Russian Journal of Developmental Biology, Vol. 36, No. 3, 2005, pp. 189–198. Translated from Ontogenez, Vol. 36, No. 3, 2005, pp. 230–240.

Abstracts of Papers at the Conference of Young Scientists, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences (Moscow, December 8, 2004)

A Study of Tissue Specificity of Biological Activity of Regulatory Proteins

A. V. Borisenko

Group of Regulatory Proteins, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, ul. Vavilova 26, Moscow, 119991 Russia

Regulatory proteins were isolated from the liver, lung, kidney, and small intestine tissues of Wistar rats using the scheme of isolation and purification developed by Yamskova *et al.* This scheme included the obtaining of tissue extract, salting-out in a saturated ammonium sulfate solution, isoelectrofocusing, and electrophoresis in polyacrylamide gel. As a result, fractions of acidic proteins (pH < 3.0) were obtained from four tissue extracts. A major component of each studied fraction was a low molecular weight protein with the molecular mass of no more than 10 kDa. The identified low molecular weigh proteins displayed biological activity in ultralow doses corresponding to 10^{-8}–10^{-10} mg protein/ml. Thus, proteins of the studied group of regulatory proteins were identified in the liver, lung, kidney, and small intestine tissues, which displayed biological activities at ultralow doses. A separate experiment was carried to assay the tissue specificity of their biological activities. Extracts of the rat liver and lung were separated via isoelectrofocusing without preliminary salting-out and fractions of the corresponding acidic proteins (pH < 3.0) were collected. The studied fractions were tested on two organ cultures, mouse liver and lung tissues *in vitro*. The extracts of liver and lung and the fractions of acidic proteins isolated from them were shown to express issue specificity of (conditional) biological effect. After salting-out, the fractions of acidic proteins lost tissue specificity of their biological actions, since they displayed membranotropic effects with respect to both live rand lung tissue *in vitro*. The data obtained suggest the presence of proteins in tissue extracts, which modulate the biological activity of the studied regulatory proteins. It can be proposed that regulatory proteins and modulator proteins form a fast enough complex in solution, which is degraded only in presence of a saturated salt solution. Apparently, under these conditions, the modulator protein passes to the precipitate. This method can be used for studying modulator proteins.

Some Aspects of Determination of Molecular Structure and Mechanism Underlying the Biological Effects of Regulatory Proteins

V. V. Vecherkin

Group of Regulatory Proteins, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, ul. Vavilova 26, Moscow, 119991 Russia

The main property that distinguished regulatory proteins from other proteins is their biological activity at ultralow doses. Regulatory proteins were found in various tissues of vertebrate animals. Regulatory proteins at doses of 10^{-8}–10^{-12} mg protein/ml affect the most important biological processes and, thus, control the organ-tissue homeostasis. Regulatory proteins of the studied group are isolated the previously developed method of their isolation and purification, including their extraction from the corresponding tissue, salting-out, electrofocusing, and electrophoresis in polyacrylamide gel. Regulatory proteins are low molecular weight proteins with the molecular mass of no more than 10kDa; they are glycosylated and their oligosaccharide chains consist of mannose and N-acetylglycosamine residues. Their amino acid composition is characterized

1062-3604/05/3603-0189

by significant amounts of glycine and serine residues and dicarbonic acids. According to the results of circular dichroism analysis, the secondary structure of regulatory proteins is described in terms of "statistical glomerus," in which regions of β-structures of several types were identified. The presence of several amino acid sequences homologous to the motifs of various cytokeratins in the regulatory protein molecule isolated from the bovine blood serum was shown using mass-spectrometry (MALDI-TOF). In tissue extracts containing regulatory proteins, the proteins modulating their biological activities were also found. One of such modulator proteins reversibly inactivating the biological activity of the serum regulatory protein was identified in the bovine blood serum. This was a 70 kDa protein, highly homologous with prealbumine of blood serum of vertebrate animals. The secondary structure of this protein was characterized by a high content of α-helices. Reversible inactivation of the regulatory protein by the modulator protein was studied using affine chromatography and circular dichroism. The data obtained suggest that inactivation is based on the formation of a molecular complex between the regulatory protein and modulator according to the principle of carbohydrate-protein interaction. Since all studied regulatory proteins are glycoprotein's, it can be proposed that their biological activities are also modulated via the complex "regulatory protein—modulator" based on carbohydrate-protein interaction.

