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BFP Protein Expression in Transfected Embryonic Stem Cells

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Cultured mouse embryonic stem cells can be transfected with a reporter gene encoding blue fluorescent protein BFP and regulated by drosophila heat shock protein 70 promoter. This gene is activated after heating and synthesizes matrix RNA. Blue protein is synthesized under these conditions. The system for transfection of stem cells allows us to activate automatically the corresponding transgenes.

Key Words: *embryonic stem cells; transfection; blot hybridization; heat shock protein*

Embryonic stem cells (ESC) possess high proliferative activity and remain undifferentiated for a long time. ESC injected into intact blastocyst participate in the formation of different tissues of the chimeric organism belonging to all germ layers [5,8,9]. Transplantation of undifferentiated ESC to adult immunodeficient mice leads to the development of teratomas and teratocarcinomas consisting of all three germ layers [11]. Culturing of ESC suspension under certain conditions results in the formation of embryoid bodies consisting of ectodermal, endodermal, and mesodermal layers, which resembles postimplantation embryogenesis [4]. When embryoid bodies adhere to the surface, the inner cells migrate across this surface and undergo spontaneous differentiation into various types of somatic cells. Directed differentiation of ESC is observed under specific culturing conditions and in the presence of exogenous growth and differentiation factors [4,10]. Analysis of gene expression during differentiation of cultured ESC into specialized cells showed that the sequence of *in vitro* expression of tissue-specific genes, proteins, and receptors corresponds to the sequence of these processes *in vivo* [4]. These data indicate that ESC are an adequate experimental object for studies of cell differentiation.

"Marker" lines of multipotent cells are required to study the pathways and mechanisms for differentiation of these cells. To this end, transfection of the gene encoding specific "colored" protein fluorescing in living cells is widely used [7]. Green (GFP) and red fluorescent proteins (RFP) are of common use. Here we studied a variant of blue fluorescent protein (BFP), cyan protein. An important step in these studies is selection of the promoter for introduction of the construct with the gene encoding colored protein into cells. Here we constructed a system for ESC transfection, which ensures automatic activation of the reporter gene at normal body temperature in mammals. Drosophila heat shock protein 70 (HSP70) gene promoter served as a part of this system [1]. We made the first attempt to develop such a system with mouse ESC.

MATERIALS AND METHODS

Experiments were performed on mouse R1 ESC presented by A. Nagy (Mount Sinai Hospital, Toronto). The cells were isolated from blastocysts of agouti-colored $(129/Sv \times 129/SvJ)F_1$ mice. ESC were cultured in α-MEM medium (Sigma) containing 15% fetal bovine serum (HyClone), 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, nonessential amino acids (ICN),

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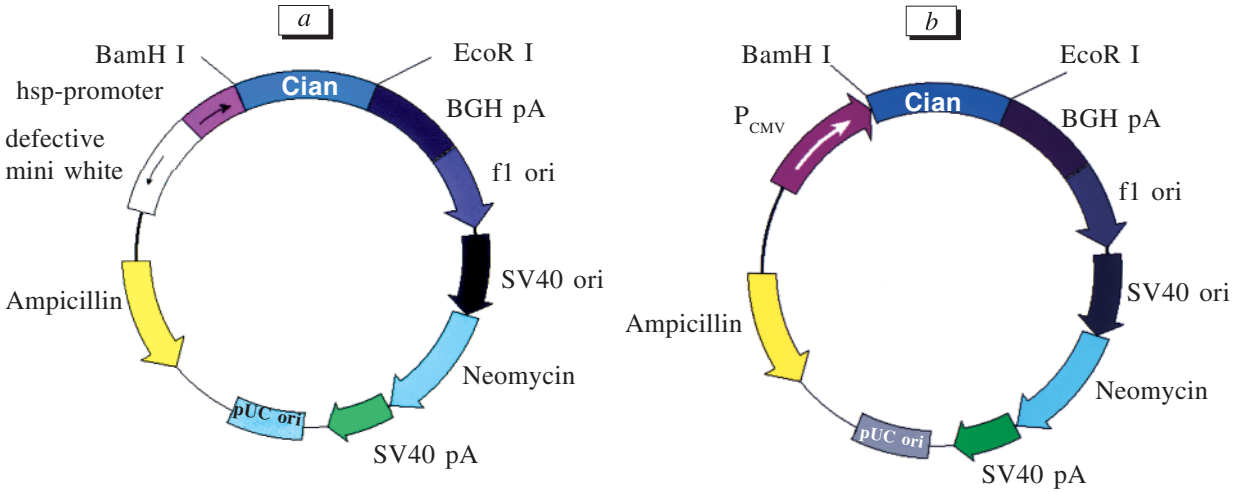

