

The *N-myc* Proto-Oncogene and IGF-II Growth Factor mRNAs Are Expressed by Distinct Cells in Human Fetal Kidney and Brain

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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 coordinately 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 epithelially 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 post-natal 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 (Brissenden 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, the two embryonic tissues 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 abortuses 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- μ g 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 pNb-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 λ DNA fragments generated with Bgl I were used as negative control probe. The inserts were isolated and purified from agarose gels by isotachopheresis (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 1×10^9 cpm/ μ g. 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- μ m-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 autoradiography emulsion (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 as the 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, 6.0, 4.9, 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

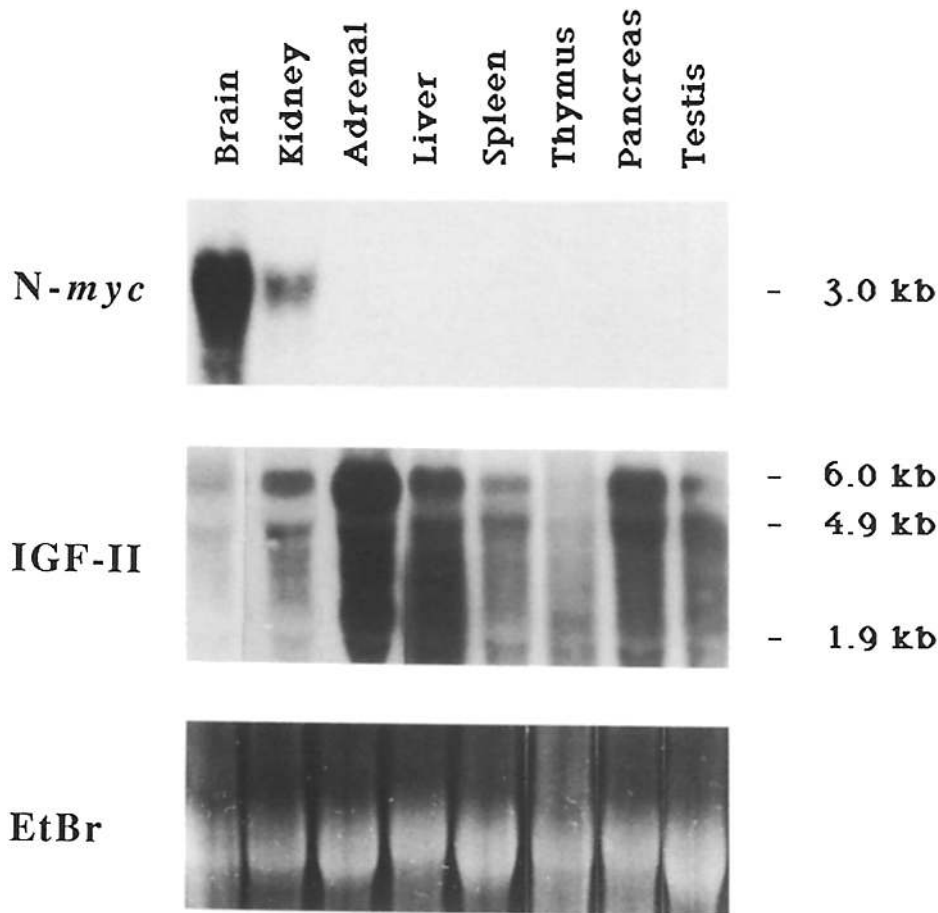


Figure 1. Analysis of size-fractionated RNAs extracted from 16–19-wk human fetal tissues and hybridized with *N-myc* and IGF-II probes. Typically, a clear *N-myc* signal is obtained from brain and kidney RNAs. Adrenal, liver, spleen, thymus, pancreas, and testis tissues gave only a very weak *N-myc* 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 (*EtBr*; showing the 28S bands) to control the amount and integrity of the RNA. The sizes of the *N-myc* and IGF-II-hybridizing RNAs are indicated in kilobases.