ACKNOWLEDGMENTS

The author expresses his sincere thanks to E.Yu. Rybakova, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, for her help in this study

Studies of Mechanisms Underlying the Effects of Protease-Activated Type 1 and 4 Receptors on Contractility of Isolated *Helix pomatia* Heart

E. S. Vinichenko and P. P. Avdonin

Laboratory of General Physiology, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, ul. Vavilova 26, Moscow, 119991 Russia

Thrombin and some trypsin-like proteases interact with specific receptors on the surface of mammalian cells, so-called protease-activated receptors (PAR). Four kinds of PARs are known: $PAR_{1,3,4}$ are activated by thrombin and PAR_2 by trypsin and trypsin-like proteases. PARs were found in many mammalian tissues, including blood vessels. The aim of this work was to establish whether phylogenetically distant animals, gastropods, have PARs. Studies were carried on the isolated *Helix pomatia* heart. Thrombin at 10^{-4} to 1 U/ml induced a dose-dependent increase of contractility of the isolated heart. Trypsin exerted a much less pronounced effect and characteristic dose dependence was absent. The contractility of the snail heart increased also under the influence of synthetic peptide ligands of $PAR_{1,4}$. Their effect was expressed in the area of 10^{-1}–10 μm. These peptides interact with receptors in mammalian tissues at the same doses. Thrombin and peptide PAR agonists increased the amplitude of heart contractions, but did not affect their frequency. At concentrations of up 100 μm, they induced the heart contraction. In order to study a possible mechanism underlying the effect of thrombin on the snail heart, the activity of adenylate cyclase was determined as the following component of the corresponding signal pathway. The treatment of the snail heart membrane preparation with thrombin and $PAR_{1,4}$ did no cause changes in the adenylate cyclase activity, while serotonin increased the cAMP synthesis. Consequently, thrombin activates the molluscan heart according to a cAMP-independent mechanism. Hence, it was proposed on the basis of the obtained data that the molluscan heart contains receptors, structurally and functionally homologous to mammalian PARs.

ACKNOWLEDGMENTS

The authors express their thanks to Dr. V.G. Solomonova, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, for her help in designing the experiments. This study was supported by the Russian Foundation for Basic research, project no. 02-04-49932.

Identification of Regulatory Protein in Bovine Eye Sclera

A. V. Kachalina

Group of Regulatory Proteins, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, ul. Vavilova 26, Moscow, 119991 Russia

Sclera is one of the most important eye structures, since it protects the internal eye tissues against mechanical damage and maintains the constant eye form for correct focusing of objects onto the retina. While pro-

viding a stable basis for the ciliary muscle contraction, sclera is involved in accommodation, as well as in contraction of external eye muscles that realize the eye movement. Vessels and nerves supplying the intraocular structures pass across sclera. This complex functioning of the scleral membrane is determined, to a great extent, by the spatial-functional organization of its extracellular matrix. The main components of the sclera extracellular matrix are collagens of different types, elastin, laminin, hyaluronans, and proteoglycans, which provide for rigidity and elasticity of this tissue. Myopia, which is determined by refraction defect, is one of the most widespread eye diseases related to changes in the sclera extracellular matrix. In case of myopia, the scleral membrane thickness decreases due to defects of the spatial organization of collagen fibers. In addition, the content of glycosamines decreases and, hence, sclera hydration decreases and its elastic properties are lost. At a high level of myopia (>6 diopters), additional pathologies may develop, which are related to chorioretinal degenerations or even retina detachment. Development of novel pharmacological drugs capable of affecting the state of sclera during myopia is a current task of ophthalmology. Taking into account the capacity of regulatory proteins of the studied group to influence the main biological processes in tissues and, thereby, tune the organ-tissue homeostasis, an attempt was undertaken to identify regulatory proteins of this group in the mammalian eye sclera. Studies were carried out on bovine eyes. For isolation and purification of regulatory proteins, the previously developed scheme was used, which comprised obtaining of scleral tissue extract, its salting-out, fractio0nation via isoelectrofocusing, and electrophoresis in polyacrylamide gel. According to the physicochemical properties and pattern of biological activity, the identified acidic protein isolated from the scleral tissue belongs to the studied group of regulatory proteins.

ACKNOWLEDGMENTS

The author would like to thank Dr. M.S. Krasnov, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, for his help in this investigation.

