Abstract. We studied the expression of the N-myc proto-oncogene and the insulin-like growth factor-II (IGF-II) gene in human fetuses of 16-19 gestational wk. Both genes have specific roles in the growth and differentiation of embryonic tissues, such as the kidney and neural tissue. Since continued expression of N-myc and IGF-II mRNAs is also a characteristic feature of Wilms' tumor, a childhood neoplasm of probable fetal kidney origin, we were particularly interested in the possibility that their expression might be linked or coordinate regulated in the developing kidney. Expression of N-myc mRNA was observed in the brain and in the kidney by Northern hybridization analysis. In situ hybridization of the kidney, N-myc autoradiographic grains were primarily located over epitheli ally differentiating mesenchyme while most of the mesenchymal stromal cells showed only a background signal with the N-myc probe. N-myc mRNA was detectable throughout the developing brain with a slight accentuation in the intermediate zone cells in between the subependymal and cortical layers. Thus, even post-mitotic neuroepithelial cells of the fetal cerebrum expressed N-myc mRNA. In Northern hybridization, IGF-II mRNA signal was abundant in the kidney but much weaker, though definite, in the brain. The regional distribution of IGF-II mRNA in the kidney was largely complementary to that of N-myc. IGF-II autoradiographic grains were located predominantly over the stromal and blastemal cells with a relative lack of hybridization over the epithelial structures. In the brain, IGF-II mRNA was about two- to threefold more abundant in the subependymal and intermediate layers than in the cortical plate and ependymal zone, respectively. The fetal expression patterns of the N-myc and IGF-II mRNAs are reflected by the types of tumors known to express the corresponding genes during postnatal life such as Wilms' tumor. However, the apparent coexpression of the IGF-II and N-myc genes in immature kidneys occurs largely in distinct cell types.

According to prevailing theories and experimental evidence, proto-oncogenes and growth factors are believed to play key roles in the growth and differentiation of embryonic tissues (Wagner and Müller, 1986; Adamson, 1987). Thus, expression of some proto-oncogenes and growth factor genes reaches a peak in early development and ceases before or shortly after birth. A failure of repression, as well as later reactivation of these genes (e.g., as a result of chromosomal translocation, deletion, point mutation, or amplification), has been suggested to have a role in preventing the terminal differentiation of cells and allowing for their continued proliferation (for reviews, see Klein, 1987; Bishop, 1987; Kahn and Graf, 1986).

The myc family of cellular proto-oncogenes consists of three known members, the c-myc, N-myc, and L-myc genes, that are believed to regulate cell growth and differentiation (Alt et al., 1986). High expression of the myc genes is frequently found in a wide variety of human tumors (Alt et al., 1986; Alitalo and Schwab, 1986). All myc genes are expressed in embryonic and fetal tissues, but distinct tissue- and stage-specific expression patterns are observed (Pfeifer-Ohlsson et al., 1985; Jakobovits et al., 1985; Zimmerman et al., 1986). This suggests that the differential expression of the myc genes may have a role in regulating multiple differentiation pathways. The N-myc proto-oncogene codes for a nuclear phosphoprotein of yet unknown function (Ikegaki et al., 1986; Mäkelä et al., 1989; Ramsay et al., 1986; Slamon et al., 1986). The gene was originally identified as an amplified, c-myc homologous DNA fragment in neuroblastoma cell lines (Schwab et al., 1983; Kohl et al., 1983), and it has been shown to have a structural organization and a protein product similar to c-myc (Kohl et al., 1986; Schwab, 1986; Stanton et al., 1986).

