Growth factors and proto-oncogenes in early mouse embryogenesis and tumorigenesis

KRESIMIR PAVELIĆ*, NIVES PECINA SLAUS and RADAN SPAVENTI

Ruder Boskovic Institute, Zagreb, Republic of Croatia, Yugoslavia

ABSTRACT Growth factors and proto-oncogenes play an important role in the regulation of embryonic growth and differentiation as well as in tumorigenesis. Insulin and insulin-like growth factor I (IGF I) are secreted by embryonic tissues during the prepancreatic stage of mouse development. Measurable amounts of these factors were found in 8- to 12-day-old embryos. Embryonic cells derived from 8- to10-day-old embryos secrete insulin and IGF I in serum-free medium. Relatively high levels of *c-myc, c-fos* and *c-H-ras* oncoproteins were also detected in 8- to12-day-old embryos. Insulin and IGF I, when added to the culture of embryonic cells, stimulate their proliferation. Similar results were obtained in some animal or human tumors. Murine myeloid leukemias and melanoma B 16 secrete a substance immunologically cross reactive with insulin (SICRI) both *in vivo* and in serum-free media. In culture, the DNA synthesis rate per leukemic or melanoma cell is proportional to cell density and is reduced by antiinsulin serum in case of leukemic cells. Human hemangiosarcoma secrete IGF I, which also plays a role as an autocrine factor. Purified IGF I efficiently induce *c-myc* and *c-fos* mRNA, which is among the earliest events following growth factor stimulation, leading to mitosis. These results lead us to the conclusion that IGF I and insulin together with oncoproteins stimulate the growth of embryonic and tumor cells, which is indirect evidence for a paracrine (or autocrine) type of action.

KEY WORDS: *proto-oncogenes, growth factors, embryogenesis, tumorigenesis*

Introduction

In recent years there has been a growing body of information contributing to our understanding of the mechanisms which regulate vertebrate cell proliferation. Many genes and their products which are capable of inducing a transformed phenotype have been identified. Among them are protooncogenes – a family of genes responsible for cell proliferation and differentiation. They show a remarkable degree of evolutionary conservation, suggesting that they serve essential functions (Kahn and Graf, 1986).

Embryogenesis requires extensive proliferation and differentiation. Growth factors and protooncogene products play an important part in this process. Identification of the specific growth factors and oncoproteins involved in embryogenesis is a difficult task, because the quantities of embryonic material available are limited. In the mouse, for example, the first 3 days after fertilization are devoted primarily to continuous multiplication. During the next 2 days, the first two differentiation steps take place to form the layers that will give rise to the fetus and to extra-embryonic structures (Jakobovits, 1986).

After implantation (at about 7 days of gestation) gastrulation begins, followed by major morphogenetic and organogenetic processes during the next 5 days. Our studies were focused on the expression and role of insulin, IGF I, *c-myc*, *c-fos*, and *c-H-ras* at early stages of mouse embryogenesis (days 8 to 12 of gestation) as well as in tumorigenesis.

Insulin and IGF I in embryonic tissue

Insulin and IGF I play an important part in the regulation of cell growth, division and differentiation in the later stages of embryogenesis, when organogenesis is completed. Our recent work presents evidence that insulin and IGF I are also secreted by embryonic tissues during the prepancreatic stage of mouse development (Spaventi *et al.*, 1990). We found measurable amounts of insulin and IGF I in 8- to 12-day-old mouse embryos (Fig. 1). Insulin activity reached maximum level in the 9-day-old embryos. To show that the activity was the result of insulin synthesis, we investigated the presence of C-peptide, the secondary product of this process. The relation between the amount of this peptide and insulin was proportional, giving indirect evidence for insulin-derived activity (Spaventi *et al.*, 1990).