Analysis of Expression of Homeobox-Containing Genes in Various Pluripotent Cell Populations

N. Yu. Krasnikova

Laboratory of Histogenesis, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, ul. Vavilova 26, Moscow, 119991 Russia

Studies of molecular-genetic and cellular mechanisms underlying proliferation and differentiation of mammalian embryonic stem (ES) cells are a current problem of developmental biology. ES cells are a unique experimental model for investigating the fundamental patterns of mammalian development. In order to establish molecular mechanisms responsible for the maintenance of pluripotency of ES cells and controlling the initial stages of their differentiation *in vitro*, studies are carried out aimed at identification and analysis of expression of the genes involved in the regulation of these processes. Specific homeobox-containing transcriptional factors play the key role in the regulation of cell proliferation, differentiation, and apoptosis and, hence, our study is aimed at investigation of expression of the genes encoding transcriptional factors of the families *POU, Nk2, Pax, Prox*, and *Ptx* in various pluripotent cell populations. For comparative analysis of expression of the studied genes, total RNA was isolated from: (1) feeder cells obtained from mouse primary fibroblasts, (2) undifferentiated ES cells growing on a feeder, (3) ES cells growing in a medium with LIF, (4) definitive embryoid bodies, (5) attached embryoid bodies, (6) ES cells growing in a medium with a low LIF content, and (7) embryonic cells of F9 carcinoma. mRNA fractions were extracted from total RNAs. cDNAs were synthesized on mRNA templates, which were used for PCR analysis of expression of the studied genes. All cDNA libraries were standardized by the level of expression of the housekeeping gene of hypoxanthine phosphoribosyl transferase. The levels of expression of the genes *oct-4*, a member of *POU* family, and *nanog*, a member of *Nk2* family, which are key regulators of the pluripotent status, did not practically differ at all stages of differentiation of ES-cells. Expression of the genes *pax-6, pax-3, prox-1*, and *ptx-2* was detected at all stages of differentiation of ES cells. The *pax-6* gene expression increased in the course of their differentiation. Two PCR products were obtained, which corresponded to two *pax-6* transcripts formed as a result of alternative splicing. The *pax-3* expression also increased in the course of differentiation of ES cells. Two PCR products were also obtained. The *prox-1* expression underwent insignificant changes in the course of differentiation of ES cells. A trend was shown to enhanced *ptx-2* expression during differentiation of ES cells, which reaches its maximum in the attached embryoid bodies. Analysis of expression of the homeobox genes in the cells growing in LIF-deficient media has not shown significant differences from the cells growing under the normal conditions despite the described morphological distinctions. The expression

of studied genes, except *nanog* and *pax-3*, was detected in the embryonic carcinoma F9 cells. The functional role of the genes *pax-6, pax-3, prox-1*, and *ptx-2* in the regulation of pluripotency and early differentiation of ES cells is not yet established and further studies are necessary for localization of expression of these transcriptional factors and investigation of signal pathways, in which they are involved.

ACKNOWLEDGMENTS

The author would like to thank Drs. R.D. Zinovieva and O.F. Gordeeva, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, for their help in this work. This study was supported by the Russian Foundation for Basic research, project no. 02-04-48434.

A Study of Protector Properties of Regulatory Protein Isolated from Bovine Lens on Experimental Model of Cataractogenesis

M. S. Krasnov

Group of Regulatory Proteins, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, ul. Vavilova 26, Moscow, 119991 Russia

This study was carried out within the framework of the concept based on defective regulation of the organ-tissue homeostasis as a primary cause of any pathology, including cataractogenesis. Lens opaqueness is related to disordered structure of lens fibers and conformation of intracellular proteins, crystallins. Hydrogen peroxide and calcium chloride were used as inducers of cataractogenesis *in vitro*, since the first agent induces opaqueness of the cortical layers due to lipid peroxidation with damage of the lens fiber membranes and their subsequent swelling. The second agent induces opaqueness due to altered activities of Ca^{2+}-bound enzyme systems in the lens cells. Similar mechanisms of damage are observed in the case of traumatic, some forms of senile, and complicated cataracts. Experiments were carried on lenses of Wistar rats cultivated *in vitro*. Regulatory protein isolated from the bovine lens according to the previously developed scheme was used as a protective drug. Acidic regulatory protein with the molecular mass of no more than 10 kDa was used, as well as two fractions of the regulatory protein obtained at two stages of purification: after isoelectrofocusing and after isoelectrofocusing and subsequent electrophoresis under the native conditions. Both fractions at a dose of 10^{-12} mg/ml were added to the culture medium. In the presence of any damaging agent, addition of the regulatory protein prevented the lens opaqueness, the effect being the most pronounced in the case of electrophoretically purified fraction. Thus, it was shown on the experimental model of cataractogenesis of rat lenses *in vitro* that the regulatory protein isolated from bovine lens exerted a protective effect and the biological activities of two fractions of the regulatory protein obtained at two stages of purification differed.

