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Pax-2 is required for mesenchyme-to-epithelium conversion during kidney development

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SUMMARY

The conversion of mesenchyme to epithelium during the embryonic development of the mammalian kidney requires reciprocal inductive interactions between the ureter and the responding metanephric mesenchyme. The *Pax-2* gene is activated in the mesenchyme in response to induction and is subsequently down-regulated in more differentiated cells derived from the mesenchyme. *Pax-2* belongs to a family of genes, at least three of which encode morphogenetic regulatory transcription factors. In order to determine the role of *Pax-2* during kidney development, we have generated a loss-of-function phenotype using antisense oligonucleotides in mouse kidney organ cultures. These oligonucleotides can specifically inhibit Pax-2 protein accumulation in kidney mesenchyme cells, where the intracellular concentrations are maximal. The kidney organ cultures were stained with uvomurulin and laminin antibodies as markers for epithelium formation. With significantly reduced *Pax-2* protein levels, kidney mesenchyme cells fail to aggregate and do not undergo the sequential morphological changes characteristic of epithelial cell formation. The data demonstrate that *Pax-2* function is required for the earliest phase of mesenchyme-to-epithelium conversion.

Key words: Pax-2, kidney development, antisense

INTRODUCTION

The interaction between mesenchyme and epithelium is a fundamental process during embryogenesis that induces the development of a variety of organs including kidney, lung, salivary glands and mammary glands. The adult kidney, or metanephros, develops from the ureteric bud, an outgrowth from the posterior Wolffian duct, and the metanephric mesenchyme (for review see Saxen, 1987). Upon induction by the ureteric bud, the mesenchymal cells aggregate and are converted to epithelium thus undergoing a series of morphological changes that generate the glomerular, proximal tubular and distal tubular epithelium (Herzlinger et al., 1992). The mesenchyme, in turn, induces the ureteric bud to branch repeatedly thus generating the collecting ducts. These inductive interactions between kidney mesenchyme and ureter can be studied in vitro (Grobstein 1953, 1956) and the subsequent changes in cell-type-specific gene expression have been well characterized, particularly with respect to extracellular matrix and epithelium basement membrane components (for review see Ekblom, 1989). However, the nature of the inductive signals and the initial genetic responses remain to be determined.

Pax-2 belongs to a family of morphogenetic regulatory genes, initially identified by homology to *Drosophila* segmentation genes (Walther et al., 1991), that encode DNA binding transcription factors. Some members of the paired box *(Pax)*-containing gene family encode developmental regulatory proteins that have been associated with a variety of known mutations including the mouse *undulated* gene (Balling et al., 1988), the mouse *splotch* (Epstein et al., 1991) and human Waardenburg syndrome (Baldwin et al., 1992; Tassabehji et al., 1992), and the mouse *small eye* (Hill et al., 1991) and human aniridia genes (Ton et al., 1991). The *Pax-2* gene is activated in the metanephric mesenchyme after induction, continues to be expressed in the early epithelial derivatives of the mesenchyme, such as the comma and S-shaped bodies, and is then rapidly turned off as the tubular epithelium matures (Dressler et al., 1990; Dressler and Douglass, 1992). *Pax-2* is not expressed in the metanephric mesenchyme in the absence of induction, as experiments with *Danforth's short tail* mice have demonstrated (Phelps and Dressler, 1993). In addition, failure to repress *Pax-2* expression during maturation of the renal tubules results in developmental abnormalitites in transgenic mice that are similar to human nephrotic syndromes (Dressler et al., 1993).

Given the transient nature of *Pax-2* expression during kidney development and the regulatory potential of the *Pax* genes, it is proposed that *Pax-2* is a determining factor for the conversion and differentiation of the kidney mesenchyme. In order to test this hypothesis, loss of function was generated in vitro by using antisense oligonucleotides directed against *Pax-2*. Phosphorothioate-substituted oligonucleotides against two different sequences of the *Pax-2* mRNA significantly reduced *Pax-2* protein levels in

embryonic kidney cultures and inhibited the formation of epithelium derived from the mesenchyme. The inhibition of *Pax-2* protein accumulation correlates directly with the intracellular oligonucleotide concentration of the mesenchymal cells. The data demonstrate that *Pax-2* is required for the differentiation of kidney mesenchyme to epithelium.

MATERIALS AND METHODS

Organ culture

Embryonic metanephric kidney rudiments from FVB/N or C57/B6/J (natural matings or superovulated) mice were obtained by microsurgical dissection of E11.5 embryos and were placed onto Nuclepore filters in a humid chamber as described (Saxen et al., 1968) and cultured for 48 hours or 96 hours. Early to mid 13-day embryonic submandibular and sublingual glands were microdissected from FVB/N mice and cultured on a Nuclepore filter in a humid chamber for 48 hours. For both kidneys and salivary glands, the medium was Dulbeccos modified eagle medium supplemented with 10% fetal calf serum, 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. All cultures were grown at 37°C with 5% CO_2.

Phosphorothioate oligonucleotides

The phosphorothioate-substituted 19-mer oligonucleotide were purchased from Oligos etc. Inc. (Oregon, USA) and HPLC purified by the manufacturer. The sequences were as follows:

AS14 (antisense) 5′- CAT CGG GAG GCA GAG GAG C -3′,
S14 (sense) 5′- GCT CCT CTG CCT CCC GAT G -3′
SCR14 (antisense scrambled) 5′- GCG AGG ACG GCG TAG AGA C -3′.
AS17 (antisense) 5′- GGG AGG CCG TGC TGG GAA C -5′,
S17 (sense) 5′- GTT CCC AGC ACG GCC TCC C-3′.
Fluorescein-labelled oligo.5′- CGA GGA GAC SGG AGG GCT AC -3′

The AS14 and S14 sequences correspond to nucleotides 309-327 in the immediate vicinity of the translation start site of *Pax-2*, as described previously (Dressler et al., 1990). The AS17 and S17 oligonucleotidesequnces correspond to nucleotides 817-835 of the *Pax-2* gene and are located between the paired domain and the paired octapeptide (Dressler et al., 1990). AS16 and S16 oligonucleotidesequences corrsepond to nucleotides 713-731 and are located in the coding region of the paired domain of *Pax-2*. Synthesis of the fluoroscein-labelled, phosphorothioate oligonucleotide was performed on an Applied Biosystems DNA-Syntheziser and the region where the fluorescein molecule is attached to the oligonucleotide sequence is underlined (fluorescein amidite, sulfurizing reagent; Glen Research, VA).

