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# Distinct isoforms of neuregulin are expressed in mesenchymal and neuronal cells during mouse development

(*neu* protooncogene/receptor tyrosine kinase/mesenchymal–epithelial interactions/neu differentiation factor/heregulin)

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**ABSTRACT** **Neuregulin, the putative ligand of the c-*neu* receptor tyrosine kinase, can induce differentiation or growth of epithelia and other cells. To gain insight into the biological role of this factor, we have analyzed the expression of neuregulin during mouse embryogenesis and in the perinatal animal by a combination of *in situ* hybridization and RNase protection experiments. We identify sites of expression that correspond to mesenchymal cells of various parenchymal organs. Our finding implies a function of neuregulin as a mesenchymal factor that acts on epithelia. The mesenchymal expression of neuregulin could thus provide a molecular basis for the biological phenomenon of mesenchymal–epithelial interactions. It also has implications on the molecular mechanism by which amplification of c-*neu* can affect tumor progression of carcinomas. In addition, neuregulin expression is found in neuronal cells during development. We show by RNase protection experiments that distinct isoforms of neuregulin are expressed in the brain. Therefore, our data indicate *in vivo* a dual role for neuregulin as mesenchymal and neuronal factor.**

Receptor tyrosine kinases and their specific ligands play essential roles in intercellular communication and thus ensure coordinated growth, differentiation, and morphogenesis during development as well as homeostasis in the adult. The important regulatory role of these ligand/receptor systems is emphasized by the fact that many genes encoding them were originally identified as oncogenes, among them the *neu* gene from the rat (1, 2). Independently, the human homologue of *neu* was isolated because of its sequence similarity with the epidermal growth factor (EGF) receptor and therefore named human EGF receptor 2 (*HER2*) or *erbB2* (3–5). Amplification and overexpression of *neu*/*HER2* can be observed in various carcinomas and have been reported to correlate with negative prognosis (6).

The putative ligand of the c-*neu* receptor, neuregulin (also named neu differentiation factor, NDF, or heregulin), was recently purified relying on an assay for rapid, factor-induced tyrosine phosphorylation of c-*neu* (7). Molecular characterization revealed that the factor contains one immunoglobulin and one EGF motif (Fig. 1; cf. refs. 8 and 9). The EGF domain expressed as a bacterial fusion protein is sufficient to induce rapid tyrosine phosphorylation of c-*neu* (9). In addition, neuregulin can be crosslinked to c-*neu* and binds with high affinity to certain cells expressing the receptor (7, 9, 10). Such binding has not been observed with all cell types that express c-*neu*, indicating that binding of neuregulin to cells requires an additional cellular component, possibly another receptor of the HER family of tyrosine kinases (10).

It has been shown that neuregulin can induce growth but also differentiation of mammary carcinoma cells (7–9). In addition, other biological activities of neuregulin have been recently characterized in cell culture: glial growth factor as well as a brain or spinal cord-derived factor that induces myotube differentiation (acetylcholine receptor-inducing activity) are products of the neuregulin gene (11, 12). Various different transcript isoforms have been characterized that arise by alternative splicing events. They differ, for example, in the presence or absence of sequences that encode a kringle domain located at the N terminus of the factor. Heterogeneity in the variants without a kringle domain is found in the sequences of the EGF motif that can be encoded by two different exon combinations ($\alpha$, $\beta$ isoforms) and in C-terminal coding sequences ($\beta_1$, $\beta_2$, $\beta_3$ isoforms) (Fig. 1; cf. refs. 8, 9, 11, and 12). Whether the different isoforms have distinct activities is unknown.

To gain insight into the biological role of neuregulin, we have determined the detailed expression pattern of the gene during murine embryogenesis and in the perinatal animal. By *in situ* hybridization we identified two different cell types that express neuregulin, mesenchymal and neuronal cells. RNase protection experiments show that distinct splice variants are expressed in the parenchymal organs and the brain. Our data thus indicate a dual role for neuregulin *in vivo* as a mesenchymal and a neuronal factor.