The concentration of IGF I varies between different days of development, but the highest values were detected on days 9 and 12 (Fig. 1). We also showed that embryonic cells derived from 8-, 9- and 10-day-old mouse embryos secrete insulin, IGF I and/or related

***Address for reprints**: Ruder Boskovic Institute, Bijenicka c. 54, P.O. Box 1016, 41001 Zagreb, Republic of Croatia, Yugoslavia. FAX: 38-41-425.497

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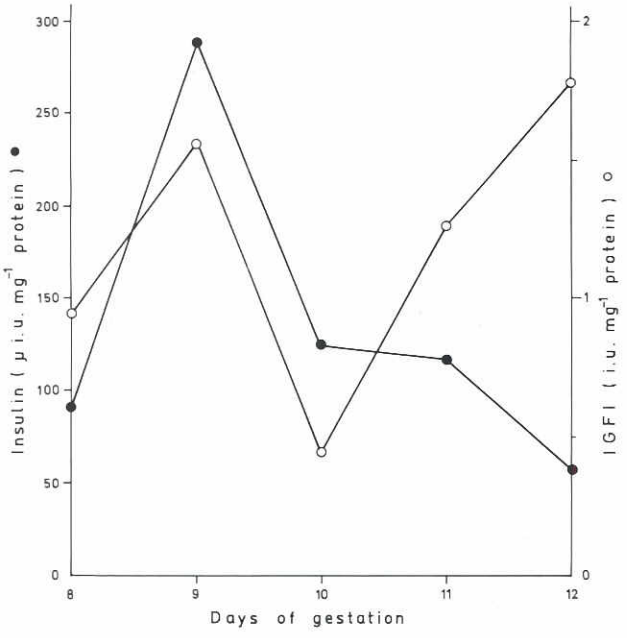

Fig. 1. Insulin (●) and IGF I (○) concentration in tissue homogenates of 8- to 12-day-old mouse embryos measured by specific RIAs. *(Spaventi* et al., *1990).*

molecules (Fig. 2). Therefore, these embryos were selected for further analysis. The cells derived from 9-day-old embryos were cultured in serum-free conditions. Either insulin or IGF I was added to these cultures at physiological concentrations. Three days later the cells were counted. The number of cells was significantly higher in the cultures with either insulin or IGF I, as compared to the control cultures where cells were grown without the investigated growth factors.

These results suggest that insulin can stimulate the growth of embryonic cells during the period when the pancreas is not yet formed, which is indirect evidence for a paracrine (or autocrine) type of action (Spaventi *et al.*, 1990).

Sufficient data from other authors have been accumulated on insulin-like growth factors to suggest their involvement in embryonic development. Rat IGF II was detected at high levels in fetal serum (Moses *et al.*, 1980), and in supernatants of fetal liver explants (Rechler *et al.*, 1979) and embryo fibroblasts (Adams *et al.*, 1983). Similarly, explants of multiple organs from mouse embryos were found to secrete IGF I (D'Ercoli *et al.*, 1980). In addition, both type I and II IGF receptors are expressed on F 9 and PC 13 cells (Nissley and Rechler, 1984).

Upon differentiation, their number decreases dramatically. The apparent loss of receptors might be explained by the differentiated cells, possibly by an autocrine mechanism (Jakobovits, 1986).

c-myc, *c-fos* and *c-ras* oncogenes in embryonal tissue

It has been presumed from the outset that protooncogenes play a role in growth control, mainly because of their potential to induce uncontrolled cell proliferation. This notion is strongly supported by evidence that the products of several protooncogenes are either growth factors or growth factor receptors (Wagner and Müller, 1986). Particular oncogenes are directly involved in the mitogenic signal – *ras*, *myc* and *fos*. To determine whether these protooncogenes are expressed in mouse embryos (8-12 days), we performed an immunohistochemical assay to localize specific oncoproteins: *c-myc* and *c-fos* in the nucleus and *c-H-ras* in the cytoplasm (Figs. 3 and 6 A,B).

Relatively high levels of *c-myc*, *c-fos* and *c-H-ras* oncoproteins were detected in 9-12-day-old embryos using specific monoclonal antibodies against these oncoproteins (Fig. 3). Our interest was focused on nuclear events, especially activation of *c-myc* oncogene. One of the most conspicuous features of this oncogene is its involvement in the regulation of cell proliferation. The *c-myc* protein is found in the nucleus and it contributes to a cell's competence to enter and progress through the cell cycle. Overexpression or unregulated expression of this protooncogene is believed to play an important role in the genesis and progression of a variety of tumors (with high levels of expression being associated with clinical aggressiveness in a variety of human neoplastic diseases), as well as in embryonal growth (Field and Spandidos, 1990).

