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Identification of novel genes expressed during metanephric induction through single-cell library screening

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Background. Development of the mature kidney is dependent on a series of inductive events between a portion of the epithelial bud at the distal end of the nephric duct and a neighboring domain of committed metanephric mesenchyme. Several genes have been identified to date that are critical in the inductive process. For example, the deletion of *Bmp7* from the mouse genome results in dysgenesis or agenesis of the kidney. These findings suggest that *Bmp7* controls the expression of genes important for nephrogenesis, but the identity of these genes has remained largely undetermined.

Methods. Single cells were isolated from mouse metanephric mesenchyme during the time of induction (between E11.0 and E11.5) and cDNA libraries constructed from induced and uninduced tissue. Subtractive hybridization was performed to isolate genes that were expressed during E11.5 but not E11.0.

Results. Using this approach, we identified eight previously known genes, three of which were known to be involved in metanephric induction, thus validating our approach, and nine novel genes. Eight of these genes were completely novel, whereas one was similar to a member of the yeast Anaphase Promoting Complex.

Conclusions. Through subtractive hybridization of mouse E11.0 and E11.5 metanephric mesenchyme single-cell cDNA libraries, we have identified novel genes that are candidates for involvement in nephrogenesis through their up-regulation during the inductive process.

The development of the definitive mammalian kidney, or metanephros, is a classic example of the inductive interaction that occurs between two neighboring tissues. Kidney formation depends on the reciprocal inductive interaction between the epithelial ureteric bud and the metanephric mesenchyme. At embryonic day 11.0 (E11.0) in the mouse, the ureteric bud emerges from the most caudal portion of the Wolffian (mesonephric) duct and grows dorsally to invade the metanephric mesenchyme, resulting in differentiation of the mesenchyme into an epithelial structure: the nephron [1]. Initial evidence of inductive interactions was obtained from transfilter culture experiments, in which embryonic inducers such as the spinal cord were placed in close proximity to metanephric mesenchyme, leading to the formation of novel structures such as primitive tubules [2, 3]. The first genetic evidence for inductive interaction was obtained from mice containing a targeted disruption of *WT1*; these mice exhibited a complete absence of kidneys. Furthermore, *WT1* was shown to be expressed in metanephric mesenchyme but not in the developing ureter [4]. Other targeted gene disruptions have since led to the identification of additional murine genes, such as *Bmp7*, that require sequential activation for renal and ureteral development to proceed normally and have provided evidence for the reciprocal nature of inductive interactions [5, 6]. Despite these advances, traditional subtractive hybridization techniques for the identification of novel regulatory genes in the developing kidney at the time of induction are often inefficient because of the paucity of biological material and the heterogeneity of the cell population. To circumvent these difficulties, we have adopted the strategy of differential screening of cDNA libraries constructed from single cells to clone genes required for mesenchymal induction. We report the construction of single-cell cDNA libraries from uninduced and induced metanephric mesenchyme and their use for the identification of 17 genes in human and mouse, several of which are novel. To our knowledge, this is the first example of the application of this technology to the study of metanephric induction.

Key words: APC complex, *BMP7*, nephrogenesis, kidney development, epithelial ureteric bud.

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METHODS

Tissue isolation

Embryos of E11.0- and E11.50-timed pregnant C57/Bl6 wild-type matings were dissected free of the uterine

muscle, decidua, and yolk sac and were placed in ice cold phosphate-buffered saline (PBS) without Ca^{++} or Mg^{++}. Kidney rudiments consisting of the Wolffian duct, ureteric bud, and mesonephric mesenchyme were dissected under a stereoscopic microscope and transferred to fresh, cold PBS without Ca^{++} and Mg^{++}, where the metanephric mesenchyme was dissociated from the ureteric bud. Single metanephric cells were ultimately picked with a pulled microcapillary tube.

cDNA synthesis and amplification

Individual cells were placed in 4 μL of ice-cold cell lysis buffer [50 mmol/L Tris-HCl, pH 8.3, 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 0.5% NP-40 containing 80 ng/mL $pd(T)_{24}$, 5 U/mL RNase inhibitor, 324 U/mL RNAguard, and 10 μmol/L of dNTPs]. Lysis and first-strand synthesis were performed according to standard protocols [7, 8]. Samples were phenol-chloroform extracted, ethanol precipitated, and resuspended in 25 μL TE. Five microliter aliquots of amplified single-cell cDNA were electrophoresed, transferred to Hybond N^+ membranes, and hybridized. Probes labeled with ^{32}P were prepared by random priming, and membranes were treated according to our standard conditions [9].

