

Differential expression of neurotrophin receptors during renal development

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SUMMARY

Early kidney differentiation is driven by local cell-cell interactions. The metanephrogenic mesenchyme stimulates the epithelial ureter bud to grow and branch, whereas the ureter bud stimulates the mesenchyme to convert into a new epithelium. These interactions may be dependent on local growth factors and their receptors. We studied the expression of receptors for nerve growth factors during kidney development. Expression of the low- and high-affinity receptors was cell-type specific. The low-affinity NGF receptor was found in the uninduced mesenchyme at early developmental stages, but in the glomerular podocytes at later developmental stages. In contrast, the high-affinity trkB receptor was found in the cortical mesenchyme cells that will differentiate into stroma. The trkC receptor was found only weakly expressed and in a few parts of the collecting ducts. The role of these receptors and c-ros, a

receptor-type kinase expressed on the tip of the ureter bud, was studied by modified antisense oligonucleotides. However, we found that both sense, antisense and nonsense phosphorothioate oligonucleotides inhibited mouse and rat embryonic kidney development in vitro. The oligonucleotides appeared to be toxic for rodent embryonic kidneys in the experimental conditions that we used. Moreover, oligonucleotides did not penetrate well into the epithelial sheets in the organ cultures. We conclude that studies with phosphorothioate antisense oligonucleotides in organ cultures of embryonic kidneys should be interpreted with caution. Our current data do not allow us to not assign a function for the low- or high-affinity NGF receptors or c-ros in kidney development.

Key words: neurotrophin receptors, renal development, phosphorothioate oligodeoxynucleotides

INTRODUCTION

Inductive cell-cell interactions are major forces for development of multicellular organisms. In a few cases, these interactions have been clarified at the molecular level. A receptor with tyrosine kinase activity, sevenless, is involved in inductive events during development of the *Drosophila* eye (Hafen et al., 1987; Rubin, 1989) and growth factors have been shown to induce the differentiation of mesoderm in *Xenopus* embryos (Smith et al., 1990). These and some other studies suggest that inductive events can be mediated by local growth factors interacting with specific cell surface receptors.

In most solid organs, inductive interactions between mesenchyme and epithelium are important for development. The developing kidney is a good model for studies of epithelial-mesenchymal interactions. Interactions between the epithelial ureter bud and mesenchyme initiate the development of the metanephric kidney. The mesenchyme stimulates the ureter to grow and branch and to differentiate into the collecting ducts. The ureter, in turn, induces a part of the mesenchyme to convert into new epithelium (Grobstein, 1955, 1967; Saxén, 1987; Ekblom, 1992). Although the impor-

tance of these epithelial-mesenchymal interactions are recognized, the molecular basis of the interactions has not yet been determined. Recently, receptors for growth factors were shown to be expressed focally in the interacting epithelium or mesenchyme. The mRNA for a tyrosine kinase receptor, c-ros, is expressed on the tip of the ureter (Sonnenberg et al., 1991) and the condensed mesenchyme was shown to express p75^{LNGFR}, the low-affinity receptor for nerve growth factor (NGF). Interestingly, antisense oligonucleotides against low-affinity NGFR retarded development of embryonic kidneys in organ cultures, suggesting that p75^{LNGFR} could indeed be involved in inductive interactions during kidney development (Sariola et al., 1991). No functional data are available for c-ros, but it is intriguing to note that c-ros is the mouse counterpart of *Drosophila* sevenless, which is involved in the inductive interactions that govern development of the compound eye (Hafen et al., 1987).

The local expression of c-ros and low-affinity NGFR in the embryonic kidney at sites of epithelial-mesenchymal interactions prompted us to test how specific antisense oligonucleotides for these mRNAs affect embryonic kidney organ culture. Neurotrophin-responsive neurons possess two

classes of receptors; one that binds the ligand with high affinity and one that binds with low affinity (Ebendal, 1992). Since no shown biological response to NGF is mediated solely by the low-affinity NGFR, we also analyzed the expression and role of high-affinity receptors for NGF in the developing kidney. High-affinity receptors have been previously found in non-neural tissues (Klein et al., 1989; Middlemas et al., 1991; Wheeler and Bothwell, 1992; Tsoulfas et al., 1993). The known high-affinity receptors belong to the *trk* family of tyrosine kinase receptors (Bothwell, 1991; Ebendal, 1992). The members, which include *trk*, *trkB* and *trkC*, can exist as different isoforms that are synthesized from differently spliced mRNAs (Martin-Zanca et al., 1989; Klein et al., 1989; Middlemas et al., 1991; Lamballe et al., 1991; Tsoulfas et al., 1993).

We show here that each member of the *trk* family of receptors is expressed at distinct sites in the developing kidney, and that none of them are found at sites where the first epithelial-mesenchymal interactions occur. Because of the expression pattern, we expected to see distinct effects in organ cultures treated with antisense oligonucleotides for mRNAs of *c-ros* and the low- and high-affinity NGF receptors. Experiments with antisense oligonucleotides in organ cultures of embryonic tissues is a promising new technology to study the role of defined genes in development and is much easier to perform than the introduction of targeted mutation into a particular gene (Capecchi, 1989). However, in our hands, all tested oligonucleotides, including sense oligonucleotides used as controls, seriously retarded kidney development in organ cultures.

MATERIALS AND METHODS

Tissues

Hybrid mouse embryos were obtained by mating C57 black and NMRI mice, and rat embryos were obtained by mating Sprague-Dawley rats. The morning of the vaginal plug was designated as day 0 of embryonic development.

Organ culture

13- and 14-day-old embryonic rat kidneys, 11.5- and 12-day-old embryonic mouse kidneys and 11-day-old embryonic mouse lungs were isolated for organ culture. We used I-MEM medium (Gibco) (Grobstein 1955; Vestweber et al., 1985) supplemented with 10% fetal calf serum and 1% L-glutamine. Phosphorothioate deoxyoligonucleotides were added to the medium, and the medium and the oligonucleotides were changed daily. After 2-4 days in culture, the tissues were embedded in Tissue Tek (Histolab), frozen in liquid nitrogen and analyzed by immunofluorescence or embedded in historesin and analyzed by staining with hematoxylin-eosin. The phosphorothioate oligonucleotides were as follows:

Antisense *trkB*, 5'-CAA GTC AAG GTG GCG GAA-3', complementary to nucleotides 914-932 of mouse *trkB* mRNA and 368-385 of rat *trkB* mRNA, 5 µM.

Sense *trkB*, 5'-TTC CGC CAC CTT GAC TTG-3', 5 µM.

Antisense *LNGFR*, 5'-AGT GGA CTC GCT GCA TAG-3', complementary to nucleotides 1356-1373 of rat low-affinity NGFR mRNA, 5 µM.

Sense *LNGFR*, 5'-CTA TGC AGC GAG TCC ACT-3', 5 µM.

Nonsense, 5'-TCT TCT TCA AGC TTT GGC-3', 5 µM.

Antisense *ros1* (A), 5'-GGC TTC ATA CTT CAG TTG C-3',

complementary to nucleotides -77 to -69 of mouse *ros1* mRNA, 1, 3, 5, 10 µM.

Antisense *ros1* (B), 5'-GCC AGC AGA TGT TCT TCA TC-3', complementary to nucleotides -1 to +19 of mouse *ros1* mRNA, 4, 5, 8 µM.

Sense (of A) *ros1*, 5'-GCA ACT GAA GTA TGA AGC C-3', 3, 5, 10 µM.

Scrambled (of A) *ros1*, 5'-GAT CTT GAC CTT GAT GTC C-3', 3, 5, 10 µM.

Anti-sense immunoglobulin (lambda chain, constant region), 5'-CCA CAT CAC TCG GGT AGA-3', complementary to nucleotides 719-736 of rat Ig lambda chain C-region mRNA, 5 µM.

For studies of cellular uptake, we used antisense NGFR and *c-ros* (oligo A) phosphorothioate oligonucleotides with the 5'-ends conjugated to fluorescein isothiocyanate (FITC).

The oligonucleotides were synthesized by Scandinavian Gene Synthesis, Sweden (purified using affinity gel filtration); Institute of Biotechnology, University of Helsinki, Finland and Biozym Diagnostic, FRG (purified by Sep-Pak). Some of the oligonucleotides from Biozym Diagnostic were purified on a Nucleosil 120-5 C18 HPLC-column (Macherey-Nagel, Düren, Germany). They were loaded in H₂O, washed in a gradient of buffer A (100-50%) (A: 25 mM triethanolamine-bicarbonate and 0-50% B (B: Acetonitril), eluted in 100% Acetonitril, lyophilized and resuspended in H₂O.

Antibodies

To detect p75^{LNGFR} in rat tissue, a mouse monoclonal antibody against p75^{LNGFR} (Oncogene Science) was used at a 1:30 dilution. The rabbit anti-laminin antiserum raised against mouse laminin and reacting equally well with A, B1 and B2 chains of laminin (Klein et al., 1988; 1990) was used at a 1:1000 dilution. FITC and rhodamine-labelled second antibodies were purchased from Jackson Laboratories. All antibodies were diluted in PBS containing 0.1% BSA.