PolyA RNA prepared from day 6, 7, 8 and 9 conceptuses containing the embryo proper and extraembryonal tissues showed high levels of *c-fos*-related sequences. More than a 10-fold lower *fos* expression was observed in embryos of later developmental stages. *c-fos* -related transcripts were found at considerably higher quantities in the placenta (Müller *et al.*, 1982) than in the embryo proper and the extraembryonal membranes. Consistent with these observations is the high level of *c-fos* expression in day 18 placenta and the absence of significant amounts of *c-fos* -related transcripts in day 12 extraembryonal membrane. These findings suggest that *c-*

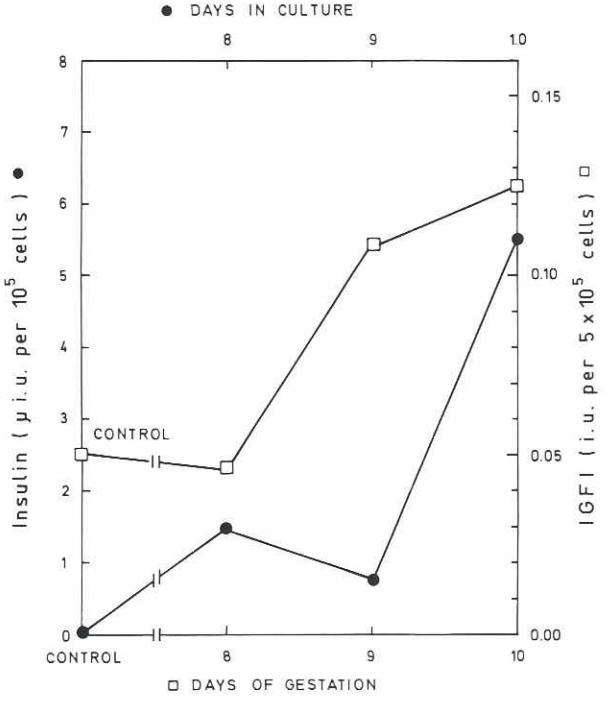

Fig. 2. Insulin (●) and IGF I (□) concentrations in the supernatants of cells derived from 8-, 9- and 10-day-old mouse embryos, and normal mature mouse fibroblasts. *In the supernatants of mouse fibroblasts there was no measurable insulin activity (control) (Spaventi* et al., *1990).*

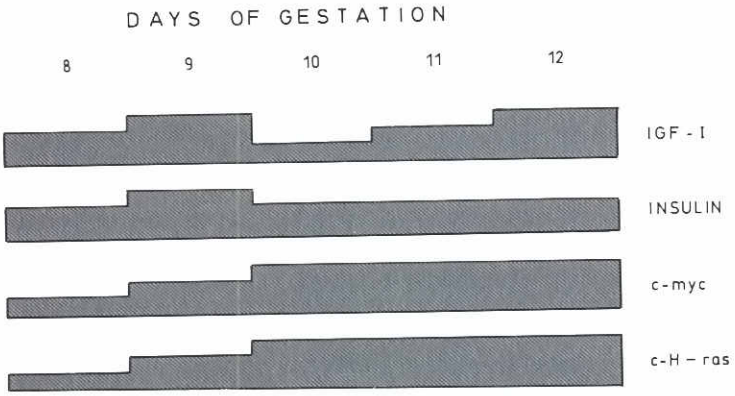

Fig. 3. *In vivo* expression of IGF I, insulin, *c-myc* and *c-H-ras* oncoproteins from 8-12 days of mouse gestation. *For IGF I and insulin see details in Fig. 1. Oncoproteins c-myc and c-H-ras were determined immuno-histochemically using the peroxidase-anti-peroxidase method with specific monoclonal antibodies raised against p62$^{c\text{-}myc}$ or p21$^{c\text{-}H\text{-}ras}$. Embryonic tissues were fixed in acetone or using the Zamboni method and embedded in paraffin. Four mm sections were performed and immunostained. Intensity of growth factors and oncogene expression was graduated from weak (+) or mild (++) to strong (+++), and shown graphically.*

fos expression at early developmental stages (days 6-9) probably occurs in cells which give rise to the placenta at later developmental stages. Expression of *c-fos* in the fetus increases about five-fold at later stages of development (after day 16 of gestation).