Library construction and screening

Amplified cDNA was *Eco*R I-cut used and ligated to suitably prepared arms of the Stratagene LamdaZap-II vector according to the manufacturer's instructions. To confirm the presence of insert in the library, 20 phage clones were picked at random and subjected to amplification using vector-specific primers. We observed inserts in 18 out of 20 clones (90%), with an average size of 500 bp. A portion of the library was subsequently amplified, plated, and transferred onto Hybond N^+ filters as described [10].

DNA sequencing and sequence analysis cDNA clones identified after library screening were sequenced with T3/T7 promoter primers on an ABI Prism 377 automated sequencer. Sequence analysis, database searches, Expressed Sequence Tag (EST) identification, and contiging were carried out as described [11].

Expression analysis

Reverse transcription-polymerase chain reaction (RT-PCR) was performed on RNA from C57/B16 wild-type embryo and neonatal mouse tissue. Northern blotting of mouse tissue was performed following RNA extraction in guanidium isothyanate buffer and ultracentrifugation in a CsCl gradient. Additional human and mouse Northern blots and multiple tissue cDNA (MTC) panels containing adult and fetal poly A^+ mRNA were purchased from Clontech. Filter hybridization, washing, and exposure were as described [11].

RESULTS

Differential screening of the E11.0 and E11.5 cDNA libraries

Single-cell amplifications were prepared from 108 individual cells collected proximally to the ureteric bud. To select suitable material for library construction, we tested for the expression of appropriate markers. To ensure a lack of contamination from ureteric bud cells, we investigated for the presence of *Ret*, which is expressed in nephric duct, mesonephric tubules, and ureteric bud but not the metanephric mesenchyme [12]. Once absence of *Ret* was established (Fig. 1), we proceeded with the hybridization of cellular cDNA with a 340 bp *Ear* I-*Pst* I fragment of *BMP7*, a growth factor known to be expressed in induced metanephric mesenchymal cells (Fig. 1) [13].

Upon library construction, the differential screening was accomplished using mRNA from the E11.0 and E11.5 time points. To identify genes preferentially expressed during metanephric induction (E11.5), 12,000 clones from the E11.5 library were plated. The primary filters were hybridized with an E11.0 cell cDNA probe, and the duplicate E11.5 filters were hybridized with the E11.5 cell cDNA probe. A total of 168 clones were identified that hybridized strongly with the E11.5 probe but not the E11.0 probe, and then a second round of screening was performed. Of the initial 168 plaques, 33 clones maintained a hybridization profile consistent with the primary screen observations.

Sequence analysis of differentially expressed cDNAs

We obtained complete sequence for all 33 clones, which was used to search all nucleotide and protein databases. This analysis identified eight previously known genes, several of which are known to be overexpressed during metanephric induction (Table 1). Upon elimination of duplicate material, nine sequences were identified that did not show an identity to any known genes/proteins and were investigated further. Evidence for an open reading frame was detected in four of these sequences, which led to the identification of four novel genes, which we termed *EMI1* through *EMI4* (expressed during mesenchymal induction 1 through 4). To ascertain whether these new transcripts were true candidates for involvement in metanephric induction, their expression profiles were determined preinduction and postinduction using the same mRNA source used for the construction of the two libraries. As shown in Figure 2, all four transcripts were present in E11.5 metanephric mesenchyme mRNA (MM), whereas two out of four were absent during E11.0, and the remaining two were weakly expressed, suggesting that these genes become up-regulated during the time of induction. These data were independently replicated using cells 7 (for E11.0) and 36 (for E11.5) to ensure that the expression profile observed was not unique