In mice, the N-myc gene is active in various fetal tissues, predominantly in the brain and in the kidney, the expression ceasing shortly after birth (Jakobovits et al., 1985; Zimmerman et al., 1986). In human fetal kidney, the overall expression of N-myc mRNA has been reported to decrease during 17-21 gestational weeks (Alt et al., 1986). In human fetal brain, N-myc mRNA was found to be expressed by both mi-
totic and undifferentiated neural cells, but no expression was seen in differentiated neurons (Grady et al., 1987). High N-myc expression was also found in tumors displaying neuroectodermal characteristics such as neuroblastomas, retinoblastomas, and small cell lung cancer (Lee et al., 1984; Schwab et al., 1984; Kohl et al., 1984; Nau et al., 1986; Wong et al., 1986). In neuroblastomas, amplification of the N-myc gene, present in ~50% of stage III-IV tumors, predicts a poor prognosis, independently of any other staging criteria (Brodeur et al., 1984; Seeger et al., 1985). Sporadic amplification of N-myc has been reported in, for example, a rhabdomyosarcoma and a lung adenocarcinoma (Garson et al., 1986; Saksela et al., 1986). Interestingly, the unamplified N-myc gene is highly expressed in Wilms' tumors originating from nephrogenic blastemal cells (Nisen et al., 1986).

IGF-II is a member of a family of peptides related to insulin by structure and function. It is synthesized by many fetal tissues (Brown et al., 1986) and may be involved in growth stimulation in the fetus. IGF-II can be detected even in adult human cerebrospinal fluid and brain, suggesting a physiological role of IGF-II in the central nervous system (Haselbacher and Humbel, 1982; Haselbacher et al., 1985). However, no IGF-II mRNA was detected by in situ hybridization with oligonucleotide probes in human and on rat fetal neural tissues (Beck et al., 1987; Han et al., 1987). Transcripts of the IGF-II gene are relatively abundant in the kidney, adrenal, liver, and striated muscle taken from first-trimester human fetuses (Scott et al., 1985; Han et al., 1987). The expression of IGF-II mRNA in these tissues decreases until birth. However, high levels of IGF-II expression, comparable to that in the fetal kidney, have been detected in Wilms' tumors (Reeve et al., 1985; Scott et al., 1985; Haselbacher et al., 1987). The IGF-II gene has been mapped to chromosomal band 11p15 (Brisseiden et al., 1984; Tricoli et al., 1984; Reeve et al., 1985). This finding is particularly interesting, because Wilms' tumors apparently carry a recessive oncogene in the adjacent chromosomal band 11p13, and a germline deletion of this band may correspond to one of the two mutations needed for the development of the tumor (Francke et al., 1979; Ladda et al., 1974; Riccardi et al., 1980; Koufos et al., 1985).

We were interested in comparing the expression of N-myc and IGF-II genes in the kidney and the central nervous system, where their specific roles in growth and differentiation and their deregulation have been implicated. Especially, we wanted to know, whether the spatial expression patterns of these genes could be coordinate and linked to cellular differentiation. For this purpose, we used Northern and in situ hybridization to analyze the tissue-, cell-, and differentiation-specific expression of the N-myc and IGF-II mRNAs in fetal human tissues.

### Materials and Methods

Fetal tissue material was obtained from therapeutic abortions of 16-19 wk of gestation. Adrenal, brain, cardiac, kidney, liver, pancreatic, spleen, testicular, and thymic tissues were identified, dissected free of surrounding tissues and immediately snap-frozen in liquid nitrogen. Samples intended for in situ hybridization analysis were fixed in 4% phosphate-buffered formaldehyde. A stage IV neuroblastoma and a Wilms' tumor, obtained before initiation of chemotherapy, were treated similarly immediately after removal at surgery. Retinal samples were studied only by in situ hybridization because of paucity of material.