ACKNOWLEDGMENTS

The author would like to thank E.P. Gurmizov, Helmholtz Institute of Eye Diseases, for his help in this study.

A Study of Adhesive Protein Isolated from Bovine Cornea

D. V. Margasyuk

Group of Regulatory Proteins, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, ul. Vavilova 26, Moscow, 119991 Russia

The fraction of acidic proteins isolated from the bovine corneas tissue extract was studied. The fraction was obtained using the previously developed method for isolation of regulatory proteins from mammalian tissues. A low molecular weight protein with the molecular mass of no more than 10 kDa was identified as the major component of this fraction. Using the adhesiometric method of biotesting regulatory proteins *in vitro*, it was demonstrated that this protein displayed biological activity at ultralow doses corresponding to 10^{-8}–10^{-10} mg protein/ml. According to its physicochemical and biological properties, this protein was similar to regulatory proteins of the studied group isolated from other tissues of vertebrate animals. It was established using immunochemical methods that the studied protein was localized in the endothelium of the *Pleurodeles waltl* (Amphibia) cornea. Taking into account the role of endothelium in maintenance of the cornea integrity, we proposed the involvement of the studied protein in repair of the cornea. In order to test this suggestion, we studied the effects of the protein in question on the proliferative activity of the *P. waltl* corneal epithelium. The choice of the object is explained by a high regenerative capacity of eye tissues and absence of pronounced inflammatory reaction in these animals. After microsurgical traumatization (transection of cornea), the heads of newts were immersed in the solution of acidic protein from the supernatant of cornea extract at 10^{-9} mg protein/ml daily for 28 days. The solution of regulatory adhesive serum protein at the

same concentration and water were used as positive and negative controls, respectively. It was shown using autoradiography on 7 μm section of the cornea that both regulatory proteins at ultralow doses statistically reliably stimulated cell proliferation in the newt cornea *in vitro*. No significant differences were found between the two groups of newts treated with regulatory proteins.

Does the Growth Zone of Fish and Amphibian Retina Correspond to the Periphery of Pars Optica Retinae of Human Fetuses?

L. A. Milyushina, D. V. Ross, and I. G. Panova

Laboratory of Problems of Regeneration, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, ul. Vavilova 26, Moscow, 119991 Russia

Studies of retina growth and differentiation in lower vertebrates have revealed the presence of the growth zone in the terminal area of retina in fish and amphibians. This zone contains little differentiated and multipotent cells capable of proliferating and replenishing the retina with new neurons throughout the life. Stem cells were found in the inner nuclear layer of the retina in adult teleostean fish (Svistunov, S.A. and Mitashov, V.I. Ontogenez, 1985, vol. 6, pp. 39–45; Otteson, D.C. *et al.*, Devel. Biol., 2001, vol. 232, pp. 62–76). The growth of human retina starting from the moment of eye anlage within the limits of the archencephalic plate and ending by the adult tissue formation lasts for a long time during the intrauterine life and first months after birth. During the period of intense growth, neuroblasts of human retina actively divide as follows from the presence of mitoses in the outer neuroblastic layer (Mann, I., The Development of Human Eye, 1949). We complemented the studies of sources of the retina growth in fish and amphibians by analysis of the human retina growth and development. This in view, we studied the retina of adult fish *Danio rerio* and 4- to 10-month old axolotls *Ambystoma mexicanum* using autoradiography. The human retina was studied at stages from 9.5 to 20 weeks of gestation via counting mitoses in different retina area on histological preparations. In addition to the known from literature sources of retina growth in the terminal zone and inner nuclear layer in teleostean fish, we described and additional source of growth in *Brachydanio rerio*: area of retina adhering to the falciform process. Labeled cells were also found in the inner plexiform layer but their nature is still unknown. In axolotls, the presence of growth zones in the terminal area of retina and in the area of eye fetal fissure was confirmed and single intensely labeled cells were found in the inner nuclear layer. The human retina is represented by two neuroblastic layers, outer and inner, at the stages of 9.5 to 12 weeks of gestation. Many mitoses could be seen all over the outer neuroblastic layer near the outer boundary membrane, with a maximum in the periphery of pars optica retinae. At the age of 20 weeks, all retina layers are already well formed. Against the general background of sharp decrease in the number of mitoses in the retina, the peripheral part of pars optica retinae stood out, where mitoses were still found. The data obtained suggest that the peripheral part of human pars optica retinae is comparable with the growth zones of the fish and amphibian terminal area at least until the 20th week of gestation.

