Mar. Biotechnol. 4, 480–486, 2002
DOI: 10.1007/s10126-002-0036-7

MARINE BIOTECHNOLOGY



# *Gal4*-Gene-Dependent Alterations of Embryo Development and Cell Growth in Primary Culture of Sea Urchins

V.P. Bulgakov,[1,*] N.A. Odintsova,[2] S.V. Plotnikov,[2] K.V. Kiselev,[3] E.V. Zacharov,[1] and Y.N. Zhuravlev[1]

[1]*Biotechnology Lab., Institute of Biology and Soil Science of the Far East Branch of Russian Academy of Sciences, Prosp, Stoletija 159, Vladivostok 690022, Russia*
[2]*Institute of Marine Biology of Far East Branch of Russian Academy of Sciences, Vladivostok 690041, Russia*
[3]*Far East State University, Vladivostok, Russia*

**Abstract:** Primary cell cultures from sea urchins have a low proliferative level that prevents the establishment of long-term cultures. To increase expression levels of the genes regulating cell growth in sea urchins, and thus enhance cell growth, we used the transcriptional activator gene *Gal4* found earlier in yeast. Sea urchin embryos were treated with plasmid DNA containing the *Gal4* gene. Expression of the transgene was confirmed by reverse transcriptase polymerase chain reaction. When the fully functional gene was used, embryos effectively formed teratoma-like structures after 50 to 55 hours of cultivation. In contrast, the *Gal4* gene, devoid of acidic activating regions, possessed little activity as a teratogen. The *Gal4*-treated cells in blastula-derived culture showed higher DNA synthesis and higher proliferative activity than control cells. We suggest that formation of the teratoma-like structures in embryos, activation of DNA synthesis, and significant increase of cell number in embryo-derived cell cultures could be attributed to *Gal4* gene action.

**Key words:** sea urchin, embryos, *Gal4* gene, transcriptional activator, cell culture.

## INTRODUCTION

Primary cell cultures of marine invertebrates are widely used to investigate particular aspects of embryology and molecular and cell biology. However, many problems of biotechnology cannot be approached without continuous cell lines from these animals. Sea urchins are an attractive object for biotechnological studies because of their ability to produce a broad spectrum of biologically active quinones (Koltsova et al., 1981). Unfortunately, primary cell cultures from echinoderms have a low proliferative level, and cell degeneration predominates over proliferation. Therefore, all efforts aimed at obtaining long-term cell lines of echinoderms have so far failed. It is well established that to obtain a continuous cell line of an animal, tissues with high growth potential such as embryonic or neoplastic tissues should be used.

Studies have revealed that expression of genes involved in the regulation of embryogenesis and development of sea urchins is mediated by a complex and extended *cis*-regulatory system (Coffman et al., 1996; Koike et al., 1998; Yuh et al., 1998). These data led us to postulate that modulation of expression patterns of these genes could be a useful tool to trigger accelerated cell growth. Activation of cell growth

Received June 1, 2001, Accepted January 22, 2002.
*Corresponding author: fax 007-4232-310193; e-mail bulgakov@ibss.dvo.ru

is thought to be sufficient for establishing a cell culture and thereby overcoming a bottleneck in marine invertebrate biotechnology. Two approaches may be used for modulation of gene expression. One approach includes using well-known transcriptional regulatory proteins, especially those that contain acidic activating domains and are considered as activators of a "universal" type, e.g. GAL4, GCN4, and VP16 (Lin and Green, 1991; Greenblatt, 1991). The other approach includes treatment of the cells by substances possessing teratogenic activity. Hopes of finding a correlation between gene activity and teratogenic activity have in some cases been fulfilled. LiCl, as a teratogen, was used to study the expression pattern of the *Endo16* sea urchin gene, which encodes a polyfunctional secreted protein of the midgut in the late embryo. LiCl treatment abolished the negative effect of the 3 repressor modules of *Endo16* gene and caused them all to act instead as transcriptional stimulators (Yuh et al., 1998).

Our experiments were carried out with the yeast *cis*-acting *Gal4* gene. *Gal4* and its derivatives, which work with high efficiency in yeast, stimulate transcription of target genes in mammalian, *Drosophila*, and plant cells (Ma et al., 1988). Regulatory elements of *Gal4* have been used to study expression patterns of the sea urchin orthodenticle-related genes (Mao et al., 1996; Kiyama et al., 1998).