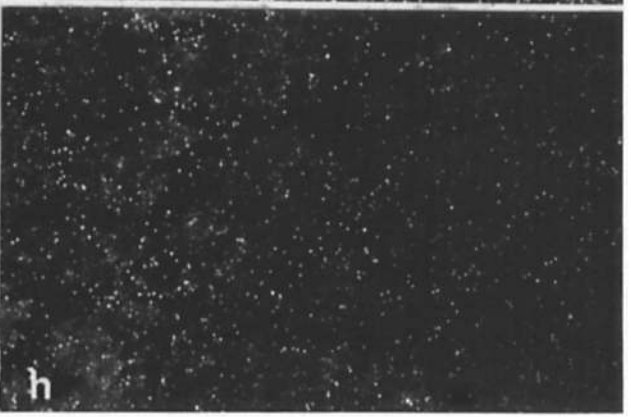
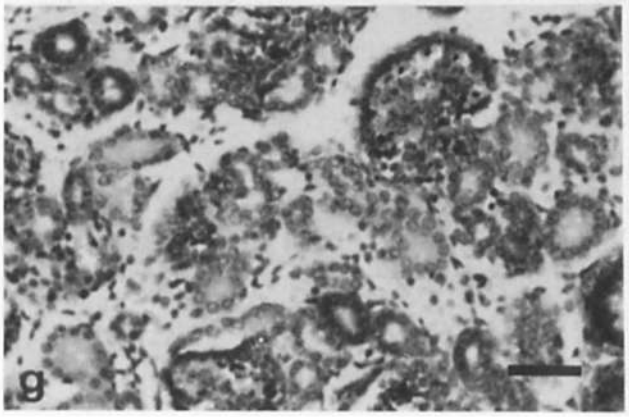
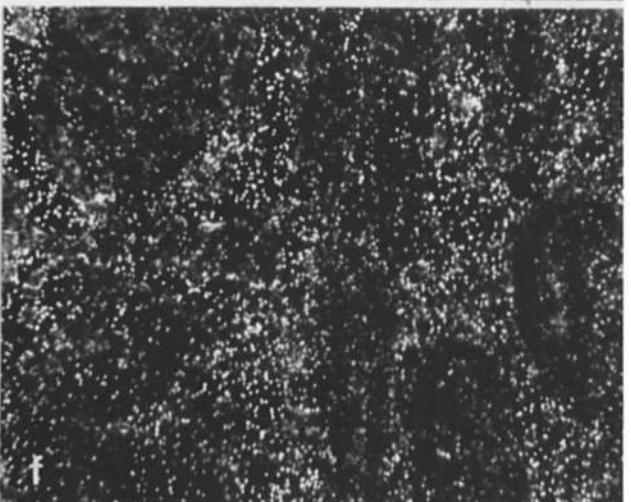
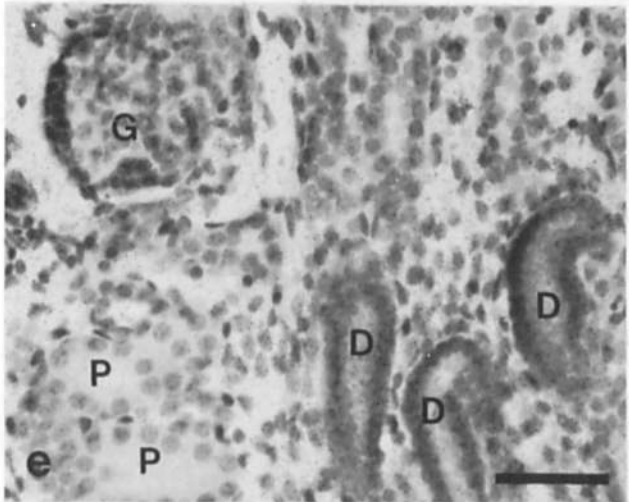
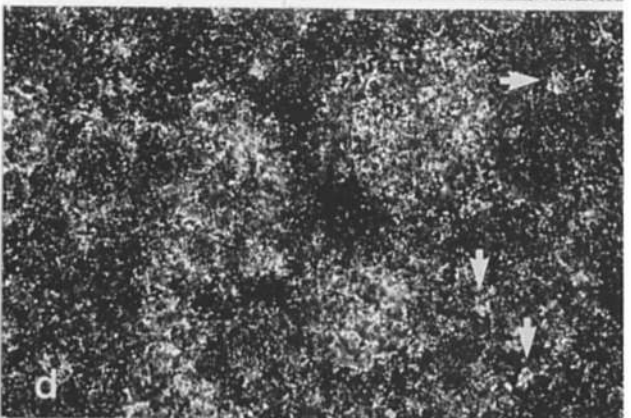
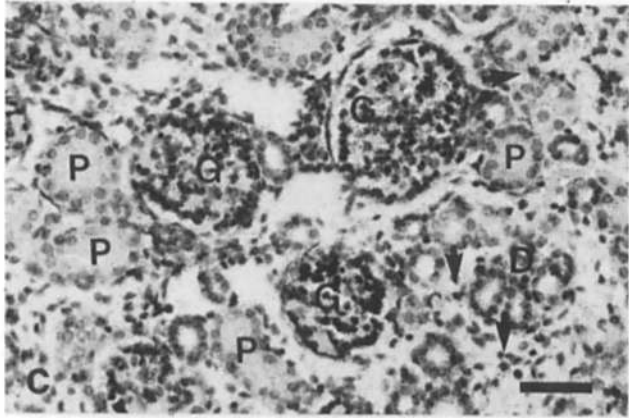
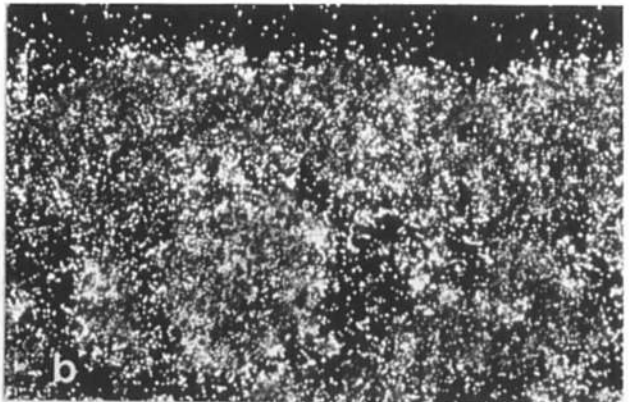
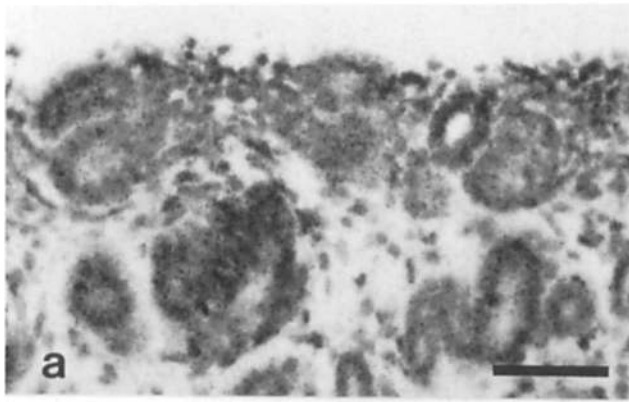
gave a low but definitive IGF-II signal. Kidney and brain were therefore chosen for in situ hybridization analysis of cells responsible for the signals obtained in Northern hybridization.

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 *N-myc* autoradiographic grains were located over the epithelially differentiating mesenchyme (i.e., mesenchymal condensates, comma- and S-shaped bodies in the cortical parts [Fig. 2, *a* and *b*]) but signal was observed also over further differentiated epithelial structures like glomeruli, convoluted tubules, and cells of the loops of Henle (Fig. 2, *c* and *d*). The visual impression of signal over glomeruli in Fig. 2 *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 *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, *e* and *f*). Control hybridization with labeled λ phage DNA fragments gave a very weak autoradiographic signal (Fig. 2, *g* and *h*).

The distribution of autoradiographic signal over different normal fetal kidney structures, hybridized with the *N-myc* and IGF-II probes, is presented in Fig. 3. It should be noted that the *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 λ phage DNA probe was less than two grains per cell and was randomly distributed over the kidney tissue (Fig. 2 *f*).

