A Retinoic Acid Responsive Gene *MK* Found in the Teratocarcinoma System Is Expressed in Spatially and Temporally Controlled Manner during Mouse Embryogenesis

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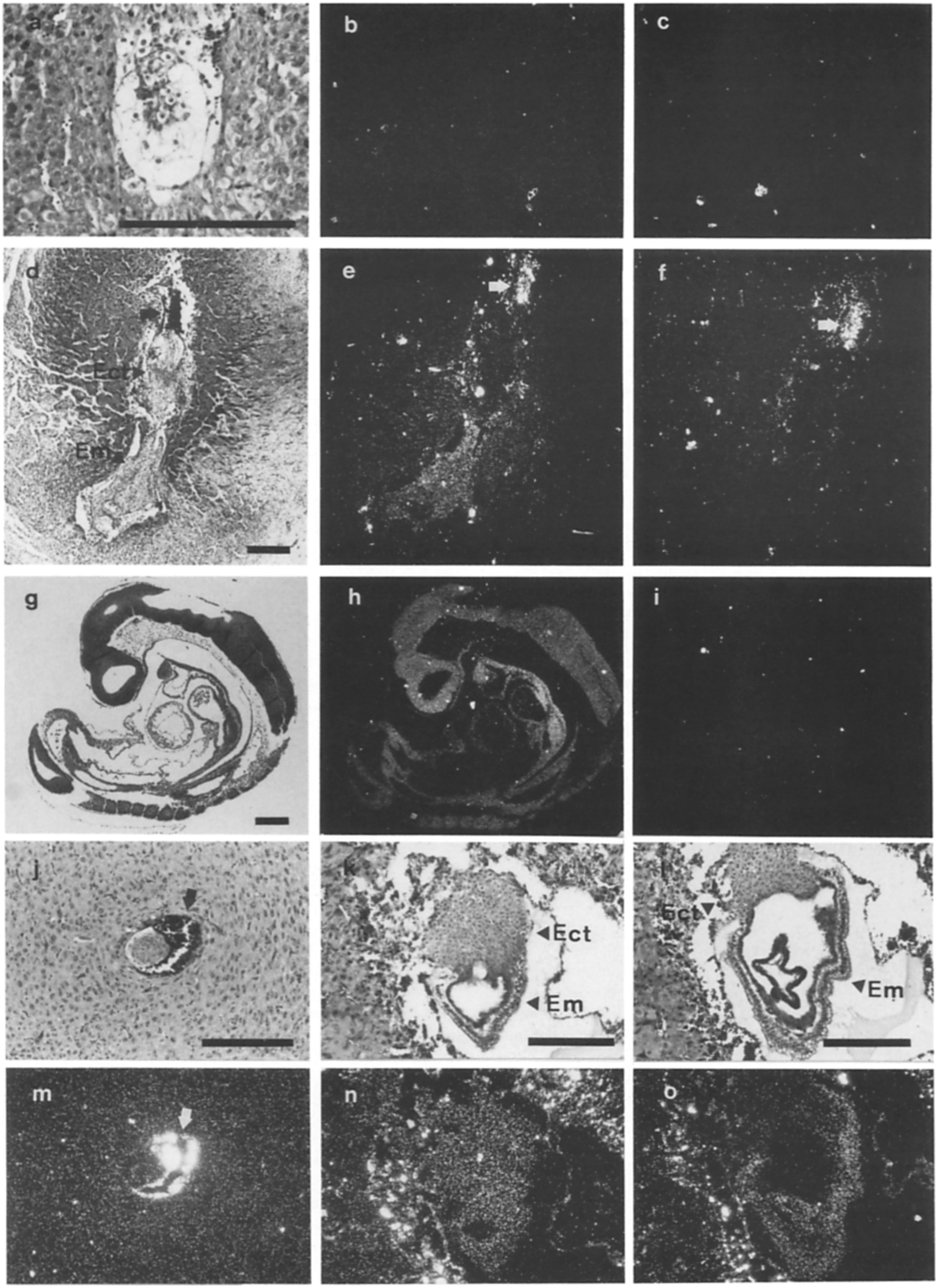
Abstract. A newly identified gene *MK* is transiently expressed in early stages of retinoic acid–induced differentiation of embryonal carcinoma cells (Kadomatsu, K., M. Tomomura, and T. Muramatsu, 1988. *Biochem. Biophys. Res. Commun.* 151:1312–1318). *MK* gene has been predicted to code a polypeptide that is rich in basic amino acids and cysteine and is not related to any other peptides so far reported. In the present study, we investigated *MK* expression during mouse embryogenesis by in situ hybridization. The *MK* transcript was detected all over the embryo proper of the 7-d embryo, while it was not detectable in the 5-d embryo. The ubiquitous expression continued in the 9-d embryo proper. On the 11th–13th d of gestation, the sites where *MK* gene was intensely expressed became progressively restricted; these sites were the brain ectoderm around the lens and brain ventricles, the anterior lobe of the pituitary gland, the upper and lower jaw, the caudal sclerotomic half of vertebral column, the limbs, the stomach, and the epithelial tissues of the lung, the pancreas, the small intestine, and the metanephros. These areas include the region where secondary embryonic induction is prominent. In the 15-d embryo, only the kidney expressed *MK* significantly. These data suggest that *MK* gene plays a fundamental role in the differentiation of a wide variety of cells; *MK* gene may also play some specific roles in generation of epithelial tissues, and remodeling of mesoderm.