## MATERIALS AND METHODS

**Isolation and Characterization of Murine Neuregulin cDNA.** Murine neuregulin cDNAs were isolated from two libraries constructed in λgt10 by standard techniques (13), using synthetic oligonucleotides as probes. The isolated cDNA clones were characterized by sequence analysis. A complete cDNA encoding the $\alpha$ subtype of neuregulin and a partial cDNA encoding the $\beta_2$ isoform were chosen for further experiments.

**RNase Protection and *in Situ* Hybridization Analysis.** RNA was isolated from various mouse tissues and analyzed by RNase protection experiments as described (14). The quantity of RNA was determined by measurements of the optical density, and quantity and quality were verified by gel electrophoresis. Probes were as follows (Fig. 1): (*i*) pNN, a transcript containing 290 nucleotides (nt) of murine neuregulin cDNA in antisense orientation (corresponding to positions 391–681 in the rat NDF cDNA; cf. ref. 8) and an additional 100 nt of plasmid-derived sequence; (*ii*) pN$\alpha$, a transcript containing 259 nt of murine neuregulin cDNA in antisense orientation (corresponding to positions 899–1158 in the rat NDF sequence; cf. ref. 8) and an additional 87 nt of plasmid-derived sequence; (*iii*) pN$\beta_2$, a transcript containing 250 nt of murine neuregulin sequences (corresponding to positions 1023–1273 in the human heregulin $\beta_2$ sequence; cf. ref. 9) and an additional 87 nt of plasmid-derived sequence.

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Abbreviations: EGF, epidermal growth factor; E, embryonic day; NDF, neu differentiation factor.

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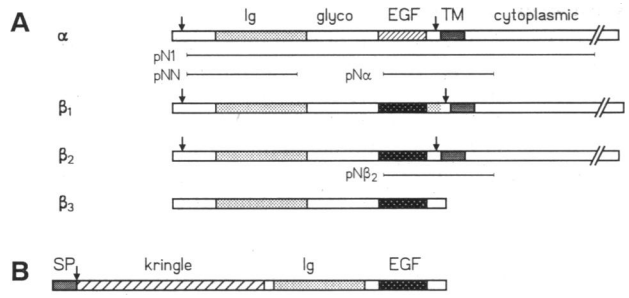


FIG. 1. Schematic representation of different isoforms of neuregulin and of murine neuregulin cDNA clones. Different isoforms of neuregulin without a kringle domain ($\alpha$, $\beta_1$, $\beta_2$, $\beta_3$) (*A*) and with a kringle domain (*B*) are drawn schematically. The different domains and sequence motifs present in the protein—i.e., kringle, Ig, and EGF motifs, sequences rich in potential glycosylation sites (glyco), transmembrane domain (TM), cytoplasmic domain, and signal peptide (SP)—are shown; putative sites of proteolytic processing are marked by arrows. As indicated, two distinct EGF domain sequences (encoded by the coding segments 6 and 7 or 6 and 8; cf. ref. 11) are present in the $\alpha$ or in the $\beta$ and kringle variants, respectively. The probes used for *in situ* hybridization (pN1) and for RNase protection experiments (pNN, pN$\alpha$, pN$\beta_2$) are depicted.

*In situ* hybridization was performed as described (14, 15). Murine neuregulin sequences (corresponding to positions 391–1458 in the rat neuregulin sequence; cf. ref. 8) in antisense orientation were used as probe. No distinct hybridization signal was observed with the corresponding sense transcript. Frozen sections of embryos on days 10–18 of development (E10–E18) or sections of paraffin-embedded embryos (E8–E11) were used for hybridization.