### Isolation and Analysis of RNA

Total RNA was extracted by the LiCl/urea-method (Auffray and Rougeon, 1980) or by the guanidine thiocyanate protocol (Chirgwin et al., 1979) from 200 to 1,000 mg of snap-frozen tissue with ultracentrifugation through cesium chloride cushions. 15-µg aliquots of total cellular RNA were fractionated in horizontal 1% agarose/formaldehyde gels, blotted onto synthetic nylon membrane (GeneScreen Plus), and hybridized under stringent conditions according to manufacturer's suggestions. The same filters were probed several times, with elution of previous probe between different experiments by incubating the filter for 30 min at +60°C in 96% formamide with 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, pH 8.0. The 2.0-kbp Eco RI insert of the clone pN6-6 (Schwab et al., 1983), carrying the second exon of the N-myc gene and flanking DNA, was labeled as such for the Northern hybridizations and for in situ hybridizations digested further with Sma I before radioactive labeling to obtain fragments shorter than 600 bp. Similarly, the insert of the phigf2 cDNA clone for IGF-II mRNA (Bell et al., 1984) was generated with Pst I and for the in situ hybridization experiments digested further with Pvu II to obtain fragments shorter than 500 bp. In in situ hybridizations, 100-790-bp bacteriophage A DNA fragments generated with Bgl I were used as negative control probe. The inserts were isolated and purified from agarose gels by isochromophoresis (Öfverstedt et al., 1984). For Northern hybridizations, the inserts were labeled with 32P dCTP (PB.10205; Amersham International, Amersham, UK) and for in situ hybridizations with 35S-dATP (SJ.1304; Amersham International) using nick-translation or the random priming method (Feinberg and Vogelstein, 1983) to achieve specific activities of 1 x 109 cpm/µg. After labeling, the probes were further extracted with phenol-chloroform, run through Sephadex G50 spin columns and ethanol precipitated to remove unincorporated nucleotides.

### In Situ Hybridizations

The in situ hybridizations were carried out on 5-µm-thick sections of formaldehyde-fixed, paraffin-embedded material, using 35S-dATP-labeled insert probes as previously described (Sandberg and Vuorio, 1987). In brief, the sections were mounted on acetylated microscope slides and deparaffinized. To facilitate the access of the probes to the sections, the slides were treated with hydrochloric acid and proteinase K, followed by an additional acetylation step to prevent electrostatic binding of the probes to the sample. The hybridizations and washes were carried out under stringent conditions. The slides were then dipped into the autodiagroemulsion (NTB 3; Kodak Laboratory and Specialty Chemicals, Rochester, NY). After 2-4 wk exposure times at +4°C, the in situ autoradiograms were developed and stained with hematoxylin-eosin. Since regional grain densities are visually difficult to correlate with variation in cell density, grain counting was used. All the quantitative analyses were corrected accordingly; i.e. the results are expressed as grains per cell.

### Immunohistochemical Techniques

Serial sections of the brain were immunostained with a polyclonal rabbit antiserum to glial fibrillary acidic protein (Dakopatts A/S, Glostrup, Denmark). Some sections of the eye were stained with an antiserum to myoglobin (Boehringer Mannheim GmbH, Mannheim, FRG). The bound primary antibodies were visualized using the avidin-biotin-immunoperoxidase technique (Vector Laboratories, Inc., Burlingame, CA), with diaminobenzidine and horseradish peroxidase as chromogen.

### Results

Total RNA was isolated from different tissues of several 16-19 wk human fetuses and analyzed for the content of N-myc and IGF-II mRNA. By Northern blotting, a strong 3.0-kb N-myc mRNA band was seen in samples from fetal brain and weaker one in the kidney samples (Fig. 1). Only a very weak N-myc signal was observed in the other tissues tested and then only after a severalfold increase of exposure times. In comparison, the signal obtained with the IGF-II probe (Fig. 1, 60, 49, and 1.9 kb) was intense in samples from kidney and adrenal tissue. IGF-II mRNA was also seen in the liver, pancreas, and spleen. Interestingly, brain RNA
Figure 1. Analysis of size-fractionated RNAs extracted from 16–19-wk human fetal tissues and hybridized with \textit{N-myc} and IGF-II probes. Typically, a clear \textit{N-myc} signal is obtained from brain and kidney RNAs. Adrenal, liver, spleen, thymus, pancreas, and testis tissues gave only a very weak \textit{N-myc} signal after a severalfold increase in exposure times (not shown) while IGF-II transcripts can be detected in all these tissues. Note that the brain RNA shows a weak but definite IGF-II signal. The RNAs loaded were visualized by ethidium bromide staining before Northern blotting \textit{(EtBr; showing the 28S bands)} to control the amount and integrity of the RNA. The sizes of the \textit{N-myc} and IGF-II-hybridizing RNAs are indicated in kilobases.