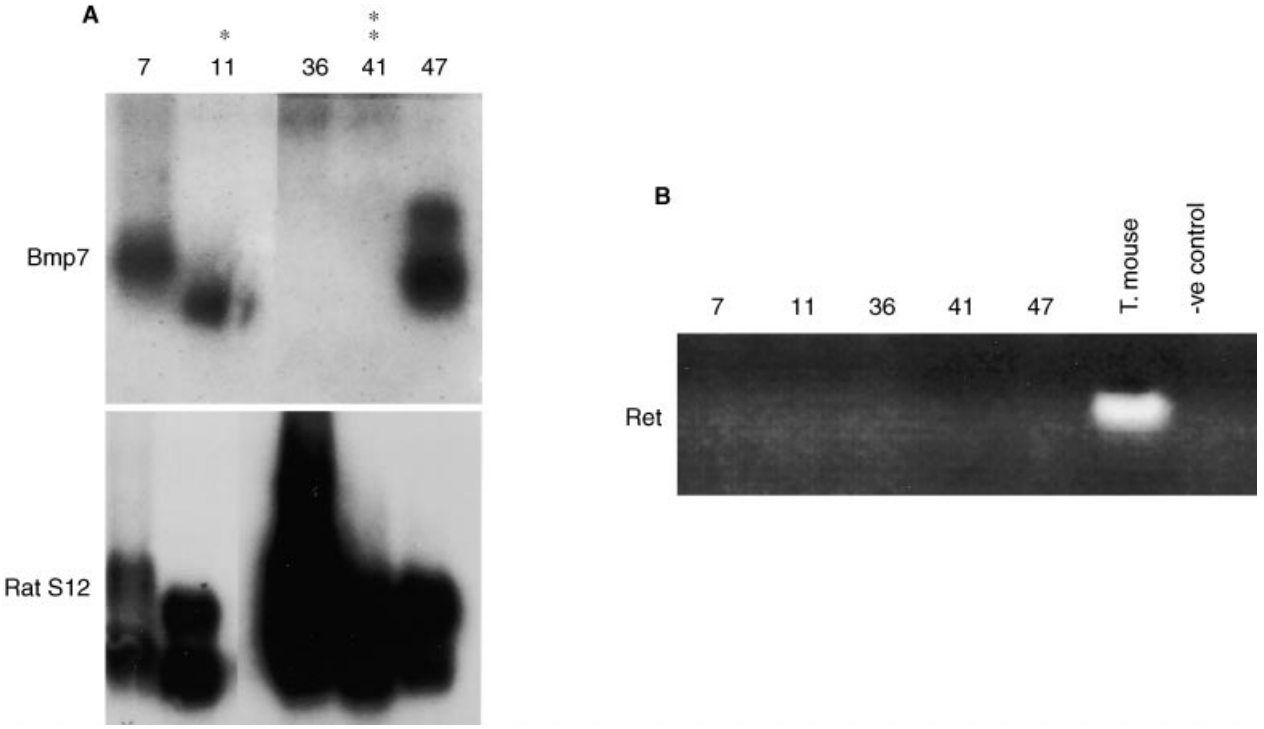

Fig. 1. (*A*) Hybridization of cDNA from single cells isolated from E11.0 and E11.5 mouse metanephric mesenchyme with *BMP7* and the cDNA for rat ribosomal protein S12. (*B*) PCR of cDNA from the same cells and whole mouse embryo mRNA with *Ret* to demonstrate absence of contamination of ureteric bud material in our sample. Cell 11 was used for the construction of the E11.5 cDNA library (single asterisk), whereas cell 41 was used for the construction of the E11.0 cDNA library (line annotated with a double asterisk).

Table 1. Known genes identified as differentially expressed during second screening

Clone 6:	Human RNA for *KIAA0164* (D79986). Similar to DBP-5, a nuclear protein that has DNA binding properties [24].
Clone 8:	Mouse laminin receptor (J02870). Up-regulated after mesenchymal induction in the metanephros [22].
Clone 10b:	Mouse mammary gland factor, *Stat5a* (U21103). Member of the STAT (signal transducer and activator of transcription) family of cytoplasmic proteins that participate in gene control in response to extracellular polypeptides [25].
Clone 24:	Mouse acidic ribosomal phosphoprotein p1 (U29402). Up-regulated in intestinal malignancy. Target molecule for lupus antibodies on membrane of proliferating cells. Regulatory role in protein expression in yeast [26].
Clone 27:	Mouse NAD-dependent methylene tetrahydrofolate dehydrogenase (M63445). Detectable in immortalized and transformed cells and in developmental tissues, but not in most adult tissues [27].
Clone 29:	Mouse rRNA regulated by bone morphogenetic protein (X95281) 3-oxoacyl reductase. Up-regulated in mesenchymal progenitor cells treated with BMP-4 (unpublished observations).
Clone 44b:	Rat thymosin beta-10 mRNA (M17698). Increased levels of transcript in malignant renal tumors and embryonic tissue [23]
Clone 105:	Mouse ribosomal protein L8 (U67771).

to the library material (data not shown). We also note that, given that the RT-PCR was performed in limited (20) cycles to ensure first-order kinetics, the relative intensity of the bands shown in Figure 2 is a measure of the relative abundance of these messages at E11.0 and E11.5.