ACKNOWLEDGMENTS

The authors would like to thank Drs. R.A. Poltavsteva and G.T. Sukhikh, Scientific Center of Obstetrics, Gynecology, and Perinatology, Russian Academy of Medical Sciences, who kindly provided the materials for studies, and N.A. Ivanova for technical assistance.

A Study of Regulatory Protein Isolated from the Bovine Prostate: Physicochemical Properties, Biological Activity, and Localization

P. A. Nazarova

Group of Regulatory Proteins, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, ul. Vavilova 26, Moscow, 119991 Russia

The prostate plays an important role in maintenance of the reproductive function in mammals. The number of various pathologies of the human prostate increases at present. The most widespread diseases include prostatitis (in various forms), adenoma, and cancer. Thus, the search of therapeutic means for treatment of these diseases remains relevant. Our work is a part of the investigation aimed at studying regulatory proteins controlling homeostasis in different mammalian tissues and deals with identification of proteins of this group isolated from the bovine prostate. Proteins were isolated using the scheme previously developed for this

purpose. The fraction of proteins with pH 4.4–5.1 was studied. It was shown using electrophoresis in polyacrylamide gel that the main component of this fraction was a low molecular weight protein with the molecular mass of no more than 10 kDa displaying biological activity at ultralow doses corresponding to 10^{-12} mg protein/ml. This fraction of proteins was used to raise a polyclonal rabbit serum. Using immunohistochemistry method, it was shown that the studied protein was localized in the secretory substance of mouse prostate. The results obtained suggest that the studied regulatory protein can affect physicochemical properties of the secretory substance, which is the main product of functioning of this gland.

Influence of Fibronectin on Actin Cytoskeleton Structure and Shape of Mitochondria in Mammalian Cells

O. E. Nekrasova

Laboratory of Biochemical Embryology, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, ul. Vavilova 26, Moscow, 119991 Russia

The method of immobilization of fibronectin and its fragments on activated slides was developed for studying the role of extracellular matrix in distribution of the mitochondria in cells and conditions were selected for attachment of CV-1 cells to the slides covered with fibronectin and its fragments in a serum-free medium. The cells were attached and spread on slides covered by fibronectin or its 120 kDa fragment in the serum-free medium. The shape and distribution of mitochondria in such cells differed from those in the cells incubated in the presence of serum: mitochondria looked short and were located near the nucleus. When the actin cytoskeleton structure was analyzed in the cells spread on immobilized fibronectin, it turned out that they were practically devoid of actin stress-fibrils, which appeared rapidly upon addition of serum to the medium. When fibronectin or its 40 kDa fragment was added to the cells spread on the slides covered by 120 kDa fragment, the shape of mitochondria underwent changes; they were elongated and their significant part was localized in the cell periphery. Hence, it was proposed that the heparin-binding fragment of fibronectin, a component of its 40 kDa fragment, plays an important role in the regulation of shape and distribution of mitochondria. In addition, it was shown that in the cells attached to fibronectin, the shape of mitochondria remained unchanged after addition of dissolved fibronectin to the medium, if microtubules had been preliminarily destroyed. Thus, it was shown that the shape of mitochondria and structure of actin cytoskeleton are regulated by external signals from extracellular matrix via certain receptors on the cell surface.

ACKNOWLEDGMENTS

The author would like to thank Drs. A.A. Minin, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, and An.A. Minin and A.V. Kulik, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, for their help in this work.

Two Isoforms of Annexin A13 in *Brachydanio rerio* Egg

S. G. Ozerova

Laboratory of Biochemical Embryology, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, ul. Vavilova 26, Moscow, 119991 Russia

Annexins are a family of calcium-binding proteins capable of binding to acidic phospholipids in the presence of Ca^{2+} *in vitro*. According to the present-day classification, there are five types of annexins: A, B, C, D, and E. They occur in different groups: from protists and fungi to higher vertebrates. Annexins of vertebrates belong to type A and are subdivided in 12 groups: A1–A13, except A12. In the cell, they can be in the cytoplasm in a free form or associated with the cytoplasmic membrane, as well as with various membrane structures, such as cell nucleus, membranes of endocytotic vesicles, endoplasmic reticulum, etc. Using the capacity of annexins to bind to acidic phospholipids in the presence of Ca^{2+} *in vitro*, a mixture of proteins, supposedly annexins, was isolated from the eggs of *Brachydanio rerio*. After two-dimensional electrophoretic separation of proteins and their subsequent identification with the help of mass-spectrometry, annexins A1, A5, and A13.1 were found in the eggs of *B. rerio*. Further work consisted of computer simulation and experimenting. The computer part comprised the following: all EST-sequences of *B. rerio* annexins were taken from the Gene Bank database, overlapping sequences were divided in groups using DNA-STAR software, and obtained reading frames were translated. The obtained amino acid sequences of *B. rerio* were complemented with all known annexin protein sequences of other vertebrates and, in this way, a dendrogram of annexins of