The distribution of the IGF-II and *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 *T* in Fig. 4 *a*), incompletely formed epithelially differentiating elements (Fig. 4, *c* and *g*), a connective tissue stroma, and the adjacent normal kidney tissue (labeled *K* in Fig. 4, *a* and *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, *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-



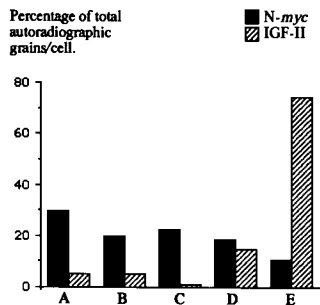


Figure 3. Summary of the in situ hybridization analysis of the kidney samples (see Fig. 2) showing relative levels of *N-myc* and IGF-II autoradiographic grains over the distinct structural components. Columns represent (A) the early cortical differentiation stages, (B) glomeruli, (C) proximal convoluted 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.

graphic grains as compared to the primitive tumor cells (Fig. 4, c and d, arrows). *N-myc* expression displayed great regional variation and was not as clearly demarcated between the tumor and normal tissue (Fig. 4, e and f) while inside the tumor mass, clusters of cells with very high *N-myc* grain density were also observed (Fig. 4, g and h, arrows). 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).

In the cerebrum, four definite zones are discernible at this stage of development (Figs. 5 and 6): (a) the highly cellular ependymal zone abounding to the ventricle; (b) the clearly less cellular subependymal zone; followed by (c) the sparsely cellular intermediate zone with radially oriented glial cells; and (d) the cortical plate with densely packed small neuroblastic cells. This developmental gradient of differentiation of the cerebral cells with cell proliferation in the ventricular zone, followed by outward migration and finally neuronal differentiation in the upper intermediate and cortical layers, is found between 5 and 34 wk of gestation (Herschkowitz, 1988). In other words, in cross sections of human fetal cerebrum at 16–19 wk of gestation, all stages of neuroepithelial cell development and differentiation are present within the distinct zones. Thus, despite the limited availability of human fetal material of different gestational ages for analysis, the histological sections that we have studied represent a pattern present for a relatively long time period of human fetal brain development. As the mitotic activity of the neuroepi-

thelial cells is limited to the ependymal zone, all the other zones consist of postmitotic neuroepithelial cells. In situ hybridization showed *N-myc* mRNA throughout all these different structural layers. The autoradiographic grains were invariably found close to the perikarya, whereas in the neuropil only background levels of grains were present. The highest autoradiographic grain counts were obtained over the cells in the subependymal and intermediate zones (Fig. 5, a–d, and Fig. 6, black bars). Somewhat fewer grains were detected in the ependymal zone adjacent to the lateral ventricles where the proliferation of the neuroepithelial cells takes place. Cells migrating along the radial glia to the cortical plate gave a strong positive reaction with the *N-myc* probe (Fig. 5, a–d). Thus, the highest level of *N-myc* expression was seen in the cells migrating to the cortical plate, and not in the proliferative cells of the ependymal zone. Some signal was also found over cells of the cortical plate (Fig. 5, b and d). These differences in grain distribution were confirmed by grain counting analysis (Fig. 6).

For comparison, we performed in situ hybridization for *N-myc* mRNA on sections of a stage IV neuroblastoma sample under identical conditions with the fetal brain sections. The tumor displayed great regional variation of the *N-myc* mRNA content (Fig. 7). High expression levels, clearly in excess of that observed in a simultaneous analysis of the human fetal brain, were found not only in the cells with the most primitive morphology, but also over some cells showing a degree of morphological differentiation, characterized by larger and paler nuclei (Fig. 7). 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 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, epithelially 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 μ m.