Immunofluorescence

Unfixed and acetone-fixed cryostat sections (7 µm) of embryonic rat kidneys were stained for double immunofluorescence using mouse monoclonal antibody against low-affinity NGFR and anti-laminin antiserum. After incubation with the primary antibodies, slides were washed in PBS and incubated with the appropriate second antibodies. Slides were washed three more times, then mounted in mounting medium and examined using a Zeiss Axiophot microscope. Results with fixed or unfixed sections did not differ. Control sections incubated in PBS containing 0.1% BSA instead of the first antibody did not show any specific fluorescence to any given structure.

Northern blot analysis

Total RNA of 16-day-old embryonic mouse kidneys and adult mouse brains was extracted by guanidium-CsCl (Chirgwin et al., 1979) from pooled deep-frozen samples. The poly(A)-containing fraction of total kidney RNA was isolated by affinity chromatography on oligo(dT)-cellulose columns. 5 µg of poly(A) RNA and 10 µg of total RNA were denatured with glyoxal and electrophoresed on a 1% agarose gel. After transfer to Zeta-Probe GT membrane (Bio-Rad), RNA was fixed by UV crosslinker with a Stratalinker (Stratagene) under conditions recommended by the manufacturer. Membranes were prehybridized for 2 hours at 68°C in solution containing 2.5× SSPE, 2× Denhardt's solution, 0.1% SDS, 50 µg/ml yeast tRNA, 50 µg/ml sheared salmon sperm DNA. Hybridization was performed in the same solution for 18-22 hours at 68°C (Sambrook et al., 1989). To detect low-affinity NGFR mRNA, we used a deoxyoligonucleotide probe (65 nt) complementary to nucleotides 822-886 of the rat low-affinity NGFR mRNA; 5'-GAG CAA TAG ACA GGA ATG AGG TTG TCG GTG GTG CCG CGG GTC ACT ACA GGC TGG GAG CTG CCC AT-3' (Radeke et al., 1987). To detect *trkB* mRNA, we used

two deoxyoligonucleotide probes, one 65-mer complementary to nucleotides 2306-2353 of the rat *trkB* mRNA: 5'-TTG TGT GTG GTT CCC GCT GCC AGC ATC CCA GCA TCA GCT CGT ACA CCT-3' (Middlemas et al., 1991), and one 63-mer complementary to nucleotides 1027-1089 of the mouse *trkB* mRNA: 5'-GGC CAT CAG GGT GTA GTC TCC GTT ATT CAT ATG AGT GGG GTT ATC CAG CTG GAG GCA GCC ATG-3' (Klein et al., 1989). The oligonucleotides were synthesized by Scandinavian Gene Synthesis, Sweden. The oligonucleotides were labelled at the 5' end with [32 P]ATP (Amersham) using T4-polynucleotidyl kinase (Northumbria Biologicals Ltd) to a specific activity of 2×10^9 cts/minute/ μ g DNA (Sambrook et al., 1989). After hybridization, the membranes were first washed in $1 \times$ SSPE, 0.05% SDS for 1-1.5 hours at room temperature and then in $2.5 \times$ SSPE, 0.1% SDS for 30 minutes at 68°C . The membranes were exposed to Kodak XAR films at -70°C in the presence of intensifying screens.

In situ hybridization

The in situ hybridisation was performed according to Ernfors et al. (1990) with a few modifications. Briefly, embryonic mouse kidneys at different stages were embedded in Tissue Tek (Histolab), frozen in liquid nitrogen, cut in $10 \mu\text{m}$ sections on a cryostat (-20°C) and thawed onto slides pretreated with poly-L-lysine ($50 \mu\text{g}/\text{ml}$). Sections were fixed in 4% paraformaldehyde, rinsed twice in PBS, dehydrated in a graded ethanol series

including a 5 minute incubation in chloroform. The same oligonucleotide probes that were used for northern blots were also used for in situ hybridization, including a 50-mer to detect *trkC* mRNA: 5'-GAA GTG GCC GTT ATG GTC TGG TTG GCT GTG CCC AGG GCA TTC TTA GCA AT-3', complementary to nucleotides 1185-1235 of the rat *trkC* mRNA (Merlio et al., 1992), and a 63-mer to detect *trk* mRNA: 5'-AGC CAG CAG CGT GTA GTT GCC GTT GTT GAC GTG GGT GGG CTG GTT GAG GCG CAG ACA CCC GTG-3', complementary to nucleotides 1147-1209 of the human *trk* mRNA (Martin-Zanca et al., 1989). The probes were labelled at the 3' end with [35 S]dATP (Amersham) using terminal deoxyribonucleotidyl transferase (Scandinavian Diagnostic Services) to a specific activity of 1×10^9 cts/minute/ μ g.

Hybridization was performed in 50% formamide, $4 \times$ SSC, $1 \times$ Denhardt's solution, 10% dextran sulfate, 0.25 mg/ml yeast tRNA, 0.5 mg/ml sheared salmon sperm DNA, 1% sarcosyl, 0.02 M Na_2HPO_4 (pH 7.0), 0.05 M dithiothreitol, using 10^7 cts/minute/ml respective probe. The sections were hybridized at 42°C for 15-18 hours in a humidified chamber with 0.1 ml of hybridization solution per slide. They were subsequently washed four times (15 minutes each) at 56°C in $1 \times$ SSC.

As controls for specific hybridisation, we used sections that were hybridised with the same amount of labelled probe plus unlabelled probe in excess. The controls did not show any specific staining. The slides were dipped in Kodak NTB-2 photo emulsion (diluted

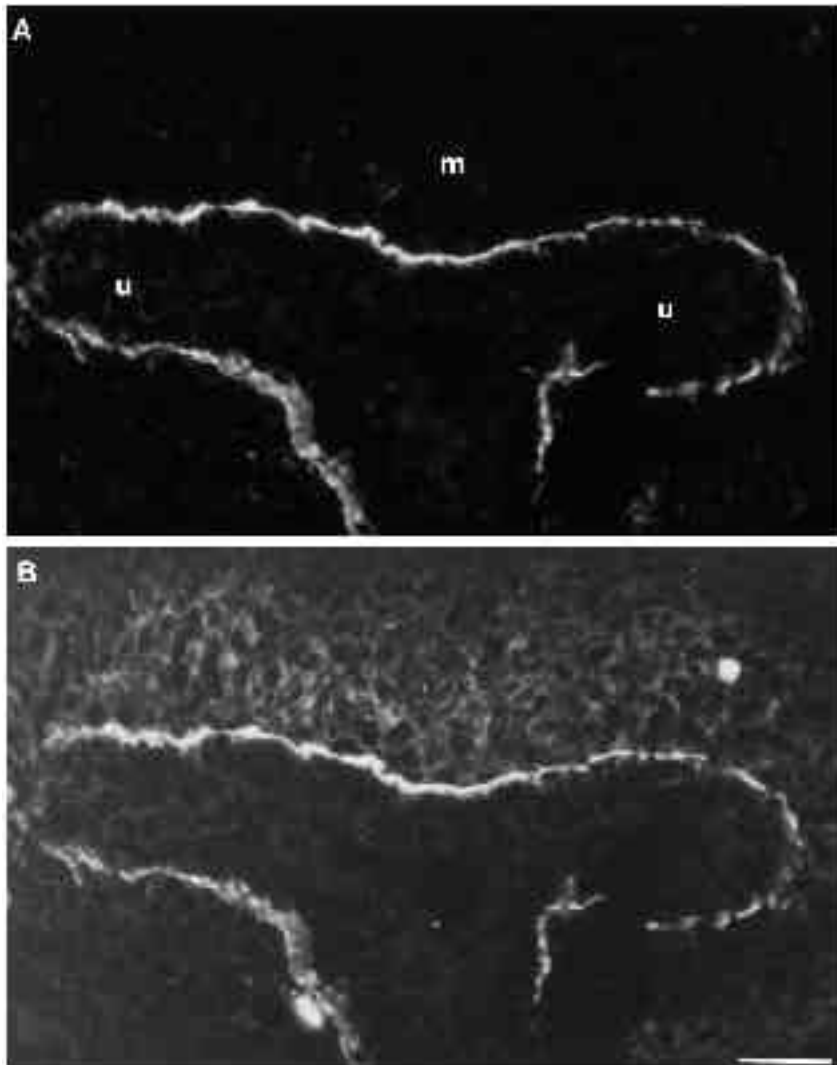


Fig. 1. Immunolocalization of laminin and p75^{LNGFR} in 13-day-old embryonic rat kidneys. Sections of 13-day-old rat kidney were double stained by indirect immunofluorescence for laminin (A) and p75^{LNGFR} (B). In 13-day-old kidneys, the antibody against laminin stains the membrane of the ureter (u) and also mesenchymal (m) cells (A). The monoclonal antibody detecting p75^{LNGFR} stains the uninduced mesenchyme (B) found on top of the T-shaped ureter. Note that no or very little p75^{LNGFR} can be seen in the condensed areas of the mesenchyme found below the T-shaped ureter. Bar, $39 \mu\text{m}$.

1:1 in water), exposed for 4-5 weeks at 4°C, developed, fixed and counterstained with cresyl violet. Photographs were taken under bright-field and dark-field illumination.