vertebrates was obtained. It can be seen that *B. rerio* lacks annexins A7 and A9, while unlike mammals, some groups of *Brachydanio* annexins are represented by several variants of proteins, for example two A13 proteins. Subsequent work was aimed at identifying, in addition to the already known form A13.1, a new predicted isoform. For this purpose, total RNA was isolated from mature oocytes. On the basis of each of two sequences of cDNA of *Danio* annexins A13, specific pairs of primers were selected. As a result of polymerase chain reaction coupled with reverse transcriptase on total RNA and specific primers, 526 and 478 kB fragments were obtained. The fragments were isolated sequenced. Each DNA corresponded to RNA with an open reading frame, which showed homology with annexins A13.1 and A13.2 upon translation.

ACKNOWLEDGMENTS

The author would like to thank Drs. A.A. Minin and N.S. Myuge, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, for their help in this work.

Characterization of Cells in Cultures of Human Embryonic Retina

O. V. Podgornyi

Laboratory of Experimental Neurobiology, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, ul. Vavilova 26, Moscow, 119991 Russia

The eye retina, which develops from cells of the prosencephalon neuroepithelium is a suitable model for studying neural developmental and plasticity. These studies are related to the solution of fundamental problems of neurogenesis and provide a possibility for development of new trends in therapy in ophthalmology. In this work, we characterize the cells of human retina at the stage of 20–23 weeks of gestation *in vivo* and *in vitro*. The native materials were characterized using polymerase chain reaction and immunohistochemical analysis. The dissociated retina cells were cultivated in a serum-containing or serum-free medium complemented with FGF, EGF, and LIF. The former cultures were growing as adhesive monolayers and the latter as free swimming spheroids for 20 days. In order to identify the cell phenotype, immunohistochemical analysis was used with antibodies to nestin, GFAP, β-III-tubulin, vimentin, neurofilaments-70, Ki67, and recoverin. The results obtained suggest that in the native retina and both culture, nestin- and vimentin-positive cells (stem and progenitor cells), photoreceptors, neuroblasts, glioblasts were present. In adhesive cultures, the βIII-tubulin-positive cells lacked long enough processes, characteristic for cells of this phenotype in adhesive culture of embryonic human brain, which we had observed earlier. However, in a selective medium, the βIII-tubulin-positive cells formed long processes, which gave rise to fibrous tracts on the surface and insides the spheres. The nestin- and vimentin-positive cells permeated the clusters of photoreceptors and formed networks of fibers in the intermediate zones. Unlike the native retina, the cultures contained many dividing cells. Hence, factors of microenvironment are present in the differentiating retina at the stage of 20–23 weeks of gestation, which blocked cell divisions.

ACKNOWLEDGMENTS

The author would like to thank his colleagues Drs. B.I. Verdiev, I.G. Panova, and M.A. Aleksandrova, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, for their help in this work. This study was supported by the Program "Molecular and Cell Biology," project no. 10002-251/11-143-150/010403-048.

Characterization of Cell Component in Living Skin Equivalent *in vitro*

O. S. Rogovaya

Laboratory of Problems of Cell Proliferation, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, ul. Vavilova 26, Moscow, 119991 Russia

Living skin and derma equivalents have been developed rather long ago and are widely used in research and as transplants in clinical practice for reconstruction of organ and tissue defects. We have already studied the properties of dermal equivalent, such as the rate and extent of contraction as a function of collagen concentration, number and properties of the cells introduced in the gel, direct interaction of cells with collagen fibrils, and medium content of serum. When keratinocytes are cultured on the gel with incorporated fibroblasts, the