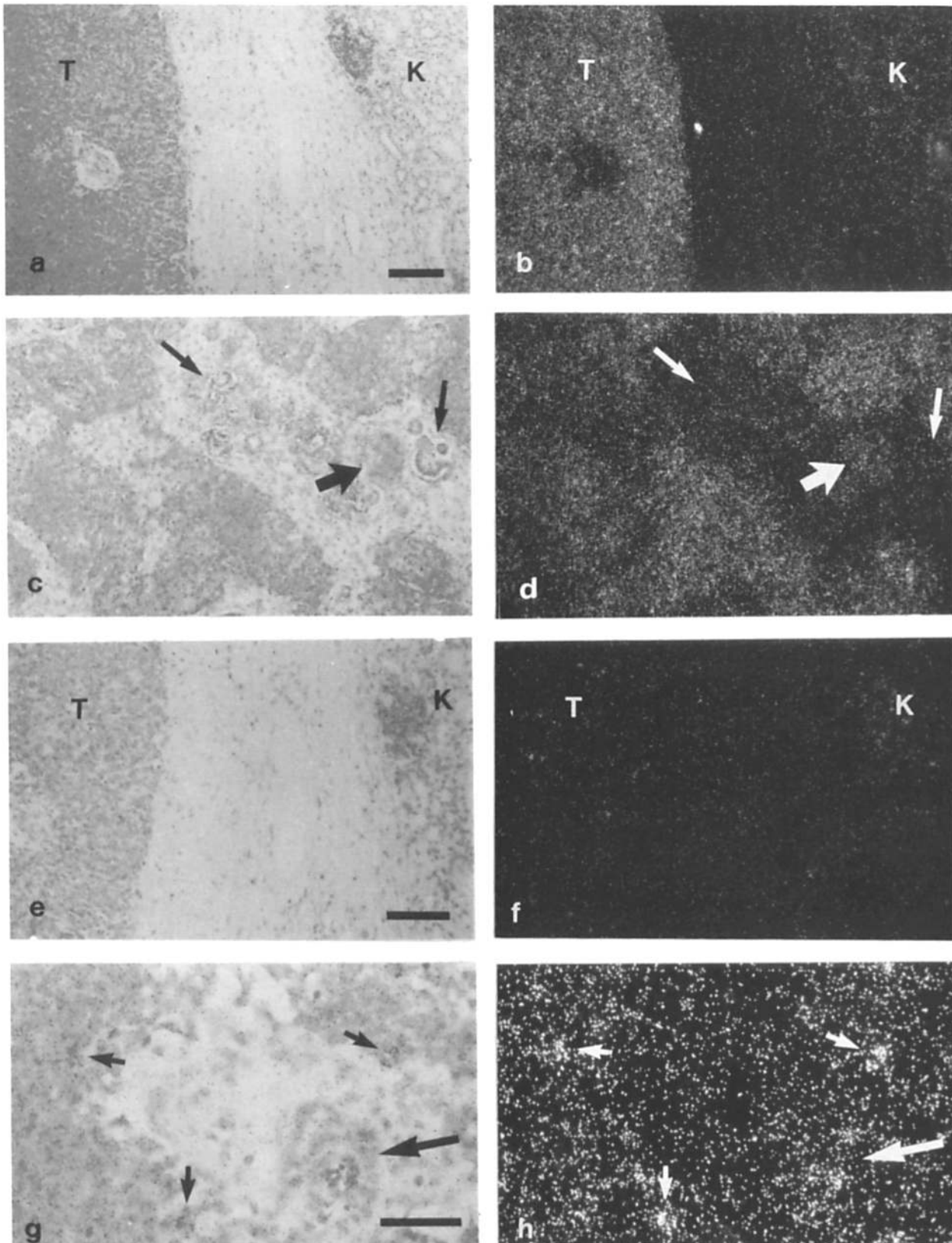


Figure 4. In situ hybridization of IGF-II (*a-d*) and *N-myc* mRNA (*e-h*) in a classical triphasic Wilms' tumor. *a, b, e,* and *f* show the peripheral expansion of the tumor against the adjacent kidney tissue. The tumor cells stain dark in the left-hand part of the photographs (*T*). The pale staining 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 μ m; (*e* and *f*) 100 μ m; (*g* and *h*) 50 μ m.

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 transfilter induction model (Eklblom, 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.

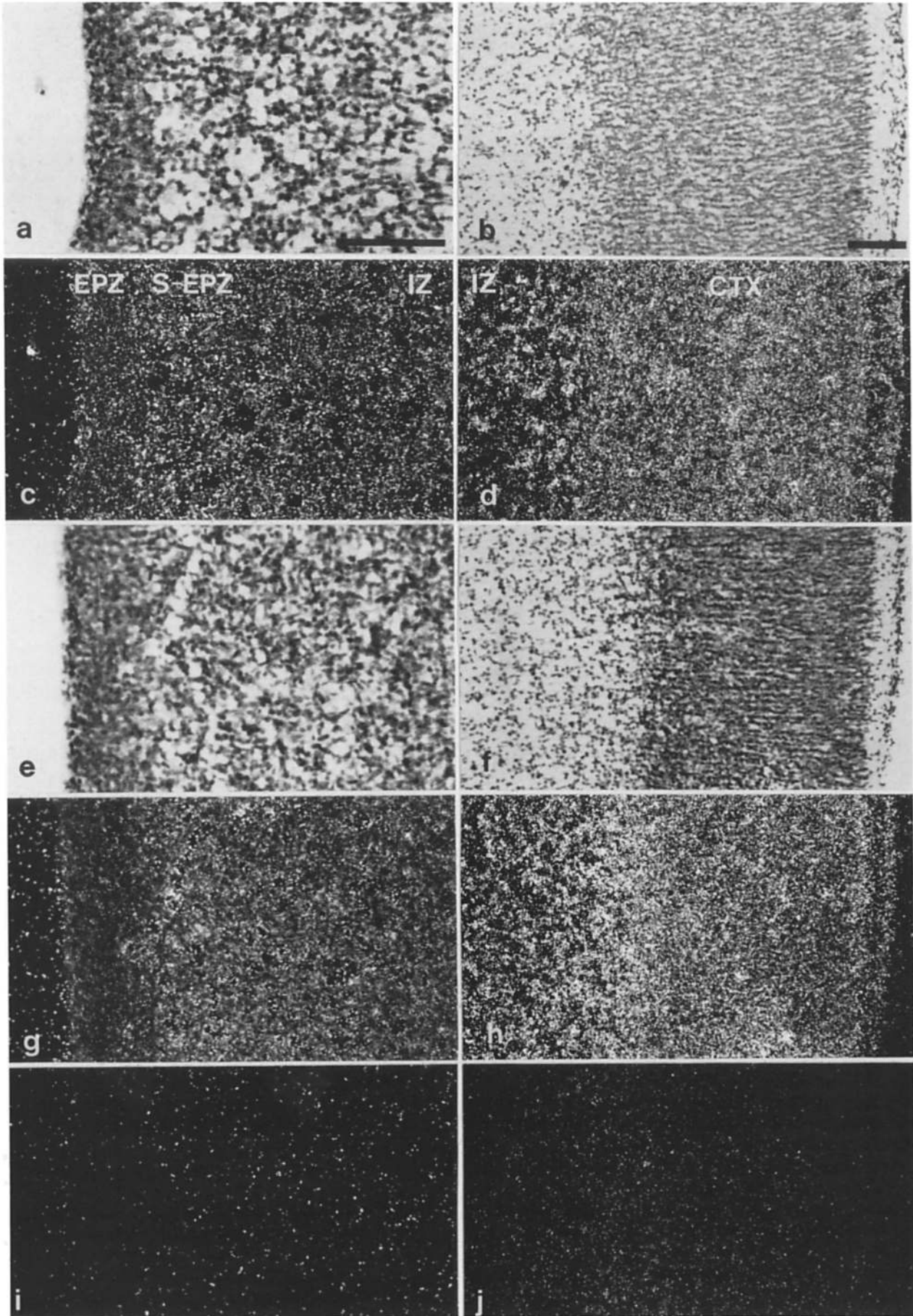
Our *in situ* hybridization results in a triphasic pediatric

Wilms' 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 Wilms' 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' tumor 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