The development of a multicellular eukaryote is accomplished by complex processes of cell differentiation and morphogenesis. All *trans*-retinoic acid, or simply retinoic acid, which is a derivative of vitamin A (retinol), is involved in diverse processes of development and appears to be one of the key molecule coordinating development. Notably, a morphogen controlling the digit pattern formation in the chick limb bud has been identified to be retinoic acid (23, 24). Furthermore, retinoic acid induces differentiation of many kinds of cells in vitro, such as embryonal carcinoma cells and HL-60 leukemia cells (9, 17, 18, 22). The direction of differentiation of EC (embryonal carcinoma) cells can be altered by changing the concentration of retinoic acid (17).

The intracellular receptor for retinoic acid belongs to a superfamily of nuclear receptors, which includes steroid hormone receptors and thyroid hormone receptors (3, 20, 25). Therefore, retinoic acid complexed with the receptor is expected to control the expression of certain genes, which regulate subsequent steps in development. To find such a gene, we used an in vitro differentiation system of a clonal line of an embryonal carcinoma cell. By the differential hybridization technique, a cDNA clone was isolated, whose corresponding RNA is transiently expressed in early differentiation stages of EC cells (11). By RNA blot analysis, the RNA is detected in midgestation mouse embryos; among the adult organs, it is detected only in the kidney. Thus we tentatively call the gene *MK* (midgestation embryo and kidney). From the cDNA structure, *MK* gene is predicted to code a novel polypeptide rich in basic amino acid and cysteine. The sequence of *MK* cDNA is not related to any genes so far reported, including *ERA-1* whose expression also increases during early stages of retinoic acid–induced differentiation of EC cells (14).

Since precise localization of gene expression among tissues is helpful in considering the function of the gene product during embryogenesis (2, 28, 29), we have analyzed *MK* gene expression in mouse embryos by the in situ hybridization technique. The temporally and spatially controlled localization of *MK* transcript that we will describe is consistent with the potentially significant role of *MK* gene product to accomplish embryogenesis.