Antibodies

The production and specificity of the *Pax-2* antibodies has been described (Dressler and Douglass, 1992; Pueschel et al., 1992).The anti-laminin antiserum, which reacts with all three laminin chains A, B1 and B2, was kindly provided by H. Kleinman.

Immunohistochemistry

Filters with mouse metanephric kidney rudiments were placed in plastic molds, covered with Tissue Tek (Miles Inc.) and frozen by floating on liquid nitrogen. Cryostat sections (Leitz Kryostat 1720) were cut at 8 μm, collected on gelatinized slides and air dried for 60 minutes. Immunostaining was as described by Harlow and Lane (1988). Sections were fixed at room temperature in 3% paraformaldehyde for 10 minutes (*Pax-2* /uvomorulin detection), washed in PBS containing 0.1% Triton X-100 for 10 minutes and washed in PBS containing 0.05% Tween 20 (PBS/Tween) for 5 minutes. In the case of tissue used for laminin/uvomorulin detection, the fixation was at −20°C in methanol for 10 minutes according to Ekblom et al. (1990). 10 μg/ml of rabbit anti-*Pax-2* IgG in a 1:250 dilution of rat anti-uvomorulin (Sigma) was prepared in 2% goat serum in PBS. Slides were incubated with 8-10 μl of the first antibody for 45 minutes in a humid chamber at room temperature. They were washed twice in PBS/Tween for 5 minutes. The second antibodies were diluted 1:32 for the tetramethylrhodamine B isothiocyanate (TRITC)-conjugated goat anti-rabbit antibody (Sigma), 1:64 for the fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse antibody (Sigma) and 1:20 for the FITC-conjugated goat anti-rat antibody (Sigma) in 2% goat serum in PBS. After a 30 minutes incubation at room temperature in a humid chamber, slides were washed twice in PBS/Tween for 5 minutes and covered. Preimmune IgGs showed no specific reactivity.

RESULTS

Titration and specificity of phosphorothioate oligonucleotides

Embryonic kidney rudiments were excised at 11.5 days gestation, just after the ureteric bud had reached the metanephric mesenchyme and branched once symmetrically. These kidney rudiments can be grown in culture for up to 14 days while displaying good morphogenesis, advanced tubular structures and glomeruli without vascularization (Saxen, 1987). The kidney rudiments were cultured with two different antisense *Pax-2* oligonucleotides, one directed against the translation start site (AS14) and one within the coding region (AS17). As controls, two corresponding sense oligonucleotides (S14, S17) and a scrambled sequence of AS14 (SCR14) were used (see experimental procedures for sequences). These results are summarized in Table 1 and representative micrographs of whole kidney rudiments are shown in Fig. 1. Both AS14 and S14 were used over a concentration range of 2.5-5.0 μM and the degree of development was assayed as a function of ureter branching. Since it was not always possible to count precisely the number of ureter branches in a given culture, the kidneys were scored on the basis of 1-6, 7-10 and more than 10 ureter branches. These estimates were initially obtained by focusing through the organ rudiment under phase contrast and were later confirmed with sectioning and immunostaining. At concentrations of 2.5-5.0 μM, AS14 and AS17 significantly inhibited ureter branching and differentiation of the mesenchyme, whereas S14, S17 and SCR14 had no differentiation inhibitory effects. At 2.5 μM, 86% and 80% of the AS14- and AS17-treated kidneys had 6 or fewer branches whereas 97% and 100% of the respective sense controls had more than 10 branches. At 5.0 μM, 90% and 100% of the AS14-and AS17-treated kidneys had 6 or fewer branches, whereas 89% and 90% of the respective controls had 7 or more branches. The effect of the scrambled antisense sequence, SCR14, was similar to the sense strand controls. Thus, differences in nucleotide composition between sense and antisense oligonucleotides could not account for the specific effects seen. A mixture of AS14 and AS17 was equally effective in limiting development of the culture, whereas again the combined sense controls had no significant effect. Concentrations of 10 μM or more appeared to be toxic as the cultures degenerated non-specifically.