![Image of Figure 1](image_url)

Gave a low but definitive IGF-II signal. Kidney and brain were therefore chosen for in situ hybridization analysis of cells responsible for the signals obtained in Northern hybridization.

The kidneys of second trimester human fetuses are relatively well differentiated. While early epithelial differentiation of the mesenchymal blastema still occurs in the outer cortical areas, the inner regions contain well-developed metanephric blastema-derived excretory units (glomeruli and proximal and distal convoluted tubules with the intervening loops of Henle) and collecting tubules derived from the ureteric bud. In in situ hybridization of the kidney, a great majority of the \textit{N-myc} autoradiographic grains were located over the epithelia differentiating mesenchyme (i.e., mesenchymal condensates, comma- and S-shaped bodies in the cortical parts [Fig. 2, \textit{a} and \textit{b}]) but signal was observed also over further differentiated epithelial structures like glomeruli, convoluted tubules, and cells of the loops of Henle (Fig. 2, \textit{c} and \textit{d}). The visual impression of signal over glomeruli in Fig. 2 \textit{d} is accentuated as a result of the high cell density in these structures. This conclusion becomes apparent in grain counting analysis shown in Fig. 3. Over the majority of the mesenchymal stromal cells only a background level of \textit{N-myc} signal was observed, but scattered, intensely labeled single cells were also present, resulting in elevated average grain counts over the mesenchymal stromal cells. In contrast, the IGF-II autoradiographic grains were associated predominantly with the stromal and blastemal cells while the epithelial structures showed a relative lack of IGF-II signal (Fig. 2, \textit{e} and \textit{f}). Control hybridization with labeled \textit{$\lambda$} phage DNA fragments gave a very weak autoradiographic signal (Fig. 2, \textit{g} and \textit{h}).

The distribution of autoradiographic signal over different normal fetal kidney structures, hybridized with the \textit{N-myc} and IGF-II probes, is presented in Fig. 3. It should be noted that the \textit{N-myc} and IGF-II grain counts are not directly comparable with each other because of differences in the complexity and specific radioactivity of the probes and slight variation in autoradiography. The background signal obtained with the \textit{$\lambda$} phage DNA probe was less than two grains per cell and was randomly distributed over the kidney tissue (Fig. 2 \textit{f}).

The distribution of the IGF-II and \textit{N-myc} in situ autoradiographic grains over the Wilms' tumor sections was very similar to their distribution in the developing kidney. Fig. 4 shows a section of a triphasic Wilms' tumor, consisting of the undifferentiated tumor tissue (labeled \textit{T} in Fig. 4 \textit{a}), incompletely formed epithelially differentiating elements (Fig. 4, \textit{c} and \textit{g}), a connective tissue stroma, and the adjacent normal kidney tissue (labeled \textit{K} in Fig. 4, \textit{a} and \textit{e}). The IGF-II mRNA signal was specific for the most primitive tumor cells with only background levels of signal over adjacent postnatal kidney tissue (Fig. 4, \textit{a–d}). Within the tumor, islets of cells showing epithelial differentiation into tubular and glomerular structures resembling the early differentiation stages of embryonic kidney showed a relative lack of IGF-II autoradio-
Percentages of total autoradiographic grains per cell.

