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Expression of the insulin-like growth factor II gene in the choroid plexus and the leptomeninges of the adult rat central nervous system

(*in situ* hybridization/fetal somatomedin)

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ABSTRACT **The rat insulin-like growth factor II gene, encoding a fetal somatomedin, expresses a multitranscript family in embryonic/fetal tissues and in the adult brain and spinal cord. By performing *in situ* hybridization on tissue sections of adult brain and spinal cord, we have found that these transcripts are not expressed in neural or glial cells but are expressed in the epithelium of the choroid plexus of each cerebral ventricle and in the leptomeninges. We propose that the choroidal epithelial cells synthesize and secrete insulin-like growth factor II into the cerebrospinal fluid.**

The somatomedins or insulin-like growth factors I and II (IGF-I and IGF-II) are mitogenic polypeptides that are structurally similar to proinsulin (for recent reviews, see refs. 1–3). We have previously shown that the single-copy rat gene encoding IGF-II (rIGF-II gene) is a complex transcription unit that generates a multitranscript family (4). Some of these transcripts have been characterized in the rat hepatic BRL-3A cell line that expresses the gene (refs. 4 and 5; M.B.S. and A.E., unpublished results). The gene uses two promoters, P1 and P2 (4–6). Transcription from P1 generates the 5′ noncoding exon E1(1) (1125 nucleotides; nt), whereas transcription from P2 generates an alternate 5′ noncoding exon, E1(2) (94 nt). The two predominant mature mRNAs [4.5 and 3.5 kilobases (kb)] consist of either E1(1) or E1(2), respectively, connected to three additional exons (E2, 163 nt; E3, 149 nt; and E4, 3.1 kb). Among the several other transcripts, a P2-specific mRNA (1.1 kb) includes only a portion of E4 (the first 650 nt) and is generated by differential polyadenylylation.

The rIGF-II gene transcripts are present in all fetal or neonatal tissues that we have examined, but they are extremely rare or undetectable in adult tissues, with the exception of the brain and the spinal cord (4). Similar observations have been made by other investigators (5, 7–9).

To precisely localize rIGF-II gene transcripts in the central nervous system, we performed *in situ* hybridization on sections from adult rat brain and spinal cord. We show that the IGF-II gene is not expressed in neural or glial cells but is expressed in the choroid plexus of the cerebral ventricles and the leptomeninges of the brain and the spinal cord.

MATERIALS AND METHODS

Enzymes and DNAs. Restriction enzymes, Klenow fragment of the *Escherichia coli* DNA polymerase, and M13 universal sequencing primers were from New England Biolabs; SP6 and T7 RNA polymerases and S1 nuclease were from Bethesda Research Laboratories; placental RNase inhibitor (RNasin) and the plasmid vector pGEM-1 (a derivative of plasmid pSP64, containing the promoters of the bacteriophage SP6 and T7 polymerases) were from Promega Biotec (Madison, WI); [α-^{32}P]dNTPs (800 Ci/mmol; 1 Ci = 37 GBq), (α-[^{35}S]thio)UTP (1200 Ci/mmol), and nylon membranes (GeneScreen*Plus*) were from New England Nuclear; pd(N)$_6$ oligomers were from P-L Biochemicals; NTB2 nuclear track emulsion, D19 developer, and fixer were from Kodak; hybridization probes were prepared from subcloned fragments of the genomic clones rIGF25 and rIGF15, and the cDNA clone 27 (see ref. 4).

RNA Analysis. Total cell RNA was prepared from BRL-3A cells (American Type Culture Collection Cell Repository Line 1442) and dissected choroid plexi or other brain regions by the guanidine thiocyanate/cesium chloride procedure (10). For RNA blot hybridization, RNA was electrophoresed on formaldehyde/1% agarose gels and then transferred onto nylon membranes. Prehybridization and hybridization were as described (11). The hybridization probes were either uniformly ^{32}P-labeled, mRNA-complementary, single-stranded DNA synthesized on M13 templates (12) or DNA fragments labeled by randomly primed synthesis using pd(N)$_6$ primers, [α-^{32}P]dNTPs, and Klenow enzyme as described (13). S1 nuclease-protection analysis was done as described (4).