## RESULTS

To obtain homologous probes for the analysis of neuregulin expression in the developing mouse, we cloned the murine cDNA from a testis library; this tissue was previously found to express the gene (8, 9). In addition, cDNAs from a library synthesized from E18 intestinal RNA were characterized, since our initial data indicated that intestinal mesenchyme is a good source for the factor. Sequence analysis of a full-length clone indicated that it corresponds to the $\alpha$ subtype of neuregulin; a partial cDNA encoding the $\beta_2$ isoform was also isolated and characterized (Fig. 1). *In situ* hybridization analysis revealed a distinct expression pattern of the neuregulin gene in mesenchymal and neuronal cell types.

**Expression of Neuregulin During Development of Parenchymal Organs.** In early organogenesis, we detect transcripts for neuregulin in mesenchymal cells of the lung, intestine, stomach, and kidney and in the genital ridge.

During development of the lung at E10–E13, we identify neuregulin transcripts in mesenchyme surrounding the epithelia of the developing bronchi (Fig. 2 *A* and *B*). Expression is confined to a small ring of mesenchymal cells in contact with the epithelia. At later stages of lung development (E18), no expression is detected by *in situ* hybridization.

The intestinal anlagen are formed early during development and consist initially of a poorly differentiated stratified epithelium surrounded by mesenchymal cells. A ring of mesenchymal cells close to the epithelia expresses neuregulin at high levels starting on E10 (i in Fig. 2 *C* and *D*). With the formation of the differentiated single-layered epithelia of the villi, this pattern changes and distinct punctuate hybridization signals are observed in the lamina propria (see arrowheads in Fig. 2 *E* and *F*). The patches of cells responsible for this signal are frequently located within the villi and are thus found at sites where no enteric ganglia are located.

Expression of neuregulin is found in the developing urogenital ridge starting on E10 (gr in Fig. 2 *C* and *D*). The gonadal primordium gives a strong hybridization signal for neuregulin, whereas no transcripts are observed in the mesonephric component. In the perinatal animal (E18), neuregulin expression is found in the as yet undifferentiated interstitial cells of the developing testis (data not shown). During early development of the metanephric kidney (E11, E13), we detect neuregulin expression in mesenchymal cells surrounding the ureter. However, no detectable signal is found by *in situ* hybridization in the kidney on E18 (data not shown).

**Expression of Neuregulin in Neuronal Development.** Onset of neuregulin expression in the neuronal system is detected on day 9 of embryogenesis. The signals are found in distinct cells of the branchial arches and in the developing spinal cord after closure of the neural tube. Starting on E10 and con-

