like structures were also formed at the early developmental stages, but their number was much less (table). Unlike the control larvae (Fig. 4c), in the embryos treated with the functional gene, the blastocoel represented a small cavity in the center, while the major part of the volume was occupied by primary mesenchyme cells, some of which had elongated shape with phyllopodia (Fig. 4d). Among them, atypical cells could be seen: larger and having rounded shape (Fig. 4d, arrows). The tumor-like aggregates were represented by a group of cells loosely positioned and with a great amount of extracellular matrix.

The dynamics of formation of the tumor-like structure in the pMA563 treated sea urchin larvae, as compared to the control, are shown in Fig. 5. The highest number of larvae with tumor-like aggregates was observed by the beginning of the third day of cultivation (48–55 h): 13.5 ± 1.4% versus no more than 2% in the control. During the following seven–nine days, the number of larvae with tumor-like structures gradually decreased and the number of separate cells increased. In the experiments with the plasmid containing the functional gene (pMA563), the formation of tumor-like structures was dose-dependent, while in those with pMA564, the number of tumor-like structures did not depend on the plasmid concentration (Fig. 6).

The control larvae and larvae transfected with the nonfunctional gene *gal4* died without additional feeding within 10–14 days, while those transfected with the functional gene dissociated into separate cells within one month of incubation in sea water without any additions. Among them, the amount of cells containing red-brown pigment reached 50–60%. The absorption spectrum of pigments isolated from the culture of cells of transfected embryos practically coincided with that of echinochrome (Fig. 7).

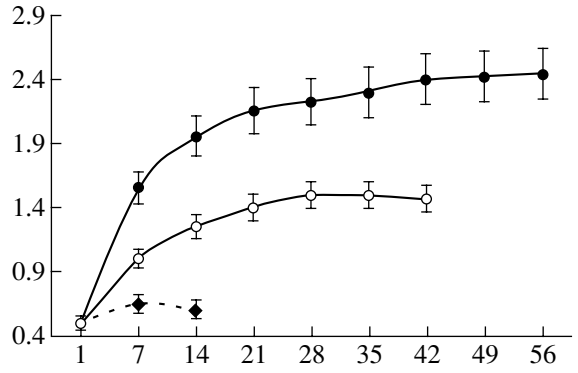

Fig. 2. Dynamics of growth of primary cell cultures obtained from the embryos of the sand dollar *S. mirabilis* at the blastula stage. Abscissa: time of cultivation, days; ordinate: number of cells per well, $\times 10^6$. Cells: control (◆) and treated with plasmids pMA563 (●) and pMA564 (○).

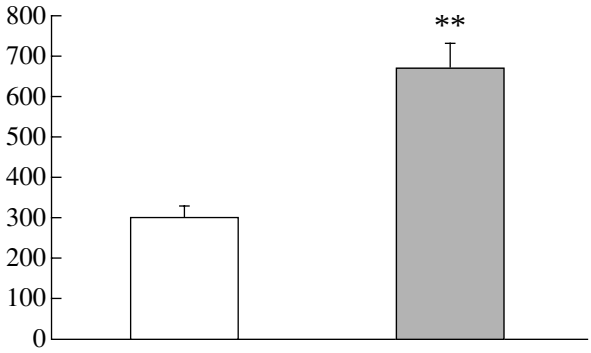

Fig. 3. Level of ^{3}H-thymidine incorporation (cps/million cells) in the cells of primary cultures after three days of cultivation. Cells: control (□) and after treatment with the plasmid containing the functional gene *gal4* (▩). ** $p < 0.01$.

Effects of plasmid DNAs (pMA563 and pMA564) on embryogenesis of sea urchins

Kind of treatment*	Number of embryos with tumor-like structures (48–55 h after fertilization), %
Control	1.3 ± 0.6
PEG	2.1 ± 0.5
pMA564	2.0 ± 0.6
pMA563	14 ± 1.4

*The eggs were treated within 15–20 min after fertilization.