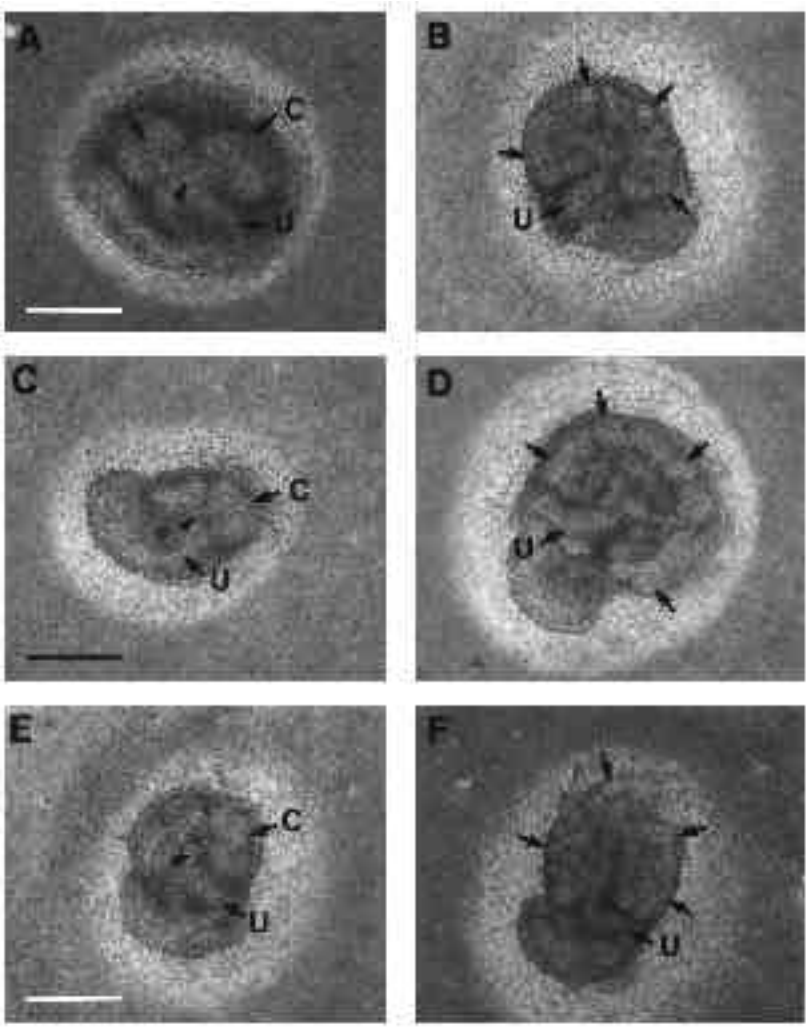

Fig. 1. Development of cultured metanephric rudiments. Phase-contrast microscopy of cultured kidney rudiments. In all panels, the ureter (u), the ureter branch point (arrowhead) and the condensed mesenchyme (C, arrows) are indicated. (A) Kidney cultured for 24 hours in media alone showing early stages of rudiment differentiation. (B) Kidney cultured for 48 hours in medium alone displays multiple branching of the ureter and accompanying mesenchymal aggregates surrounding the ureter tips. (C) Kidney rudiments cultured for 48 hours with AS17 (2.5 μM). Note similarity to control kidney cultures after 24 hours. (D) Kidney cultured for 48 hours with SCR 14 (2.5 μM). Note similarity to kidney cultured in medium alone, shown in B. (E) Kidney cultured for 48 hours with AS 14 (2.5 μM). Note similarity to A and C. (F) The same kidney as shown in E allowed to recover for 48 hours in medium alone. Note branching of ureter and multiple mesenchymal aggregates as observed in B and D. Bars represent 375 μm for all panels.

Table 1. Effect of phosphorothioate-substituted oligonucleotides on ureter branching in kidney rudiments cultured for 48 hours

			Number of kidneys		
Oligo	Concentration (μM)	Total kidneys	1-6	7-10	>10 branches
AS14	2.5	44	38	5	1
AS14	3.75	6	5	1	0
AS14	5.0	10	9	1	0
AS17	2.5	20	16	4	0
AS17	5.0	10	10	0	0
AS14/AS17	1.25*	15	12	2	1
AS14/AS17	2.5*	15	15	0	0
S14	2.5	29	1	3	25
S14	3.75	7	0	0	7
S14	5.0	9	1	5	3
SCR14	2.5	40	1	4	35
SCR14	5.0	6	1	2	3
S17	2.5	8	0	1	7
S17	5.0	10	1	2	7
S14/S17	1.25*	15	1	2	12
S14/S17	2.5*	6	1	2	3

*Concentration of each oligonucleotide.

Fig. 1 shows representative micrographs of whole-organ cultures incubated with the indicated oligonucleotides. At 24 hours postexplantation, the kidney cultures exhibit a symmetrically branched ureter surrounded by mesenchyme (Fig. 1A). After 48 hours in culture with medium alone (Fig. 1B) or the control oligonucleotides, S14, S17 or SCR14 (Fig. 1D), all kidney rudiments exhibited growth and differentiation as evidenced by the number of ureteric bifurcations (Table 1) and the condensations presented around the tips of the ureter branches (Fig. 1B,D). These cultures did not differ significantly from those grown in medium alone. The kidneys incubated with antisense oligonucleotides, AS17 (Fig. 1C), AS14 (Fig. 1E), or a mixture of both, remained essentially unchanged for the first 24-48 hours in culture and failed to exhibit multiple branched ureters and associated condensations to the same extend as controls. In fact, in AS14- and AS17-treated cultures, the demarcation between epithelium and mesenchyme becomes increasingly more difficult to distinguish by phase-contrast microscopy (Fig. 1C,E). Moreover, the effect of the antisense oligonucleotides is reversible. If the kidney is grown for 48h in the presence of AS14 (Fig. 1E) and subsequently allowed to recover for 48 hours in medium alone, the kidney cultures begin to develop normally again (Fig. 1F). Thus, the effect of the antisense oligonucleotides is not due to premature cell death.

As an additional control, a developing organ that did not express the *Pax-2* gene was cultured in the presence of the antisense and sense *Pax-2* oligonucleotides. For these experiments, salivary glands were chosen because they grow and differentiate well in culture and consist of epithelial and mesenchymal components (Takahashi and Nogawa, 1991). After 48 hours in culture, E13 embryonic submandibular salivary glands exhibited repeated cell proliferation and branching of lobules (Fig. 2); epithelial cleft formation was numerous and roughly 50 lobules derived from the submandibular and more than 10 lobules derived from the sublingual gland can be discerned. As shown in Fig. 2, the submandibular and sublingual salivary gland rudiments grow equally well with *Pax-2* antisense oligonucleotides as with medium alone. These data demonstrate a specific effect for the antisense *Pax-2* oligonucleotides in developing kidney organ cultures.

Immunostaining of kidney organ cultures

A detailed developmental profile and protein expression analysis was done by immunostaining frozen sections of embryonic kidneys cultured for 48 hours. Sections were stained for *Pax-2* and uvomorulin, an epithelium-specific adhesion molecule expressed in the ureter and in developing epithelial cells derived from the mesenchyme (Vestweber et al., 1985). Kidneys incubated in medium alone (Fig. 3A) or with control oligonucleotides (Fig. 3B), show similar patterns of *Pax-2* expression throughout the