***In Situ* Hybridization.** Tissues from Sprague–Dawley rats were sectioned and prepared for *in situ* hybridization as described (14). Hybridization, followed by washing under stringent conditions, autoradiography, exposure to photoemulsion, development, and counterstaining with hematoxylin/eosin were as described (15). The RNA hybridization probes were prepared as follows. A 545-base pair (bp) coding region fragment from rIGF-II cDNA clone 27 (4) was isolated. This fragment extends between a *Bam*HI site located one nucleotide downstream from the ATG initiator and an *Eco*RI linker present at the end of the fragment three nucleotides downstream from the TGA terminator. The fragment was subcloned into the *Bam*HI and *Eco*RI sites of the pGEM-1 vector polylinker. Transcription with T7 polymerase from *Bam*HI-linearized plasmid generated antisense probe, whereas transcription with SP6 polymerase from *Eco*RI-linearized plasmid generated sense (control) probe. Transcription reactions with these polymerases (10-μl vol each) were performed according to the manufacturer's specifications, using 25 μM ^{35}S-UTP, 500 μM each of the other three dNTPs, 20 units of RNasin, 1 μg of linearized template, and 20 units of polymerase.

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Abbreviations: IGF-I and -II, insulin-like growth factors I and II, respectively; rIGF-II, rat IGF-II; CSF, cerebrospinal fluid.

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RESULTS

We had previously demonstrated by RNA blot hybridization that rIGF-II gene transcripts are present in the steady-state population of RNA extracted from embryonic/fetal and adult brain and from adult spinal cord (4). To localize these RNA species in the nervous system of adult rats we performed *in situ* hybridization studies. Sagittal and coronal sections of whole rat brain at 50-μm intervals were hybridized to ^{35}S-labeled, single-stranded, coding-region RNA probes (sense and antisense; see *Materials and Methods*). Autoradiography of the sections that were hybridized with antisense probe revealed intense hybridization signals localized within each of the four cerebral ventricles, whereas there was no evidence of hybridization above background in the remainder of the brain tissue (Fig. 1 *a–d*). Hybridization signal outlining the edges of the sections was also detected (Fig. 1 *b–d*). The control (sense) RNA probe yielded negative results (Fig. 1 *c'* and *d'*). These observations suggested that the rIGF-II gene is active in the choroid plexus of the ventricles and in the leptomeninges.

The choroid plexus is a villous structure that projects into each cerebral ventricle (refs. 16–21 and refs. therein) and is largely responsible for the production and secretion of the cerebrospinal fluid (CSF). The plexus consists of choroidal epithelium (a single layer of cuboidal cells) covering a stromal core containing blood vessels (Fig. 1*e*). The epithelium is modified ependyma (the lining of the ventricular cavities), whereas the core is thought to be derived from pia mater.

The leptomeninges (Fig. 1*e*) are traditionally described as two membranes, the arachnoid mater and the pia mater, separated by the subarachnoid space, which is filled with CSF. They are composed predominantly of flat leptomeningeal cells, organized in different locations into cell layers of variable thickness (refs. 16, 22–28, and refs. therein). Generally, the arachnoid is several (usually three) cell layers thick, whereas the pia consists of a single layer of cells. Some cells of the inner layer of the arachnoid are connected to cells of the pia by thin cytoplasmic processes (trabeculae), which traverse the subarachnoid space (Fig. 1*e*). Pial cells accompany the walls of the larger blood vessels as they enter the cerebral parenchyma from the subarachnoid space, thereby forming a perivascular coat (Fig. 1*e*).

Histological examination of the brain sections after exposure to a nuclear track emulsion confirmed our interpretation of the autoradiographic results: numerous silver grains were present throughout the cytoplasm of the choroidal epithelial cells (Fig. 2 *a–c*) at approximately the same density in the plexus of each ventricle. However, we cannot exclude from these data the contribution of stromal cells to the hybridization signal. In contrast, silver grains above background were absent from the ependymal cells; a sharp transition between hybridizing and nonhybridizing epithelium was evident at the point of attachment of the choroid plexus to the ependymal lining (Fig. 2 *a* and *b*).