In an attempt to identify cells expressing *c-fos* transcripts, polyA RNA from various postnatal tissues was analyzed (Müller *et al.*, 1982). In all tissues investigated low levels of *c-fos* expression were observed. However, RNA isolated from «bone» (sternum, ribs and vertebra of 1-3-day-old mice, including bone marrow, muscles and part of the pleura) and «skin» (including the subcutaneous connective tissue, muscles and part of the peritoneum) from 1-3-day-old mice showed a 5- to 20-fold greater hybridization to the *fos*-specific probe than did RNA from all other tissue. Little transcriptional activity of *c-fos* could be detected in any hemopoietic tissue, including fetal liver. Moreover, elevated levels of *c-fos* expression were also observed in the «skin» suggesting that the enhanced *c-fos* transcription occurs in cells of mesenchymal origin.

The role of the *c-fos* gene in normal bone differentiation would be of particular interest, as its viral homologue (*v-fos*) has been implicated in the induction of osteosarcomas by FBJ-MSU in newborn mice (Finkle *et al.*, 1966; Curran and Teich, 1982).

A *ras-H* encoded protein of molecular weight 21K has been identified in both virus-transformed and normal mouse cells. In the present study c-*H-ras* was found to be expressed at considerable, but similar, levels at all stages of prenatal development, both in the embryo proper and in extraembryonal tissues. High levels of *c-H-ras* expression were also observed in various tissues of newborn or 10-day-old mice, particularly in the «bone», brain, kidney and «skin». Analysis of polyA RNA indicates a heterogeneous class of *c-H-ras*-related transcripts with an average size of 1.4 kb for all pre- and postnatal tissues. A portion of guanosine triphosphate binding protein (G protein) is highly homologous with the corresponding region in the *ras* protein (Hurley *et al.*, 1984). Whereas the precise function of the *ras* proteins is not known, their biochemical properties are similar to the properties of the I subunit of the G proteins. Both *ras* and GI are GTP-binding proteins that are associated with the cell membrane (Willingham *et al.*, 1980). Both have GTPase activity and both are substrates for phosphorilation. Both proteins appear to function by interaction with the cytoplasmic portion of cell surface receptors (Kamata and Feramisco, 1984). *Ras* and I subunit of G protein both seem to be encoded by small families of homologous genes (Chang *et al.*, 1982). These similarities suggest that G proteins and *ras* proteins may have analogous functions.

Concept of autocrine growth regulation

The role of insulin, IGF I, c-myc, c-fos *and* c-H-ras *in cell proliferation*

It is over 20 years since the first autocrine models of tumorigenesis were proposed, but recent years have brought some stunning molecular support for these hypotheses. Our lab was one of the first to present such molecular support for three tumor-derived growth

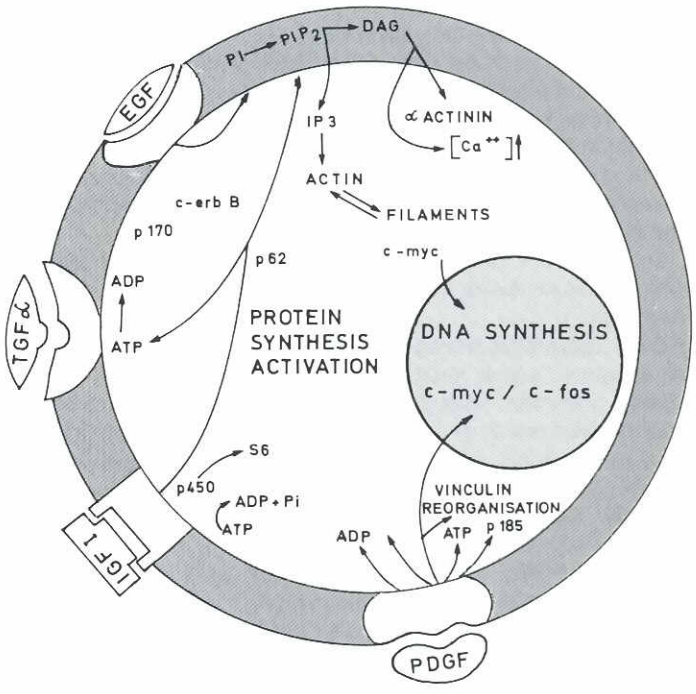

Fig. 4. Involvement of growth factors, cell-surface receptors and oncogene products in cell division. *Growth factor receptors have specific binding sites for ligands and their interaction leads to second messenger activation, inositol lipid metabolism or gene activation. PDGF- platelet-derived growth factor, EGF- epidermal growth factor, TGFα - transforming growth factor α, IGF I- insulin-like growth factor I, PI- phosphatidylinositol, PIP2- phosphatidylinositol-4,5-diphosphate, DAG- diacylglycerol, IP3- inositol-1,4,5-triphosphate, ATP - adenosine triphosphate, ADP- adenosine diphosphate, Pi- phosphate group.*