RESULTS

The expression of NGFR in embryonic rat and mouse kidney

At day 13 in the embryonic rat kidney, nephrogenesis is initiated around the ureter bud and the mesenchyme condenses to form the first nephrons. A basement membrane

separates the two tissue compartments, as shown here by immunofluorescence for laminin (Fig. 1A). By immunofluorescence, p75^{NGFR} was detected in the uninduced stem cells. Interestingly, no or very little p75^{NGFR} could be seen in the condensed areas of the mesenchyme (Fig. 1B). The condensates subsequently form comma-shaped bodies and then S-shaped bodies consisting of three major parts; developing distal tubule, proximal tubule and glomerulus. To form a complete nephron, the tubules elongate, the glomerular podocyte epithelium becomes folded and the distal tubules merge with the ureter. New condensates form in the upper cortex, while the more differentiated areas are found

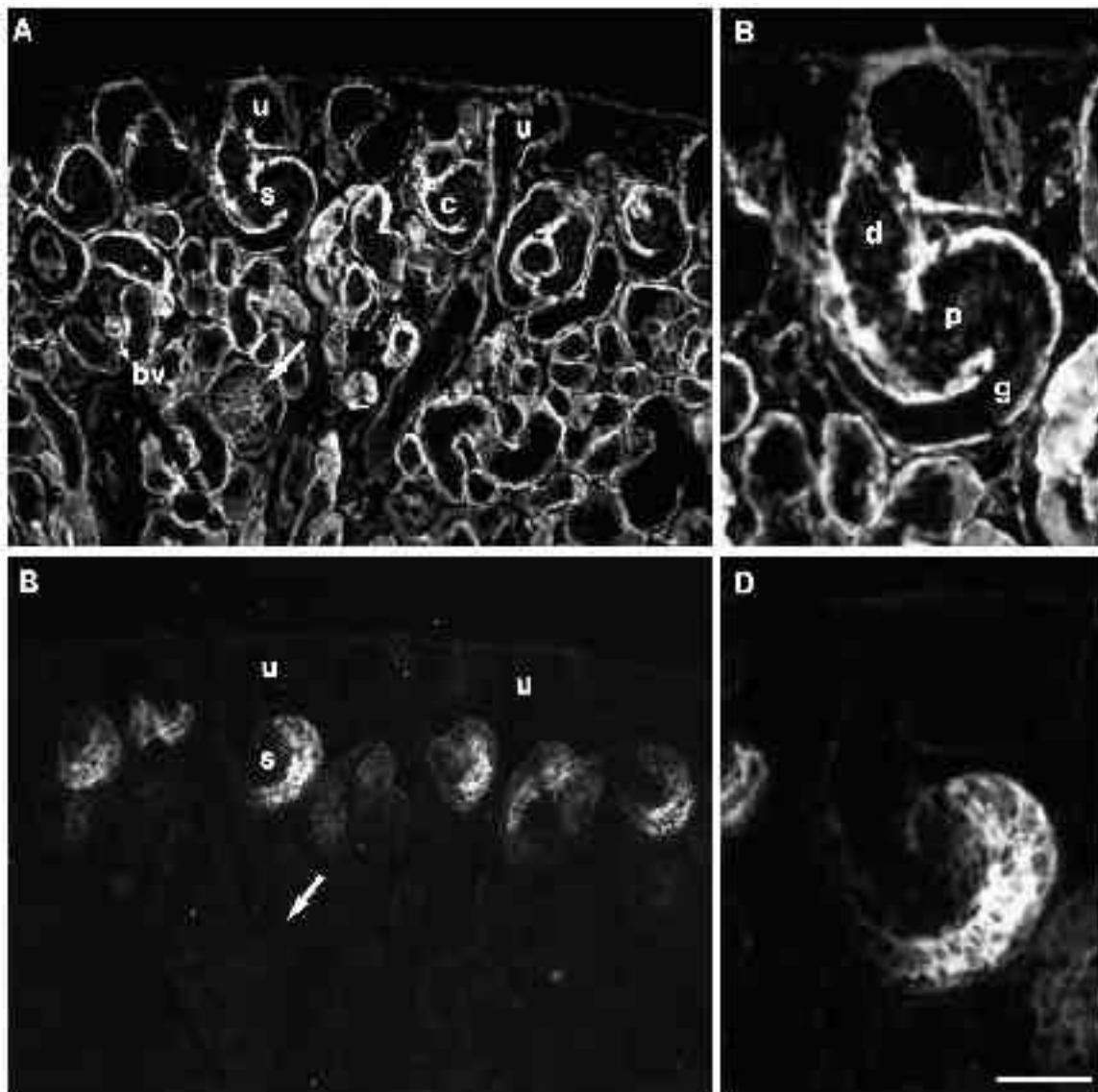


Fig. 2. Immunolocalization of laminin and p75^{NGFR} and distribution of low-affinity NGFR mRNA during in vivo development of rat and mouse kidney. In newborn rat kidneys laminin is expressed in all basement membranes including those of the ureter (u), the comma-shaped body (c), the S-shaped tubuli (s) and blood vessels (bv) (A,B). p75^{NGFR} is expressed in the glomerular part of the S-shaped tubuli (C,D). Note the lack of p75^{NGFR} when the glomerulus is fully differentiated (arrow). Sections of 15-day-old embryonic mouse kidneys were analyzed by in-situ hybridisation with an ³⁵S-labelled antisense oligonucleotide probe complementary to nucleotides 822-886 of the rat low-affinity NGFR mRNA. The autoradiographs are shown in bright field (E,F) and dark field (G,H). The expression of low-affinity NGFR mRNA is congruent with the expression of p75^{NGFR}. A strong signal is seen in the glomeruli. For controls of specific hybridisation; see materials and methods. d, developing distal tubule; p, proximal tubule; g, glomerular podocytes. Bar, 79 µm (A,C,E,G); 57 µm (B,D); 43 µm (F,H).

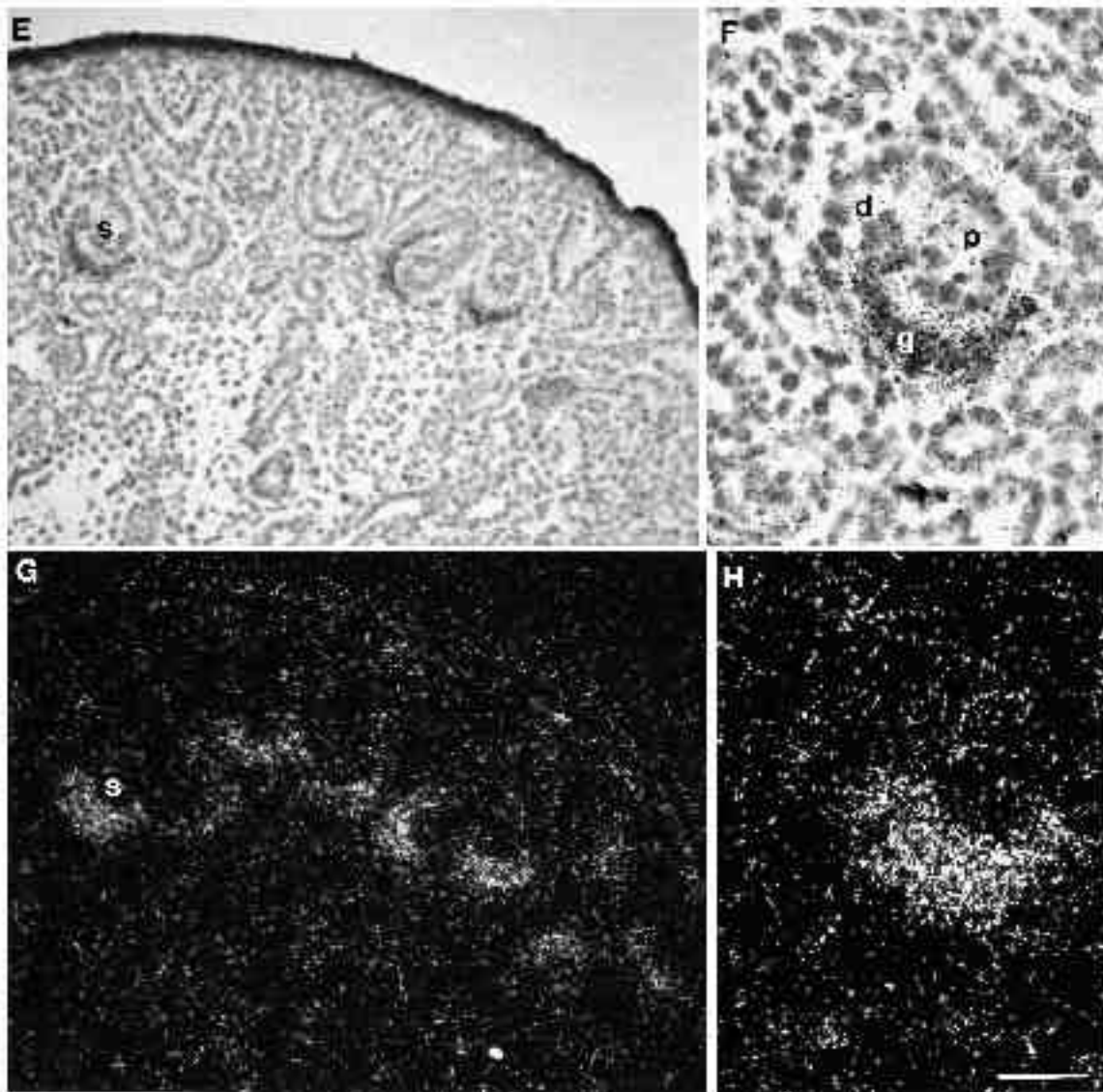
in the medullary part. The epithelial structures can be well visualized by immunostaining for laminin (Fig. 2A,B). In S-shaped tubules, p75^{NGFR} was seen almost exclusively in the glomerular part (Fig. 2C,D) but was no longer seen in more differentiated glomeruli found in the medullary part of the kidney. No p75^{NGFR} was detected in the new condensates in the upper cortex (Fig. 2C). The restricted expression in the glomerular podocytes was also noted at the mRNA level by in situ hybridisation (Fig. 2E-H). Northern blot analysis showed the presence of a single 3.7 kb low-affinity NGFR transcript in 16-day-old embryonic mouse kidney (Fig. 3, lane 1).

