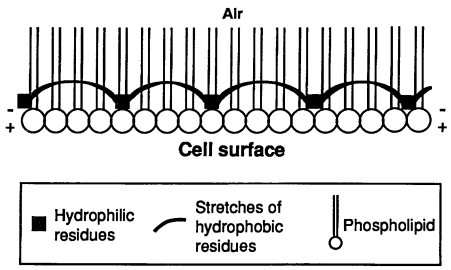

Fig. 5. Two-dimensional theoretical representation of the mechanism of function of SP-B protein in the PL monolayer. The hydrophilic, charged residues interact with polar head groups of the PL, whereas the hydrophobic stretches of the peptide interact with the acyl side chains. These interacting forces induce stabilization of the PL layer, providing greater capacity to resist surface tension or alveolar collapse.

spectroscopic data, published separately (*11*), showing that RL_4 and SP-B peptide 59-81 complex with PL to form a denser head group packing order than that demonstrated by PL alone and provide increases in intermolecular and intrachain order of the PLs. These data are consistent with the observations of Baatz *et al.* (*12*) showing that bovine SP-B induces an increased ordering of the polar surface of DPPC-DPPG membrane bilayers.

The data support a concept that electrostatic charges of the intermittent hydrophilic residues of SP-B interact with the PL polar head groups and that stretches of hydrophobic residues interact with acyl side chains of the PLs to induce increased lateral intermolecular order. The electrostatic interactions are essential for surfactant function. These relationships are depicted in Fig. 5. The combination of these simplified peptides and DPPC-POPG provide excellent surfactant in 27-day-old fetal rabbits and 130-day-old fetal rhesus monkeys (*13*). The simplified peptide surfactant could serve as an excellent material for replacement therapy in respiratory distress syndromes.

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16. Synthetic peptides in chloroform-methanol (generally 50% chloroform and 50% methanol) were added to mixtures of DPPC and PG (3:1) in glass tubes. Relative to the total amount of PL, the peptide was 1%, 3%, or 5% by weight as noted. After a 10-min incubation at 43°C, the samples were dried under N_2 and held for 15 min under vacuum. Water was added, and the tubes were incubated at 43°C with periodic vortexing for 1 hour. Small volumes (usually 10% of final volume) of concentrated NaCl solutions were added to yield a final salt concentration of 0.9%. The final concentration of PL was 10 mg/ml for studies performed in the pulsating bubble surfactometer and 20 mg/ml for fetal rabbit studies. Vesicle preparations used for tryptophan fluorescence studies differ slightly; the dried lipid-peptide mixtures were rehydrated directly in saline (0.9% NaCl), and the final peptide concentration was held constant at 50 μM with a peptide to lipid ratio of 1:20, resulting in PL concentrations of 2.6 to 3.2 mg/ml.
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28 May 1991; accepted 20 August 1991

Phosphorylation of c-Src on Tyrosine 527 by Another Protein Tyrosine Kinase

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The protein tyrosine kinase activity of the cellular Src protein is negatively regulated by phosphorylation at tyrosine residue 527 (Tyr^{527}). It has not been established whether this regulatory modification of Src is mediated by autophosphorylation or by another cellular protein kinase. The phosphorylation of a modified form of c-Src that lacks kinase activity was examined in mouse cells that do not express endogenous Src (because of the targeted disruption of both *src* alleles). Phosphorylation of the inactive form of Src on Tyr^{527} occurred to a similar extent in cells lacking endogenous Src as it did in cells expressing Src. Therefore, Tyr^{527} phosphorylation, and thus negative control of Src kinase activity, is mediated by another cellular protein tyrosine kinase.

THE C-*SRC* PROTEIN IS A 60-KD CELLULAR protein tyrosine kinase that has served as the prototype member of a family of cytoplasmic protein tyrosine kinases. The c-*src* protein is the cellular homolog of the v-*src* oncogene product encoded by the Rous sarcoma virus. The specific activity of c-Src is 4 to 50 times lower than that of v-Src and other oncogenic variants of Src (*1, 2*). Wild-type c-Src is phosphorylated on Tyr^{527} (*3*), whereas oncogenic, activated variants of c-Src show severely reduced or undetectable phosphorylation at this site (*2, 4, 5*). The kinase activity of c-Src appears to be negatively regulated by phosphorylation at Tyr^{527}. Dephosphorylation at this site causes a 10- to 20-fold enhancement of catalytic activity (*6, 7*), and amino acid substitutions at residue 527 cause an increase in kinase activity and an activation of the transforming potential of c-Src (*8*). Lastly, the activated form of c-Src that is associated with middle T antigen of polyoma virus is not phosphorylated on Tyr^{527} (*9*).