EMI1. When clone 89 was used to search dbEST, it showed identity to ESTs from a library constructed from rat PC-12 cells induced with nerve growth factor [19], implying that it too may be differentially regulated. Conceptual translation of the cDNA sequence did not reveal any homology to known proteins. A domain search did not yield any known motifs except for a putative myristoylation domain close to the 3′ UTR, which may suggest a role in cytoplasmic or membrane transduction pathways [20]. To ascertain the expression profile of *EMI1*, we performed Northern blot and RT-PCR analysis in human and mouse. Using a 312 bp fragment of the 3′ untranslated terminal region (UTR), we identified a 2.2 kb mRNA in most tissues tested (Fig. 3A). In the adult state, we found *EMI1* to be expressed most strongly in the bladder and lung (Fig. 3B). RT-PCR analysis also showed the presence of the transcript at E9.5 but complete absence at E10.5, with a re-emergence at E11.5 (Fig. 3C). It should be noted that differences in the expression profile are most likely due to either sample age

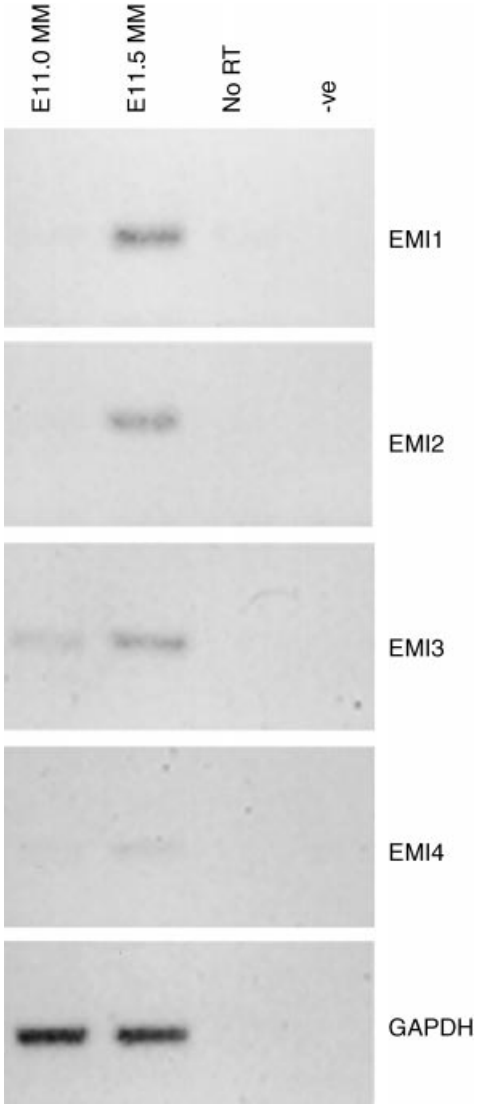

Fig. 2. RT-PCR of *EMI1* through *EMI4* on mRNA from E11.0 and E11.5 metanephric mesenchyme (MM) to investigate mRNA levels preinduction and postinduction. The *GAPDH* control reaction is also shown.

difference (for example, human lung between Northern and master blot where the age of the tissue ranges from the teens to 70s) or cross-species differences (for example, the different levels of *EMI1* expression in human and mouse heart).

EMI2. Through EST assembly and cDNA library screening, we identified a 1357 bp human transcript and a 1355 bp mouse transcript encoding a 246 and 248 amino acid protein, respectively, which exhibited 85% identity at the amino acid level extending over the entire length of the protein (Fig. 4A). Database searches did not identify any similarity to known proteins, nor did they yield known motifs. The closest similarity of *EMI2* to a known sequence was a 39% similarity with a yeast predicted ORF, YOR057w (Z74965) of unknown function. Northern analysis on adult human tissues with a 212 bp fragment from the 3′ UTR of the human locus identified a 1.4 kb mRNA species in all tissues tested, although the levels of expression in the lung and liver were substantially reduced (Fig. 5A). A similar expression pattern was seen in mouse adult tissues (data not shown). We also investigated the expression profile of *EMI2* in fetal human tissues in which a high level of expression was seen in fetal kidney, as well as other embryonic tissues and times (Fig. 5B).