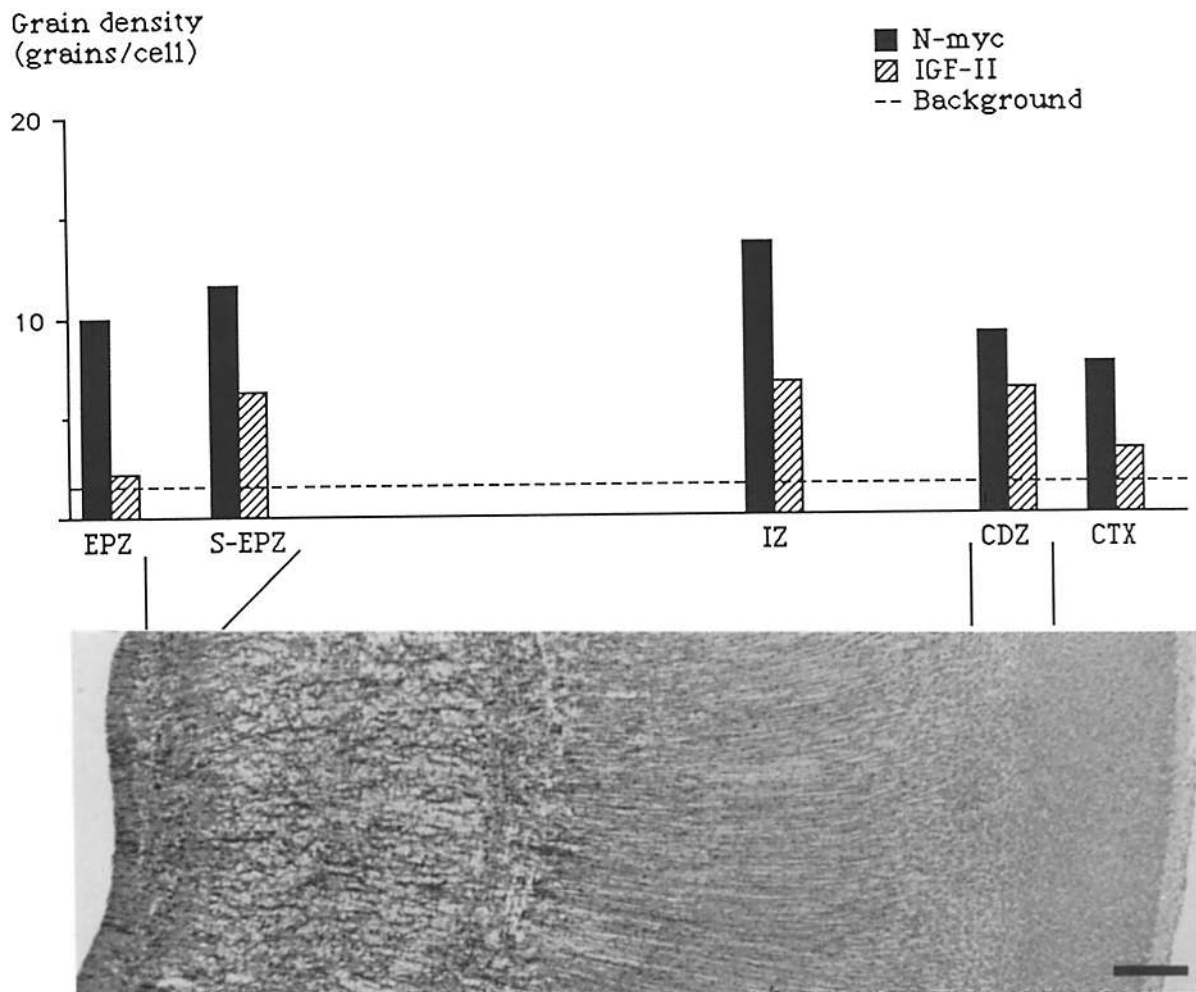


Figure 6. Grain counting analysis of the in situ hybridization results in human fetal brain with the *N-myc*, IGF-II, and control probes. The photomicrograph shows a GFAP-stained sagittal section of human fetal brain at 17 gestational wk. The zones marked correspond to the columns given in the upper 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 .

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 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 .

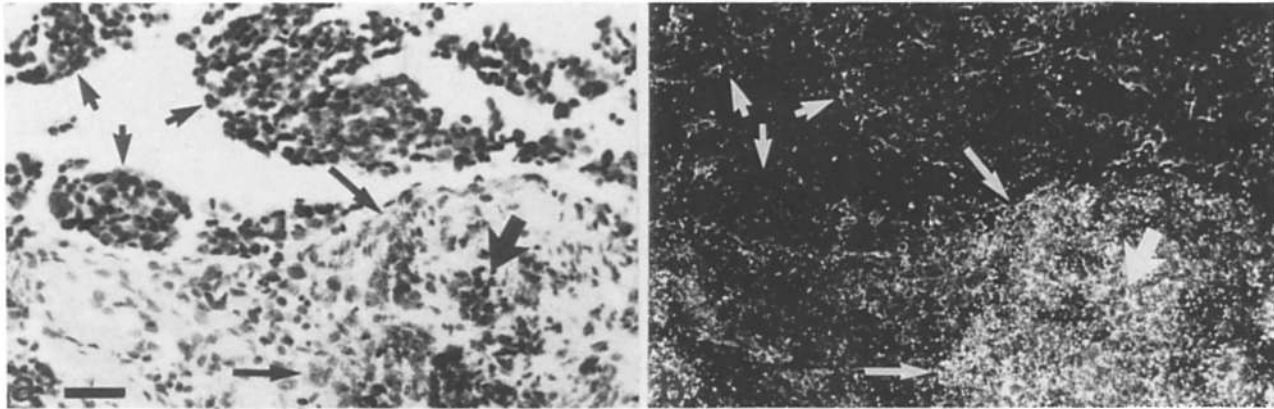


Figure 7. Bright field (a) and dark field (b) photomicrographs of a section of a stage IV neuroblastoma hybridized with the *N-myc* probe. The heterogeneity of *N-myc* expression by tumor cells is apparent. *N-myc* autoradiographic grains, best visualized in dark field microscopy (b), are located over 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 .

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,

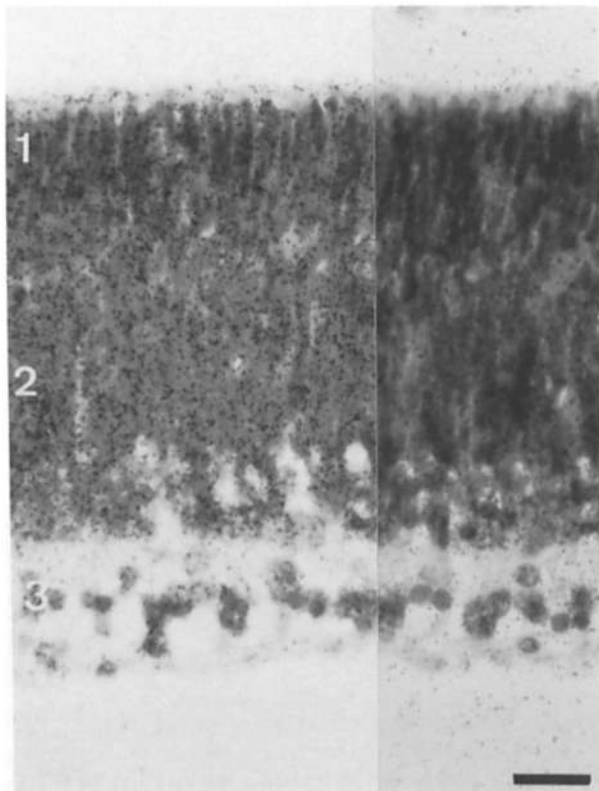


Figure 8. A section of fetal retina sample hybridized with the *N-myc* probe shows abundant signal over all cell layers. Because of the section thickness, the background is shown on the right in a separate picture focused on the background. (1) Outer nuclear layer; (2) inner nuclear layer; (3) ganglion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