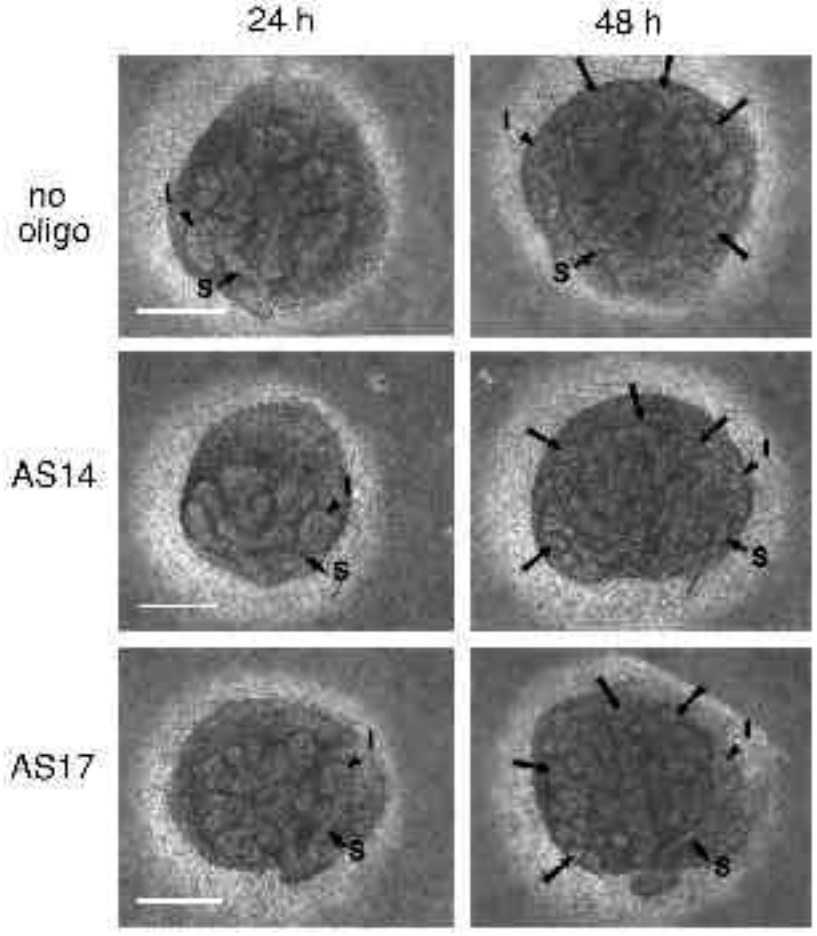

Fig. 2. The effect of *Pax-2* antisense oligonucleotides on mouse embryonic salivary gland development. Phase-contrast micrographs of E13 salivary gland rudiments cultured for 24 and 48 hours. Indicated are the sublingual stalk (s), the sublingual lobules (l) and the increasing number of submandibular lobules derived by branching morphogenesis (arrows). Salivary glands were cultured in media alone, AS14 (2.5 μM) or AS17 (2.5 μM) as indicated and identical cultures are shown after 24 and 48 hours. Note no apparent difference in the numbers of lobules observed in glands treated with either *Pax-2* antisense oligonucleotides (AS14, AS17) compared to the control culture (no oligo). Bars represent 375 μm in all panels.