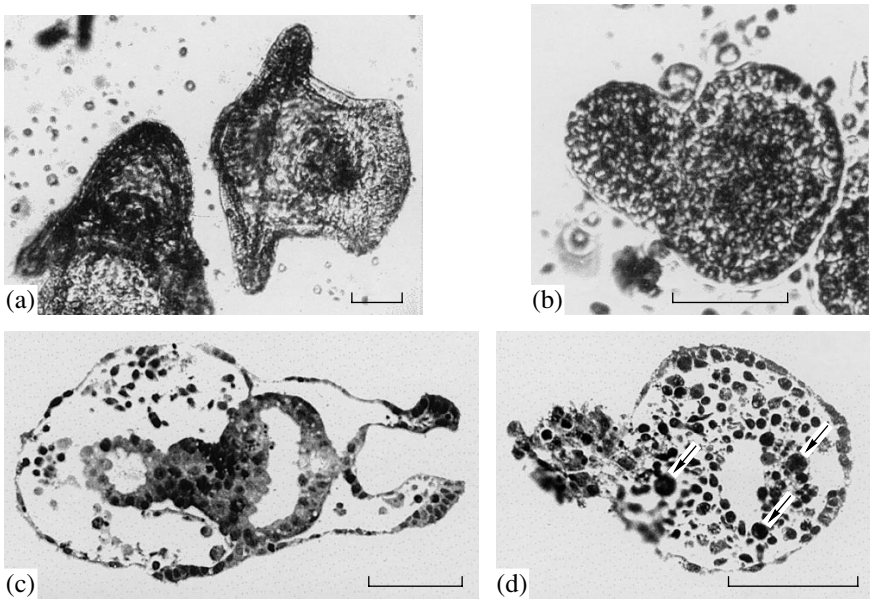

Fig. 4. Larvae of *S. mirabilis* within 55 h after fertilization: (a, c) control (pluteus); (b, d) larvae after treatment with plasmid pMA563; (a, b) external appearance; (c, d) semithin sections. Scale: 50 μm.

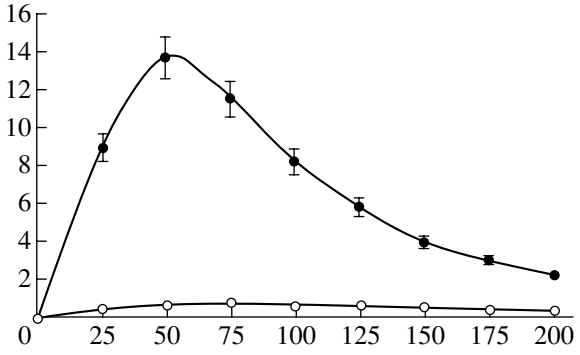

Fig. 5. Dynamics of formation of tumor-like structure during cultivation of embryos transfected by plasmids pMA563 (●) and pMA564 (○). Abscissa: time of cultivation, h; ordinate: embryos with tumor-like structures, %.

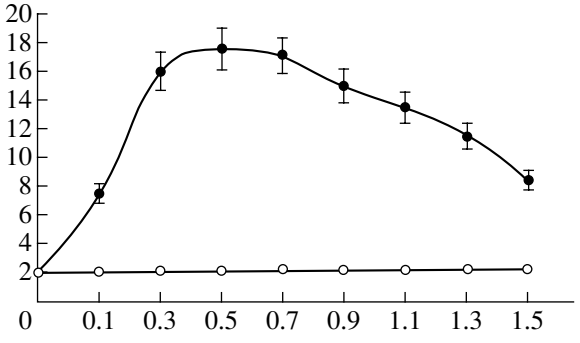

Fig. 6. Influence of the plasmid DNA concentration (abscissa, μg/sample) on the amount of tumor-like structures (ordinate, %) in the embryos of *S. mirabilis*. Plasmids: (●) pMA563, (○) pMA564.