It is not clear whether Tyr^{527} is phosphorylated by an autophosphorylation event or by another tyrosine kinase. Partially purified c-Src is inefficiently phosphorylated on Tyr^{527} in vitro (even after removal of the endogenous phosphate from Tyr^{527}), indicating that Tyr^{527} may be phosphorylated by another cellular enzyme (*7, 10*). Modified forms of c-Src lacking kinase activity were

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phosphorylated on Tyr^{527} to the same extent as was wild-type c-Src (*11, 12*). However, because endogenous c-Src was expressed in the cells used in these experiments, the possibility remained that the endogenous active enzyme could be responsible for intermolecular phosphorylation of inactive exogenous c-Src.

Chicken c-Src has also been expressed in lower eukaryotic cells so that the phosphorylation of c-Src could be investigated in phyletically distant cells that do not express a Tyr^{527} kinase. The results were equivocal: wild-type chicken c-Src expressed in yeast cells was phosphorylated at Tyr^{527}, albeit to a limited extent relative to the amount of phosphorylation in avian cells (*13, 14*). The low stoichiometry of phosphorylation at this site [5 to 10% (*13*), compared to >90% in avian cells (*3*)] could be interpreted as evidence that another kinase is responsible for the majority of phosphate incorporation at Tyr^{527} in avian cells or that yeast cells do not express factors that facilitate autophosphorylation in avian cells.

To examine the mechanism of Tyr^{527} phosphorylation in vertebrate cells without the interference by endogenous Src, we established cell lines from mouse embryo fibroblasts lacking endogenous Src (*15*). Cells from such embryos, 1 and 6, and a normal embryo, 8, were subsequently immortalized at passage three by infection with a recombinant retrovirus encoding the large T antigen of SV40 (*16*). After passage 17, these cell lines (designated 1T, 6T, and 8T) were tested for expression of c-Src. The c-Src protein immunoprecipitated with monoclonal antibody to Src (anti-Src 327) undergoes autophosphorylation at Tyr^{416} in the presence of adenosine triphosphate (ATP) and Mg^{2+} (*10, 17*). Such autophosphorylation of Src was not detected in cells derived from either clone 1T or clone 6T (Fig. 1A) but was found in immunoprecipitates from clone 8T, which expresses c-Src. This result confirmed that there was no c-Src activity in the 1T and 6T cell lines (*18*).

The 1T, 6T, and 8T cell lines were infected with a murine retrovirus encoding a chicken c-Src variant that contains an arginine substitution for Lys^{295}. This substitution inactivates the catalytic activity of c-Src by altering the ATP binding site (*12, 19, 20*). Assays of in vitro autophosphorylation were performed after immunoprecipitation with an antibody to Src (anti-Src EC10) that exclusively recognizes the avian form of Src (*21*). No autophosphorylation of Src was detected in the Src-EC10 immunoprecipitates from lysates of any of the cells expressing the c-SrcK295R variant (Fig. 1B). As positive controls, the lysates were incubated with anti-Src 327 or the polyclonal antiserum, SrcII, which recognizes the COOH-terminal sequences of the Src, Fyn, and Yes protein tyrosine kinases (*22*). The 60-kD band immunoprecipitated with SrcII antiserum showed diminished activity in the 1T and 6T cell lysates, consistent with the loss of Src activity and with the detection of residual Fyn. (p62^{Yes} was not detected in these cells with this antiserum.) These assays confirmed the absence of endogenous Src in these infected cells and verified the lack of activity of the exogenous c-SrcK295R protein.