A useful prerequisite to our work was fulfilled in the publication of the nucleotide sequence of the sea urchin epidermal growth factor gene, which could contain a GAL4-binding site in the upstream promoter region (Delgadillo-Reynoso et al., 1989). Two derivatives of the *Gal4* gene were used: pMA563 and pMA564 (Figure 1). The first construction contains the functional *Gal4* gene, which consists of both nucleotide regions responsible for transcriptional activation, according to the Ptashne-Gann model (Ptashne and Gann, 1990). The second construction, pMA564, which has been used as a control plasmid, lacks acidic activation domains and fails to activate transcription in yeast, mammalian, and plant cells (Ma et al., 1988). We chose to use a recombinant *Gal4* gene containing acidic activating domains linked with DNA-binding sites because the small protein encoded by this gene is stable in heterogeneous systems (Ma and Ptashne, 1987; Ma et al., 1988; Ornitz et al., 1991). We transformed sea urchin embryos as well as embryo-derived cells with the *Gal4* gene and showed that treatment resulted in formation of teratoma-like structures possessing neoplastic features and enhanced cell growth potential.

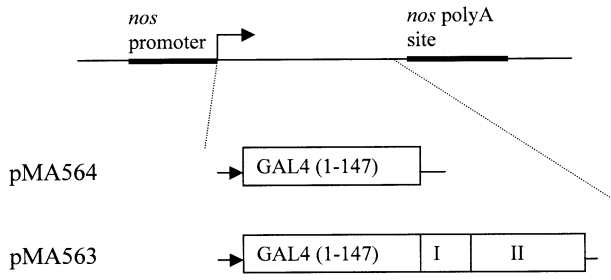


**Figure 1.** Maps of plasmids pMA563 and pMA564. DNA constructs encode the amino-terminal portions of the 881 amino acid GAL4 protein (1–147 amino acid residues, DNA-binding site), activator region I (residues 148–196), activator region II (residues 768–881). I and II are regions that encode acidic activator domains, interacting with a component of the transcriptional machinery (Ma et al., 1988; Ma and Ptashne, 1987; Lin and Green, 1991). The term *nos* promoter indicates promoter of the nopaline synthase gene of *Agrobacterium tumefaciens*; *nos* polyA site, polyA site of nopaline synthase gene (Bevan et al., 1983). The constructs are not drawn to scale.

## Materials and Methods

### Animals

Sea urchins (*Strongylocentrotus intermedius* and *Scaphechinus mirabilis*) were collected from Vostok Bay in the Sea of Japan. Experiments were carried over a 4-year period (1998–2001) at the Sea Experimental Station of the Institute of Marine Biology. Animals were washed 2 to 3 times with UV-irradiated seawater and maintained in tanks with aerated seawater as described (Odintsova et al., 1999).

### Plasmids and Reagents

Plasmid DNA samples (pMA563 and pMA564) were kindly provided by Jun Ma (Harvard University, Cambridge, MASS.). Standard techniques were used to transform *Esherichia coli* Tg2 strain by these plasmids (Maniatis et al., 1982). The *E. coli* TG2/pMA563 and TG2/pMA564 were grown in LB medium at 37°C with the addition of tetracycline (15 mg/L) and ampicillin (50 mg/L). The presence of plasmids in *E. coli* strains was confirmed by plasmid isolation via CsCl gradient centrifugation and restriction analysis. For the transformation experiments we used freshly isolated plasmid DNA. The *E. coli* strains were grown overnight, and plasmid DNA was isolated by alkaline lysis and purified as described (Maniatis et al., 1982). Reagents were purchased from Sigma Chemical Company

(St. Louis, MO.) and Serva Feinbiochemica GmbH & Co. (Heidelberg, Germany). Polyethylene glycol M 4000 (PEG) was obtained from Loba Feinchemie (Fischamend, Austria).

### Transformation of Embryos

Concentrated suspensions of sea urchin fertilized eggs or embryos at different stages of development (15 minutes, 45 minutes, 6 hours, and 12 hours after fertilization) were centrifuged at 500 *g* for 45 seconds and resuspended in sterile seawater (2500–3000 embryos/ml). The embryo suspension 500 µl was incubated with a solution of plasmid DNA (0.01–1 µg) for 1 minute. A further equal volume of 20% PEG solution (wt/vol, molecular weight 4000) in seawater was added drop by drop, and the embryos were incubated for 20 minutes at 19°C, washed stepwise in 9 vol of seawater, and centrifuged at 500 *g* for 1 minute. The pellet of embryos was resuspended in sterile seawater (250–300 embryos/ml), cultured in Petri dishes at 18°C, and observed to estimate the percentage of growth induced. Embryos without PEG and plasmid DNA treatment were used as controls.