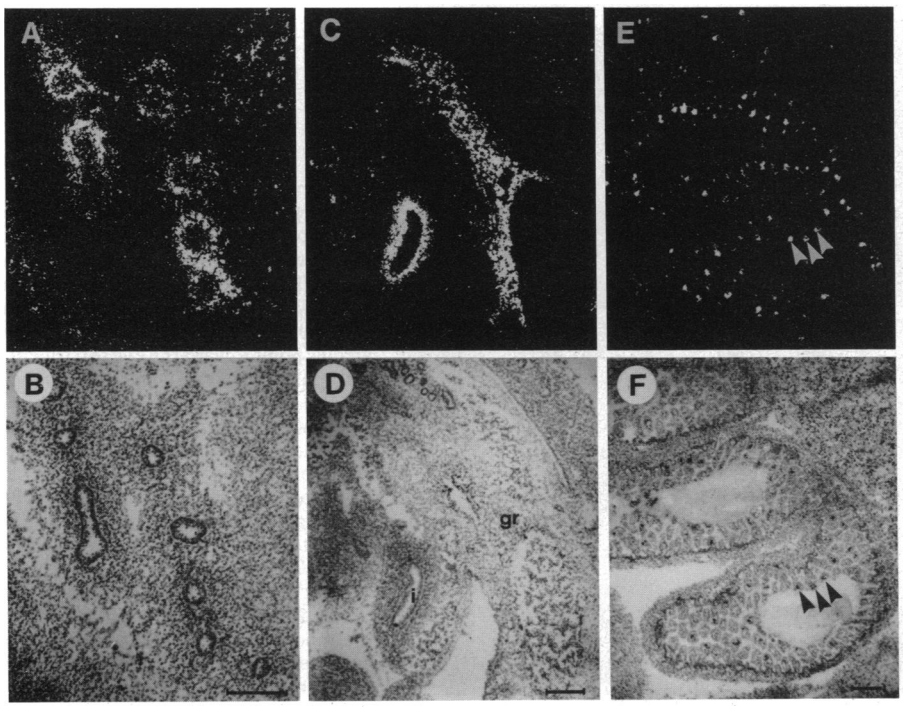


FIG. 2. Neuregulin expression in mesenchymal cells during development of parenchymal organs. Sections through murine embryos at various developmental stages were hybridized to a neuregulin probe. The developing lung on day 11 of embryogenesis is shown in dark-field (*A*) and bright-field (*B*). (Bar in *B* = 100 $\mu$m.) The genital ridge (gr) and intestine (i) on day 11 of embryogenesis are shown in dark-field (*C*) and bright-field (*D*). The developing intestine on day 18 of embryogenesis is shown in dark-field (*E*) and bright-field (*F*); arrowheads point to patches of cells inside the villi that express neuregulin. (Bars in *D* and *F* = 200 $\mu$m.)

tinuing during further development, we detect specific transcripts in the dorsal root ganglia (drg in Fig. 3 *A* and *B*) and in the ganglia of the branchial arches (tg, fg, and gg in Fig. 3 *A* and *B*). Additional prominent sites of expression are the ventral horns of the spinal cord where the developing motoneurons are located (data not shown). Distinct cells in the brain also express the gene; early in the development of the cerebral cortex, a narrow layer of superficial cells in the marginal zone shows a positive hybridization signal (mz in Fig. 3 *A* and *B*). At later developmental stages, expression in the central nervous system is more widespread. For example, transcripts are then found in the ventricular zone, in the cortical plate, and in a narrow layer of cells in the marginal zone of the telencephalon. In addition, distinct nuclei of the pons, thalamus, and hypothalamus and cells of the cerebellum express the neuregulin gene as well (data not shown).

**Expression of Neuregulin in Sensory Organs.** In the early development of the eye, we detect neuregulin transcripts in the neural layer of the optic cup (e in Fig. 3 *A* and *B*). After onset of differentiation of distinct cell types in the eye, the specific signal is confined to the innermost layer of the retina where the differentiating ganglion cells are located (Fig. 3 *C* and *D*).

The nasal cavities develop from the olfactory pits, an ectodermal invagination, and differentiate into two functionally and histologically distinct units, an olfactory part and a respiratory part. We detect neuregulin transcripts in the entire developing nasal cavities starting with their appearance on E11 (data not shown). In the prenatal mouse on E18, when terminal differentiation of the distinct cell types in this complex epithelium has occurred, expression is confined to neuronal cells—i.e., the olfactory receptors (Fig. 3 *E* and *F*; cf. ref. 16 for an assignment of olfactory receptors in the epithelium).

**Mesenchymal and Neuronal Splice Variants.** To verify the specificities of the observed *in situ* hybridization signals, RNase protection experiments were performed using a labeled antisense RNA probe transcribed from a cDNA subclone (pNN indicated in Fig. 1). Protected fragments of distinct size were observed when RNA from various embryonal organs—e.g., lung, intestine, and brain—was used. This demonstrates the presence of neuregulin-specific transcripts in such organs (Fig. 4). A very weak signal was observed with RNA from embryonic liver (Fig. 4).