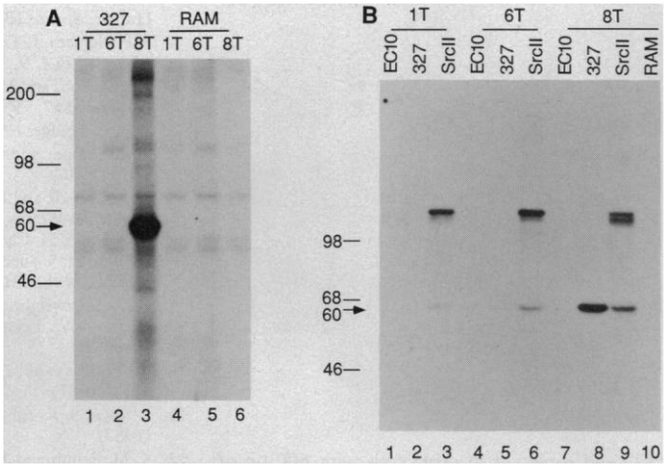

Fig. 1. (**A**) Analysis of c-Src kinase activity in Src cells containing or lacking c-Src. Cells from clones lacking Src, 1T (lanes 1 and 4) and 6T (lanes 2 and 5), and from the clone containing endogenous Src, 8T (lanes 3 and 6), were lysed and immunoprecipitated with anti-Src 327 (lanes 1 to 3) or with antibody to mouse immunoglobulin G (IgG) bound to formalin-fixed *Staphylococcus aureus* (Pansorbin, Calbiochem, La Jolla, California) (lanes 4 to 6). Primary embryonic mouse fibroblasts were isolated from E18 stage embryos as described (*26*). Animal care was provided in accordance with Baylor College of Medicine guidelines. Genotypes were determined from DNA isolated from fetal liver using the Src probe A (*15*). After two passages, cells were infected at multiplicity of 0.1 with ZipTex, a retroviral vector transducing the SV40 large T antigen under the control of the Moloney murine leukemia virus long terminal repeat (*16*). Immortalized cells were established after multiple passages and maintained by passage at 1:20 dilution every 3 days. Cell cultures at 90 to 100% confluency were lysed in radioimmunoprecipitation assay (RIPA) buffer (*17*), and c-Src was immunoprecipitated from 750 μg of protein extract with 0.1 μg of anti-Src 327 (from ascites fluid) and 1 μg of antibody to mouse IgG from rabbit (RAM) (Cappel, Westchester, Pennsylvania) bound to Pansorbin (*17*). In vitro kinase reactions were performed after immunoprecipitations by resuspension of the c-Src bound to Pansorbin in reaction mix {5 μCi [γ-^{32}P]ATP (New England Nuclear, 5000 Ci/mmol), 10 mM tris (pH 7.5), 5 mM $MnCl_2$, 1 μM ATP} for 15 min at 4°C, as described (*17*). The reaction products were subjected to electrophoresis on polyacrylamide gels (7.5%). The gel was dried and then exposed at −70°C to XAR-5 film (Kodak) and enhanced with DuPont Cronex Lightning-Plus intensifying screens for 4 days. (**B**) Analysis of chicken c-SrcK295R in vitro kinase activity from infected 1T, 6T, and 8T cells. Cells (10^6) were infected with 1 ml of a stock solution of murine retrovirus, LNCX c-SrcK295R (*27*). RIPA lysates (750 μg of protein) from infected 1T (lanes 1 to 3), 6T (lanes 4 to 6), and 8T (lanes 7 to 10) cells were immunoprecipitated with 1 μl of 1:100 dilution of ascites fluid containing the antibody specific to Src from chicken (EC10) and 1 μg of RAM (lanes 1, 4, and 7), 0.1 μg of anti-Src 327 and 1 μg of RAM (lanes 2, 5, and 8), 5 μl of polyclonal rabbit antiserum SrcII (lanes 3, 6, and 9), or 1 μg of RAM alone (lane 10). These immunoprecipitates were resuspended in the in vitro kinase reaction mix as described above and separated by a polyacrylamide gel electrophoresis (7.5% gel). The dried gel was exposed for 20 hours at −70°C. The mobility of molecular size markers (in kilodaltons) are noted on the left of each figure. The identity of the 110-kD protein in lanes 3, 6, and 9 is unknown, but its abundance does not correlate with the amount of Src expression.

1T, 6T, and 8T cells infected with the retrovirus encoding c-SrcK295R were metabolically labeled with ^{32}P-labeled orthophosphate, and the avian c-SrcK295R protein was immunoprecipitated from cell lysates with anti-Src EC10 (Fig. 2A). The 60-kD c-SrcK295R protein was precipitated from the infected cultures and was not detected in the uninfected cultures. The 60-kD bands containing c-SrcK295R labeled in vivo were excised from the gel and subjected to partial proteolytic digestion with staphylococcal V8 protease (Fig. 2B). Three fragments were detected: V2, a 26-kD fragment that contains Tyr^{527} and Tyr^{416} [the site of both in vitro phosphorylation (*10*) and the site of in vivo phosphorylation in oncogenic variants of Src (*2, 4, 5*)], and V3 and V4, 18-kD and 16-kD fragments that contain Ser^{17}, the site of phosphorylation by adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (*23*). In cells that contain endogenous c-Src, c-SrcK295R is phosphorylated on Tyr^{527} (*11, 12*). In this experiment, the V2 fragment from c-SrcK295R was labeled with ^{32}P in both cell lines that lack endogenous c-Src. The ratios of ^{32}P labeling of V3 and V4 relative to V2 was almost identical in cells expressing and lacking c-Src. This result indicates that the catalytically inactive form of c-Src is phosphorylated on a site within the COOH-terminal fragment of this protein.