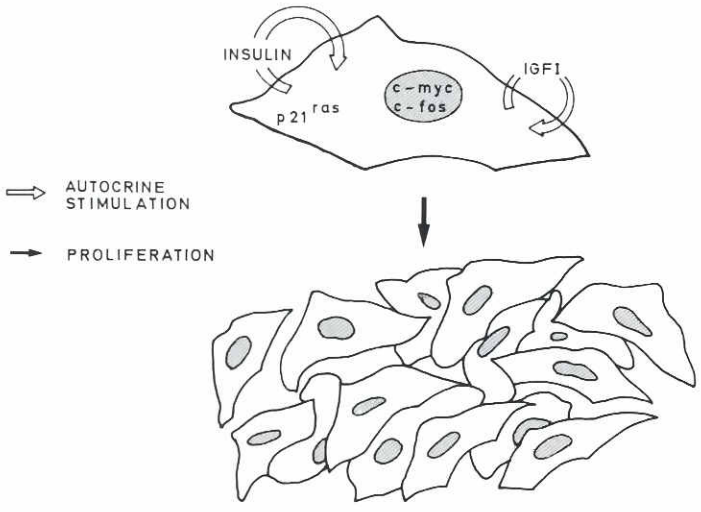

Fig. 5. Hypothetical schematic representation of the growth factor and oncoprotein role in autocrine embryonic growth control.

factor-like molecules: IGF I (somatomedin C) (Pavelic *et al.*, 1986), epidermal growth factor (EGF) (Cabrijan *et al.*, 1988) and substance immunologically cross reactive with insulin (SICRI) (S. Vuk-Pavlovic *et al.*,1986; Z. Vuk-Pavlovic *et al.*, 1986).

Autocrine regulation is defined as a mechanism of self-control in growth and differentiation which involves several events: autonomously controlled production and secretion of autocrine mediators, distribution of autocrine mediators among cells, expression by cells of functional receptors for autocrine mediators, transduction and intracellular integration of signals mediated by autocrine mediators, growth response and maintenance of autonomous control of the state of growth and/or differentiation in the progeny (Bajzer and Vuk-Pavlovic, 1990).

Some tumors have been found to produce growth factors whereby they stimulate their own growth and maintain transformed phenotype.

Once a growth factor has been activated by the binding of ligand, the mitogenic signal must travel through the cytoplasm to the nucleus, ultimately leading to a round of DNA synthesis and to cell division (Kahn and Graf, 1986). Many growth factor receptors and oncogene products possess protein–tyrosine kinase activity with tyrosine phosphorylation, which is important in the mitogenic response. These kinases lead to the phosphorylation of a large number of cellular proteins.

Insulin interacts with the extracellular I subunit of the receptor, activating the protein kinase of the cytoplasmic b subunit. Ligand occupation possibly induces receptor aggregation thereby altering the conformation of the b subunit or the two I-b dimers of the receptor tetramer essential for insulin-dependent activation of the receptor kinase (Kahn *et al.*, 1978). The most rapid postbinding effect of insulin is receptor kinase activation. At least two mechanisms are proposed by which the insulin-dependent receptor kinase transduces the insulin signal (Rosen, 1987). The most obvious is by catalyzing the phosphorylation of cellular protein substrates. Autophosphorylation would allow the receptor to interact with or dissociate from one or more cellular constituents, thereby initiating biochemical processes involved in insulin action.