1. *Abbreviations used in this paper*: EC, embryonal carcinoma; pc, post coitum.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Materials and Methods

Embryos and Organs

Embryos were obtained from naturally mated ICR mice. The day when the vaginal plug was detected was taken as day 0 of pregnancy. Pregnant females were killed by cervical dislocation, and the uterus was excised and put into ice-cold Dulbecco's PBS. For the fixation, decidua containing 5- or 7-day post coitum (pc) embryos were immediately transferred to ice-cold 4% paraformaldehyde in Dulbecco's PBS and fixed overnight at 4°C. Later-stage embryos were separated from decidual and extraembryonic tissues, and were treated as above. Organs were obtained from mice at birth, at 44 d old and later stages. The excised organs were immediately treated for RNA extraction or immediately transferred into liquid nitrogen and stored at −80°C until RNA extraction.

RNA Extraction and RNA Slot Blot Analysis

Total cellular RNA was extracted by the guanidine, cesium chloride method (16). RNA was applied to RNA slot blot apparatus of Schleicher & Schuell Inc. RNA blots on a nitrocellulose filter were then hybridized with a ^{32}P-labeled *MK* probe (from Bal I site at residue 278 to 3′ end of MK1 cDNA) (11) prepared by using random oligonucleotide primer. β-Actin cDNA used as a reference probe was given by Dr. K. Shimada.

In Situ Hybridization

In situ hybridization was performed essentially as described by Wilkinson et al. (28). Briefly, fixed embryos were embedded in paraffin, and sections of 6-μm thickness were cut. Three sections at 6–18 μm intervals were applied for the hybridization with antisense or sense probe and the hematoxylin eosin staining. For in situ hybridization, sections were transferred to albumin-coated slides and dried overnight at 50°C. Then the slides were treated with 20 μg/ml proteinase K (Sigma Chemical Co., St. Louis, MO) and acetic anhydride before hybridization with probes (8). For preparation of the probe, the fragment of *MK* or β-actin cloned into SP64 or SP65 were transcribed in vitro with ^{35}S-UTP (∼800 Ci/mmole; Amersham), and the ^{35}S-labeled RNA was degraded to an average length of 100 bases (1). The hybridization solution consists of 0.03 ng/μl probe in 50% formamide, 0.3 M sodium chloride, 20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 10 mM sodium phosphate (pH 8.0), 10% dextran sulfate, 1× Denhardt's solution, 0.5 mg/ml yeast tRNA, and 20 mM DTT. The solution was applied to slides and covered with siliconized baked coverslips. Slides were immersed in prewarmed mineral oil and incubated overnight at 50°C. Slides were washed as described including the wash with 20 μg/ml ribonuclease A (Sigma Chemical Co.) for 30 min at 37°C (1). Slides were coated with nuclear emulsion (NR-M2; Konica), diluted one to one with distilled water, by the dipping method. These slides were stored under the low humidity at 4°C for 7-14 d or 3 wk (Figs. 2, *f* and *j*, 3 *j*, and 5 *f*), and developed with Rendol (Fuji Photofilm, Tokyo, Japan) for 4 min at 20°C.

Results

MK Transcript Is Broadly Distributed in 7- and 9-d Mouse Embryos

By in situ hybridization experiments, *MK* expression was not detected in 5-d mouse embryos. The density of silver grains on adjacent sections of 5-d embryos hybridized with either antisense or sense *MK* probe was similar and uniform in both the embryo and the maternal decidual tissue (Fig. 1, *b* and *c*).

However, *MK* expression was clearly detected in 7-d embryos. Using antisense *MK* probe, silver grains accumulated in the embryoblast (embryo proper), but not in the trophoblastic tissue (ectoplacental cone) nor in the maternal decidual tissue (Fig. 1 *e*). On the other hand, the sense probe scarcely gave the positive reaction (Fig. 1 *f*). Thus, *MK* expression was specific to the embryoblast. *MK* expression in the 7-d embryo appeared to be uniform and was not specific to a germ layer.

Uniform expression of *MK* was observed also in 9-d embryos; at the stage, the embryo just starts organogenesis. Closer examination did not reveal stronger expression (the otic vesicle, brain, somite, gut) (Fig. 1 *h* and data not shown). The sense probe gave a negligible reaction (Fig. 1 *i*).