To examine whether the V2 fragment was phosphorylated on Tyr^{527}, Tyr^{416}, or another site, ^{32}P-labeled c-SrcK295R immu-

noprecipitated from cell lysates was cleaved with cyanogen bromide (Fig. 3). Tyr^{527} is contained within a 4-kD fragment, Tyr^{416} within a 10-kD fragment, and Ser^{17} within a 31-kD peptide (*12*). Wild-type c-Src was radiolabeled in vitro to provide a marker for the 10-kD peptide containing Tyr^{416}. The c-SrcK295R from cells both lacking and containing endogenous Src displayed similar amounts of ^{32}P incorporation into the 4-kD fragment containing Tyr^{527}, indicating that both proteins were phosphorylated on Tyr^{527}.

Phosphorylation of Tyr^{527} was not detectably diminished under conditions where intra- and intermolecular autophosphorylation could not occur. Intramolecular autophosphorylation by wild-type c-Src may account for some of the phosphorylation at Tyr^{527} in vivo; however, the data indicate that another cellular tyrosine kinase is responsible for the majority, if not all, of the in vivo phosphorylation at this site.

There is a threefold reduction in Tyr^{527} phosphorylation of inactive c-Src molecules during mitosis (relative to phosphorylation of wild-type Src during mitosis) (*20*). One interpretation of these data is that intramolecular autophosphorylation may be responsible for Tyr^{527} phosphorylation. However, our data indicate that if intramolecular autophosphorylation is responsible for Tyr^{527} phosphorylation, the involvement of this mechanism must be restricted to mitotic cells. Alternate explanations for the reduced phosphorylation of inactive Src from mitotic cells, such as inaccessibility to a Tyr^{527} kinase or increased susceptibility to Tyr^{527} phosphatase, are tenable.

Identification of the Tyr^{527} kinase is now critical for elucidation of the mechanisms involved in this regulatory modification of Src. Other members of the Src family of tyrosine kinases may phosphorylate Tyr^{527} on c-Src, or, alternatively, another cellular tyrosine kinase may be responsible for this activity. A candidate Tyr^{527} kinase isolated from brain extracts phosphorylates c-Src at this site in vitro (*24*). The gene encoding this kinase is homologous to the Src-family kinases, but it lacks tyrosine residues at positions analogous to Tyr^{527} and Tyr^{416} (*25*). Whether this enzyme functions in vivo to regulate c-Src through Tyr^{527} phosphorylation remains to be determined. Because each of the kinases in the Src family contain a Tyr residue in a position analogous to that of Tyr^{527}, it is possible that a Tyr^{527} kinase could regulate the activity of this family of kinases.