### Transformation of Embryo-Derived Cells

For obtaining primary cell cultures, sea urchin embryos at the mesenchyme blastula stage (13 hours after fertilization) were collected onto a fine-meshed gauze, washed in $Ca^{2+}$-free and $Mg^{2+}$-free artificial seawater containing 100 U penicillin and 100 µg/ml streptomycin, twice rinsed with sterile seawater containing the same antibiotics, and then dissociated in a 0.25% collagenase solution for 20 minutes 19°C (Odintsova et al., 1994). Cells were resuspended at a concentration of $25 \times 10^6$/ml in a modified Leibovitz medium (1100 mOsmol), supplemented with 2% fetal calf serum, insulin (50 mg/L), α-tocopherol-acetate (1.75 mg/L), glutamine (100 mg/L), and gentamycin (40 mg/L). The resulting cell suspension was treated with PEG and plasmid DNA as described above and cultivated in 24-multiwell plates (Corning) at 19°C. Seeding concentration was 0.5 to $0.6 \times 10^6$ cells per well.

### RT-PCR Analysis

Total RNA from sea urchin embryos was extracted with TRIzol reagent (Life Technologies, Rockville, Md.) following the instructions of the manufacturer. Extracted RNA was digested with DnaseI (Amresco, Solon, Ohio) and purified with BlueSorb (Clonogen, St. Petersburg, Russia). Complementary DNAs were synthesized from 1 µg of RNA using RNA PCR Kit (Silex M, Moscow, Russia). The reactions were performed in 25-µl aliquots of reaction mixture containing RT buffer, 0.25-µl each of the 4 dNTPs, 1.5 µg of random 6-mer primer, 5U of Tth-polymerase, 1.2 mM $MnCl_2$ at 37°C for 1 hour. The 10-µl samples of reverse transcription products were then amplified by polymerase chain reaction (RT-PCR) with the following primers:

Primer 1 5′-TCCAACCAGGTGACAG-3′
Primer 2 5′-CCTCGAGAAGACCTTG-3′

The 2 primers were designed to flank a 720-bp fragment of the *Gal4* gene in pMA563 (regions 1–147 and II, see Figure 1). Amplification reactions were performed in volumes of 25 µl containing 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 2.0 mM $MgCl_2$, 0.01% gelatin, 0.1 mM Triton X-100, 0.2 mM of each dNTP, 0.2 µM of primer, and 1.0 U of *Taq* DNA polymerase. Analysis was performed in an UNO Thermoblock thermal cycler (Biometra, Göttingen, Germany) programmed for an initial denaturation step of 60 seconds at 94°C followed by 45 cycles of 60 seconds at 94°C, 60 seconds at 45°C, 90 seconds at 72°C, and a last cycle of 72°C for 420 seconds, using the fastest available transitions between each temperature. The PCR products were separated by electrophoresis in 1.5% agarose gel (DNA grade, Pharmacia, Piscataway, N.J.). The size of the fragment was estimated by comparison with synthetic markers.

### DNA Synthesis

The level of [$^3$H]thymidine incorporation in cells was determined as described previously (Odintsova et al., 1999).

### LiCl Treatment

LiCl (final concentration, 25 mM) was added as an aqueous sterile solution to pMA563-treated and twice-washed embryos immediately after the transformation procedure.

## Results and Discussion

### Transformation of Embryos

The first indication of *Gal4*-gene action could be detected within 55 to 60 hours of plasmid DNA treatment of *S. intermedius* and *S. mirabilis* embryos. Some of the