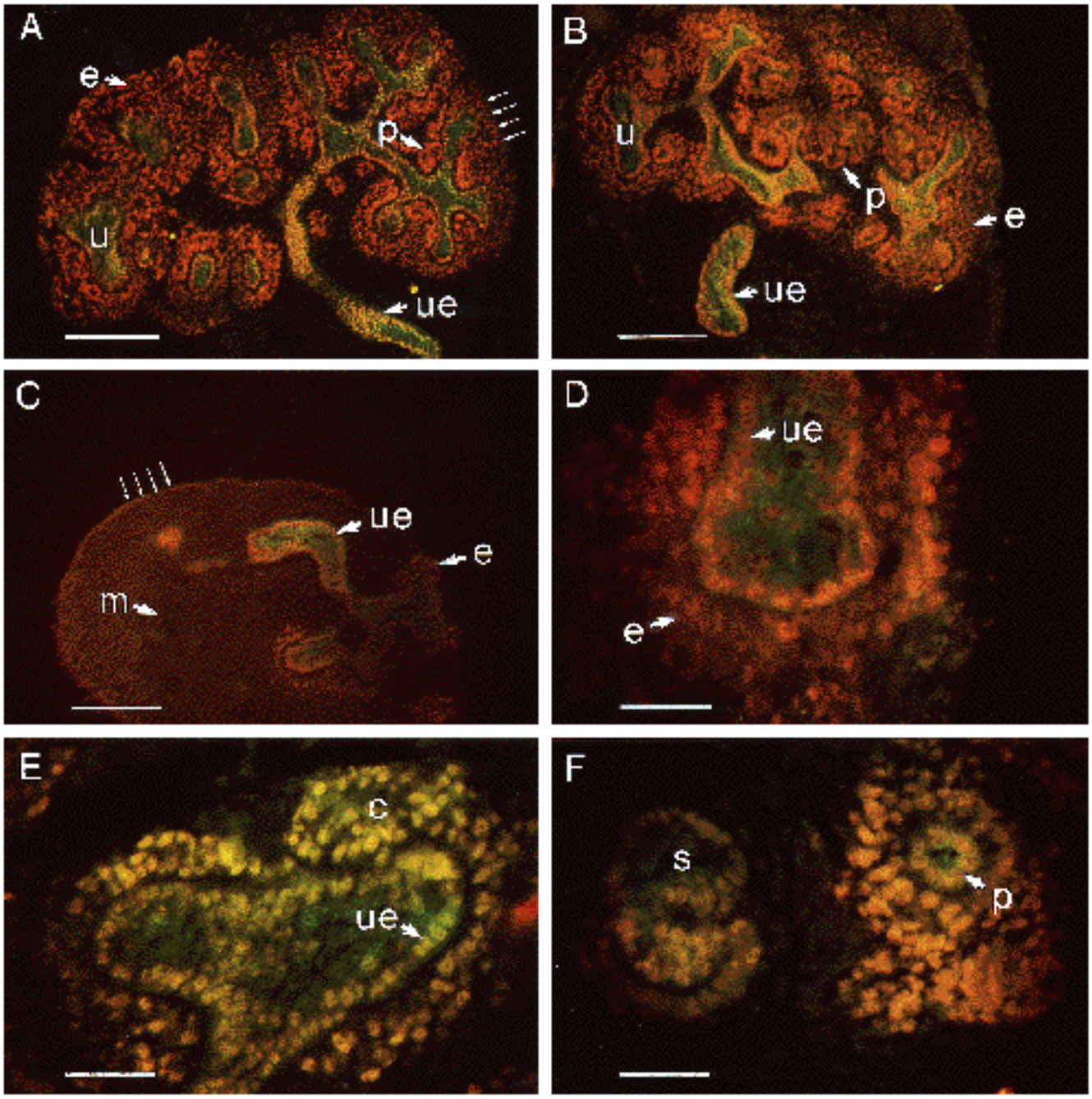

Fig. 3. *Pax-2* and uvomorulin expression in kidney rudiment cultures after 48 hours. For all panels, *Pax-2* staining is red/orange and uvomorulin is green. (A) In medium alone, kidney cultures express *Pax-2* in the ureteric epithelium (ue), the early mesenchymal condensations (e) surrounding the tips of the ureter branches, and in the pretubular condensations (p) located proximal to the older branches of the ureter (u)). Note the widespread expression of *Pax-2* throughout the culture and along the periphery (arrows) and the frequent ureter bifurcations, stained for uvomorulin. (B) Kidneys cultured with a mixture of the sense oligonucleotides S14/S17 (2.5 μM each), showed no significant morphological differences compared to A. (C) Kidneys cultured with a mixture of the antisense oligonucleotides AS14/AS17 (2.5 μM each), exhibit severly reduced ureter branching and *Pax-2* expression. *Pax-2* expression is still evident in the ureter epithelium (ue) and in some early mesenchymal condensations (e) near the ureter tips. Note that *Pax-2* is not expressed in the uninduced mesenchyme (m) throughout the kidney, nor near the periphery (arrows) and the absence of pretubular condensations, and comma- and S-shaped bodies. D) Higher magnification of a ureteric tip from a kidney cultured with the antisense oligonucleotide AS17 (2.5μM). *Pax-2* expression is present in the ureter epithelium (ue) but is more diffuse in the few condensations (e) observed. (E) Higher magnification of kidney cultured with sense oligonucleotide S17 (5.0 μM) showing the early renal vesicle, including a comma-shaped body (c). The arrangement of *Pax-2*-expressing cells surrounding the ureter is more structured when compared to the diffuse expression pattern seen in D. (F) The formation of S-shaped body (s) and pretubular condensations (p) in a kidney cultured with the scrambled (control) antisense oligonucleotide SCR14 (5.0 μM) is apparent. Pretubular condensations represent the early renal vesicle stages, when the cells proliferate very actively (Jokelainen, 1963). Bar represents 160 μm in A, B and C and 25 μm in D, E and F.

culture up to the organ periphery, in the induced mesenchyme and the early mesenchymal condensations (Fig. 3A,B), in the ureter epithelium (Fig. 3A,B,E), in the pretubular condensations surrounding the ureter buds (Fig. 3A,B) and in the comma (Fig. 3E) and S-shaped bodies (Fig. 3F), which are the precursors of the glomeruli and the proximal convoluted renal tubules. The morphology and *Pax-2* expression patterns are markedly different in kidneys cultured with AS14, AS17 (Fig. 3D), or a mixture of both (Fig. 3C). The ureter has clearly failed to branch repeatedly and the mesenchymal condensations are limited (Fig. 3C). Most of the surrounding mesenchyme does not stain with the *Pax-2* antibodies, although Pax-2 can still be detected in the ureter and in the limited condensations surrounding the buds (Fig. 3C,D). The morphology of these cultures has changed little from the first 24 to 48 hours. It should also be stressed that *Pax-2* is already expressed in the ureter and in that portion of mesenchyme that has been induced by 11.5 days gestation, at the time of explant. In the antisense oligonucleotide cultures, the small amount of mesenchyme that still expressed *Pax-2* (Fig. 3C,D), was more loosely organized and never formed the early epithelial-like sheets of elongated adjacent cells surrounding the ureter (Fig. 3E,F), nor were comma- or S-shaped bodies observed.

To support the hypothesis that *Pax-2* specifically affects the conversion of mesenchyme to epithelium, a polyclonal antibody that recognizes all three laminin isoforms (A, B1 and B2, gift of H. Kleinman) was used as a marker to stain basement membranes generated by the developing epithelium. Although the B1 and B2 chains are already expressed in the uninduced mesenchyme at the first day in culture, induction results in increased expression of B1 and B2 and activation of the A isoform (Klein et al., 1988a; Ekblom et al., 1990). Whereas in uninduced mesenchyme, laminin B1 and B2 staining is weak and diffuse, the newly formed epithelium, which appears during the second day in culture (Grobstein, 1956), exhibits strong laminin staining in the basement membranes (Ekblom et al., 1990). Adjacent or similar cryosections of kidney cultures were immunostained with laminin and uvomorulin (Fig. 4B,D,F) or *Pax-2* and uvomorulin (Fig. 4A,C,E) antibodies. Kidneys cultured for 48 hours without oligonucleotides or with control oligonucleotides (S14, S17, or a mixture of both) showed strong laminin staining in the basement membranes of the ureteric epithelium (Fig. 4B), weaker laminin staining around the pretubuar condensations (Fig. 4B) and also strong laminin staining in the basement membranes of the S-shaped bodies (Fig. 4B,D). In the antisense oligonucleotide-treated kidney cultures (AS14, AS17 or a mixture of both), only weak general laminin staining was observed throughout the organ culture and some filamentous or "droplet" staining of laminin near the ureter (Fig. 4F). As in previous experiments, *Pax-2* staining was significantly reduced, mesenchyme aggregates were not evident and ureter branching was limited, in kidneys cultured with antisense oligonucleotides (Fig. 4E) compared to the control cultures (Fig. 4A,C).

Differential intracellular oligonucleotide levels

It was not altogether clear why some cells still maintained high levels of *Pax-2* protein even after 48 hours in the presence of the *Pax-2*-specific antisense oligonucleotides. It was postulated that the cells in the interior of the organ cultures might not have the same accesibility to high concentrations of oligonucleotides from the medium as cells in the rudiment exterior. Therefore, the intracellular levels of oligonucleotides in the cultures were assayed by using a fluorescent tagged sequence. After 48 hours incubation with a phosphorothioate-substituted fluorescent control oligonucleotide (see experimental procedures), the cultures developed normally, were sectioned and counterstained for *Pax-2* expression. The intensity of fluoroscent staining varied depending on the cell type in the kidney organ culture (Fig. 5). The mesenchymal cells on the periphery and in the interior of the organ stained brightest with the fluorescein-labelled oligonucleotide, whereas the ureteric epithelium appears significantly darker (Fig. 5A,B,E). The mesenchymal aggregates adjacent to some of the ureter tips also did not stain as brightly (Fig. 5A,B,E), suggesting a decrease in oligonucleotide uptake or an increase in oligonucleotide degradation. Precisely those cells that continue to express *Pax-2* in the presence of *Pax-2*-specific oligonucleotides, such as the ureteric epithelium and induced mesenchyme, appear to have lower intracellular oligonucleotide levels (Fig. 5C-F). Examination at higher magnification indicates that there is a clear demarcation between cells that harbor high amounts of fluorescein-labelled oligonucleotide and those that do not (Fig. 5E,F). Thus, the reduced levels of oligonucleotides in the epithelium and induced mesenchyme are consistent with the residual pattern of *Pax-2* expression present in the antisense oligonucleotide cultured kidneys.