Various neuregulin isoforms were previously characterized, which differ, for example, in the presence or absence of a N-terminal kringle domain (11). The probe employed here, pNN, distinguishes transcripts encoding these two classes of factor (see Fig. 1); it contains N-terminal sequences (290 nt) entirely present in the $\alpha$ and $\beta$ subtypes of neuregulin that do not contain the kringle domain; only a part of these sequences (240 nt) is found in isoforms encoding the kringle domain. RNA isolated from many embryonal tissues (e.g., heart, kidney, lung, intestine) protects a single fragment 290 nt in length (Fig. 4, lanes 2–5 and 8). Therefore, the transcripts produced by most embryonal tissues encode $\alpha$ or $\beta$ subtypes of neuregulin, but no isoforms with the kringle domain. In contrast, RNA from embryonic or adult brain protects an additional fragment 240 nt in length (Fig. 4, lanes 7, 10, and 11). Among the characterized isoforms, only transcripts encoding neuregulin with a kringle domain are expected to give rise to a protected fragment of this size. Such transcripts are thus expressed in a tissue-specific manner. However, other, as yet uncharacterized, isoforms might exist, which protect a fragment of identical size.

To assess the tissue-specific expression of $\alpha$ and $\beta$ types of neuregulin, we used the two cDNA fragments pN$\alpha$ and pN$\beta_2$ (indicated in Fig. 1) as probes. They correspond to equivalent sequences present in $\alpha$ and $\beta_2$ neuregulin but encode the distinct EGF motifs found in the $\alpha$ and all $\beta$ isoforms. In addition, the probes contain sequences for the transmembrane and part of the cytoplasmic domains that are identical in $\alpha$ and $\beta_2$ neuregulin (see Fig. 1). We have observed two major fragments protected by each probe: (*i*) the fragments containing the entire coding sequences of the probes (a length of 250 nt or 241 nt with probe pN$\alpha$ or pN$\beta_2$, respectively; compare also schematic drawing in Fig. 5) and (*ii*) a smaller fragment containing coding sequences for the transmembrane and cytoplasmic domains only (a length of 120 nt with both probes). With RNA from intestine (which contains