Uniformly distributed silver grains at high density were also detected in the leptomeninges covering the cerebral hemispheres (Fig. 2 *d–f*), the cerebellum, and the brain stem (data not shown). Notably, the cytoplasm of the trabeculae, which connect the arachnoid and pial membranes, also showed intense hybridization (Fig. 2*f*). Although we could not distinguish histologically between leptomeningeal cells and arachnoidal fibroblasts, the prominent trabecular hybridization strongly suggests that at least the former cells contain IGF-II transcripts. We found that assessment of silver grains over the pia mater was more difficult than for the arachnoid because the pia is thinner and can be easily distorted. Thus, in some regions we did not detect hybridization above background along the parenchymal perimeter corresponding to the pia mater, but in other regions silver grains were clearly present, albeit at lower density than in the arachnoid. Additional evidence, suggesting that cells of the pia layer of the leptomeninx also synthesize IGF-II transcripts, was provided by the observation that many larger-diameter intraparenchymal blood vessels were encircled by a ring of silver grains presumably corresponding to the pial perivas-

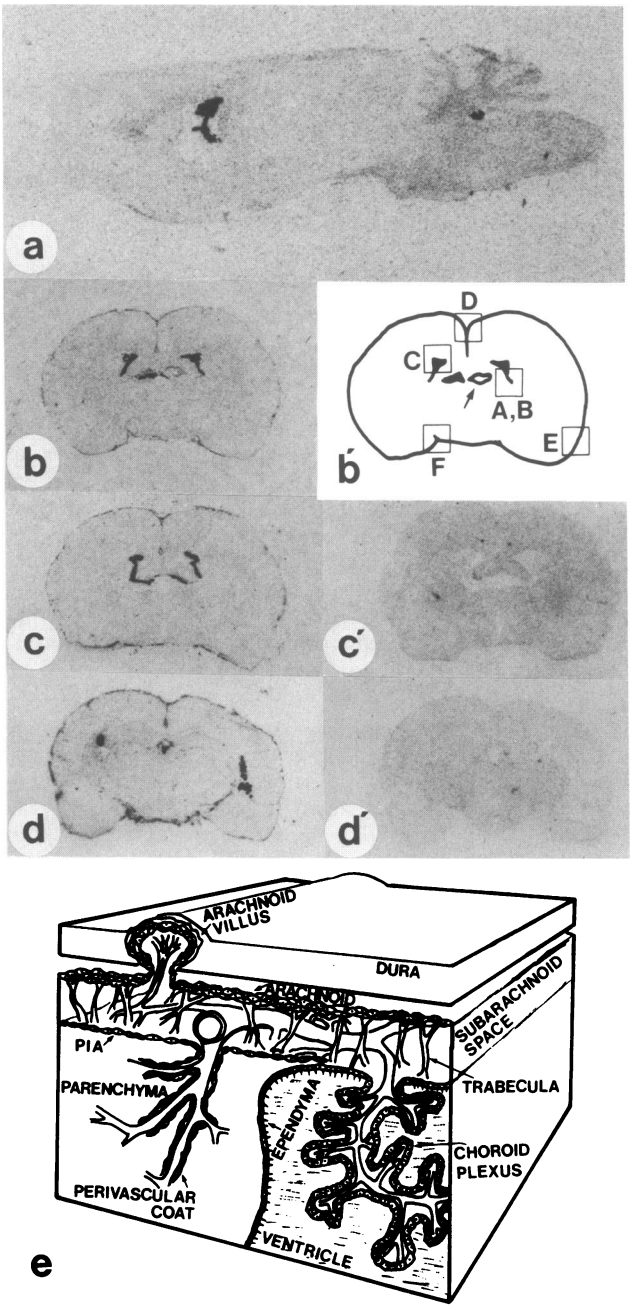

FIG. 1. Autoradiograms of brain sections hybridized with ^{35}S-labeled RNA probes, antisense (*a–d*) or sense (*c'* and *d'*). The hybridization signal is localized within one of the lateral ventricles (at left) and the fourth ventricle (at right) seen in sagittal section (*a*); and within the lateral (left and right) and third (center) ventricles (*b–d*) seen in coronal sections at the levels of the anterior third ventricle (*b*), foramen of Monroe (*c*), and posterior third ventricle (*d*). Hybridization signal is outlining the perimeter of the sections and the interhemispheric fissure in *b–d*. (*c'* and *d'*) Sections adjacent to *c* and *d*, respectively. (*b'*) Diagram of the section in *b*. The squares A–F indicate, respectively, the regions shown in *a–f* of Fig. 2. An arrow indicates the transverse cerebral fissure, the perimeter of which is outlined by hybridization signal in *b*. (*e*) Schematic representation of the relationships between the meninges, choroid plexus, ependyma, and brain parenchyma.