The expression of *trkB* in embryonic mouse kidney

TrkB is expressed in the nervous system as multiple mRNAs, some of which encode truncated receptors. Our results suggest that *trkB* mRNA in the embryonic kidney corresponds to truncated transcripts, lacking coding sequences for the intracellular tyrosine kinase domain (Klein

et al., 1989, Middlemas et al., 1991). Northern blot analysis with an oligonucleotide probe against the extracellular domain revealed two transcripts; 6.5 kb and 2.7 kb in 16-day-old embryonic mouse kidney (Fig. 3, lane 2); when an oligonucleotide probe against the kinase domain was used, no specific transcripts in the embryonic kidney was observed (Fig. 3, lane 3). The results are not due to a failure of the probe to recognize *trkB*-specific transcripts since we detected several transcripts in the adult mouse brain with the same probe. These transcripts included the full-length 7.5 kb mRNA and mRNAs of 6.5, 3.7, 2.7 and 2.0 kb. The mRNA encoding the full-length receptor is the transcript of 7.5 kb (Fig. 3, lanes 4 and 5).

In situ hybridisation with a probe against the extracellular domain of *trkB* mRNA showed *trkB* in the uninduced mesenchyme in the upper cortex of the kidney (Fig. 4A-C). In the developing kidney, the cortical area consists of uninduced mesenchyme and early differentiation stages while the central parts of the kidney contain late and terminal stages of epithelial differentiation surrounded by



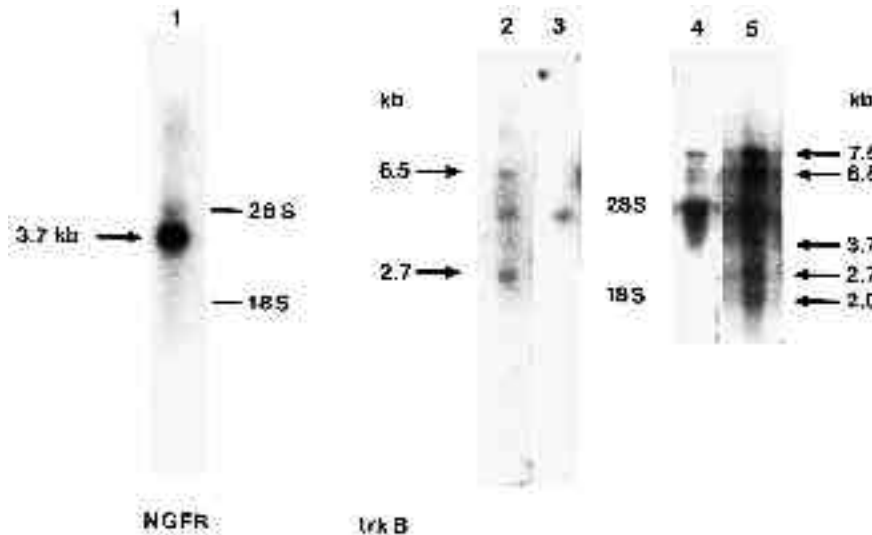
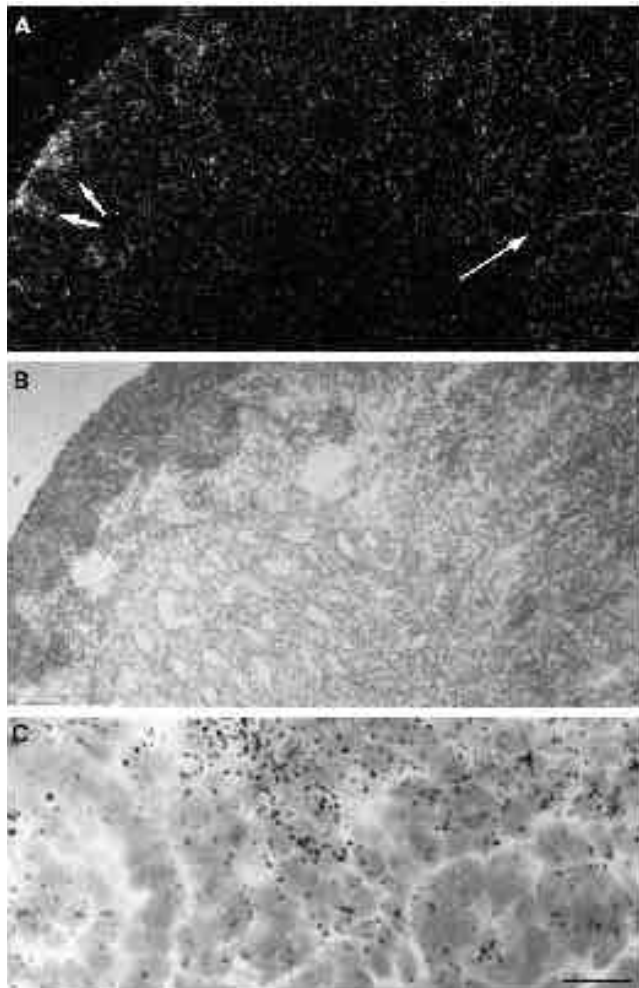


Fig. 3. Northern blot analysis of low-affinity NGFR mRNA in embryonic mouse kidney and trkB mRNA in embryonic mouse kidney and adult mouse brain. 5 µg of poly(A) RNA of 16-day-old embryonic mouse kidney (lanes 1, 2 and 3) and 10 µg of total RNA of adult mouse brain was used (lanes 4 and 5). The filters were hybridised with antisense oligonucleotide probes corresponding to low-affinity NGFR (lane 1) and trkB (lanes 2-5) mRNA. A single band of 3.7 kb representing low-affinity NGFR mRNA was detected in embryonic kidney (lane 1). For the analysis of trkB mRNA, two different oligonucleotide probes were used; one probe against the extracellular domain (lanes 2 and 5) and one probe against the kinase-binding domain (lanes 3 and 4). Two bands of 6.5 and 2.7 kb representing trkB mRNAs (truncated forms) were detected (lane 2) whereas no signal

representing full-length trkB could be identified (lane 3) in embryonic kidney. In adult mouse brain, several trkB transcripts were observed. These transcripts included RNA species of 7.5 kb, which encodes the full-length receptor (lane 4) and 6.5, 3.7, 2.7 and 2.0 kb, which encode truncated receptors (lane 5). 28S rRNA is also detected presumably since oligodeoxynucleotides hybridize unspecifically to the ribosomal RNA. Exposure times were 6 days for low-affinity NGFR and 14 days for trkB.



a fully differentiated stroma (Mugrauer and Ekblom, 1991). Some trkB mRNA was also detected in the medullary part of the kidney (Fig. 4A,B). However, due to the rather weak expression, the cells that express trkB mRNA in the medullary part could not be defined with certainty.

The expression of trkC in embryonic mouse kidney

TrkC is also expressed in the nervous system as multiple mRNAs, encoding full-length receptors and a truncated receptor that lacks the catalytic domain (Tsoulfas et al., 1993).

The expression of trkC during mouse kidney development was analysed by in situ hybridisation, using an oligonucleotide probe against the transmembrane region. trkC mRNA could not be detected in 13-day-old embryonic mouse kidney (data not shown) whereas in 15-day-old kidneys, clusters of silver grains with higher density than the background were visible (Fig. 5A,B). Higher magnification of these clusters shows that trkC transcripts are localised in parts of the collecting ducts (Fig. 5C).

Fig. 4. Expression of trkB mRNA in 2-day-old postnatal mouse kidney. Sections of 2-day-old postnatal kidneys were analysed by in situ hybridisation with an ³⁵S-labelled antisense oligonucleotide probe against the extracellular domain of the mouse trkB mRNA. Dark-field (A) and bright-field (B,C) micrographs are shown. TrkB mRNA is expressed in the uninduced mesenchyme in the upper cortex of the kidney (A,B; indicated by thick arrows); weak expression is found in the medullary part of the kidney (A,B; indicated by a thin arrow). Higher magnification of another section shows that the epithelial structures in the upper cortex are negative. Bar, 150 µm (A,B); 25 µm (C).