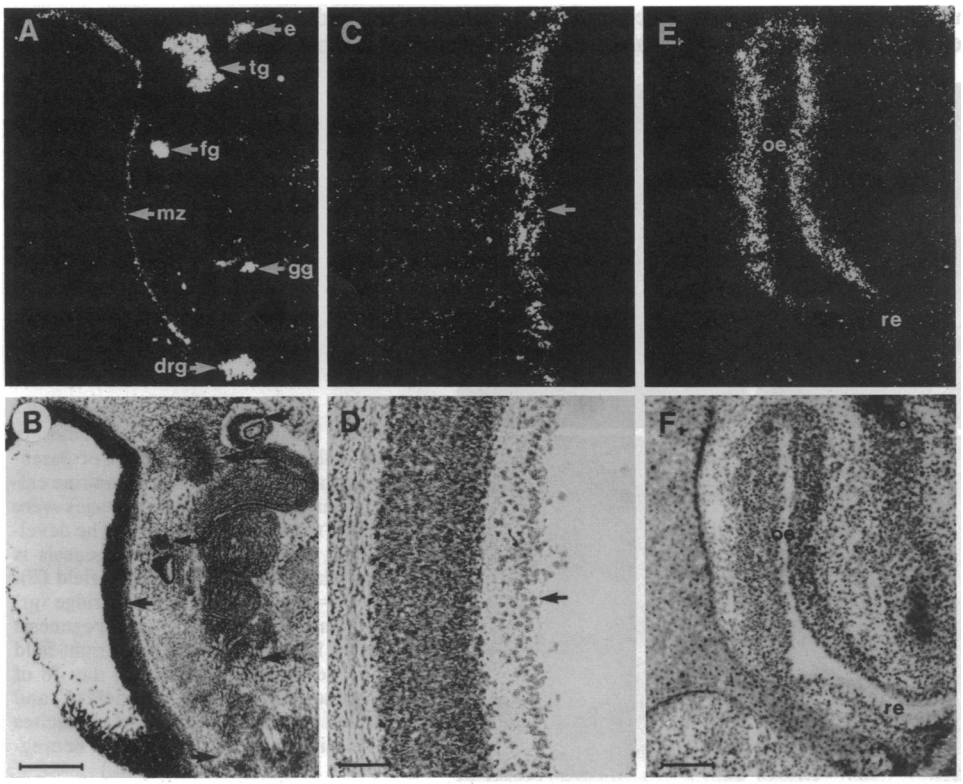


FIG. 3. Neuregulin expression in developing neuronal cells and in sensory organs. Sections through murine embryos at various developmental stages were hybridized to a neuregulin probe. The head region of an embryo on day 11 of development is shown in dark-field (*A*) and bright-field (*B*). The arrows point toward the eye (e), trigeminal (tg), facioacustic (fg), glossopharyngeal (gg), and dorsal root (drg) ganglia as well as the marginal zone (mz) of the hindbrain. A section showing the embryonal eye on day 18 (*C* and *D*) of embryogenesis is shown in dark-field and bright-field. The arrow in *C* and *D* points toward the ganglion layer of the retina. The nasal epithelium on day 18 of embryogenesis is shown in dark-field (*E*) and bright-field (*F*); the olfactory (oe) and respiratory (re) epithelia are indicated. (Bar in *B* = 500 $\mu$m; bar in *D* = 40 $\mu$m; bar in *F* = 100 $\mu$m.)

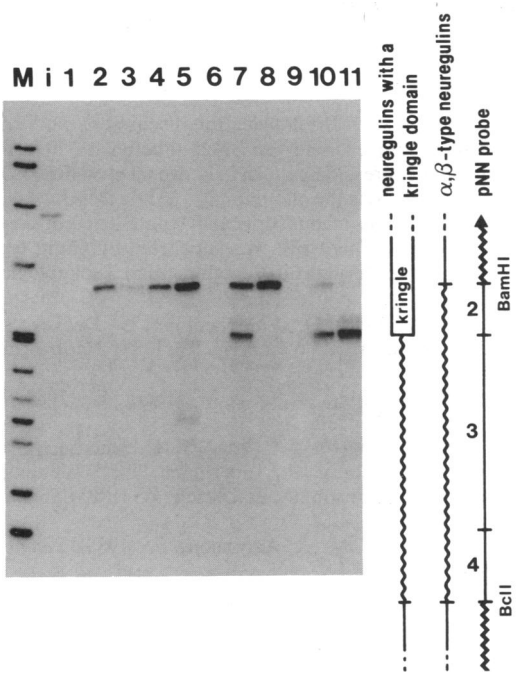


FIG. 4. Neuregulin expression in various murine tissues analyzed by RNase protection. Total RNA (75 μg) was analyzed by hybridization to $^{32}$P-labeled run-off transcripts synthesized from the neuregulin cDNA probe pNN (see Fig. 1). As markers, end-labeled *Msp* I-cut pBR322 DNA (lane M) and an aliquot of the undigested probe (lane i) were run in parallel. Tissue sources of RNA used for hybridization are as follows: lane 1, tRNA from yeast; lane 2, embryonal heart (E14); lane 3, embryonal kidney (E14); lane 4, embryonal lung (E14); lane 5, embryonal intestine (E14); lane 6, embryonal liver (E14); lane 7, embryonal brain (E14); lane 8, embryonal intestine (E18); lane 9, embryonal liver (E18); lane 10, embryonal brain (E18); lane 11, adult brain. The coding segments of the neuregulin cDNA present in the probe (segments 2, 3, and 4; cf. ref. 11) are schematically shown on the right; the transcript isoforms that encode neuregulin with or without a kringle domain and the fragments of the probe protected by such transcripts are indicated. Wavy lines correspond to protected fragments observed and serrated lines correspond to plasmid sequences present in the probes.