EMI3. Screening of dbEST with clone 5 from the subtractive hybridization experiments resulted in the generation of two mouse sequences of 1540 and 1382 bp, which were, in turn, used to search the human subset of dbEST. After EST contiging, we generated a 1173 bp human sequence. A comparison of the human and mouse sequences revealed an 81% identity at the nucleotide level, with the exception of 173 bp between nucleotides 441 and 613, which were present in some mouse but not human clones. Conceptual translation yielded a single ORF of 206 and 212 amino acids in human and mouse, respectively (Fig. 4B). This analysis also revealed that the additional mouse sequence is likely to represent a splice isoform, which results in the formation of a truncated version of the protein, containing 121 residues. BLAST analysis revealed a 40% similarity between the long protein isoforms of our sequence with the *M. jannaschii* hypothetical protein MJ0443 (Accession No. Q57885; http://www.ncbi.nlm.nih.gov/Entrez). No other similarities were found, nor were we able to discern any known motifs. Northern hybridization of the human 3′ UTR of *EMI3* identified a 1.6 kb mRNA species in heart, brain, and kidney (Fig. 5C). Amplification of the same portion of the 3′ UTR in human embryonic tissues also yielded evidence for expression in several embryonic tissues, embryonic kidney being one of the more prominent ones (Fig. 5D).

EMI4. EST analysis yielded a 1381 bp contig of mouse and of a 1260 bp contig of human cDNA sequence (Fig. 3C). Expression studies were performed using a 212 bp fragment from the human 3′ UTR onto Northern blots containing adult human and mouse mRNA from several tissues. A 1.4 kb mRNA fragment was detected in all tissues tested, which closely resembles the size of the isolated mouse cDNA (Fig. 5 E, F). Examination of the predicted protein structure identified a region close to the carboxy terminus of the protein (amino acids 353 to 362; Fig. 3C), which bears similarity to an aminoacyl-transfer RNA synthetases class II signature. A search of the Swissprot database also indicated 49% homology with a predicted ORF from *C. elegans* (P34513). Finally, a search of the yeast nucleotide database revealed 43% similarity to a *S. cerevisiae* gene, Anaphase Promoting Complex (APC) component (Z73299). The APC complex