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

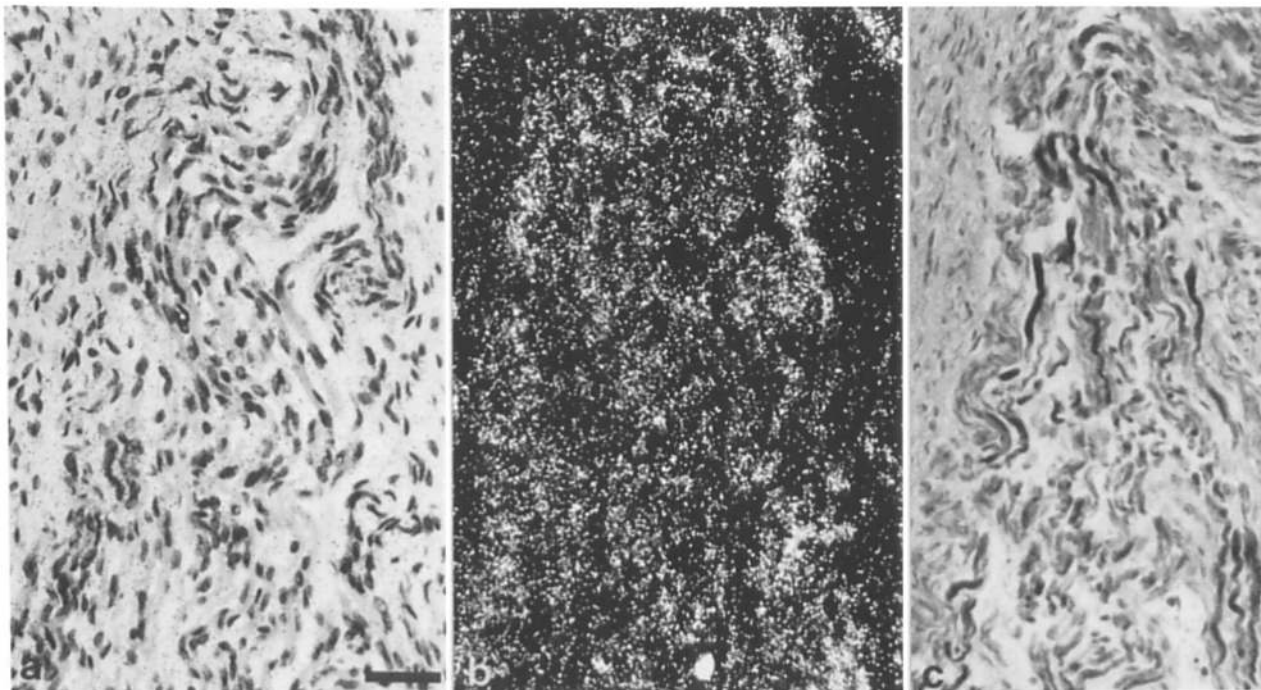


Figure 9. *N-myc* autoradiographic grains over a section of the extrinsic ocular striated musculature (a and b), identified morphologically and by immunostaining for myoglobin (c). Bar, 100 μm .

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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References

- Adamson, E. D. 1987. Oncogenes in development. *Development*. 99:449-471.
- Alitalo, K., and Schwab, M. 1986. Oncogene amplification in tumor cells. *Adv. Cancer Res.* 47:235-281.
- Alt, F. W., R. A. DePinho, K. Zimmerman, E. Legoy, K. Hatton, P. Ferrier, A. Tesfaye, G. Yancopoulos, and P. Nisen. 1986. The human *myc* gene family. *Cold Spring Harbor Symp. Quant. Biol.* 51:931-942.
- Amatruda, T. T., N. Sidell, J. Ranyard, and H. P. Koeffler. 1985. Retinoic acid treatment of human neuroblastoma cells is associated with decreased *N-myc* expression. *Biochem. Biophys. Res. Commun.* 126:1189-1195.
- Auffray, C., and F. Rougeon. 1980. Purification of mouse immunoglobulin heavy chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem.* 107:303-314.
- Beck, F., N. J. Samani, J. D. Penschow, B. Thorley, G. W. Tregear, and J. P. Coghlan. 1987. Histochemical localization of IGF-I and -II mRNA in the developing rat embryo. *Development*. 101:175-184.
- Bell, G. I., J. P. Merryweather, R. Sanchez-Pescador, M. M. Stempien, L. Priestley, J. Scott, and L. B. Rall. 1984. Sequence of a cDNA clone encoding human preproinsulin-like growth factor II. *Nature (Lond.)*. 310:775-777.
- Bishop, J. M. 1987. The molecular genetics of cancer. *Science (Wash. DC)*. 235:305-311.
- Brissenden, J. E., A. Ullrich, and U. Francke. 1984. Human chromosomal mapping of genes for human insulin-like growth factors I and II and epidermal growth factor. *Nature (Lond.)*. 310:781-784.
- Brodeur, G. M., R. C. Seeger, M. Schwab, H. E. Varmus, and J. M. Bishop. 1984. Amplification of *N-myc* in untreated human neuroblastomas correlates with advanced disease state. *Science (Wash. DC)*. 224:1121-1124.
- Brown, A. L., D. E. Graham, S. P. Nissley, D. J. Hill, A. J. Strain, and M. M. Rechler. 1986. Developmental regulation of insulin-like growth factor II mRNA in different rat tissues. *J. Biol. Chem.* 261:13144-13150.
- Cavenee, W. K., and M. F. Hansen. 1986. Molecular genetics of human familial cancer. *Cold Spring Harbor Symp. Quant. Biol.* Vol. 51(2):829-836.
- Cavenee, W. K., A. L. Murphee, M. M. Schull, W. F. Benedict, R. S. Sparkes, E. Kock, and M. Nordenskjold. 1986. Prediction of familial predisposition to retinoblastoma. *N. Engl. J. Med.* 314:1201-1206.
- Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry*. 18:5294-5299.
- Comings, D. E. 1973. A general theory of carcinogenesis. *Proc. Natl. Acad. Sci. USA*. 70:3324-3328.
- Eklom, P. 1984. Basement membrane proteins and growth factors in kidney differentiation. In *The Role of Extracellular Matrix in Development*. R. L. Trelstad, editor. Alan R. Liss Inc., New York. 173-206.
- Feinberg, A. P., and B. Vogelstein. 1983. A Technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6-13.
- Francke, U., L. B. Holmes, L. Atkins, and V. K. Riccardi. 1979. Aniridia-Wilms tumor association: evidence for specific deletion of 11p13. *Cytogenet. Cell Genet.* 24:185-192.
- Garson, J. A., J. Clayton, P. McIntyre and J. P. Kemshead. 1986. *N-myc* oncogene amplification in a rhabdomyosarcoma at relapse. *Lancet*. i:1496.
- Grady, E. F., M. Schwab, and W. Rosenau. 1987. Expression of *N-myc* and *c-src* during the development of fetal human brain. *Cancer Res.* 47:2931-2936.