DISCUSSION

The data presented in this report demonstrate that *Pax-2* function is required at the earliest phase of mesenchyme-to-epithelium conversion. Although *Pax-2* is still expressed in the epithelial cells of the ureter, the ureter fails to branch. We believe that this is an indirect effect due to the inhibition of condensation and polarization of the mesenchyme. Upon induction, the condensing mesenchyme reciprocally induces the ureter to branch (Grobstein, 1956). These inductive interactions are repeated as the multiple branches of the ureter grow outward, eventually forming the network of collecting ducts. Without appropriate signals emanating from the induced mesenchyme, ureter branching is inhibited.

Validity of antisense technology

The inhibition of epithelium formation is observed with two different antisense *Pax-2* oligonucleotides, one directed to the translation start site (AS14) and a second directed to the coding region (AS17), localized between the conserved paired domain and the conserved octapeptide. However, not all oligonucleotides tested inhibited differentiation and *Pax-2* expression, as the antisense oligonucleotide AS16 (see experimental procedures) did not produce a specific effect. The differential accessibility of mRNA sequences due to secondary structure may account for these observations. The validity of antisense oligonucleotide technology has been

tested in a large number of culture systems (for review see Neckers et al., 1992). More specifically in kidney organ cultures, antisense oligonucleotides directed against the low affinity nerve growth factor receptor inhibit formation of epithelial tubules (Sariola et al., 1991). Woolf et al. (1992) have demonstrated mRNA cleavage and degradation in *Xenopus* oocytes, mediated by RNAseH, even though the antisense oligonucleotides contained multiple mismatches. Thus given the length of our oligonucleotides (19-mer), imperfect hybrid formation may affect other mRNAs as well. We believe that the effects on non-targeted genes is minimal for a number of reasons. Primarily, two different sequences directed against the *Pax-2* mRNA have essentially the same effect and it is unlikely that these different sequences would react non-specifically with the same mRNAs. Secondly, the sense-strand oligonucleotides would be equally capable of non-specific interactions, due to imperfect hybrid formation, although they would react with a different mRNA pool. Thirdly, the antisense oligonucleotides have no effect in salivary glands, thus any additionally affected mRNAs would have to be kidney specific, this effectively reduces the complexity of the targeted gene pool in kidneys. Finally, the stability of the phosphorothioate-substituted oligonucleotide/RNA hybrid is less than a phosphodiester/RNA hybrid, as measured by a decrease of 5-10°C in melting temperatures (Agrawal et al., 1989; Chang et al., 1989). This should not affect the stability of perfectly matched hybrids at physiological temperatures, but may decrease the tolerance for mismatches.

Using a fluorescein-tagged control oligonucleotide, we have demonstrated that the intracellular concentration of oligonucleotides in the kidney cultures varies depending on the cell type. The uninduced mesenchymal cells have the highest relative levels of intracellular oliognucleotides, whereas the more developed structures such as the ureteric epithelium and the condensing mesenchyme exhibit reduced fluorescence. These differences may be due to differential uptake efficiencies among the cell types, or differential ability to metabolize the phosphorothioate oligonucleotide. In fact, the adult mouse kidney and liver have an unusually high capacity to degrade phosphorothioate-substituted oligonucleotides. Up to 50% of intravenously injected oligonucleotides were degraded in liver and kidney, compared to approximately 15% in stomach, heart, intestine and plasma (Agrawal et al., 1991). This would suggest that the lower oligonucleotide levels observed in the more developed structures, in kidney cultures, are due to higher degradation rates. The fluorescein staining cannot distinguish between intact and partially degraded oligonucleotides. However, it is a positive indicator of uptake. By the time of kidney explant at E11.5, the ureter has generally branched once and some *Pax-2*-expressing mesenchyme has already condensed around the two buds of the ureter. After 48 hours in culture with antisense Pax-2 oligonucleotides, *Pax-2* expression is still detected in the ureter and in a limited number of mesenchymal cells, precisely those cells that exhibit lower intracellular oligonucleotide levels. Thus, the inhibition of *Pax-2* expression by antisense oligonucleotides is most striking in that population of mesenchymal cells that exhibit high levels of intracellular oligonucleotides and that would be induced after explantation.

Pax 2 and kidney development

The activation of potential transcription factors in the kidney mesenchyme, in response to induction by the ureter, has not been extensively characterized. *Pax-2* is one of the earliest markers of induced metanephric mesenchyme and is quickly repressed as the mesenchyme derived epithelium matures (Dressler and Douglass, 1992). Thus, it is possible that *Pax-2* regulates genes required for cell aggregation and cell polarization, the two processes that define the early stages of epithelium formation. The Pax proteins bind DNA through the paired domain and paired type homeodomain and can potentiate transcription from heterologous promoter sequences (Chalepakis et al., 1992). In developing B lymphocytes, the *Pax-5* gene was cloned based on its binding capacity to specific target genes (Adams et al., 1992). However, genes regulated by other Pax proteins have not been identified to date.