Figure 3. Summary of the in situ hybridization analysis of the kidney samples (see Fig. 2) showing relative levels of N-myc and IGF-II autoradiographic grains over the distinct structural components. Columns represent (A) the early cortical differentiation stages, (B) glomeruli, (C) proximal convoluted tubules, (D) distal part of the nephron, and (E) mesenchyme. Total values of average N-myc and IGF-II autoradiographic grain counts per cell over these structures were normalized to 100 after subtracting background counts, and are expressed as percentages found over each structure. N-myc signal located over mesenchymal stroma (E, solid black bar) accounts for only 10% of total N-myc grain density, whereas the IGF-II signal (E, hatched bar) over mesenchyme accounts for 74% of total IGF-II grain density, implying a relative lack of N-myc mRNA and an abundance of IGF-II mRNA in the stroma as compared to epithelial structures.

Figure 2. Histology and autoradiography of sections of human fetal kidney hybridized with the N-myc probe (a-d), IGF-II probe (e and f), and bacteriophage λ DNA control probe (g and h). N-myc signal is present particularly over cortical, epithelioidly differentiating mesenchyme (a and b), but signal is present also over epithelial cells forming the distal part of the nephron (D) as well as cells of the developing glomeruli (G), and to lesser extent in the proximal convoluted tubules (labeled P in c and d). Most cells in the mesenchymal stroma show only background signal with some isolated cells (arrows) showing strong N-myc signal. IGF-II signal is located primarily over the mesenchymal cells with a relative lack of signal over the tubular epithelial structures (labeled P and D) and glomeruli (labeled G in e and f). Very few grains are seen in the sections hybridized with the λ-phage DNA fragments (g and h). The N-myc and the control autoradiograms were exposed for 28 d and the IGF-II slides for 21 d. Bar, 50 μm.

Figure 1. Photomicrographs of brain sections (a-d) showing N-myc and IGF-II autoradiographic grains. In contrast to the IGF-II signal, N-myc autoradiographic grains were present also over the islets of epithelially differentiating cells (Figs. 4, c, d, and h, arrows). The N-myc signal was also found over cells of the cortical plate (Fig. 5, b and d). Again, some cells with a more primitive morphology were apparently devoid of signal. No c-myc expression was detected in the tumor by Northern hybridization or by in situ hybridization in conditions where most fetal tissues gave a readily detectable signal (data not shown).

IGF-II autoradiographic signal in the brain was weak in the ependymal zone adjacent to the lateral ventricles where a definitive N-myc autoradiographic signal was detected (Fig. 5, e and g). In contrast, IGF-II signal in the subependymal and intermediate cell layers was approximately threefold higher than in the ependymal zone (Figs. 5 and 6). A visual accentuation of IGF-II signal was associated with the transitional zone between the intermediate zone and cortical plate as compared to the intermediate zone (Figs. 5, f and h). However, grain counting analysis showed that this was a visual artefact due to increased cell density in the intermediate zone just adjacent to the cortical plate (Fig. 6). The cortical plate again had a reduced IGF-II grain density (Fig. 5, f and h). A summary of the quantitation of grain density in the different structural zones of the 17-wk fetal brain is presented in Fig. 6.
Figure 4. In situ hybridization of IGF-II (a–d) and N-myc mRNA (e–h) in a classical triphasic Wilms' tumor. a, b, e, and f show the peripheral expansion of the tumor against the adjacent kidney tissue. The tumor cells stain dark in the left-hand part of the photographs (T). The pale staining region in the middle is connective tissue, and the normal kidney parenchyma can be seen in the right-hand side (K). c, d, g, and h depict areas of the tumor showing all three components of differentiation typical for a triphasic tumor. Long arrows in c and d point to the tumor-derived structures showing some degree of epithelial differentiation. Note that these structures have a relative lack of IGF-II signal (c and d) as compared to condensates of primitive tumor cells (short arrows). However, the epithelially differentiating structures express elevated levels of N-myc mRNA (long arrow in g and h). Within the mass of primitive tumor cells, scattered cells reacting very strongly with the N-myc probe are observed as a characteristic pattern (short arrows in g and h). Compare also to the normal expression pattern of the developing kidney shown in Fig. 2. Bars: (a–d) 100 μm; (e and f) 100 μm; (g and h) 50 μm.
In retinal tissue analyzed concomitantly with the brain sections, an intense N-myc signal was obtained in the ganglionic cells as well as in the inner and outer nuclear layers. The grains were numerous over the cellular layers while the nerve fiber layers and the pigment layer displayed background levels of signal (Fig. 8, a and b). Interestingly, a weak but definitive N-myc signal was seen over a section of the extrinsic eye muscles (Fig. 9, a and b), identified as striated muscle morphologically and by antimyoglobin immunoperoxidase staining (Fig. 9 c).