- Han, V. K. M., A. J. De'Ercole, and P. K. Lund. 1987. Cellular localization of somatomedin (insulin-like growth factor) messenger RNA in the human fetus. *Science (Wash. DC)*. 236:193-197.
- Haselbacher, G., and R. Humbel. 1982. Evidence for two species of insulin-like growth factor II (IGF-II) in human spinal fluid. *Endocrinology*. 110:1822-1824.
- Haselbacher, G. K., M. E. Schwab, A. Pasi, and R. E. Humbel. 1985. Insulin-like growth factor II (IGF-II) in human brain: regional distribution of IGF-II and of higher molecular mass forms. *Proc. Natl. Acad. Sci. USA*. 82:2153-2157.
- Haselbacher, G. K., J.-C. Irminger, J. Zapf, W. H. Ziegler, and R. E. Humbel. 1987. Insulin-like growth factor-II in human adrenal pheochromocytomas and Wilms' tumors: expression at the mRNA and protein level. *Proc. Natl. Acad. Sci. USA*. 84:1104-1106.
- Herschkowitz, N. 1988. Brain development in the fetus, neonate and infant. *Biol. Neonate*. 54:1-19.
- Ikegaki, N., J. Bukovsky, and H. Kennet. 1986. Identification and characterization of the NMYC gene product in human neuroblastoma cells by monoclonal antibodies with defined specificities. *Proc. Natl. Acad. Sci. USA*. 83:5929-5933.
- Irminger, J.-C., K. M. Rosen, R. E. Humbel, and L. Villa-Komaroff. 1987. Tissue-specific expression of insulin-like growth factor II mRNA's with distinct 5' untranslated regions. *Proc. Natl. Acad. Sci. USA*. 84:6330-6334.
- Jakobovits, A., M. Schwab, J. M. Bishop, and G. R. Martin. 1985. Expression of N-myc in teratocarcinoma stem cells and mouse embryos. *Nature (Lond.)*. 318:188-191.
- Kahn, P., and T. Graf. 1986. *Oncogenes and growth control*. Springer-Verlag, New York. 369 pp.
- Klein, G. 1987. The approaching era of tumor suppressor genes. *Science (Wash. DC)*. 238:1539-1545.
- Kohl, N. E., N. Kanda, R. Schreck, G. Bruns, S. Latt, and F. Gilbert. 1983. Transposition and amplification of oncogene related sequence in human neuroblastomas. *Cell*. 35:359-367.
- Kohl, N. E., C. E. Gee, and F. W. Alt. 1984. Activated expression of the N-myc gene in human neuroblastomas and related tumors. *Science (Wash. DC)*. 226:1335-1337.
- Kohl, N. E., E. Legouy, R. A. DePinho, P. D. Nisen, R. K. Smith, C. E. Gee, and F. W. Alt. 1986. Human N-myc is closely related in organization and nucleotide sequence to c-myc. *Nature (Lond.)*. 319:73-77.
- Koufos, A., M. F. Hansen, M. G. Copeland, N. A. Jenkins, B. C. Lampkin, and W. K. Cavenee. 1985. Loss of heterozygosity in three embryonal tumours suggests a common pathogenetic mechanism. *Nature (Lond.)*. 316:330-334.
- Ladda, R., L. Atkins, J. Littlefield, P. Neurath, and K. M. Marimuthu. 1974. Computer-assisted analysis of chromosomal abnormalities: detection of a deletion in Aniridia/Wilms' tumor syndrome. *Science (Lond.)*. 185:784-787.
- Lee, W. H., A. L. Murphee, and W. F. Benedict. 1984. Expression and amplification of the N-myc gene in primary retinoblastoma. *Nature (Lond.)*. 309:458-460.
- Lehtonen, E. 1976. Transmission of signals in embryonic induction. *Med. Biol. (Helsinki)*. 54:108-128.
- Lehtonen, E., and L. Saxén. 1986. Control of differentiation. In *Human Growth*. F. Falkner and J. M. Tanner, editors. Plenum Publishing Corp., New York. 27-51.
- Mäkelä, T. P., K. Saksela, and K. Alitalo. 1989. Two N-myc polypeptides with distinct amino termini encoded by the second and third exons of the gene. *Mol. Cell. Biol.* In press.
- Nau, M. M., B. J. Brooks, D. N. Carney, A. F. Gazdar, J. F. Battey, E. A. Sausville, and J. D. Minna. 1986. Human small-cell lung cancers show amplification and expression of the N-myc gene. *Proc. Natl. Acad. Sci. USA*. 83:1092-1096.
- Nisen, P. D., K. A. Zimmerman, S. V. Cotter, F. Gilbert, and F. W. Alt. 1986. Enhanced expression of the N-myc gene in Wilms' tumors. *Cancer Res.* 46:6217-6222.
- Nowakowski, R. S., and P. Racik. 1981. The site of origin and route and rate of migration of neurons to the hippocampal region of the rhesus monkey. *J. Comp. Neurol.* 196:129-154.
- Öfverstedt, L.-G., K. Hammarström, N. Balgobin, S. Hjerten, U. Petterson, and J. Chattopadhyaya. 1984. Rapid and quantitative recovery of DNA fragments from gels by displacement electrophoresis (isotachopheresis). *Biochim. Biophys. Acta*. 782:120-126.
- Pfeifer-Ohlsson, S., J. Rydner, A. S. Goustin, E. Larsson, C. Betsholtz, and R. Ohlsson. 1985. Cell-type specific pattern of myc proto-oncogene expression in developing human embryos. *Proc. Natl. Acad. Sci. USA*. 82:5050-5054.