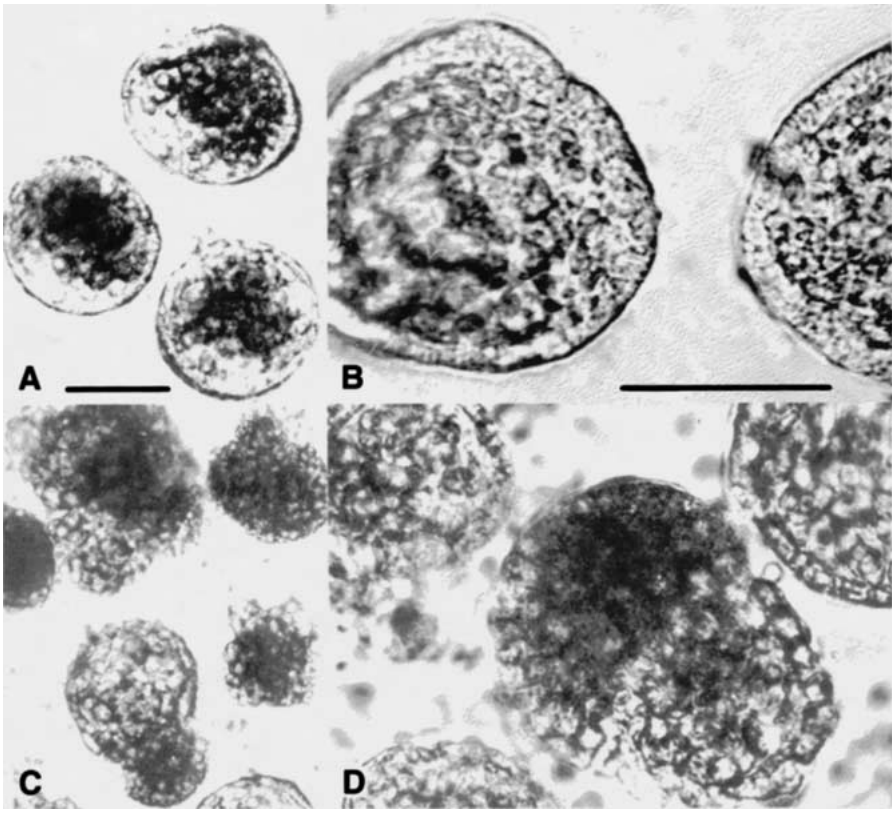


**Figure 2.** Formation of teratoma-like structures on embryos transformed by *Gal4* gene. **A, B:** Embryos treated with PEG. **C, D:** Embryos treated with PEG + pMA563. Embryos were cultivated for 60 hours after fertilization. Bar indicates 50 μM.

pMA563-treated embryos developed growths (Figure 2) consisting of undifferentiated cells with basophilic cytoplasm, large nuclei, and poorly differentiated nucleoli. We designated these growths "teratoma-like structures" (TLSs). During 4 years of work at the Vostok Experimental Station, 25 independent transformation experiments were carried out, and development of TLSs after transformation was repeatedly observed. The count of embryos based on a single typical experiment is given in Table 1. The control embryos did not form abnormal structures, with one exception (Table 1). PEG alone could cause abnormal development of embryos; up to 2% of treated embryos formed TLS. The effect of *Gal4* derivatives on development of the embryos of both sea urchin species was dependent on the time of treatment, and maximal efficiency of TLS formation was observed at 45 minutes after fertilization. Treatment of the embryos with pMA563 DNA resulted in the maximal effect, with approximately 1 of 3 embryos forming the teratoma-like structures. Some activity, however, showed pMA564: 6% of *S. intermedius* and 9% of *S. mirabilis* embryos formed TLS. Older embryos (6 and 12 hours after fertilization) were noncompetent for plasmid DNA treatment. The finding that pMA563 possessed much more activity than pMA564 provided evidence that the *Gal4*-gene effect was specific. In addition, pMA563 activity was shown to be dose-dependent, unlike pMA564 activity (data not shown).

Gene-specific PCR analysis revealed that DNA samples of pMA563- and pMA564-treated embryos contained *Gal4* gene sequences (data not shown). Using RT-PCR, we found that the *Gal4* gene was expressed in pMA563-treated embryos (Figure 3).

## Effect of LiCl

LiCl has been shown to interact with sea urchin regulatory genes on the transcriptional level (Koike et al., 1998; Yuh et al., 1998). To further characterize the putative role of *Gal4* on embryo development, we studied the effect of LiCl on TLS formation after *Gal4* treatment. LiCl, having being added to the incubation mixture, caused different abnormalities of embryo development. Some of them were morphologically similar to those caused by the *Gal4* gene. When used at a concentration of 25 mM after the *Gal4* treatment, LiCl decreased the formation of TLSs substantially (Table 2). The interaction between *Gal4* and LiCl is of particular interest: a clear LiCl response reinforces the likelihood that *Gal4* acts on the transcriptional level.