forming structure is called living skin equivalent. In order to characterize the epidermal layer grown on the collagen gel surface, antibodies against intermediate filaments specific for keratinocytes, such as cytokeratins 10, 14, and 19, were used. In addition, marker p63 and marker of proliferating cells Ki67 were used for characterization of keratinocyte populations used for the formation of living skin equivalent. Immunohistochemical staining of histological sections of a living skin equivalent with antibodies against Ki67 revealed stained cells uniformly distributed in the basal layer of the epithelial sheet. This suggests a high proliferative activity of this culture. All keratinocytes of the basal layer contained keratin K14, while keratinocytes of the suprabasal layer were stained with antibodies to K10, which corresponds to the distribution of these keratinocyte-specific intermediate filaments in the human skin. Single cells stained with antibodies to K19 could be seen in the basal layer. In the human skin preparations, K19-positive cells were found only in the hair follicle outer root sheath. The basal layer of the living skin equivalent also contained p63-positive keratinocytes arranged in groups. In the human skin, groups of p63-positive cells were present in the papillary layer, which corresponds to the published data. Based on the published data and our results, one can speak that the model of living skin equivalent is adequate for cultivation of histotypically formed highly differentiated epidermal sheet. Specifically, we use living skin equivalents for cultivation of cells that retain BrdU label for a long time. The epidermal sheet grown in such system was then separated from the sublayer and examined under a confocal microscope. The living skin equivalent is widely used for treatment of burns, healing of chronic wounds and as a transplant for restoration of trachea and urethra epithelium.

ACKNOWLEDGMENTS

The author would like to thank Drs. E.A. Vorotelyak, A.V. Vasiliev, and V.V. Terskikh, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, for their help in this work.

A Possible Mechanism Underlying the Method of Biotesting *in vitro* of Regulatory Proteins Displaying Biological Activity at Ultralow Doses

E. Yu. Rybakova

Group of Regulatory Proteins, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, ul. Vavilova 26, Moscow, 119991 Russia

Biotesting of regulatory proteins displaying biological activity at ultralow doses is realized by means of the adhesiometric method. It is based on estimation of an experimentally determined parameter reflecting viscoelastic properties of the tissue, which depend, in turn, on the state of cell and microenvironment macrostructures, such as Ultrastructure of specialized cell contacts, plasma membrane, and cytoskeleton. Interaction of the plasma membrane receptors with components of cell microenvironment induces spatial reorganization not only of the plasma membrane, but also of the cytoskeleton. Thus, the membranotropic effect of specific ligands is capable of developing cascade reorganizations of the main macromolecular structures of the cells and its microenvironment, which make a significant contribution to the viscoelastic properties tissues. Carbohydrates play an exceptionally important role in cell adhesion and "recognition." Using the adhesiometric method, we studied the membranotropic effects of some carbohydrate-containing substances in order to establish dependence between molecular structure and biological activity at ultralow doses. α-Methylmannoside, α-methylgalactoside, and sucrose were studied, as well as glycoproteins, such as α1-acidic glycoprotein of blood serum (orosomucoid), ovalbumin, fibronectin, and serum albumin. All studied glycoproteins had mannose-containing glycans of varying structure as components. Membranotropic effects were exerted only by α-methylmannoside, α-methylgalactoside, and fibronectin at ultralow doses. At the next stage, the mebranotropic effects of two mannose-containing glycans, penta- and nonamannozyl-di-N-acetylchitobiose, were studied, which differed in the character of bond between mannose terminal residues. The membranotropic activity was displayed only by the glycan containing bonds (α1-2) between mannose terminal residues. Thus, it was shown that the adhesiometric method developed for biotesting of regulatory proteins displaying biological activity at ultralow doses makes it possible to identify mannose-containing substances with oligomannosid chains of a certain structure.

ACKNOWLEDGMENTS

The author would like to thank Dr. V.E. Piskarev, Nesmeyanov Institute of Elementoorganic Compounds, Russian Academy of Sciences, who kindly provided mannose-containing glycans.

Genetic Structure of *Drosophila littoralis* Population as Revealed by Analysis of Polymorphism of Mitochondrial DNA

S. Yu. Sorokina

Laboratory of Genetics, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, ul. Vavilova 26, Moscow, 119991 Russia

Studies of genetic subdivision of populations and changes in population structure are essential for investigation of microevolutionary processes. On the one hand, genetic heterogeneity of a population provides for the population stability to changing environmental conditions and, on the other, it is of important evolutionary significance, since it can serve as the beginning of divergence with subsequent speciation. In this work, the polymorphism of mitochondrial DNA was studied in a natural *D. littoralis* population from the Rostov District (Rybnoe) using the method of polymorphism of lengths of restrict fragments. *D. littoralis* belongs to phylade *montana* of the group of closely related *virilis* species, which, beginning with the studies of Patterson and Stone, is actively used for investigation of speciation. During the period of four years, we collected and analyzed 75 isofemale lines. As was shown by us earlier, polymorphism for the sites of restriction *HinfI* in the group *virilis* is informative at the inter- and intraspecific levels, therefore this enzyme was used analysis of the collected lines. Mitochondrial DNA of *Drosophila* contains 9 to 14 *HinfI* sites. Native mitochondrial DNA is cleaved in fragments, 30 to 5150 bp in size. Coincidence for seven or more fragments corresponds to the intraspecific level of variation. In this sample, nine mitochondrial *HinfI* haplotypes (Hi–H9) were identified two of which, H1 and H2, are major haplotypes and constitute 90% of the total number of analyzed lines. The ratio of H1/H2 haplotypes is approximately 1 : 1. This ratio remained stable during the entire period. The observed stability was not related to the infection with reproductive parasites of the *Wolbachia* type. Only two haplotypes from those found in the Rostov population were found also beyond the limits of this population. Haplotype H7 occurs in a closely located Northcaucasian population. Haplotype H1 is widespread over the entire range and occurs in East Siberia, North Caucasus, Finland, Iran, and Canada.