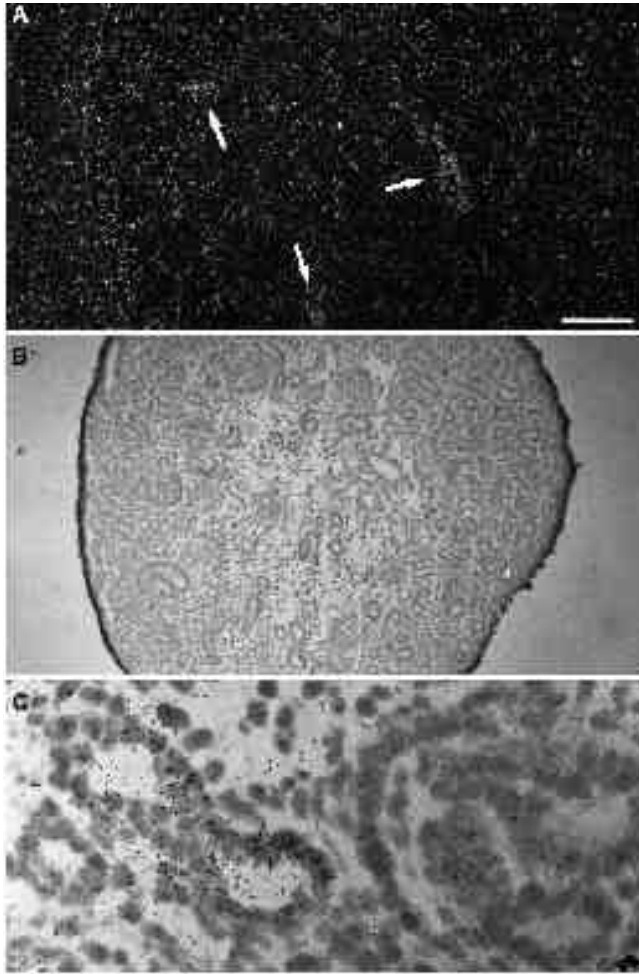


Fig. 5. Localisation of trkC in embryonic mouse kidney. Dark-field (A) and bright-field (B,C) micrographs are shown. In 15-day-old embryonic kidneys, clusters with higher density than the background are visible (A,B). Higher magnification shows that trkC mRNA is expressed in some parts of the collecting ducts (C). Bar, 158 μ m (A,B); 25 μ m (C).

Unspecific inhibition of metanephric development in organ culture by phosphorothioate oligonucleotides

Initially, we asked whether the function of c-ros (Sonnenberg et al., 1991) and the trk receptors in the embryonic mouse kidney could be studied by applying antisense oligonucleotides to organ culture. The specific inhibition of kidney development observed previously by antisense but not by sense low-affinity NGFR oligonucleotides were intended as positive and negative controls. Phosphorothioate oligonucleotides were produced as described (Sariola et al., 1991) by Biozym Diagnostics (Hameln, Germany). The differentiation stages of mouse kidney development in vitro have been well described previously (Grobstein, 1955; Saxén, 1987; Ekblom, 1992).

We first tested two different antisense phosphorothioate oligonucleotides (A and B) complementary to mouse c-ros mRNA and two control oligonucleotides, sense of oligonucleotide A and scrambled of A. The phosphorothioate

Table 1. Effects of ros1 antisense, sense and scrambled phosphorothioate oligonucleotides on branching morphogenesis of cultured 12-day-old embryonic mouse kidneys

Oligo	Total no.	Branching morphogenesis		
		--	+-	++
Antisense ros1 (A)	30	29	1	0
Sense ros1 (A)	15	13	2	0
Scramb. ros1 (A)	4	4	0	0
Without oligonucleotide	23	0	0	23

5 μ M phosphorothioate oligonucleotide was used in each case.

--, branching severely inhibited; +-, branching inhibited to some extent; ++, normal branching and growth.

oligonucleotides were tested at 5 μ M according to the protocol of Sariola et al. (1991). All tested oligonucleotides perturbed kidney development equally well. No tubular development was seen and ureter branching was severely affected in a 2-day culture (Table 1). We then asked whether the oligonucleotides would be more specific inhibitors of development than sense oligonucleotides at lower concentrations. At 1 μ M neither sense nor antisense probes perturbed development, but at 3 μ M both probes were equally good inhibitors of kidney development. Careful purification of sense oligonucleotides by HPLC (Matsukura et al., 1987) did not abolish the inhibitory effect. We nevertheless also tested sense and antisense c-ros oligonucleotides produced by another manufacturer (Scandinavian Gene Synthesis), but both probes at 5 μ M severely retarded kidney development. Histology of kidneys incubated with sense oligonucleotides showed that no tubules were formed. An extensive cell death was seen in the mesenchymal compartment in the kidneys treated with the oligonucleotides (Fig. 6A-C), suggesting toxic effects of the oligonucleotides.

Phosphorothioate sense oligonucleotides against low-affinity NGFR, reported not to perturb rat kidney development (Sariola et al., 1991), also severely inhibited mouse kidney development and were equally effective as the antisense low-affinity NGFR oligonucleotides at 5 μ M.

A slight difference between 11.5- and 12-day-old kidneys was seen. Both antisense and sense oligonucleotides to low-affinity NGFR severely inhibited development of 11.5-day-old kidneys whereas both the sense and antisense oligonucleotides were less potent inhibitors of development when tested on kidneys from 12-day embryos.

Restricted uptake of oligonucleotides in mouse metanephric organ cultures

c-ros mRNA is in all developmental stages found exclusively in the tip of the ureter bud (Sonnenberg et al., 1991), whereas low-affinity NGFR is found in the uninduced mesenchyme in the early developmental stages. It was important to show that antisense oligonucleotides reached the target mRNAs in organ cultures. However, antisense oligonucleotides for c-ros and low-affinity NGFR labelled with fluorescent markers were taken up exclusively by mesenchymal cells of 12-day embryonic kidneys and neither probe

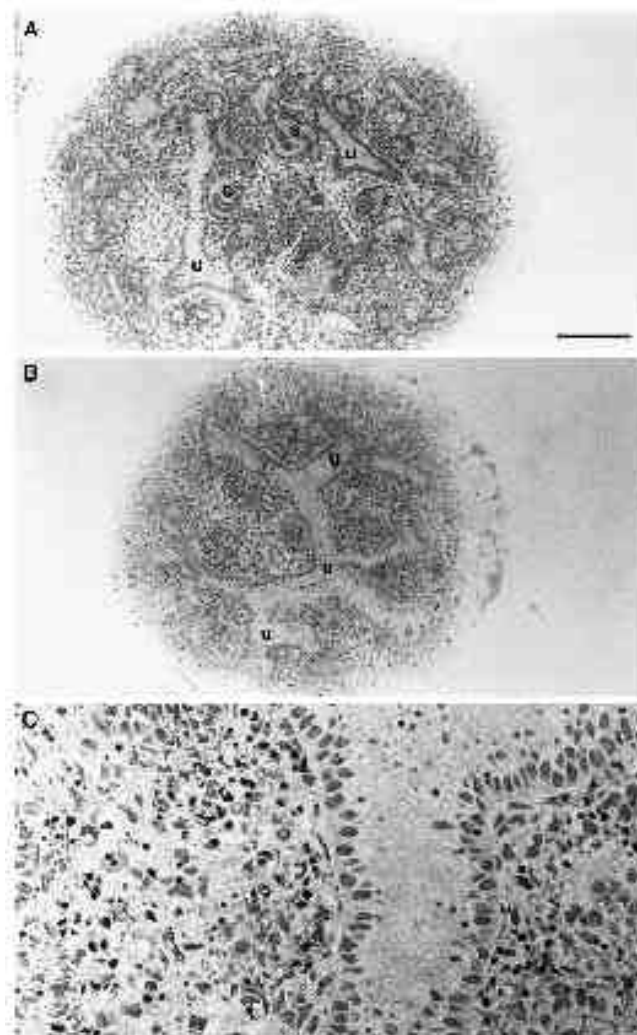


Fig. 6. Branching morphogenesis in the presence of sense *ros1* phosphorothioate oligonucleotides. 12-day-old mouse kidneys were cultured for 2 days whereupon they were embedded in historesin and stained with hematoxylin-eosin. In kidneys incubated without phosphorothioate oligonucleotides, normal tubular branching occurs (A). Kidneys incubated with sense *ros1* phosphorothioate oligonucleotides are growth inhibited and the tubular branching is also inhibited (B). Also, an extensive cell death (note the black dots) is found in the mesenchyme (C) while the epithelial cells seems to be unaffected. u, ureter; c, comma-shaped body; s, s-shaped body. Bar, 161 μ m (A,B); 40 μ m (C).

reached the epithelium (Fig. 7A,B). These results suggest that the inhibitory effect of antisense *c-ros* was not due to binding to the target mRNA.