DISCUSSION

The data presented suggest that the gene of yeast universal activator of transcription *gal4* is capable of affecting cell differentiation in sea urchins. The molecular mechanisms of this process are as yet unknown. According to the model of Ptashne and Gann (1990), in order to ensure the functioning of *gal4*, an upstream activation sequence for GAL4 should be located in the above lying part of the promoter of the gene to be activated (Giniger *et al.*, 1985). This sequence consists of several repeating 17-nucleotide sequences (17-meres) providing for recognition of GAL4, which contain the sequence TGACA binding to the N-terminal domain of GAL4. Unfortunately, the above lying promoter regions of the genes of growth factors of sea urchins have been sequenced only partially, to approximately 150 bp (Delgadillo-Reynoso *et al.*, 1989). These regions contain binding sites for GAL4, but no 17-meres. It cannot, however, be excluded that the binding sites for GAL4 are inside the regulatory genes of marine invertebrates. This suggestion is corroborated by a significant difference between the activities of functional (pMA563) and nonfunctional (pMA564) gene *gal4* (Bulgakov *et al.*, 2002). Computer search in GenBank has shown that 17-meres are present in DNA of mammals and plants. However, such regions were not found in DNA of sea urchins, possibly, due to insufficient knowledge about their regulatory genes. Our further work along these lines will consist in identification of the protein GAL4 in sea urchin embryos, determination of its stability, and search for gene(s) with which the activator interacts.

We propose that in the beginning of embryogenesis, when the cells of marine invertebrates are most suscep-

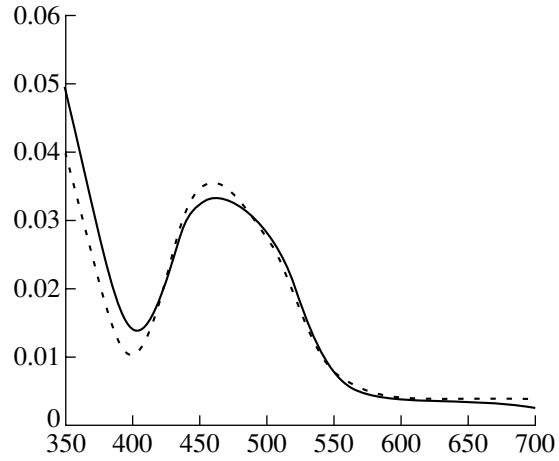

Fig. 7. Absorption spectra of echinochrome (- - -) and pigments (–) from cells of the transfected embryos. Abscissa: wavelength, nm; ordinate: optic density.

tible to the influence of external factors (Dixon, 1983), transfection of the activator and its subsequent expression enhance continuation of cell proliferation and lead to cell dedifferentiation and formation of tumor-like structures in sea urchin embryos or significantly increased number of embryonic cells in culture. The observed increase of cell number in the cultures treated with the nonfunctional gene *gal4* indicates to a possible cell growth as a result of stimulating effect of plasmid pMA564. Further experiments are needed to disclose the causes of this phenomenon.

As the time of cultivation increases, the relative amount of larvae with tumor-like structures decreases, while that of separate cells covering the dish floor by a dense layer increases. We explain this effect by gradual degradation of rather "fragile" structures. The efficiency of transfection as was earlier shown by Bulgakov *et al.* (2002) is the highest at the early stages of sea urchin development. This appears to be due to the fact that in this case, the area of dividing cells is maximal, while their amount is minimal. This is of especial significance in the case of transfection with the use of PEG, since this agent enhances the increased adhesion of the plasmid DNA to the surface of cells under treatment (Maniatis *et al.*, 1982). The dose-dependent patter of formation of the tumor-like structures (in the case of pMA563) confirms that the increase in the number of tumor-like structures is related to the activity of the functional gene *gal4*.

The red-brown coloration of cultured cells from the embryos transfected by the functional gene is similar to that appearing *in vivo* in the embryos at the prism stage due to the synthesis of naphtochinoid pigments (Koltsova *et al.*, 1981). The absorption spectrum of pigments from the cultured cells practically coincides with that of echinochrome, while the amount of naphtochinoid pigments in cells obtained from the transfected embryos, as calculated per one cell (Kominami and Takata, 2002), increased 9–10-fold, as compared to the cells of normal pluteus larvae *in vivo* (Koltsova *et al.*, 1981).

The regulation of proliferative activity of the cells of marine invertebrates by the genes–activators of transcription capable of increasing the expression of growth factors can be considered as a new promising approach in marine biotechnology, which can lead in future to the production constant cell lines of marine invertebrates.

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