It may be possible to consider further insulin action within the framework of other pathways used by polypeptides to signal across membranes. The adenylate cycle system utilizes a number of different gene products (among them are G proteins) to generate mitogenic signal. A rapid stimulation in the turnover of phosphatidylinositol (PI) (Masters and Bourne, 1986) is a crucial cascade of events in the transduction of signal from membrane to nucleus (Beridge and Irvine, 1984). This cascade (or pathway) involves the phosphorylation of phosphatidylinositol (PI), in two steps, to yield phosphatidylinositol diphosphate (PIP_2), which is then hydrolized by phospholipase C to form diacylglycerol (DG) and inositol triphosphate (IP_3). Diacylglycerol is an activator of the Ca^{++} (phospholipid-dependent serine) threonine-specific protein kinase, protein kinase C (Nishizuka, 1984). One of the early nuclear events in these processes is activation of *c-myc* and *c-fos* oncogenes.

Recently, we described production and secretion of substances which cross-react with insulin-specific antibodies (SICRI) in several human (Pavelic, 1979; Pavelic *et al.*, 1981; Pavelic *et al.*, 1982a; Pavelic *et al.*, 1982b; Pavelic and Vuk-Pavlovic 1983; Baltic *et al.*, 1985) or animal tumors, and IGF I in human hemangiosarcoma (S. Vuk-Pavlovic *et al.*, 1986; Z. Vuk-Pavlovic *et al.*, 1986; Cabrijan *et al.*, 1988).

A human primary hemangiosarcoma was derived from a patient with severe hypoglycemia. Cell line established from that tumor secreted IGF I in serum-free culture media and this, in turn, stimulated cell proliferation within the culture. By modulating IGF I secretion with human GH or cortisol we were able to stimulate or reduce tumor proliferation. Our results suggest that clinical therapy designated to lower the amount of IGF I released by tumor cells may be beneficial in inhibiting tumor growth *in vivo*.

Similar results were obtained with two different growth factor molecules: EGF (Cabrijan *et al.*, 1988) and SICRI (Vuk-Pavlovic *et al.*, 1986). We found that progression of melanoma B16, growing in diabetic and nondiabetic C57BL/6 mice, is paralleled by the increase of SICRI levels in the serum of both diabetic and nondiabetic animals; this increase correlates with a decreased concentration of circulating glucose and an elevated concentration of growth hormone in blood (S. Vuk-Pavlovic *et al.*, 1986). Murine myeloid leukemia secretes SICRI both *in vivo* and in serum-free media, but high SICRI concentrations in peripheral blood of tumorous animals do not affect circulating glucose levels (S. Vuk-Pavlovic *et al.*, 1986).

Concept of competence and progression

The commitment to growth may be dependent upon two different group factors: factors of competence (PDGF) and factors of progression (IGF I, EGF) (Bravo and Müller, 1986). The first group of factors decide between quiescence and proliferation competence. Under the influence of progression factors, cells can progress toward DNA synthesis. We found that the presence of these factors in mouse embryonal fibroblasts can influence proliferation capacity of the cells. The addition of IGF I or insulin into media increases proliferation capacity of these cells. These peptides act as stimulatory factors inducing and controlling a number of biochemical events which are needed to duplicate all cell components. The final consequence is exponential growth. Therefore, we presume that the presence of growth factors is responsible for mitotic events in these cells in an autocrine fashion. One of the earliest nuclear events in mitogenic signal is *c-myc* protooncogene expression.