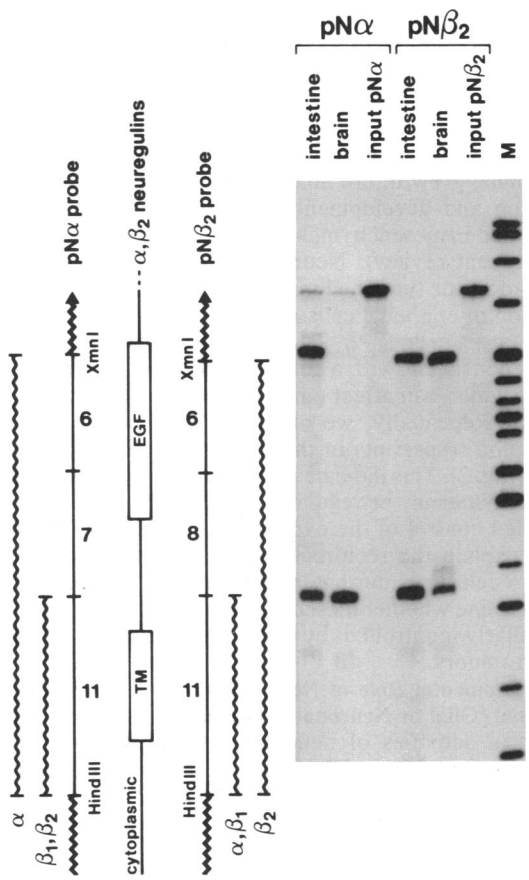


FIG. 5. Characterization of different isoforms of neuregulin transcripts produced in murine tissues. Total RNA (100 μg from E18 embryonic intestine and 40 μg from E18 brain) was analyzed by hybridization to $^{32}$P-labeled run-off transcripts synthesized from the neuregulin cDNAs plasmid pNα or pNβ$_2$ (see Fig. 1). End-labeled *Msp* I-cut pBR322 DNA (lane M) and aliquots of the undigested probes were run in parallel. Undigested pNα probe (input) and digested pNα probe after hybridization to brain and intestinal RNA as well as undigested pNβ$_2$ probe (input) and digested pNβ$_2$ probe after hybridization to brain or intestinal RNA are shown. The length of the marker fragments is indicated. The two probes (pNα and pNβ$_2$,) and the neuregulin coding segments present in the probe in antisense orientation (coding segments 6, 7, and 11 or 6, 8, and 11; cf. ref. 11) are shown schematically; the fragments protected from RNase digestion by different transcript isoforms are indicated. Wavy lines correspond to observed protected fragments and serrated lines correspond to plasmid sequences present in the probes.

mesenchymal cells expressing high amounts of neuregulin), probe pNα or pNβ$_2$ give rise to both protected fragments (Fig. 5). This indicates that α and β$_2$ isoform RNAs are major transcripts in mesenchymal cells. In contrast, brain RNA protects both fragments of probe pNβ$_2$ and only the small fragment of probe pNα (Fig. 5). Therefore, the isoform encoding the α type neuregulin is not produced in E18 or adult brain.

## DISCUSSION

We have determined the expression pattern of the neuregulin gene during murine development by a combination of RNase protection and *in situ* hybridization experiments. We detect strong expression of neuregulin in mesenchymal cells of various parenchymal organs. In addition, we identified neuregulin transcripts in neuronal cell precursors, as described previously (11, 12, 17). In parenchymal organs, we found neuregulin transcripts in the mesenchyme of the developing lung, intestine, stomach, and kidney and the genital ridge (Fig. 2). This finding implies a function of neuregulin in the development of epithelia known to respond to the factor by growth or differentiation and is also of interest in the context of the role of c-*neu*/*HER-2* in the genesis of human carcinomas. Amplification of the receptor, but not activation of the transforming potential by mutation, is observed frequently in mammary carcinomas; neuregulin increases tyrosine phosphorylation of c-neu/HER-2 and thus signaling via this receptor in such cells (7–9). An autocrine signaling modus seems to be of minor importance since carcinoma cell lines rarely express neuregulin (8). Thus, mesenchyme in vicinity of the carcinoma cells is the putative source for the ligand. Neuregulin binds to heparin, a property used also for the purification of the factor. Proteoglycans of the heparin sulfate type are found in high concentration in the basement membranes (18), which are formed in developing organs and separate in the adult mesenchymal and epithelial cell compartments. Therefore, an undamaged basement membrane could present a barrier for neuregulin in differentiated tissues. During metastasis, the basement membrane is damaged and invading carcinoma cells gain unregulated access to the mesenchymal neuregulin, which could profoundly influence further tumor progression.