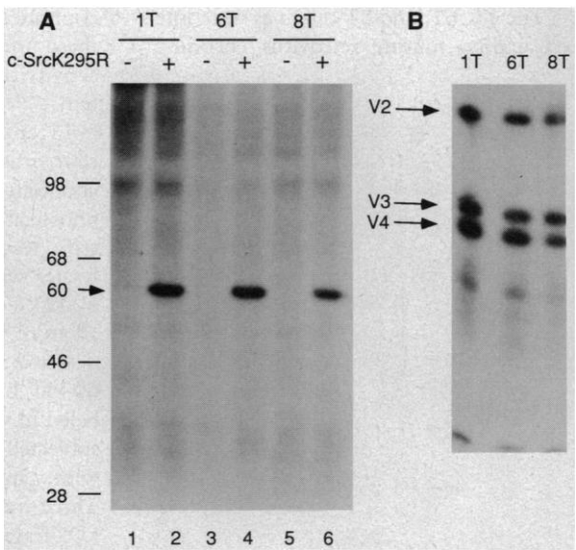

Fig. 2. Analysis of phosphopeptides after partial proteolysis of Src proteins from 1T, 6T, and 8T cells infected with LNCXc-SrcK295R. (**A**) Confluent cultures of LNCXc-SrcK295R–infected cells were starved in serum-free and phosphate-free Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, Maryland) for 40 min and then labeled for 3.5 hours with carrier-free [^{32}P]orthophosphate (1 mCi/ml) in phosphate-free DMEM. Cells were lysed in RIPA buffer and Src proteins were immunoprecipitated with anti-Src EC10 and RAM and separated on polyacrylamide gels (7.5%), as described in Fig. 1B. Lanes 1, 3, and 5 correspond to uninfected clones 1T, 6T, and 8T, respectively. Lanes 2, 4, and 6 correspond to LNCXc-SrcK295R–infected clones 1T, 6T, and 8T, respectively. (**B**) The band containing c-SrcK295R was excised from the gel in (A) and subjected to proteolysis with 600 ng of staphylococcal V8 protease (*12*) during electrophoresis on a polyacrylamide gel (12.5%). The dried gel was exposed for autoradiography for 3 days. The ratios of V2 to V3 and V4 were quantitated by direct scanning of radioactivity with a Molecular Dynamics Phosphorimager: 1T, 0.41; 6T, 0.38; and 8T, 0.38.

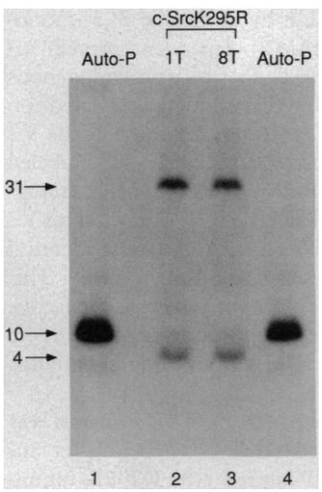

Fig. 3. Phosphopeptide analysis of SrcK295R after digestion with cyanogen bromide. Confluent cultures (100-mm plates) of 1T and 8T LNCX-SrcK295R–infected cells were labeled with [^{32}P]orthophosphate (1 mCi/ml). The c-SrcK295R proteins were immunoprecipitated with anti-Src EC10, and RAM, separated on a polyacrylamide gel (7.5%) as described in Fig. 1B, eluted from the gel, cleaved with cyanogen bromide, and separated on a polyacrylamide gel (27.5%) as described (*12*). Lanes 2 and 3 correspond to cyanogen bromide–cleaved c-SrcK295R from 1T and 8T cells, respectively. Lanes 1 and 4 contain c-Src labeled in vitro and immunoprecipitated from extracts of NIH 3T3 cells infected with a murine retrovirus encoding the avian c-Src cDNA. The autoradiograph was exposed for 6 days. Molecular size markers are in kilodaltons.

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28. We thank D. Livingston for the ZipTex virus; D. Shalloway for the pcR295 plasmid; J. Schmidt for construction of LNCXc-SrcK295R; B. Meyers and S. Thomas for preparation of virus stock; S. Parsons for the EC10 antibody; J. Bolen for SrcII antiserum and antiserum to Fyn, Lyn, Yes, Hck, Fgr, Blk, and Lck; and S. Thomas, C. Seidel-Dugan, and J. Bolen for critical evaluation of the manuscript. J.E.T. is supported by a fellowship from the NIH and P.S. is a Pew Scholar in the Biomedical Sciences. Supported by the National Cancer Institute (J.S.B.) and the National Institutes of Child Health and Human Development (P.S.).

26 April 1991; accepted 9 July 1991

Dependence of Kidney Morphogenesis on the Expression of Nerve Growth Factor Receptor

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Nerve growth factor receptor (NGFR) serves as the binding site for the neurotrophic growth factors. Although NGFR has been found in several embryonic tissues outside the nervous system, the function of NGFR in embryogenesis of non-neuronal organs remains unknown. NGFR is transiently synthesized by embryonic rat kidney and disappears from nephrons upon their terminal differentiation. Anti-sense oligonucleotide inhibition of NGFR expression inhibits kidney morphogenesis. Therefore, NGFR is required not only for development of the nervous system, but also for differentiation of the kidney tubules.

A FAMILY OF NEUROTROPHIC GROWTH factors, including nerve growth factor β (βNGF), brain-derived neurotrophic factor, and neurotrophin 3 (hippocampus-derived neurotrophic factor), is likely important for development and maintenance of various sets of neurons (*1*). It is possible that all of these factors bind to the same nerve growth factor receptor (NGFR) (*2*). NGFR is expressed on some neuronal and glial cells (*3*), as well as in several adult and embryonic tissues (*4–8*), including glomeruli of the permanent or metanephric kidney (*4, 8*). The function of NGFR in the development of these non-neuronal organs remains unknown.