Control experiments using β-actin probe supported the above conclusions: the antisense probe reacted with the 5-d embryo (Fig. 1 *m*) and ectoplacental cone of the 7-d embryo (Fig. 1 *n*), which was unreactive with *MK* antisense probe (Fig. 1, *e* and *o*).

MK Gene Expression Becomes Restricted in the 11–13-d Embryos

A complex expression pattern of *MK* was observed in 11-d embryos. Although *MK* transcript was detected at some level over the entire conceptus, there were several regions expressing *MK* distinctly greater than the surrounding portion (Fig. 2 *b*). They are the brain ectoderm around the lens and brain ventricles, the anterior lobe of the pituitary gland, the upper and lower jaw, the lung, the caudal sclerotomic half of the vertebral column, the limbs, the small intestine, the stomach, the pancreas, and the metanephros (the rudiment of the adult kidney) (Figs. 2 *b*, 3, 4, 5, and 6). In the 13-d embryo, the expression appeared to be more strictly restricted to the regions listed above (Figs. 2 *d*, 3, 4, 5, and 6). Using the sense probe, no significant signal was detected in these periods. In the mouse, 11-13-d pc coincides with the early organogenesis stage. Some of the organs expressing *MK* (e.g., the pituitary gland, the eye, the lung, the limbs, the pancreas, and the metanephros) are known to be formed by characteristic interactions of proximate tissues, called secondary embryonic induction (5). From this view, the restricted mode of *MK* expression in this period is provocative, and needs detailed description. Different sections to be described below were done on one 11th d embryo and on one 13th d embryo, although we confirmed the reproducibility of the result using different embryos.

In the pancreas, the endodermal epithelium outpocketing from the gut into the mesenchyme forms exocrine ducts and acini, interacting with the mesenchyme (5, 21, 27). *MK* transcripts were detected with a patchlike pattern that corresponded to the endodermal epithelium (Fig. 3, *d* and *f*). A similar mode of expression was observed in the small intestine (Fig. 3 *f*). Intense expression of *MK* took place in the inner surface, which corresponded to the endodermal epi-

Figure 1. MK expression on a 5-, 7-, and 9-d embryo. *MK* antisense (*b*, *e*, *h*, and *o*) and sense (*c*, *f*, and *i*) probes were hybridized to sections of a 5-d (*b* and *c*), 7-d (*e*, *f*, and *o*) and 9-d (*h* and *i*) embryo. As the positive control β-actin antisense probe was also hybridized with sections from a 5-d (*m*) and 7-d (*n*) embryo. Results are shown by dark field photomicrographs. Bright field photomicrographs (*a*, *d*, *g*, *j*, *k*, and *l*) represent the adjacent sections stained with hematoxylin and eosin. *a* corresponds to *b* and *c*; *d* to *e*, and *f*; *g* to *h* and *i*; *j* to *m*; and *k* to *n* and *l* to *o*. The blood cells are seen as large bright grains in both *MK* sense and antisense hybridization at 7-d pc (*e* and *f*, *arrows*) and in β-actin antisense hybridizations at 5-d pc (*m*, *arrow*). *Em*, embryoblast; *Ect*, ectoplacental cone. Bars, 200 μm.