In addition, various neuronal cells express neuregulin: ganglia of the branchial arches and spinal roots and cells of the central nervous system (cf. ref. 17). We also identify sites of expression in sensory organs (Fig. 3).

**Developmental Role of Neuregulin in Mesenchymal–Epithelial Interactions.** Mesenchymal signals are major driving forces in organ development since they are essential for growth, morphogenesis, and differentiation of epithelia. Recently, evidence has accumulated that tyrosine kinase receptors and their ligands, which are commonly associated with controlling growth, are able to play decisive roles in differentiation and development. In particular, they have been implicated in mesenchymal–epithelial interactions (cf. ref. 19 for a recent review). Neuregulin, a putative ligand of the c-*neu* receptor tyrosine kinase, can induce growth or differentiation of epithelial cells *in vitro*, depending on the appropriate target cells (7–9). The expression pattern observed *in vivo* is consistent with a role of neuregulin as mesenchymal factor, which can affect embryonal epithelia during organogenesis. Repeatedly, we observe a high concentrations of neuregulin transcripts in the immediate vicinity of the epithelia (Fig. 2). This indicates that neuregulin expression in the mesenchyme may be regulated by epithelial factors. Such an epithelial control of the expression of mesenchymal factors could explain the reciprocal interaction observed between the two cell types during development. It will be of interest to determine whether mesenchymal expression of neuregulin is similarly controlled by carcinoma cells in developing human tumors.

**Developmental Role of Neuregulin in Interactions Between Neuronal/Glial or Neuronal/Muscular Cells.** Two additional biological activities of neuregulin, stimulation of glial cell growth and myotube differentiation, have been found by *in vitro* experiments (11, 12). The expression of neuregulin in developing motoneurons and various other neuronal cell types described here and observed by others (11, 12, 17) is in accordance with such functions *in vivo*. Interestingly, onset of neuregulin expression in neural structures during development occurs early in embryogenesis (E9) and thus before myotube differentiation or the major burst of glial growth and differentiation occurs. It is therefore feasible to postulate that neuregulin, besides functioning as glial growth or myotube differentiation factor, determines other early steps in neurogenesis.

**Mesenchymal and Neuronal Isoforms of Neuregulin.** An amazing variety of neuregulin cDNAs have previously been molecularly characterized; they were isolated from libraries synthesized from brain, spinal cord, and pituitary tissue RNA as well as from RNA of carcinoma cells and *ras*-transformed fibroblasts (8, 9, 11, 12). We show here that most tissues of the mouse embryo do not produce all of these isoforms. For instance, we find no transcripts that encode the N-terminal kringle domain in parenchymal organs. In contrast, such transcripts are produced in brain tissue. Thus, our data show that mesenchymal and neuronal cells produce distinct neuregulin variants. Although the genomic structure of neuregulin has not been fully characterized yet, two independent promoters seem to exist that initiate transcripts coding for the isoforms with and without kringle variants (11). Our results therefore suggest that *in vivo* both promoters function in the brain, whereas only a single promoter is used in parenchymal organs.

**Note Added in Proof.** Since preparation of this manuscript, it was shown that neuregulin also interacts directly with the HER-4/p180$^{erbB4}$ receptor. Moreover, in the absence of HER-4/p180$^{erbB4}$, no interaction between c-neu/HER-2 and neuregulin can be observed (20).

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