**Table 1.** Effect of pMA563 and pMA564 on Sea Urchin Embryo Development

| Treatment | Time after fertilization | *S. intermedius* embryos, % | | | *S. mirabilis* embryos, % | | |
|---|---|---|---|---|---|---|---|
| | | Normal | Teratoma-like | Under-developed | Normal | Teratoma-like | Under-developed |
| Control | | 86 | 0 | 14 | 91 | 1 | 8 |
| PEG | | 0 | 0 | 100 | 92 | 1 | 7 |
| PMA564 | 15 min | 67 | 5 | 28 | 87 | 1 | 12 |
| PMA563 | | 55 | 10 | 35 | 88 | 4 | 8 |
| Control | | 81 | 0 | 19 | 95 | 0 | 5 |
| PEG | 45 min | 12 | 2 | 86 | 90 | 1 | 9 |
| PMA564 | | 42 | 6 | 52 | 42 | 9 | 50 |
| PMA563 | | 31 | 28 | 41 | 35 | 30 | 35 |
| Control | | 96 | 0 | 4 | 94 | 0 | 6 |
| PEG | 6 h | 15 | 2 | 83 | 98 | 0 | 2 |
| PMA564 | | 43 | 1 | 56 | 94 | 0 | 6 |
| PMA563 | | 84 | 0 | 16 | 94 | 1 | 5 |
| Control | | 100 | 0 | 0 | 94 | 0 | 6 |
| PEG | 12 h | 94 | 0 | 6 | 96 | 0 | 4 |
| PMA564 | | 99 | 0 | 1 | 95 | 0 | 5 |
| PMA563 | | 100 | 0 | 0 | 94 | 0 | 6 |

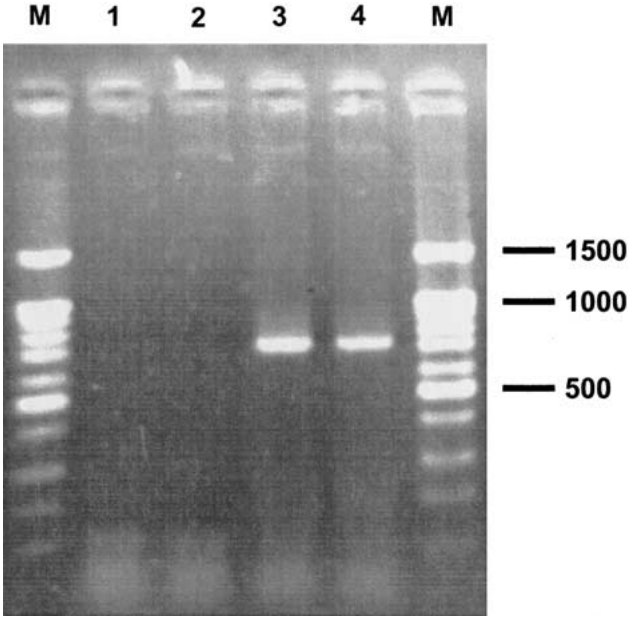


**Figure 3.** RT-PCR detection of messenger RNA of the *Gal4* gene in transformed sea urchin embryos. RNA samples were isolated at 55 hours after transformation from control *S. mirabilis* embryos (lane 1) and control *S. intermedius* embryos (lane 2); transformed by pMA563 *S. mirabilis* embryos (lane 3), and transformed by pMA563 *S. intermedius* embryos (lane 4). Lane M, synthetic marker.

Somewhat of a paradox exists with regard to the *Gal4*-LiCl effect on embryo formation since both *Gal4* and LiCl could act as transcriptional activators. It is possible that LiCl affects the permeability of sea urchin membranes and thus decreases uptake of *Gal4* plasmid DNA. However, this is thought to be unlikely because embryos were washed before LiCl treatment. An alternative explanation for the *Gal4*-LiCl interaction exists. It is known that an excess of one activator can inhibit activity of another (Ptashne and Gann, 1990). In our experiments an excess of LiCl could abolish activity of the *Gal4* gene, and vice versa.