- Ramsay, G., L. Stanton, M. Schwab, and J. M. Bishop. 1986. Human proto-oncogene N-myc encodes nuclear proteins that bind DNA. *Mol. Cell. Biol.* 6:4450-4457.
- Reeve, A. E., M. R. Eccles, R. J. Wilkins, G. I. Bell, and L. J. Millow. 1985. Expression of insulin-like growth factor-II transcripts in Wilms' tumour. *Nature (Lond.)*. 317:258-260.
- Riccardi, R. M., H. M. Hittner, U. Francke, J. J. Yunis, D. Ledbetter, and W. Borges. 1980. The Aniridia-Wilms' tumor association: the critical role of chromosome band 11p13. *Cancer Genet. Cytogenet.* 2:131-137.
- Rotwein, P., S. K. Burgess, J. D. Milbrandt, and J. E. Krause. 1988. Differential expression of insulin-like growth factor genes in the rat central nervous system. *Proc. Natl. Acad. Sci. USA*. 85:265-269.
- Saksela, K., J. Bergh, and K. Nilsson. 1986. Amplification of the N-myc oncogene in an adenocarcinoma of the lung. *J. Cell. Biochem.* 31:297-304.
- Sandberg, M., and E. Vuorio. 1987. Localization of types I, II and III collagen mRNAs in developing human skeletal tissues by in situ hybridization. *J. Cell Biol.* 104:1077-1084.
- Sara, V. R., C. Carlsson-Skwirut, C. Andersson, E. Hall, B. Sjögren, A. Holmgren, and H. Jörnvall. 1986. Characterization of somatomedins from human brain: identification of a variant form of insulin-like growth factor I. *Proc. Natl. Acad. Sci. USA*. 83:4904-4907.
- Sariola, H. 1984. *Angiogenesis of the kidney*. Ph.D. thesis. Department of Pathology, University of Helsinki, Helsinki, Finland. 96 pp.
- Saxén, L., H. Sariola, and E. Lehtonen. 1986. Sequential cell and tissue interactions governing organogenesis of the kidney. *Anat. Embryol.* 175:1-6.
- Schwab, M. 1986. Amplification of proto-oncogenes and tumor progression. In *Oncogenes and Growth Control*. P. Kahn and T. Graf, editors. Springer-Verlag, New York. 332-339.
- Schwab, M., K. Alitalo, L. Klempenauer, H. Varmus, J. Bishop, F. Gilbert, G. Brodeur, M. Goldstein, and J. Trent. 1983. Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumor. *Nature (Lond.)*. 305:245-248.
- Schwab, M., J. Ellison, M. Busch, W. Rosenau, H. E. Varmus, and J. M. Bishop. 1984. Enhanced expression of the human gene N-myc consequent to amplification of DNA may contribute to malignant progression of neuroblastoma. *Proc. Natl. Acad. Sci. USA*. 81:4940-4944.
- Scott, J., J. Cowell, M. E. Robertson, L. M. Priestley, R. Wadey, B. Hopkins, J. Pritchard, G. I. Bell, L. B. Rall, C. F. Graham, and T. J. Knott. 1985. Insulin-like growth factor-II gene expression in Wilms' tumour and embryonic tissues. *Nature (Lond.)*. 317:260-262.
- Seeger, R. C., G. M. Brodeur, H. Sather, S. E. Siegel, K. Y. Wong, and D. Hammond. 1985. Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. *N. Engl. J. Med.* 313:1111-1116.
- Slamon, D. J., T. C. Boone, R. C. Seeger, D. E. Keith, V. Chazin, H. C. Lee, and L. M. Souza. 1986. Identification and characterization of the protein encoded by the human N-myc oncogene. *Science (Wash. DC)*. 232:768-772.
- Stanton, L. W., and J. M. Bishop. 1987. Alternative processing of RNA transcribed from NMYC. *Mol. Cell. Biol.* 7:4266-4272.
- Stanton, L. W., M. Schwab, and J. M. Bishop. 1986. Nucleotide sequence of the human N-myc gene. *Proc. Natl. Acad. Sci. USA*. 83:1772-1776.
- Thiele, C. J., P. C. Reynolds, and M. A. Israel. 1985. Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma. *Nature (Lond.)*. 313:404-406.
- Tricoli, J. V., L. B. Rall, J. Scott, G. I. Bell, and T. B. Shows. 1984. Localization of insulin-like growth factor genes to human chromosomes 11 and 12. *Nature (Lond.)*. 310:784-786.
- Wagner, E. F., and R. Müller. 1986. A role for proto-oncogenes in differentiation? In *Oncogenes and Growth Control*. P. Kahn and T. Graf, editors. Springer-Verlag, New York. 18-26.
- Wong, A. J., J. M. Ruppert, J. Eggleston, S. R. Hamilton, S. B. Baylin, and B. Vogelstein. 1986. Gene amplification of c-myc and N-myc in small cell carcinoma of the lung. *Science (Wash. DC)*. 233:461-464.
- Zimmerman, K., G. D. Yancopoulos, R. G. Collum, R. K. Smith, N. E. Kohl, K. A. Denis, M. M. Nau, O. N. Witte, A. Toran-Allerand, C. E. Gee, J. D. Minna, and F. W. Alt. 1986. Differential expression of myc family genes during murine development. *Nature (Lond.)*. 319:780-783.