Discussion

In our screening of human fetal tissues, abundant N-myc mRNA expression was found in the brain and the kidney by Northern hybridization in accordance with earlier studies (Grady et al., 1987; Zimmerman et al., 1986). IGF-II mRNA was expressed by various fetal tissues including brain and kidney. These tissues as well as retina were chosen for the topographic analysis by in situ hybridization. In the kidney, N-myc mRNA appeared to be expressed by early epithelially differentiating mesenchyme as well as by further differentiated epithelial structures, i.e., the loops of Henle, convoluted tubules, and the developing glomeruli. In contrast, the expression of IGF-II mRNA was largely confined to the stromal and blastemal cells of the kidney. In the developing brain, N-myc mRNA was detected in all the different developmental zones while IGF-II mRNA was most abundant in the intermediate and subependymal zones.

In the kidney, localization of N-myc mRNA into epithelially differentiating mesenchymal cells originating from the metanephric blastema implies that high level N-myc expression is not limited to cells of neuroectodermal origin only. It is possible, though unproven, that the induction of differentiation of the mesenchymal blastema cells (Lehtonen, 1976; Saxén et al., 1986) is associated with the induction of high level N-myc expression. Alternatively, the expression of N-myc is elevated already in the undifferentiated blastema cells, but, at some point during development, the expression ceases in the mesenchymal, stromally differentiating cells. To distinguish between these possibilities, in situ hybridization studies are needed in suitable experimental systems where the inductive events can be observed and manipulated such as in the transfiltre induction model (Ekblom, 1984; Lehtonen and Saxén, 1986; Sariola, 1984).

The blastema-derived cells also give rise to Wilms' tumors where high N-myc expression levels are detected (Nisen et al., 1986). It has been claimed that the expression of IGF-II mRNA by mesenchymal cells presents a form of paracrine growth factor expression as most of the IGF-II immunoreactivity is not found in the mesenchymal or connective tissues, but it is present in the kidney in the renal tubular cells (Han et al., 1987). Our finding of the complementary expression of the IGF-II and N-myc mRNAs in the developing kidney stroma and epithelially differentiating structures, respectively, is thus compatible with the idea that the expression of IGF-II by blastemal and stromal cells provides a paracrine stimulus for the growth and differentiation of the epithelial structures, a stimulus that could also regulate N-myc expression.

Our in situ hybridization results in a triphasic pediatric Wilm's tumor are similar to our findings in the developing normal kidney. IGF-II expression in the tumor is decreased in cells showing features of epithelial differentiation while these abortive epithelial structures resembling the corresponding early differentiation stages of the normal embryonic kidney still expressed N-myc mRNA. These results suggest a downregulation of IGF-II mRNA expression in the epithelially differentiating structures, which is analogous to the findings in normal embryonic kidney cells. Also, N-myc expression in the Wilm's tumor occurs in a pattern predicted by N-myc expression in the normal kidney: in addition to most primitive blastemal cells, those undergoing epithelial differentiation also show N-myc expression. Therefore, the expression of IGF-II and N-myc mRNAs in Wilms' tumors may not be caused by a deregulation of the mechanisms controlling their expression, but it is likely to reflect the normal developmental pattern of gene expression, which is maintained because of a failure of complete differentiation of the tumor cells.