That Pax genes are morphogenic control factors is underscored by the three mouse mutations *undulated, splotch* and *small eye*, all of which are due to mutations or deletions of Pax genes (Gruss and Walther, 1992). The expression patterns of *Pax-2* in kidney development and *Pax-1* in vertebral column development share features that may reflect similar functions. *Pax-1* is expressed in a subset of sclerotome cells that surround the notochord and form the intervertebral discs (Deutsch et al., 1988). The aggregation of *Pax-1* expressing cells and their clear delineation from sclerotome cells destined to become the vertebral bodies suggests that *Pax-1* may be important in defining boundaries between groups of adjacent cells. Indeed, a mutation in *Pax-1* results in the *undulated* phenotype (Balling et al., 1988), which exhibits alterations in the size of the vertebral discs relative to the vertebral bodies (Gruneberg, 1954). Similarly, *Pax-2* may drive aggregation of induced kidney mesenchymal cells and delineate the boundaries of individual aggregates. Disruption of cell movement is also a feature of the *splotch* phenotype, a *Pax-3* mutation that affects primarily the neural tube and neural crest cell derivatives (Epstein et al., 1991). The semi-dominant nature of the mouse mutations *undulated*, *splotch* and *small eye* indicates a strict quantitative requirement for Pax proteins during development. Thus, it is not suprising that the kidneys cultured with antisense *Pax-2* oligonucleotides show limited development even though *Pax-2* proteins are not completely eliminated.

The changes in extracellular matrix gene expression following the conversion of kidney mesenchyme to epithelium have been well characterized. The specific induction of the laminin A chain in the newly forming basement membrane (Klein et al., 1988a) and its growth factor activity (Panayotou et al., 1989), together with the shift in cellular adhesion molecules (Vestweber et al., 1985; Klein et al., 1988b), may provide the morphogenetic forces driving epithelium formation. However, these events occur at least 24 hours postinduction and do not reflect a primary genetic response to the inductive signal. Loss of N-*myc* function results in hypoplastic mesenchyme surrounding the metanephric tubules in homozygous mutant mouse embryos and, in extreme cases, only epithelial condensation occured while mesonephric tubules were absent (Stanton et al., 1992). Thus, N-*myc* is also an important factor for early epithelium formation in the mammalian kidney. In addition,

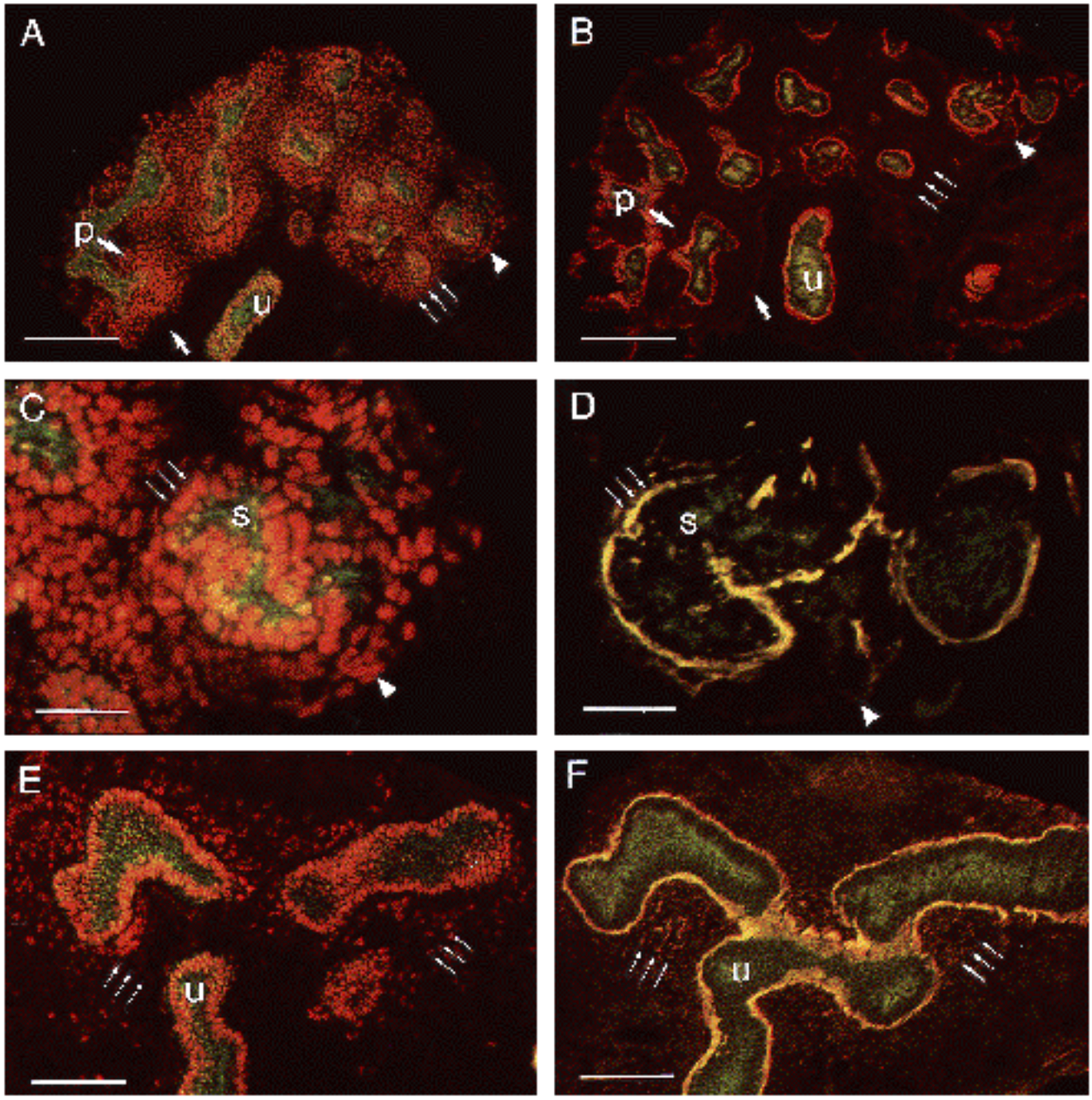

Fig. 4. Laminin, *Pax-2* and uvomorulin expression in kidneys cultured for 48 hours. (A,B) *Pax-2* (red, A), laminin (red, B) and uvomorulin (green, A and B) staining in adjacent sections of a kidney rudiment cultured with the scrambled (control) antisense oligonucleotide SCR14 (2.5 μM). Development of the rudiment is normal. Early and pretubular condensations (p) and one S-shaped body (arrowhead) are observed. The single arrow and the three smaller arrows indicate the area where *Pax-2* is expressed (A) and the basement membrane (B) is generated surrounding the early condensed mesenchyme. (C,D) Higher magnification of adjacent sections A and B respectively. Arrowhead and three smaller arrows point to *Pax-2*-expressing cells at the border of the S-shaped body (s) in C. Laminin staining is evident in the basement membrane surrounding the whole structure (arrowhead, D), as well as in the convolusions of the S-shaped body (small arrows, D). (E,F) Adjacent sections of kidney cultured with AS14 (5.0 μM) and stained for *Pax-2* (red, E), laminin (red, F) and uvomorulin (green, E and F). Ureter (u) development is limited to a single branch point. *Pax-2* is expressed near the ureter branches and in the ureterepithelium. Small arrows (F) point to regions where laminin is detected as filamentous staining, or droplets, around the ureter near the first branch point. Note the basement membrane around the ureteric epithelium, but the lack of basement membrane formation in the mesenchymal cells. Bars represent 160 μm in A and B, 25 μm in C and D, and 80 μm in E and F.