Inhibition of rat kidney development in organ culture by sense and antisense oligonucleotides to rat *LNGFR*, *trkB* and immunoglobulin

Since it was not possible to obtain specific inhibition of mouse kidney development with any of the tested antisense probes and Sariola et al. (1991) had used rat embryos, various oligonucleotides obtained from different sources were tested on rat embryonic kidneys in culture. Kidneys

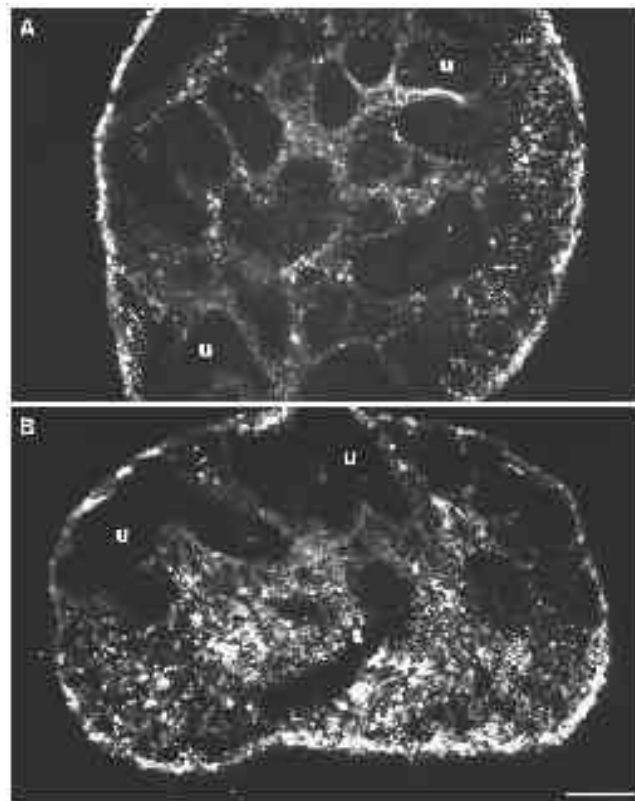


Fig. 7. Uptake of fluorescently labelled oligonucleotides in metanephric organ cultures. 12-day-old mouse kidneys were cultured for 24 hours in the presence of FITC- antisense *c-ros* (A) and FITC antisense low-affinity NGFR (B) phosphorothioate oligonucleotides. Cryostat sections of the incubated kidneys show that the oligonucleotides are taken up by mesenchymal cells. Note that no signal can be seen in the tips of the ureter. u, ureter. Bar, 78 μ m.

from 13-day-old rats resemble kidneys from 12-day-old mice, and are composed of mesenchyme and a largely unbranched ureter. When such kidneys were cultured without oligonucleotides an abundant branching of the ureter into the expanding mesenchyme was seen after 2 days in culture and condensates formed around the tips of the ureter. On day 3, the condensates had formed S-shaped tubuli. In contrast, sense and antisense phosphorothioate oligonucleotides (5 μ M) complementary to *trkB* or low-affinity NGFR mRNA severely inhibited development. The sense oligonucleotides affected tubular branching to the same extent as the antisense probe (Table 2). It is unlikely that the oligonucleotides obtained from Scandinavian Gene Synthesis were contaminated with impurities because of the purification protocol, but we also tested oligonucleotides produced by the Institute of Biotechnology, University of Helsinki, the source used by Sariola et al. (1991). However, the nonsense and sense oligonucleotides for low-affinity NGFR obtained from the other source also affected tubular branching and growth to the same extent as the antisense oligonucleotides. We can exclude that the poor development was due to suboptimal culture conditions because kidneys incubated without oligonucleotides were cultured in parallel

Table 2. Effects of low-affinity NGFR antisense, sense, nonsense; trkB antisense, sense and immunoglobulin antisense phosphorothioate oligonucleotides on branching of cultured embryonic rat kidneys

Oligo	Embryonic stage (days)	Total no.	Branching morphogenesis			Growth		
			--	+-	++	--	+-	++
Antisense LNGFR	13	20	20	0	0	20	0	0
Sense LNGFR	13	18	18	0	0	18	0	0
Nonsense LNGFR	13	10	10	0	0	10	0	0
Antisense LNGFR	14	23	21	1	1	7	15	1
Sense LNGFR	14	22	14	7	1	2	15	5
Antisense trkB	13	7	7	0	0	7	0	0
Sense trkB	13	7	7	0	0	7	0	0
Antisense Ig (, C)*	13	10	10	0	0	5	5	0
Without oligonucleotide	13	26	0	2	24	0	2	24
Without oligonucleotide	14	29	0	2	27	0	2	27

*Immunoglobulin; lambda chain, constant region. 5 μ M phosphorothioate oligo-nucleotide was used in each case. --, branching and growth severely inhibited; +-, branching and growth inhibited to some extent; ++, normal branching and growth.

in each experiment; they grew well and the conversion of mesenchymal condensates into comma- and S-shaped bodies could be seen (Fig. 8A-D). In contrast, immunofluorescence staining for laminin demonstrated that ureter branching was inhibited by antisense, sense and nonsense low-affinity NGFR oligonucleotides (Fig. 9A,B). Contrary to this report of Sariola et al. 1991), staining for p75^{LNGFR} showed the presence of some p75^{LNGFR} in kidneys incubated with antisense oligonucleotides (Fig. 9C). Hence, all tested oligonucleotides seemed to perturb rat kidney development severely although complete inhibition of p75^{LNGFR} expression was not obtained.

We also tested kidneys from 14-day rat embryos. Both sense and antisense oligonucleotides affected the development to some extent. The kidneys incubated with sense oligonucleotides expressed small amounts of p75^{LNGFR}, more than the kidneys incubated with antisense oligonucleotides but less than the kidneys incubated without oligonucleotides. Although the kidneys incubated with sense low-affinity NGFR oligonucleotides express slightly more p75^{LNGFR} than the kidneys incubated with antisense low-affinity NGFR oligonucleotides, the growth was still inhibited and the tubular branching was also affected (Table 2).

To test further whether our results were due to non-specific toxicity of the oligonucleotides, we incubated 13-day-old embryonic rat kidneys (early T-stage) with antisense phosphorothioate oligonucleotides (5 μ M) complementary to rat immunoglobulin mRNA (lambda chain, constant region), which is not expressed in the kidney. A severe inhibition of development was seen in all tested kidneys (Table 2), with a partial inhibition of growth in 50% of the kidneys and a severe inhibition of growth in 50% (Fig. 10A,B).

Effect of oligonucleotides on lung development

Although the development of embryonic mouse or rat kidneys could be perturbed by all tested oligonucleotides, other embryonic tissues may be more resistant. Indeed, lung rudiments cultured in the presence of 3 μ M antisense, sense or scrambled phosphorothioate oligonucleotides for c-ros developed well, and an extensive branching of the lung epithelium was observed. However, at 10 μ M lung differentiation was affected to some extent. It is noteworthy that c-

ros mRNA is not expressed on day 11 of mouse lung development (Sonnenberg et al., 1991). Hence, the results suggest that phosphorothioate oligonucleotides cause some non-specific inhibition of lung development as well, although at least a 3-fold to 5-fold higher concentration of the oligonucleotides is required to obtain toxic levels in lung than kidney.

DISCUSSION

Our current study on the expression of the low- and high-affinity receptors in the developing kidney shows that the expression of each receptor is cell-type specific. The high-affinity trkB receptor is found in the cortical mesenchymal cells that will differentiate into stroma, the trkC receptor in the medulla in the collecting ducts, whereas the low-affinity NGF receptor is found in the uninduced mesenchyme and later in the glomerular parts of the S-shaped body. We also report our efforts to study the function of these mRNAs using modified specific antisense oligonucleotides.

The presence of the low-affinity NGF receptor in the mesenchymal condensates has been reported previously (Sariola et al., 1991). However, we found a different and rather complex expression pattern of the low-affinity receptor. Immunohistology showed no or very little p75^{LNGFR} in the condensing mesenchyme but rather in the uninduced stem cells. When the first condensates differentiated into S-shaped tubules, it was found predominantly in the glomerular podocytes. Wheeler and Bothwell (1992) have also shown that the low-affinity NGF receptor in later developmental stages is exclusively found in the glomerular epithelium. The expression results argue against a general role for the low-affinity NGF receptor in branching morphogenesis of the embryonic kidney. The actions of NGF through the low-affinity NGF receptor is enhanced if the receptor subunit is complexed to high-affinity receptors, and we therefore studied whether any of these receptors were coexpressed with the low-affinity receptor. According to the *in situ* hybridisations, trkB and trkC but not trk were expressed in the developing kidney. However, neither trkB or trkC were coexpressed with the low-affinity receptor. TrkB mRNA was found in the uninduced mesenchyme in the