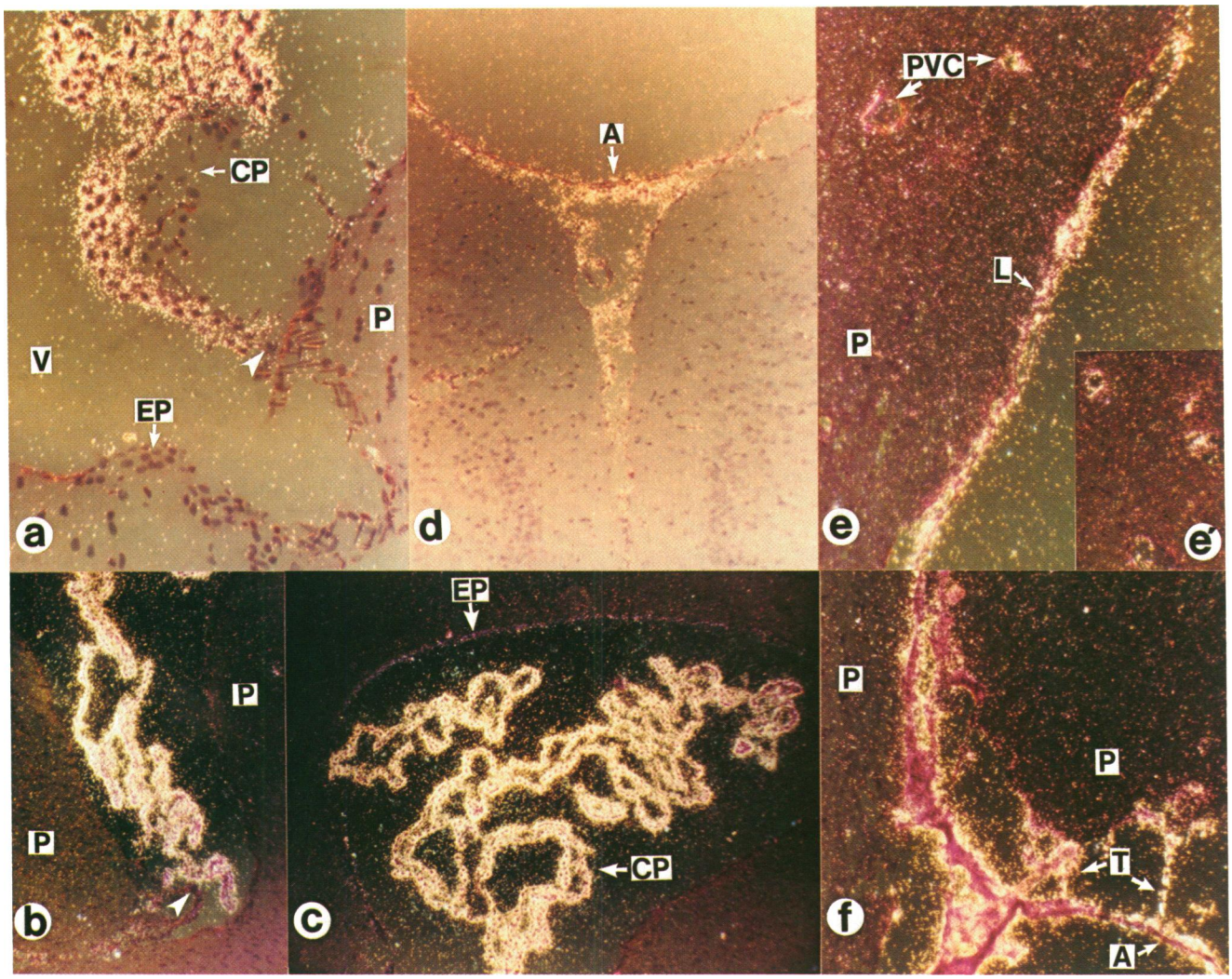

FIG. 2. *In situ* hybridization of regions of brain sections (see Fig. 1*b'*). Silver grains are seen in the epithelial cells of the choroid plexus (CP) of the lateral ventricle (*a–c*) and in the leptomeninx (L) or its arachnoid component (A) (*d–f*). The abrupt loss of hybridization signal in the ependymal (EP) cells is indicated by an arrowhead (*a* and *b*; different sections of the same region in different magnification). V, P, and T indicate the ventricular space, the parenchyma, and trabeculae. Arrows in *e* indicate rings of hybridization signal in the perivascular coat (PVC) of parenchymal vessels (see also inset *e'*).

cular coat (Fig. 2*e* and inset *e'*). Autoradiographic and histological examination of sections of the spinal cord also demonstrated an intense hybridization signal localized exclusively in the leptomeninx (data not shown).

Previously, rIGF-II gene transcripts were detected in total RNA extracted from various brain regions by RNA blot hybridization using coding region probes (4, 5, 7). These results should now be interpreted as due to the presence in the preparations of RNA from the choroid plexus and/or the leptomeninx, portions of which presumably remained associated with parenchyma during tissue dissection. This interpretation is consistent with estimates of the level of IGF-II transcripts by S1 nuclease-protection analysis; the concentration of IGF-II RNA species is ≈200-fold higher in total RNA extracted from dissected choroid plexi than in RNA prepared from parenchyma of various brain regions still associated with the leptomeninges (data not shown). Unfortunately, microdissection of the leptomeninges away from neural parenchyma was difficult in our attempt to prepare RNA derived exclusively from neural tissue for comparative analysis. Nevertheless, should neural cells also express the rIGF-II gene, the level of transcripts must be below detection limits by *in situ* hybridization, a method with adequate sensitivity to easily detect 150–300 mRNA molecules per cell (29) and probably as few as 5–10 molecules per cell (30).