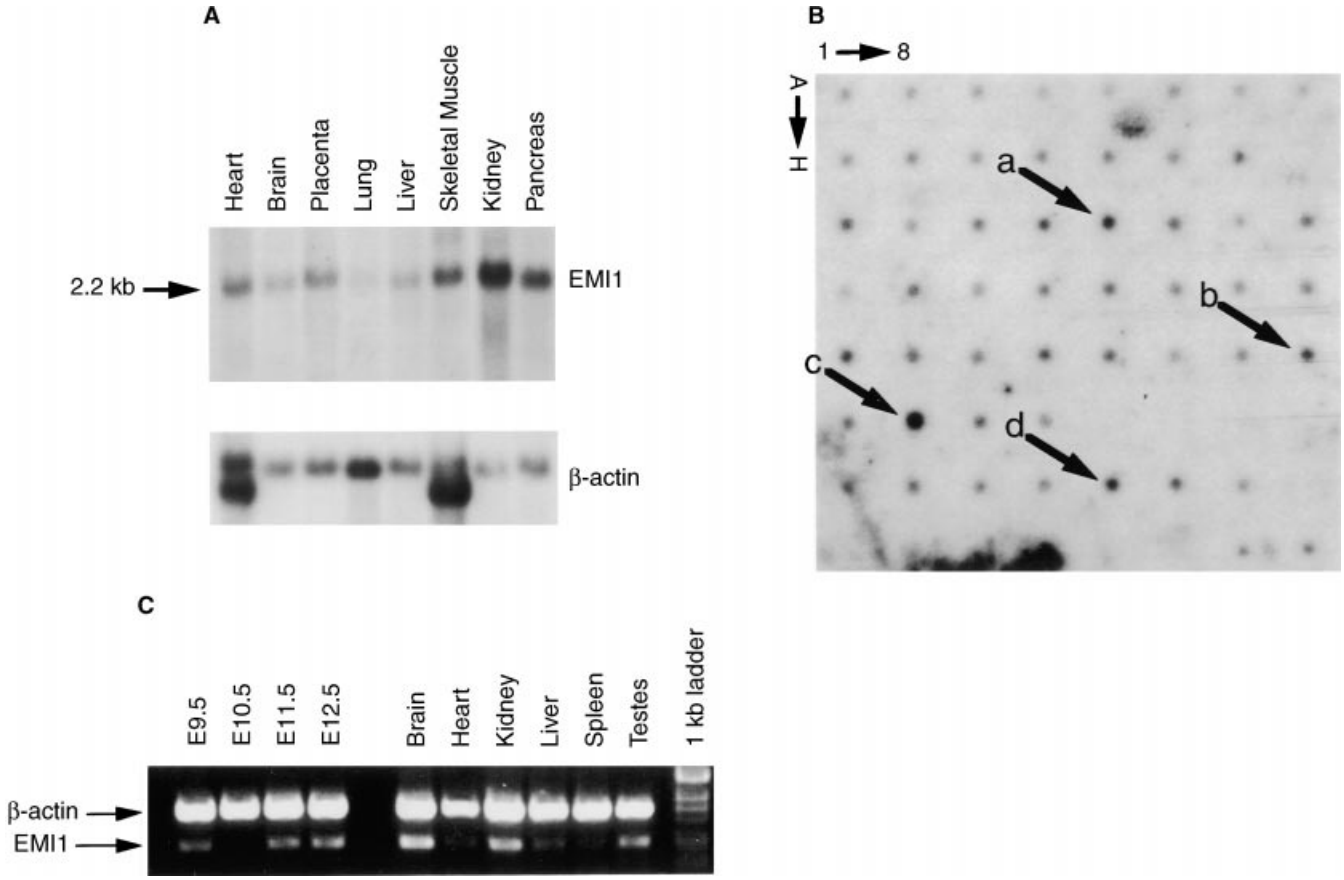

Fig. 3. Expression analysis of *EMI1*. (*A*) Hybridization of a 212 bp fragment from the 3′ UTR of *EMI1* to an adult tissue Northern blot. *EMI1* is ubiquitously expressed with a prominent hybridization signal in adult kidney. (*B*) Hybridization of the same probe to a human dot blot containing adult and fetal poly A^+ mRNA. The highest intensity signals are indicated with an arrow and are as follows: (a) bladder, (b) bone marrow, (c) lung, and (d) fetal spleen. The expression levels in adult and fetal kidney can be seen at coordinates A5 and C7, respectively. (*C*) RT-PCR analysis of mouse *EMI1* on embryonic and neonatal mouse tissue. β-actin was amplified in the same reaction as an internal control.

ubiquitinates a broad range of proteins and is essential for the progression of the cell cycle through anaphase [21].

DISCUSSION

Targeted gene disruption experiments in mice have started to unravel the molecular mechanisms of nephrogenesis. The cumulative evidence suggests that this process is mediated by regulatory genes at a series of critical checkpoints [1]. However, despite the approximately 14 proteins that have been shown to be absolutely required for renal development [5], many more genes remain to be identified. To this end, we chose to isolate cDNA from single uninduced (E11.0) and induced (E11.5) metanephric cells, using a modification of the technique reported by Dulac and Axel and Brady and Iscove [22, 23]. The single-cell strategy solved two experimental problems: the paucity of biological material and the heterogeneity of the starting cell population.

Microsurgical selection of a single cell from the appropriate anatomic location combined with analysis with cell-type specific markers allowed us to choose two cells from which to construct an E11.0 metanephric and an E11.5 metanephric library. Differential screening of these libraries identified 33 clones that represent genes expressed during metanephric induction. Sequencing analysis of these identified eight known genes and nine novel sequences, four of which we have characterized further. Three of these genes have been shown to be involved in kidney morphogenesis, thus suggesting that our approach was successful.

We also describe the sequence and basic expression profile of four novel genes, *EMI1* through *EMI4*. Little functional insight can be gained from nucleotide and protein analysis, since all but one of the proteins do not exhibit similarity to sequences of known function. The only gene related to a protein of known function is *EMI4*, by virtue of its 43% similarity to a yeast APC component, suggesting that it may be involved in the progression of the cell through mitosis. The similarity does not extend over the entire length of the human sequence, however, suggesting that *EMI4* is not the ortholog of the yeast locus, but shares some functional features.