The presence of N-myc mRNA in cells of the intermediate zone and cortical plate of fetal brain is of particular interest because these cells have ceased to proliferate, and yet they express N-myc mRNA. Since N-myc mRNA has a short half-life of ~20 min (Stanton and Bishop, 1987) and the migration of cells into the cortical plate takes 4–6 d (Nowakowski and Rakic, 1981), we exclude the possibility that the signal seen in the nonmitotic cell layers represents mRNA maintained from transcription within the ependymal zone. This implies that the expression of N-myc is not associated with proliferative activity only. On the other hand, N-myc expression decreased in the cortical plate suggesting an association of this downregulation with differentiation. This finding is in line with experimental data obtained from retinoic acid-induced differentiation of primitive neuroblasts that showed decreasing levels of N-myc mRNA before morphological differentiation (Thiele et al., 1985; Amatruda et al., 1985). However, a growth arrest was also detected in the retinoic acid–differentiated cells, which thus partly disables the assessment of the eventual differential effects of decreasing N-myc expression on proliferation and differentiation of the cells. Our estimate for the level of N-myc expression differs somewhat from that obtained by Grady et al. (1987) in that we find slight accentuation of N-myc signal per cell in the intermediate layers when compared with ependymal and cortical layers. However, eventual differences of the brain regions studied by Grady et al. and by us disable direct comparison of the results. Similarly to Grady et al., we detect elevated N-myc mRNA in the inner nuclear and ganglionic cell layers of the fetal retina.

The presence of N-myc mRNA in the striated extrinsic eye muscle may reflect a role for N-myc during normal myogenesis since N-myc mRNA has been detected also in the cardiac muscle in developing mice (Zimmerman et al., 1986). Again, it may reflect the fact that cranial muscles, unlike other striated musculature, arise from the neural crest, being thus of neuroectodermal origin. Interestingly, Garson et al. (1986) have reported amplification of N-myc in a recurrent rhabdomyosarcoma arising as a palatal mass; i.e., in structures derived from the neural crest. This may indicate similarities between cranial rhabdomyosarcomas and some other neural crest–derived tumors in their tendency to gain a growth advantage through increased dosage of the N-myc gene.

The finding of N-myc expression not only in the most
Figure 6. Grain counting analysis of the in situ hybridization results in human fetal brain with the N-myc, IGF-II, and control probes. The photomicrograph shows a GFAP-stained sagittal section of human fetal brain at 17 gestational wk. The zones marked correspond to the columns given in the upper part of the figure: EPZ, ependymal zone; S-EPZ, subependymal zone; IZ, intermediate zone; CDZ, transitional zone between the intermediate layer and the cortical plate, with increasing cell density; and CTX, cortical plate. The radial glia directing the migration of neuroepithelial cells from the ependymal zone to the cortical plate is apparent from the GFAP-stained section. Bar, 200 μm.

Figure 5. Bright corresponding dark field photomicrographs of in situ hybridization of human fetal brain sections with the N-myc (a–d), IGF-II (e–h), and bacteriophage λ probes (i and j). The ependymal region is shown in the left column and the cortical surface in the right column. Expression of N-myc mRNA is apparent throughout the brain (b and d). The proliferative cells in the ependymal zone (EPZ) show fewer autoradiographic grains than the subependymal layers (S-EPZ) (a and c). The IGF-II mRNA is almost totally absent from the ependymal zone (e and g) and present predominantly in the cells of the intermediate zone (IZ). The apparent accentuation of the IGF-II signal between the intermediate zone and the inner margin of the cortical plate (CTX) is a visual artefact caused by local increase in cell density (see Fig. 6). The few grains in Fig. 5 g adjacent to the brain tissue represent nonspecific binding of the probe to the margins of the tissue section, an artefact seen in some sections even with the control probe. Bars: (a, c, e, g, and i) 100 μm; (b, d, f, h, and j) 100 μm.

primitive neuroblastoma cells but also in cells showing some degree of neuronal differentiation bears a direct analogy to our results of hybridization in the fetal brain samples because expression was detected also in cells that had undergone some degree of neural differentiation, as judged by morphology. An earlier report by Schwab et al. (1984) documented the highest N-myc expression in the most primitive portions of the neoplasm. However, the biological variation of neuroblastoma tumors is great and regional variations in them may result from subtle differences in gene expression not apparent morphologically.