The involvement of *c-myc* gene in cell proliferation has been

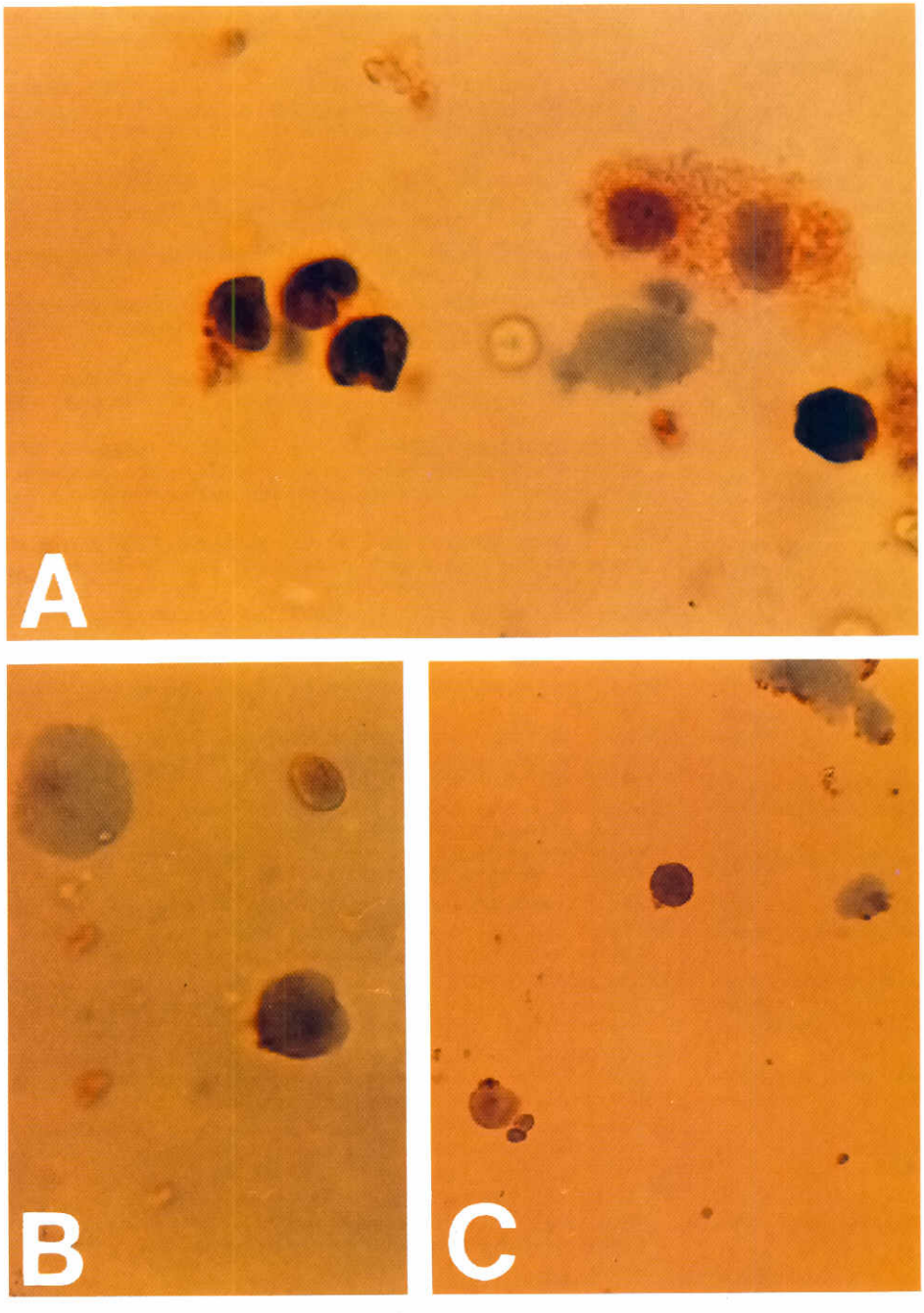

Fig. 6. Immunohistochemical localization of p62$^{c\text{-}myc}$ oncoprotein in the nucleus and p21$^{c\text{-}H\text{-}ras}$ oncoprotein on the inner part of the membrane and in the cytoplasm of embryonic cells derived from 8-day-old-embryo.

investigated by studying its expression following growth stimulation of tumor, as well as embryonal cells. In these experiments cells were stimulated with different growth factors. One hour after stimulation, the steady-state level of *c-myc* mRNA shows an increase followed by a slow decrease reaching basal levels of expression at approximately 16 h (Bravo and Müller, 1986). Purified growth factors (EGF, IGF I, FGF and PDGF) efficiently induce *c-myc* in leukemia cells (unpublished data), as well as *c-myc* and *c-fos* in hemangiosarcoma cells. The observation that *c-myc* (and *c-fos*) induction is among the earliest events following growth factor stimulation of tumor and embryonal cells suggests that both proto-oncogene products play an important role in conferring competence on stimulated cells. There is some evidence that *c-fos* and *c-myc* may function in concert to promote cell division. The fact that IGF I can also induce competence genes (*myc* and *fos*) in tumor cells and embryonal fibroblasts, suggests that IGF I and insulin in some cells can probably bypass the need to PDGF or EGF (competence factors) in the induction of competence.

Besides these facts, our understanding of the role that growth factors play in mouse embryogenesis is still in a preliminary phase. Little is known about which cells secrete these growth factors, their *in vivo* targets, or their mechanism of action.

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