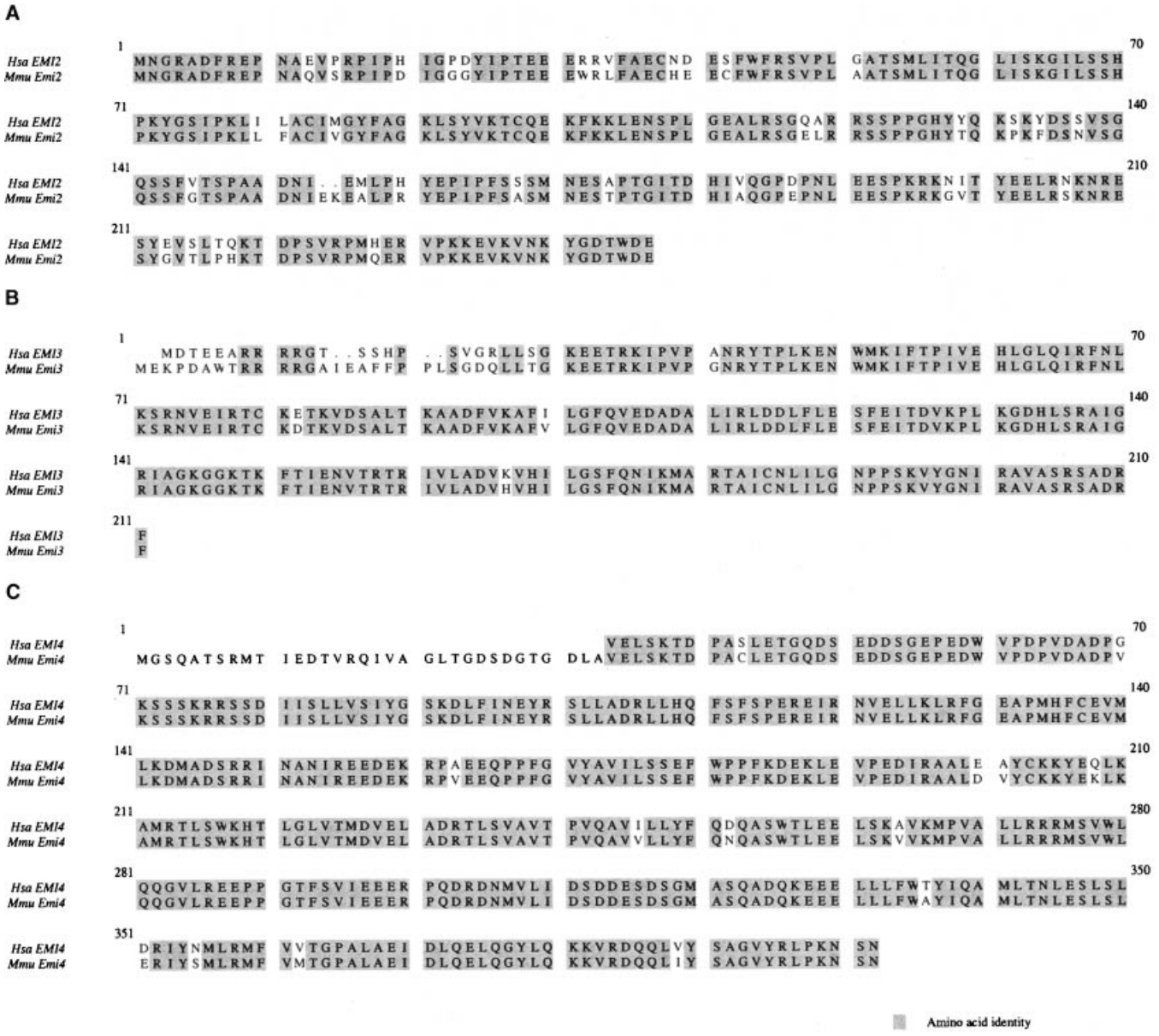

Fig. 4. Comparison of the amino acid sequence of human and mouse EMI2 (*A*), EMI3 (*B*), and EMI4 (*C*). The shaded boxes represent amino acid identity. Gaps have been introduced to optimize the alignment.

All genes isolated by the single-cell differential screening strategy are expressed in the developing as well as the adult kidney. RT-PCR analyses of these transcripts in preinduction and postinduction metanephric mesenchyme reveals that the message is either absent (*EMI2*) or minimal (*EMI3, EMI4*) at E11.0, but more prominent at E11.5. Furthermore, RT-PCR of *EMI1* indicates that this locus may be down-regulated at E10.5 and again reactivated on E11.5 in the mouse, an observation also consistent with our hypothesis that these loci may be involved in metanephric induction. The remaining transcripts are expressed both during development and adulthood. This is not surprising, since all genes known to be involved in renal development are also found in a range of other tissues. For example, *BMP7* mRNA is found in a wide range of embryonic and adult tissues, from the developing heart to the nasal epithelium [24]; the same is true for *Wnt1*, *Wnt4*, *Ret*, and others.