Another interesting finding obtained in the present study...
Figure 7. Bright field (a) and dark field (b) photomicrographs of a section of a stage IV neuroblastoma hybridized with the N-myc probe. The heterogeneity of N-myc expression by tumor cells is apparent. N-myc autoradiographic grains, best visualized in dark field microscopy (b), are located over primitive tumor cells (short arrow) as well as cells displaying morphological features of neuronal differentiation such as increased cell size, and large, paler nuclei (long arrows). In addition, some primitive appearing cells are relatively devoid of signal (short arrows). Bar, 100 μm.

Figure 8. A section of fetal retina sample hybridized with the N-myc probe shows abundant signal over all cell layers. Because of the section thickness, the background is shown on the right in a separate picture focused on the background. (1) Outer nuclear layer; (2) inner nuclear layer; (3) ganglion cell layer. Bar, 20 μm.

is the low but definitive IGF-II mRNA signal in the fetal brain. This can very likely be accounted for by a better sensitivity in our study using a nearly full-length cDNA probe, whereas in an earlier study by Han et al. (1987) short oligonucleotide probes were used. Our analysis, then, gives the first spatial information on IGF-II synthesis in the human fetal brain. It was noteworthy to find different patterns of distribution of N-myc and IGF-II mRNAs in the ependymal and subependymal zones. Although the levels of IGF-II expressed must be low, IGF-II mRNA has been shown to be present in the developing rat central nervous system (Brown et al., 1986; Sara et al., 1986; Rotwein et al., 1988), and a physiological role for IGF-II as neuroregulator and/or brain growth factor has been suggested. The absence of the IGF-II mRNA signal in the proliferative ependymal zone and its appearance in the migratory and differentiating zones agrees well with this view. Our results are also consistent with the finding that cultured astroglial but not neuronal cells produce IGF-II (Rotwein et al., 1988) as IGF-II mRNA is most abundant in the intermediate zone, where astroglia is the prevailing cell type. A further complexity of IGF-II mRNA expression has recently emerged from studies of the differential splicing pattern of IGF-II transcripts in different tissues (Irminger et al., 1987). The two promoters that have been found in the human IGF-II gene could very well be regulated in a tissue-specific manner, a matter that will be best resolved in studies of hybrid gene constructs in transgenic mice.

Mechanisms responsible for the emergence of childhood neoplasms present a problem to pathologists, embryologists, and basic scientists. Some common childhood neoplasms, such as neuroblastoma and Wilms' tumor, appear to represent a failure of proper maturation of their tissues of origin with a variable degree of differentiation. Molecular analysis of these tumors has revealed specific deletions of chromosomal DNA that may be associated with an activation of recessive oncogenes in, for example, neuroblastomas, retinoblastomas, and Wilms' tumors (for review, see Cavenee and Hansen, 1986; Cavenee et al., 1986). It has been postulated that the recessive oncogenes act as suppressor genes downregulating the expression of "transforming" genes, which are active during embryogenesis but suppressed during differentiation (Comings, 1973; Klein, 1987). Loss of
both copies of these genes may lift the suppression, thus maintaining the expression of the transforming genes and leading to tumorigenesis. Such events might be fundamental in the genesis of, for example, Wilms' tumor displaying N-myc oncogene and IGF-II growth factor overexpression. Comparative analysis of fetal tissues and embryonally derived tumors may illuminate these questions. Since fetal tissues, unlike most tumors expressing the N-myc gene, display coexpression of the myc family genes, it will be interesting to extend these studies to the localization of c-myc and L-myc transcripts in the same tissues; these ongoing experiments will probably help to clarify the significance of each myc gene and their regulation in cellular proliferation and differentiation during normal human fetal development.

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