## Embryo-Derived Primary Cell Culture of *S. intermedius*

Cells of primary culture were obtained from *S. intermedius* embryos by collagenase treatment and transformed as described in "Materials and Methods." Transformed cells demonstrated rapid growth (Figure 4) and formed a monolayer during 3-day cultivation. In control wells the cell number increased much more slowly, and cell granulation and general deterioration followed by cell lysis occurred after 14-day cultivation. Nine subcultivations of the actively growing transformed cells were carried out for 3

**Table 2.** Effect of LiCl Treatment on the *Gal4* Gene Effect*

| | *S. intermedius* embryos, % | | | *S. mirabilis* embryos, % | | |
|---|---|---|---|---|---|---|
| Treatment | Normal | Teratoma-like | Under-developed | Normal | Teratoma-like | Under-developed |
| Control | 75 | 0 | 25 | 36 | 0 | 64 |
| pMA563 | 21 | 20 | 59 | 21 | 21 | 58 |
| LiCl | 15 | 14 | 71 | 38 | 9 | 53 |
| pMA563 + LiCl | 14 | 5 | 81 | 40 | 0 | 60 |

*Embryos were treated at 45 minutes after fertilization.

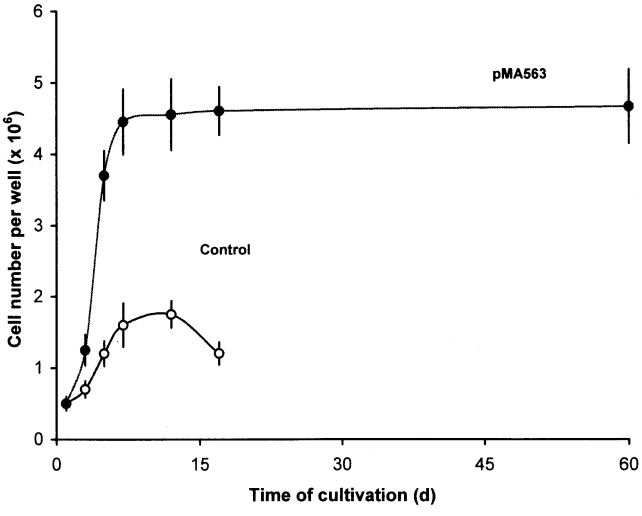


**Figure 4.** Growth of blastula-derived *S. intermedius* non-transformed cells control(s) and cells cultures transformed with the full-length *Gal4* gene (pMA563). Values are means ± SE based on 5 replicate samples obtained in a single experiment.

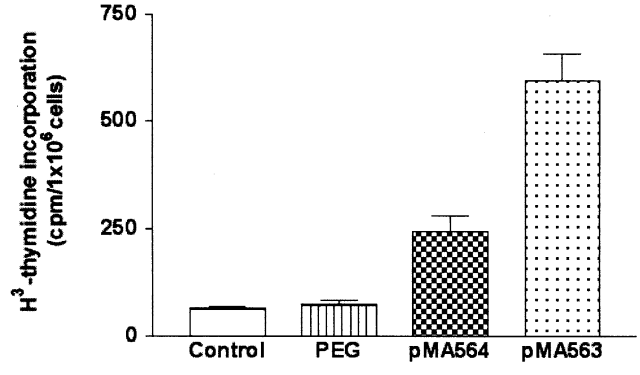


**Figure 5.** Activation of DNA synthesis in sea urchin cells treated by plasmids in blastula-derived 3-day culture. Mean values ± SE from 2 independent experiments.

months. Further growth of transformed culture was delayed, and cells become granulated, as in the control culture. However, we observed rare aggregates of nongranulated cells in 3-month-transformed cultures and found that the time of cell duplication was approximately 10 days. Unfortunately, these cells were subsequently lost owing to microbial contamination. Nevertheless, the effect of transformation was clearly noted and supported by the observation that the *Gal4*-transformed culture demonstrated a higher level of DNA synthesis than the control culture (Figure 5).

An unexpected feature of truncated *Gal4* gene was its weak but clear and reproducible stimulatory effect on TLS formation and DNA synthesis (Table 1, Figure 5). This effect is difficult to unravel at present, and possible function of the truncated *Gal4* gene will be examined in our future work.