The conclusion that the parenchymal cells do not actively transcribe the rIGF-II gene is based on our assessment of silver-grain density in coronal and sagittal brain sections. For comparison we examined five different microscopic fields of parenchyma from different brain regions in four pairs of adjacent sections hybridized with antisense or sense probe. In each field, grains were counted in five different areas, each corresponding to 3.77 μm^2. The mean ± SD of the calculated ratios of the experimental and control values for each field was 1.02 ± 0.06, which demonstrates that the appearance of grains in neural parenchyma is due to background hybridization. The corresponding value in parallel examination of the choroid plexus was 19.34 ± 3.55.

The mechanism that maintains the activity of the rIGF-II gene in the adult choroid plexus is not known. However, we have excluded the operation of developmental stage-specific promoters (see *Discussion*). Detailed analysis of the blot hybridization profiles of IGF-II transcripts in choroid plexus RNA, using not only coding region probe but also probes from the alternate 5′ noncoding regions and the 3′ noncoding region, demonstrated transcription from both the P1 and P2 promoters of the gene and did not reveal the occurrence of

any transcripts different from those observed previously (4) in BRL-3A cells and in rat fetal tissues (data not shown).

DISCUSSION

IGF-II is clearly an embryonic/fetal somatomedin in the rat (31), although its exact developmental role is still not known. IGF-II transcripts are expressed during the neonatal period, but these transcripts disappear from most adult rat tissues (4, 9, 32). Although transcripts from the IGF-II gene have also been detected by *in situ* hybridization in several human fetal tissues (33), the developmental expression of this gene apparently differs between humans and rats. In humans, IGF-II appears present in serum at lower concentrations during fetal development than in adults (34). Moreover, IGF-II transcripts are certainly synthesized in human liver during adult life as a consequence of the operation of an adult promoter (35). We have been unable thus far to identify a corresponding adult promoter in rats, and we do not know whether it exists. However, our analysis indicates that the appearance of rIGF-II transcripts in the choroid plexus and the leptomeninges during adult life is due to the operation of the same two promoters (P1 and P2) that function during the embryonic stages.