We have investigated the expression and effect of NGFR in the developing kidney. The metanephric kidney is formed by the interaction between ureter bud epithelium and the surrounding nephrogenic mesenchyme. The ureter bud induces nephrogenic mesenchyme to form the secretory part of the nephrons (*9, 10*). Reciprocally, nephrogenic mesenchyme causes the ureter bud to branch, thereby forming collecting tubules. The ureter bud can be microsurgically separated from mesenchyme and replaced by several neural or non-neural embryonic tissues that effectively trigger differentiation of kidney tubules (*10, 11*). Thus, the unknown morphogens of kidney tubulogenesis are widespread among embryonic tissues.

By in situ hybridization with a ^{35}S-labeled rat NGFR riboprobe, NGFR mRNA was first detected in the developing rat kidney at embryonic day 13 in cell condensates that later form nephrons (Fig. 1A). Expression was abundant in the embryonic nephrons (so called S-shaped bodies, Fig. 1, B and C) and ceased a few days after birth upon terminal differentiation of nephrons. A similar pattern of NGFR expression was also revealed by indirect immunoperoxidase staining with monoclonal antibodies (MAbs) to rat NGFR (Fig. 1, D and E), as well as by iodinated-βNGF binding and subsequent autoradiography (Fig. 1, F and G). In the adult kidney, NGFR expression is limited to specific non-epithelial cell types such as mesangial cells of the glomerulus, nerve fibers, and the adventitia of blood vessels (*4*). Expression of NGFR in the nephrons was transient and began during induction of the nephron.

Two classes of NGFR have been character-

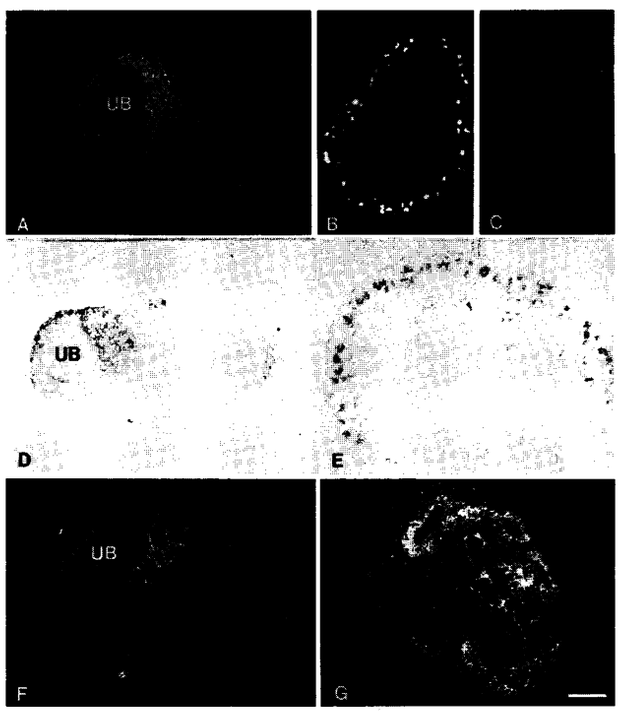

Fig. 1. Expression of NGFR in embryonic rat kidneys. (**A** and **B**) In situ hybridization (*20*) with the ^{35}S-labeled antisense riboprobe to NGFR mRNA. At day 13 (A), nephrogenesis is initiated around the ureter bud (UB) and the mesenchyme condenses to form the first nephrons. The grains are clustered in the condensing mesenchyme. At day 18 (B), nephrogenesis continues only in the outer-most cortex of the embryonic kidney. Each cluster of grains represents an embryonic nephron. (**C**) In situ hybridization with the ^{35}S-labeled sense riboprobe to NGFR mRNA shows no binding to 18-day embryonic kidney. Immunoperoxidase staining with MAb to NGFR on (**D**) day 13 and (**E**) day 19. Binding of iodinated βNGF (*21*) on (**F**) day 13 and (**G**) day 14. Kidneys incubated in 1000-fold excess cold βNGF did not reveal specific binding. Bar represents 60 μm in (A), (D), and (F); 300 μm in (B) and (C); 700 μm in (E); 120 μm in (H).

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