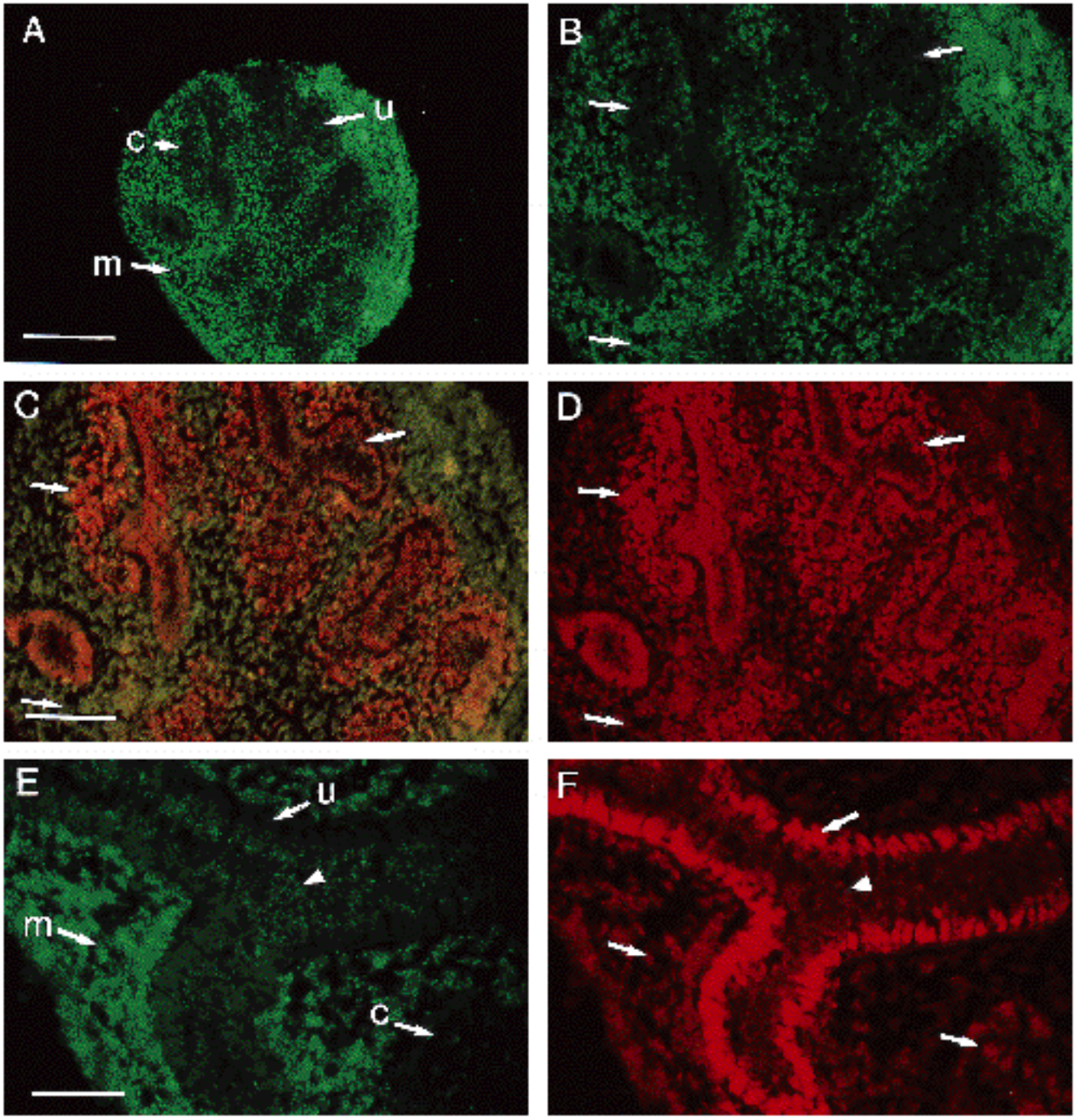

Fig. 5. Intracellular oligonucleotide levels and *Pax-2* expression. Kidneys cultured for 48 hours with a fluorescein-labeled control oligonucleotide and stained for *Pax-2* expression (red). (A) Whole kidney culture exhibits high fluorescent staining in mesenchymal cells (m), weaker fluorescent staining in mesenchymal condensations (c) around the ureter, and little staining of the ureter epithelium (u). (B) Higher magnification of A. (C,D) Comparison of fluorescent staining (C only) and *Pax-2* expression (C and D) reveals less oligonucleotide in *Pax-2*-expressing cells of the mesenchymal condesations and ureter (arrows). (E,F) Higher magnification shows clear demarcation between cells with high fluorescent staining (E) and *Pax-2*-expressing ureteric epithelium (F). Identical structures are indicated in E and F as mesenchyme (m), ureter epithelium (u), condensed mesenchyme (c) and lumen surface (arrowhead). Bars represent 160 μm in A, 80 μm in C, B and D, and 25 μm in E and F.

programmed cell death may help to pattern the eventual mesenchyme-derived structures of the kidney by eliminating populations of uninduced mesenchymal cells (Koseki et al., 1992). By generating a loss of *Pax-2* function in vitro, we have shown that *Pax-2* is required for the earliest phase of mesenchyme-to-epithelim transition. Thus, *Pax-2* may help to initiate a genetic cascade, controlling a complex series of events that results in the conversion of embryonic kidney mesenchyme to epithelium.

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