Fig. 1. Gene engineering constructs for transfection of embryonic stem cells (ESC): *bfp* gene regulated by drosophila heat shock protein 70 (HSP70) gene promoter (*a*); *bfp* gene regulated by CMV promoter (*b*).

nucleosides, vitamins, and antibiotic gentamicin (20 μg/ml) at 37°C and 5% CO_2. Primary fibroblasts were isolated from mice on days 11-12 of embryogenesis and served as a feeder layer for ESC. Proliferation of fibroblasts was blocked with mitomycin C (Sigma) in a concentration of 5 μg/ml. DMEM medium (ICN) containing 10% fetal bovine serum was used as a growth medium for the primary culture of fibroblasts. ESC were cultured in Petri dishes (diameter 35 mm, 2 ml). The medium was inoculated with 100,000 cells per 1 ml. During culturing of ESC without a feeder layer, leukemia inhibitory factor (LIF) in a final concentration of 10 ng/ml was added to the medium to block spontaneous cell differentiation. Subculturing and medium replacement were performed at 3-day intervals.

R1 ESC were transfected by electroporation on a SUM4 electroporator (Institute of Molecular Biology, Russian Academy of Sciences) under specific experimental conditions (1.5 msec pulse duration, 400 V voltage) using 10 μg plasmid DNA per 1 million cells.

Two constructs with the pcDNA 3.1 vector were prepared for transformation of ESC with BFP gene (Fig. 1, *a*, *b*). This vector includes genes determining resistance to ampicillin and neomycin. The fluorescent protein gene was introduced under drosophila HSP70 promoter (CMV, Fig, 1, *a*) and cytomegalovirus promoter (Fig. 1, *b*). In the first construct insertion was performed by stick ends EcoRI/Bamhl. The second construct is based on the first construct; the vector was restricted by Spe and blunted. Restriction by BamHI allowed us to obtain the construct restricted by Spe/BamHI. The HSP70 promoter was obtained from the CaSper-hs-act promoter restricted by Sma/BamHI. The promoter was introduced into this vector by the blunt and stick ends.

R1 cells transfected with the pcDNAneo plasmid served as the control. The cells were selected using antibiotic G418 in a dose of 200 μg/ml for 14 days. The frequency of transformation was 2×10^{-4}. BFP expression was detected using an Axioscope-2 microscope (Carl Zeiss). Transfection and selection of ESC yielded neomycin-resistant cells that characterized by stably high expression of BFP.

Insertion of the construct into the BFP genome was studied by the Southern blot hybridization technique.

RESULTS

Southern blot hybridization showed that transfected cultures of ESC contain a construct with colored protein gene and, hence, the corresponding transgene (Fig. 2).

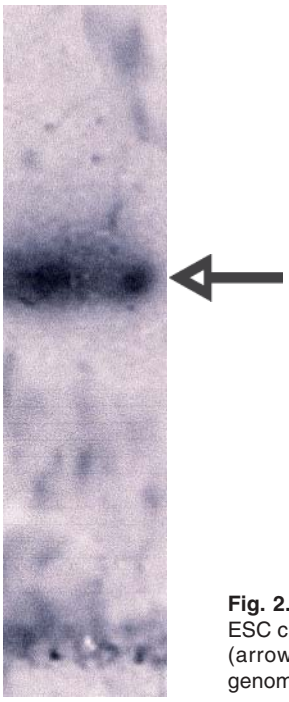

Fig. 2. Southern blot analysis of transfected ESC culture. Positive reaction to blue protein (arrow) reflects its incorporation into the genome of transfected ESC.