The single-cell strategy is a powerful tool for the identification of novel transcripts involved in developmental processes, since it offers the possibility of examining changes in gene expression at the single-cell level during differentiation/induction without the need for large amounts of material or the complication of heterogeneous cellular populations, both being problems inherent in other techniques. The identification of genes in which

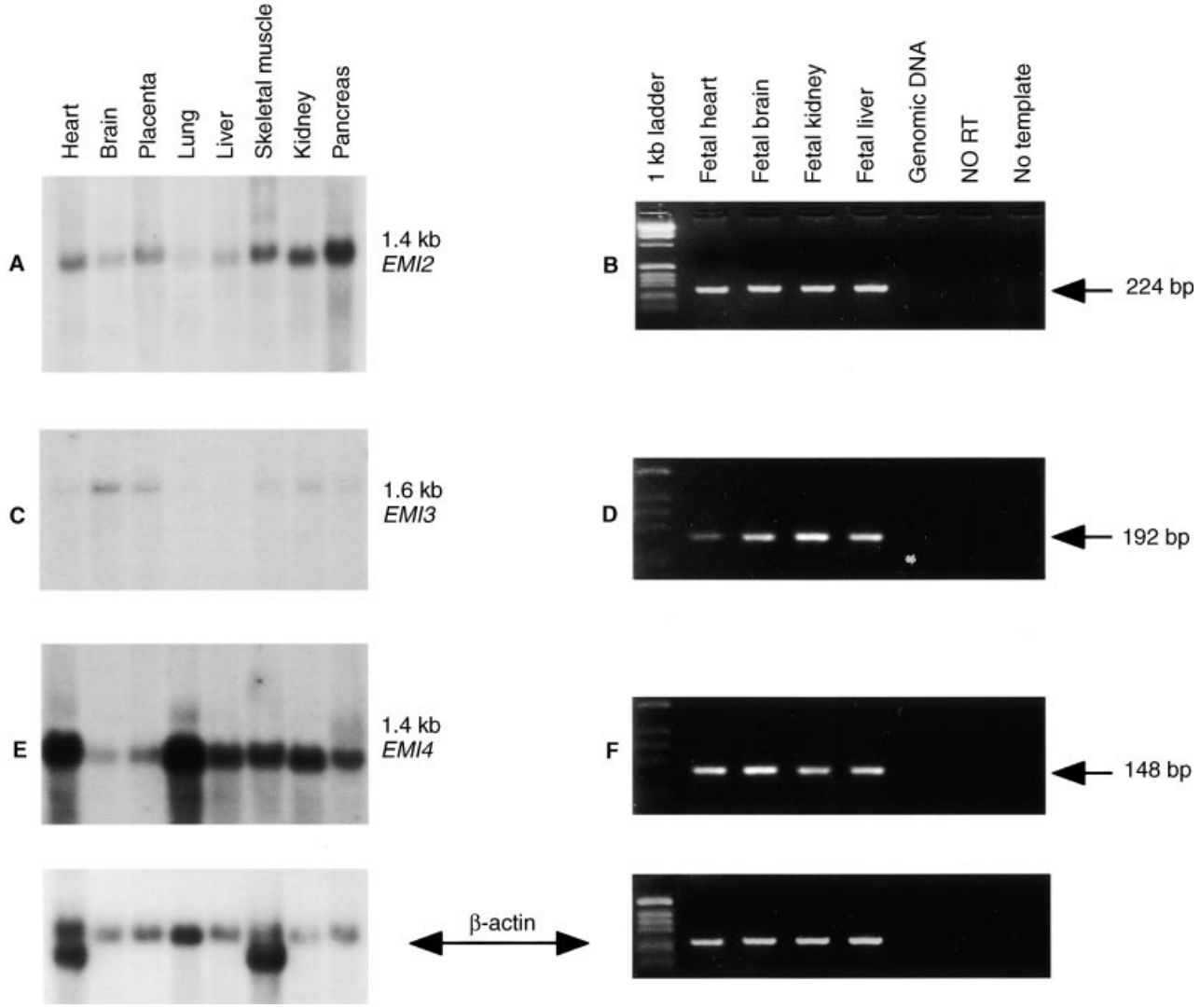

Fig. 5. Expression analysis of *EMI2* through *EMI4*. (*A*, *C*, and *E*) Northern analysis of the three genes on adult human tissues using 3′ UTR sequences as probes. (*B*, *D*, and *F*) RT-PCR profiles of the three genes on human 16- to 32-week-old embryonic tissues.

a priori involvement in kidney development is known validates the methodology and increases our confidence that the novel genes isolated are good candidates for the involvement in metanephric induction and are worthy of further study. Such genes may also be important in understanding common birth defects of the kidney, such as renal agenesis and other developmental abnormalities.

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