During development, the cells of the choroidal epithelium differentiate distinctly from the ependymal cells, despite common embryological origin from the neuroepithelial cells that line the internal surface of the neural tube (18). Their morphological and functional differences can now be correlated with the presence or absence of two molecular markers; choroidal, but not ependymal, cells transcribe the rIGF-II and transthyretin genes. Transthyretin (prealbumin), a transport protein for retinol and thyroxin, is synthesized *de novo* in the choroidal epithelium and then secreted into the CSF (36–39).

Our evidence, demonstrating that the rIGF-II gene is active in the epithelial cells of the choroid plexus and in the pia/arachnoid cells throughout the neuraxis, establishes a common biochemical marker for these cell types, although their embryological relationship is uncertain. The origin of the leptomeningeal cells is still a matter of controversy. It is believed that they are derived from neuroectoderm (possibly the neural crest), or from mesoderm, or from both (see refs. 27, 40–43, and refs. therein). Nevertheless, the plexus and the leptomeninges are linked physiologically as a functional unit. The CSF is produced and secreted into the ventricles by the choroid plexus, exits through foramina in the roof of the fourth ventricle, percolates through the subarachnoid space, and is reabsorbed into the venous system via the arachnoid villi (see Fig. 1*e*). Thus, the plexus and the leptomeninges constitute the major interfaces of the blood and CSF compartments (16).

The presence of IGF-II in human CSF has been demonstrated (44), and its CSF-to-plasma ratio has been shown to be significantly higher than that expected if IGF-II were transported into CSF via nonspecific routes (44, 45). In humans the IGF-II gene is also probably expressed in the choroid plexus. Interestingly, the IGFs, which are found in the circulation associated with binding proteins (46), are also bound to carrier polypeptides in the CSF (47). One of these carriers, a 34-kDa polypeptide, is enriched in the CSF as compared with plasma and exhibits selective affinity for IGF-II (47). From these observations in conjunction with our results, we propose that the choroid plexus synthesizes and secretes IGF-II into the CSF.

IGF-II has been detected by radioimmunoassays or radioreceptor assays in adult human brain regions (48, 49). These results (48) indicated that the concentration of IGF-II was on the average at least 10 times higher in the pituitary than in any brain region. In the rat we observed abundant IGF-II transcripts in the embryonic pituitary by *in situ* hybridization (unpublished results), but we were unable to detect any such transcripts in the pituitary of adult animals by RNA blot hybridization or *in situ* hybridization (data not shown).

The detection of transcriptional activity of the rIGF-II gene in both the arachnoid and pial cells, which cannot be discriminated morphologically (22–25), suggests that they share at least some common function. However, our data cannot demonstrate whether the IGF-II gene is active in all of the leptomeningeal cells, or in a specific subset [at least two, and possibly more, cellular types constitute the cell population of the leptomeninges (23, 24, 42)]. Also, our results do not prove that these cells are secretory, although this function is not unlikely. Comparative examination of arachnoid and meningioma cells has suggested, from a series of morphological and biochemical criteria, that such cells exhibit both epithelial and mesenchymal features (42). The secretory nature of tumor cells in some meningiomas, which are of leptomeningeal origin, has been suggested (50).

The local production of IGF-II in structures associated with the embryonic and adult nervous system and its circulation in the CSF suggest that this growth factor is functionally important for neurons and/or glial cells. Receptors for both IGF-I and IGF-II are present in adult human and rat brain (51). However, the function of IGF-II in the central nervous system is not known, and direct immunocytochemical evidence for the localization of the growth factor in nervous tissue is currently not available. Nevertheless, the identification of IGF-II receptors in the brain implies that the choroid plexus, and possibly the leptomeninges, may constitute a paracrine system. Several observations are consistent with this notion. For example, a glia maturation factor (an acidic 14-kDa polypeptide), which has no mitogenic effect in the absence of serum, can stimulate the growth rate of normal astroblasts cultured in a defined serum-free medium containing physiological concentrations of IGF-II (52). Moreover, IGF-II stimulates the incorporation of tritiated thymidine into DNA in primary cultures of fetal rat brain cells (53) and in a cell line of human neuroblastoma cells that proliferate in the presence of the mitogen in a defined medium (54). Finally, IGF-II enhances neurite outgrowth in the same cloned human neuroblastoma cells (55) and in primary cultures of sensory and sympathetic neurons from embryonic chick ganglia (56, 57).

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