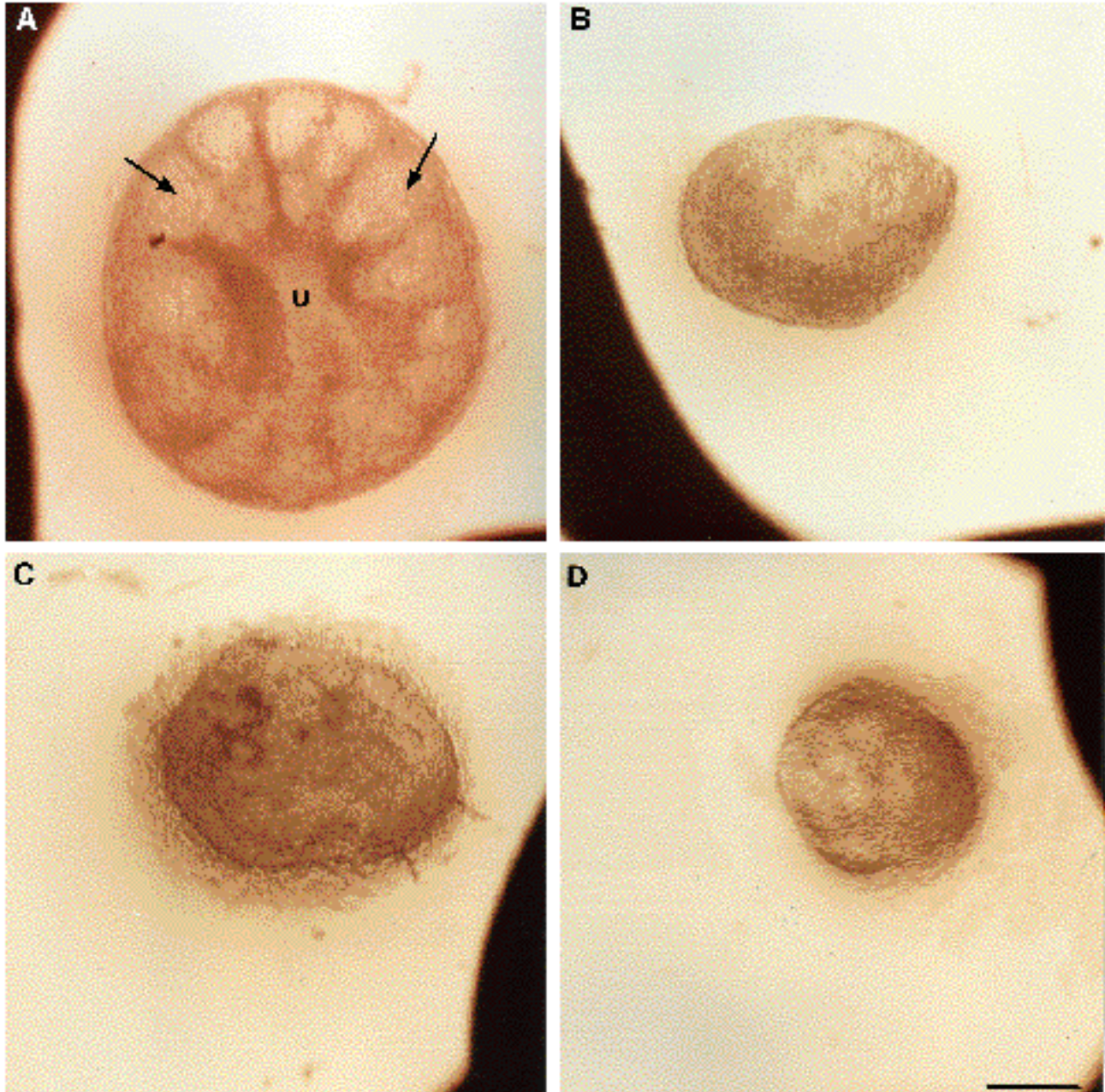


Fig. 8. Branching morphogenesis in the presence of low-affinity NGFR antisense, sense and nonsense phosphorothioate oligonucleotides. 13-day-old embryonic rat kidneys were examined under a stereomicroscope after 4 days in culture, where they had been incubated in the absence or presence of phosphorothioate oligonucleotides. An abundant branching of the ureter (u) into the growing mesenchyme can be seen in the kidneys incubated without phosphorothioate oligonucleotides (A) and mesenchymal condensates around the tips of the ureter are formed (arrows). No ureter branching and no condensates can be seen in the kidneys incubated with low-affinity NGFR antisense (B), low-affinity NGFR sense (C) and nonsense (D) phosphorothioate oligonucleotides. Bar, 56 μ m.

upper cortex of the kidney in areas where cells will not differentiate into tubules, but some *trkB* mRNA was also found in the medullary part where differentiated tubules are found. Interestingly, *trkB* mRNA in the embryonic kidney was truncated, lacking the part encoding the intracellular tyrosine kinase domain. The expression pattern of *trkC* was quite different from those of low-affinity NGFR and *trkB*; *trkC* mRNA was localised in some parts of the collecting ducts only. Our observation that *trkC* is expressed in the

embryonic kidney is supported by Tsoulfas et al. (1993) who showed the presence of transcripts encoding full-length *trkC* receptors in mouse kidney. The results of the expression studies argue against a role of the studied high-affinity NGF receptors in the inductive cell-cell interactions that initiate kidney epithelial cell development.

The family of neurotrophins (nerve growth factor NGF; brain derived neurotrophic factor, BDNF; neurotrophin 3, NT-3; neurotrophin 4, NT-4 and neurotrophin 5, NT-5 are

also expressed in several non-neural cell-types and tissues (Barde et al., 1982; Levi-Montalcini, 1987; Leibrock et al., 1989; Hohn et al., 1990; Maisonpierre et al., 1990; Hallböök et al., 1991; Thoenen, 1991; Berkmeier et al., 1991). NGF is

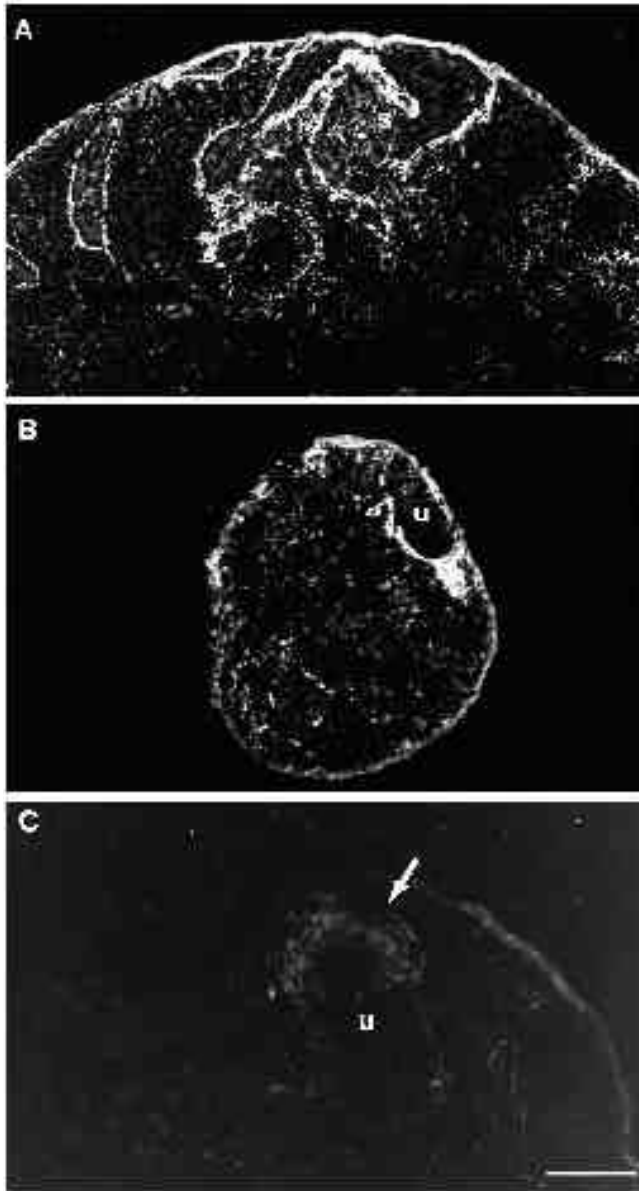


Fig. 9. Indirect immunofluorescence showing laminin in kidneys incubated without phosphorothioate oligonucleotides, and with nonsense phosphorothioate oligonucleotides and showing p75^{LNGFR} in kidneys incubated with antisense phosphorothioate oligonucleotides. 13-day-old rat kidneys were cultured for 4 days whereupon cryostat sections were cut. Sections of kidneys incubated without phosphorothioate oligonucleotides were stained for laminin (A) which is expressed in the basement membranes of the ureter and the developing S-shaped (s) tubules and also in the mesenchymal cells. Sections of kidneys incubated with nonsense phosphorothioate oligonucleotides stained for laminin (B). Note that no tubular branching can be distinguished. Sections of kidneys incubated with low-affinity NGFR antisense phosphorothioate oligonucleotides were stained for p75^{LNGFR} (C) which is expressed in small amounts (arrow). u, ureter. Bar, 96 μ m (A,B); 48 μ m (C).

expressed in several adult tissues including kidney, BDNF displays a more restricted pattern of expression (adult brain, heart, lung, muscle), whereas NT-3 is expressed in almost every tissue and it is noteworthy that the level of NT-3 mRNA in adult kidney is substantially higher than in any other tissue (Maisonpierre et al., 1990). NT-5 is also expressed in several non-neuronal adult tissues including kidney (Berkmeier et al., 1991). Since several neurotrophins are expressed in the kidney, it would be interesting to study their expression and possible functions in early kidney differentiation.