The results presented in this report provide evidence that the yeast transcriptional activator can regulate cell differentiation processes in sea animals. Apparently, rapid development of TLSs in the embryos, activation of DNA synthesis, and significant increase of cell number in embryo-derived cell cultures can be attributed to the *Gal4* gene action. We suggest that after transformation the transcriptional activator gene provides a signal allowing continued cell division, which inhibits the process of normal cell differentiation. However, if the activator function is prevented, then embryos fail to receive this molecular signal and develop normally, as shown for the truncated *Gal4* gene (Figure 1, Table 1). Despite restricted knowledge about the gene regulatory systems of marine invertebrates, regulation of the proliferative potential of cells of these animals by transcriptional activator genes emerges as a novel approach for research in the field of marine biotechnology.

## Acknowledgments

We thank Dr. Jun Ma for providing samples of plasmid DNA. The authors also thank Dr. Phillip Ledger for helpful comments on the manuscript. The research described in this publication was made possible in part by Award 99T-03 of the U.S. Civilian Research and Development Foundation for the Independent States of the Former Soviet Union (CRDF).

## References

Bevan, M., Barnes, W.M., and Chilton, M.-D. (1983). Structure and transcription of the nopaline synthase gene region of T-DNA. *Nucleic Acids Res* 11:369–385.

Coffman, J.A., Kirchhamer, C.V., Harrington, M.G., and Davidson, E.H. (1996). SpRunt-1, a new member of the Runt domain family of transcription factors, is a positive regulator of the aboral ectoderm-specific *CyIIIA* gene in sea urchin embryos. *Dev Biol* 174:43–54.

Delgadillo-Reynoso, M.G., Rollo, D.R., Hursh, D.A., and Raff, R.A. (1989). Structural analysis of the uEGF gene in the sea urchin *Strongylocentrotus purpuratus* reveals more similarity to vertebrate than to invertebrate genes with EGF-like repeats. *J Mol Evol* 29:314–327.

Greenblatt, J. (1991). Roles of TFIID in transcriptional initiation by RNA polymerase II. *Cell* 66:1067–1070.

Kiyama, T., Akasaka, K., Takata, K., Mitsunaga-Nakatsubo, K., Sakamoto, N., and Shimada, H. (1998). Structure and function of a sea urchin orthodenticle-related gene (HpOtx). *Dev Biol* 193:139–145.

Koike, H., Akasaka, K., Mitsunaga-Nakatsubo, K., and Shimada, H. (1998). Proximal *cis*-regulatory elements of sea urchin arylsulfatase gene. *Dev Growth Differ* 40:537–544.

Koltsova, E.A., Boguslavskaya, L.V., and Maximov, O.B. (1981). On the functions of quinonoid pigments production in sea urchin embryos. *Int J Invertebr Rep* 4:17–28.

Lin, Y.-S., and Green, M.R. (1991). Mechanism of action of an acidic transcriptional activator in vitro. *Cell* 64:971–981.

Ma, J., Przibilla, E., Hu, J., Bogorad, L., and Ptashne, M. (1988). Yeast activators stimulate plant gene expression. *Nature* 334:631–633.

Ma, J., and Ptashne, M. (1987). Deletion analysis of GAL4 defines two transcriptional activating segments. *Cell* 48:847–853.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory.

Mao, C.A., Wikramanayake, A.H., Gan, L., Chaung, C.K., Summers, R.G., and Klein, W.H. (1996). Altering cell fates in sea urchin embryos by overexpressing SpOtx, an orthodenticle-related protein. *Development* 122:1489–1498.

Odintsova, N.A., Ermak, A.V., and Tsal, L.G. (1994). Substrate selection for long-term cultivation of marine invertebrate cells. *Comp Biochem Physiol* 107A:613–619.

Odintsova, N.A., Belogortseva, N.I., Ermak, A.V., Molchanova, V.I., and Luk'yanov, P.A. (1999). Adhesive and growth properties of lectin from the ascidian *Didemnum ternatanum* on cultivated marine invertebrate cells. *Biochim Biophys Acta* 1448:381–389.

Ornitz, D.M., Moreadith, R.W., and Leader, P. (1991). Binary system for regulating transgene expression in mice: targeting *int-2* gene expression with yeast GAL4/UAS control elements. *Proc Acad Sci USA* 88:698–702.

Ptashne, M., and Gann, A. (1990). Activators and targets. *Nature* 346:329–331.

Yuh, C.-H., Bolouri, H., and Davidson, E.H. (1998). Genomic *cis*-regulatory logic: experimental and computational analysis of a sea urchin gene. *Science* 279:1896–1902.