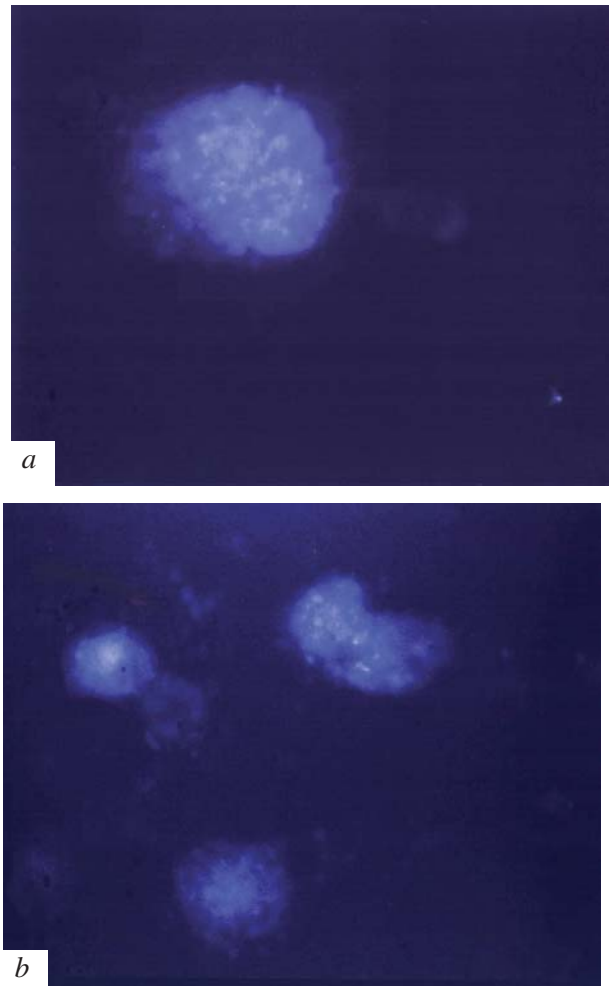

Fig. 3. Fluorescence of transfected cultured ESC: drosophila HSP70 gene promoter (*a*) and CMV promoter (*b*, ×200).

Examination of transformed ESC cultures under an Axioscope-2 fluorescence microscope showed that cells transfected with the BFP gene under the control of CMV promoter exhibit blue fluorescence, which attests to the synthesis of blue protein in these cells (Fig. 3, *b*).

Microscopic examination revealed no fluorescence in cells transfected with BFP gene under drosophila HSP70 promoter. However, after preheating of cultured ESC to 40°C for 30 min transfected cells exhibited a strong blue fluorescence (Fig. 3, *a*).

Our results suggest that mammalian heat shock-inducing factor (HSF) can interact and activate HSP70 gene promoter, which agrees with previous data [2,3].

Under normal conditions the exposure to heat shock temperature induced HSF release from the HSF/HSP90 complex, formation of HSF trimer [6], its translocation into the nucleus, and activation of the HSP70 promoter. In our experiments the HSF/HSP90 complex was disintegrated upon heating to 40°C. Non-preheated cells transfected with BFP regulated by drosophila HSP70 promoter cannot synthesize BSF mRNA. In contrast to drosophila HSF/HSP83 complex, activity of the HSF/HSP90 complex in humans and animals does not depend on body temperature and, therefore, temperature of culturing. Disintegration of the HSF/HSP90 complex at 40°C is followed by HSF transport into the nucleus. We found that mammalian HSF interacts with drosophila transgene-linked promoter.

Our findings indicate that it is possible to obtain stem cell culture carrying a system responding to body temperature in mammals by activation of the corresponding transgenes (regulated by HSP promoters) necessary for the therapy of various disorders, including neurodegenerative diseases.

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