The specific roles of the neurotrophin receptors in development of non-neuronal organs is not yet known. The possible role of low-affinity NGFR has been studied for the kidney but the results are conflicting. Antisense oligonucleotides experiments have suggested that p75^{LNGFR} could be important for early stages of branching ureter morphogenesis and for formation of kidney tubules (Sariola et al., 1991). Contrary to that report, Lee et al. (1992) could not show any morphological or physiological defects in kidneys of mutant mice lacking the gene product of low-affinity NGFR. In order to clarify the issue, it is important to study the methodologies used in detail. We reasoned that the distinct expression patterns of the different NGF receptors and c-ros would give us a good opportunity to study their function since specific antisense oligonucleotides should cause distinct morphological abnormalities in kidney organ cultures.

Sariola et al. (1991) reported that antisense oligonucleotides can be used to inhibit branching epithelial morphogenesis specifically in kidney organ culture, and application of antisense oligonucleotides in organ culture might thus be an excellent assay to define molecules of importance for kidney differentiation. However, we could not show specific inhibition of development with the antisense low-affinity NGFR oligonucleotides. Instead, our results suggest that phosphorothioate oligonucleotides cause nonspecific toxicity in organ cultures of embryonic mouse and rat kidneys. Antisense, sense and nonsense phosphorothioates oligonucleotides of the low-affinity NGFR sequence affected tubular branching equally well. Histological sections of the kidneys treated with sense or antisense oligonucleotides showed widespread necrosis, especially in the mesenchymal compartment. The same result was obtained with oligonucleotides from three different sources, and purification of the oligonucleotides with HPLC did not lead to diminished inhibition. Since some of the oligonucleotides were obtained from the same source as those used by Sariola et al. (1991), we have no explanation for the discrepancy.

It is possible that phosphorothioate oligonucleotides taken up by mesenchymal cells cause some non-specific toxicity. Phosphorothioates are more resistant to nuclease degradation than unmodified oligonucleotides. On the other hand, they are more slowly accumulated within cells and seem to possess more non-specific toxicity in a variety of cell-culture system (Neckers et al., 1992). Breakdown of phosphorothioate oligonucleotide may lead to incorporation of phosphorothioate mononucleotides into cellular DNA which can lead to obscure side effects (Eckstein, 1985). Phosphorothioate oligonucleotides might also act as non-specific inhibitors of protein synthesis at high concentrations, and the difference between sense and antisense oligonucleotides might become apparent only at low concentrations. We

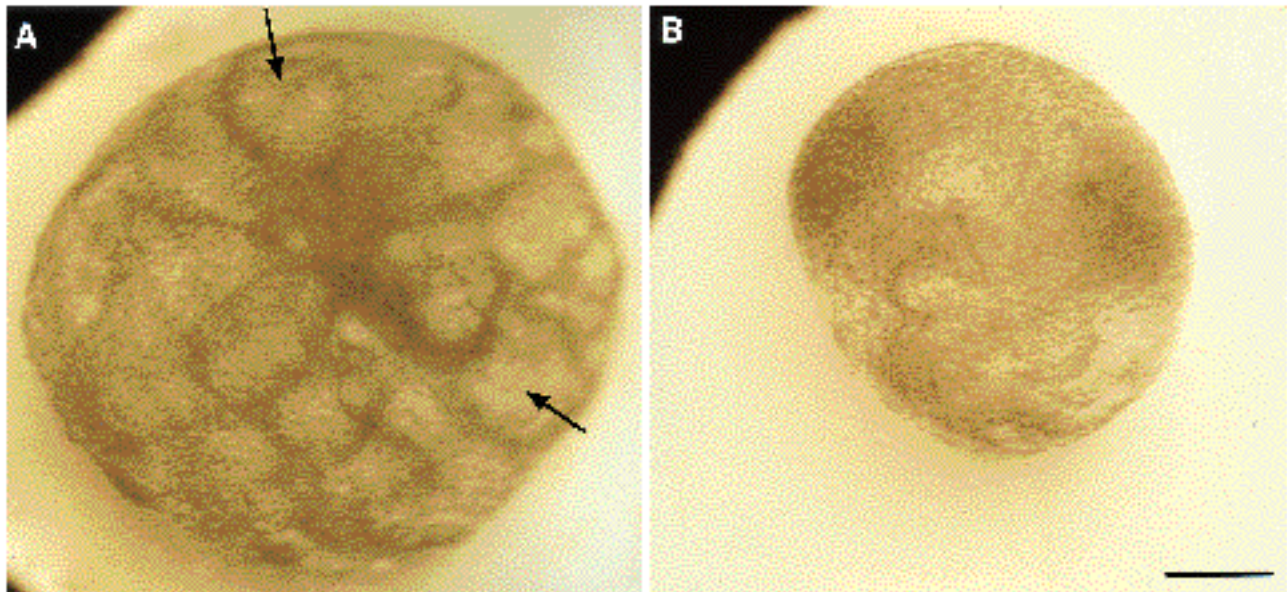


Fig. 10. Branching morphogenesis in the presence of antisense phosphorothioate oligonucleotides corresponding to rat immunoglobulin (lambda chain, constant region) mRNA. 13-day-old rat kidneys cultured in the absence of phosphorothioate oligonucleotides develop normally (A) whereas the tubular branching of the kidneys cultured in the presence of antisense immunoglobulin phosphorothioate oligonucleotides is strongly inhibited (B). Arrows denote condensates. Bar, 56 μ m.

therefore carefully compared the effect of various concentrations of sense and antisense oligonucleotides to the c-ros gene. In our hands, both the antisense and sense oligonucleotides were inhibitory over the same concentration range; 1 μ M (antisense) ros did not affect kidney development, 3 μ M (antisense, sense) was partially inhibitory and 5 μ M or 10 μ M (antisense, sense, scrambled) were clearly inhibitory.

The unspecific nature of the inhibition is further supported by the studies of the uptake of the antisense c-ros and low-affinity NGFR oligonucleotides. Fluorescent oligonucleotides entered only the mesenchymal cells and not the epithelial cells. The mRNA for c-ros is exclusively expressed by the tip of the epithelial ureter (Sonnenberg et al., 1991). The antisense c-ros oligonucleotides thus do not reach the target mRNA and yet sense and antisense oligonucleotides for c-ros inhibited kidney development. The fact that phosphorothioate oligonucleotides cause non-specific toxicity in kidney organ culture is also shown by the experiments with rat embryonic kidney incubated with antisense oligonucleotides for immunoglobulin, which also inhibited tubular branching. Toxic effects of the oligonucleotides are also suggested by the findings that all tested sense, antisense and other oligonucleotides used in the current study caused a similar type of profound inhibition of kidney development and growth.

Antisense oligonucleotides have recently become popular tools to study the role of specific genes in organ development. Yet, the usefulness of this approach is far from proven in all cases and the lack of proper controls is sometimes evident. Our current study suggests that toxic effects can be a serious problem and the validity of the methodology can be questioned. The nature of the toxic effects are unclear at the moment but it has recently been described that oligonucleotides in *Xenopus* oocytes cause cleavage of not only the targeted RNA but also of many nontargeted RNAs (Woolf

et al., 1992). It is therefore possible that many of the currently available oligonucleotides can not be used to study the function of specific genes in *Xenopus* oocytes, and our current study suggests that they are not useful tools for the study of rodent kidney development in organ culture.

It is possible that the embryonic kidney is particularly susceptible to toxic effects, and we cannot exclude that the oligonucleotide approach can give meaningful results when used on other embryonic organs. Indeed, in preliminary experiments, we have noted that the embryonic lung is more resistant to toxic effects of the oligonucleotides used in this study. While this does not prove that the probes inhibit specific target mRNAs in the lung, it shows that the toxic levels of the probes may differ from tissue to tissue. The toxicity of oligonucleotides might differ depending on the chemical modification and we are currently investigating this issue in more detail. A number of studies have suggested that antisense oligonucleotides can be used to study function not only in cell cultures but also in organotypic cultures or in vivo (Represa et al., 1991; Simons et al., 1992; Diekwisch et al., 1993). In most cases, control studies with sense oligonucleotides have been performed. Our study suggests that more rigorous controls should be used in addition. We note that the antisense approach in some cases leads to different results than null mutations introduced into the gene of interest (Represa et al., 1991; Sariola et al., 1991; Lee et al., 1992; Mansour et al., 1993). This discrepancy could be due to the unspecific effects of oligonucleotides in culture. In complex tissues containing epithelial cells, it is important to verify that the probes reach the cells expressing the target mRNA, and a large number of unrelated oligonucleotides should be used to show that the inhibition indeed is specific. Moreover, the study of Woolf et al. (1992) suggest that it might be useful to monitor whether nontargeted mRNAs are

also degraded. We conclude that studies with oligonucleotides should be interpreted with caution unless such control experiments have been performed. There is no doubt that antisense oligonucleotides can be extremely useful tools to study the role of specific mRNAs in cultured cells, and new modifications of oligonucleotides might also be less toxic and hence useful in complex organ cultures.

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