ACKNOWLEDGMENTS

This study was supported in part by the Russian Foundation for Basic Research, project no. 02-04-50021, and Program "Dynamics of Gene Pools," project no. 10002-251/P-24,154-150/200404-111.

Divergence of Sibling Species of the Group *Drosophila virilis* According to Wing Shape

L. M. Temkina

Laboratory of Genetics, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, ul. Vavilova 26, Moscow, 119991 Russia

The shape of wing plate was studied in 11 closely related species of the group *virilis* using the method of geometric morphometry. According to the classical taxonomy of this group, it consists of two phylades: *virilis* and *montana*. Studies of wing shape variation in the group *virilis* are interesting, since the wing posses a distinct adaptive function providing for flight and species specific nuptial behavior of males. A total of 1780 wings was analyzed, whose shapes were described with the help of 20 marker dots that determine the beginning and end of veins, and the sites of their branching. The images were processed and analyzed using the Thin Plates Splines software. The images of wings are superimposed and equalized by the studied marker dots. This leads to the calculation of consensus wing shape, which is used to calculate new characters: Procrustes distances and axes of deformation. These characters were analyzed using the multidimensional statistics methods, such as dispersion and discriminant analysis, method of main components, and cluster analysis. Dispersion analysis of specific deformations has shown the absence of sexual dimorphism for the wing shape in the studied *Drosophila* group and species specificity of all studied marker dots. The contribution of different marker dots to the extent of differentiation of species by the wing shape and the differences between sibling species were evaluated using the discriminant analysis. This method allowed identification of species within the limits of the studied group with a high precision (98–100%). Analysis of the obtained discriminant functions has shown that both cross veins and site of intersection of costal and radial 2 + 3 veins are involved in the formation of wing species specific characters. The degree of closeness of the studied species by characters of con-

sensus wings calculated for all 11 species was estimated using the cluster analysis. The obtained picture of divergence of the studied species by the wing shape characters radically differs from the classical topology of phylogenetic trees based on such characters as inversion polymorphism or neutral molecular features.

ACKNOWLEDGMENTS

This study was supported in part by the Russian Foundation for Basic Research, projects nos. 02-04-50021, and 04-04-63035 and Program "Dynamics of Gene Pools," project no. 10002-251/P-24,154-150/200404-111.

Use of Isolated Neuron for Monitoring of Neuroactive Factors of Environment of the Generator of Motor Behavior

I. A. Chistopol'skii

Laboratory of Comparative Physiology, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, ul. Vavilova 26, Moscow, 119991 Russia

Feeding behavior of the pond snail *Lymnaea stagnalis* is generated in the paired buccal ganglia, one of the functions of which is to control rhythmic movements of the scraping mouth apparatus. The standard motor cycle comprising three phases is provided by alternate activities of the central generator neurons. During long-term experiments on preparations of the isolated snail CNS, we followed changes in the medium composition near the isolated buccal neuron B4 (or B4CL) placed side by side with the chosen region of ganglion surface (the neuron was isolated from CNS of another animal). The generator activity was also followed by *in situ* recording of the electrical activity of neuron B4, which was a part of one of the tested buccal ganglia. The neuron activity *in situ* contained, besides the proper motor rhythm with a period of ca. 7 s, slow rhythms with periods of the order of 50 and 500 s. The results obtained suggest that the electrical activity of the isolated neuron used as a sensor correlates with the activity of neuron *in situ* during the periods of both slow rhythms. A strong chemical effect of the ganglion on membrane potential and frequency of spikes of the isolated neuron suggests the involvement of periodic chemical emissions realized by the ganglion cells in the formation of rhythmic activity of the generator of feeding motility.

ACKNOWLEDGMENTS

This study was supported by the Russian Foundation for Basic research, project no. 02-04-48901.