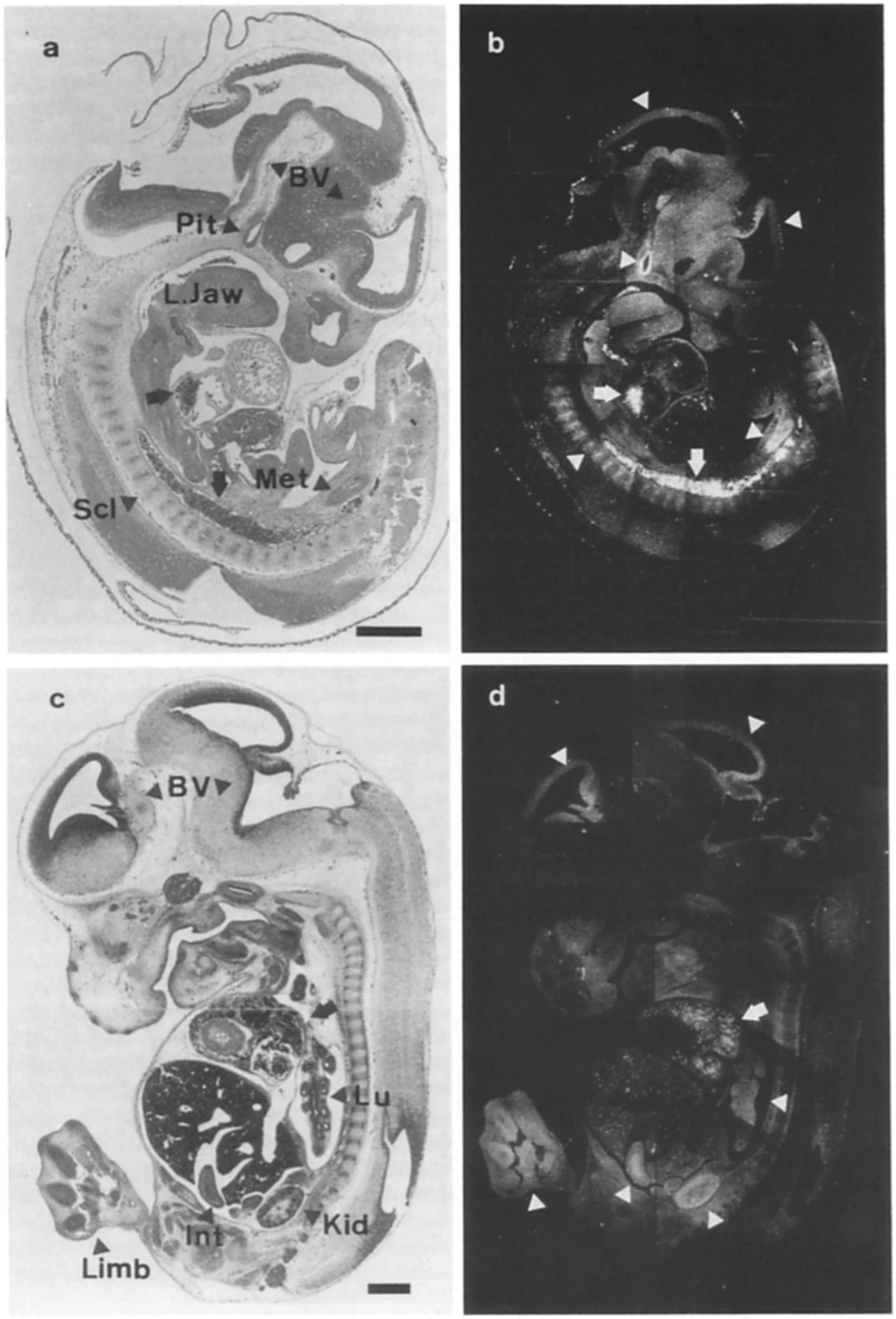


Figure 2. MK expression on an 11-d and 13-d embryo. *MK* antisense probe was hybridized to sections of an 11-d (*b*) and 13-d (*d*) embryo. Several pieces of dark field photomicrograph were combined to show the whole embryo (*b* and *d*). Bright field photomicrographs (*a* and *c*) represent adjacent sections stained with hematoxylin and eosin. Arrowheads indicate the areas of special interest whose anatomical names are described in *a* and *c*. Arrows indicate blood cells, thus, nonspecific signals. *BV*, brain ventricle; *Pit*, pituitary gland; *L. Jaw*, lower jaw; *Met*, metanephros; *Scl*, sclerotome; *Lu*, lung; *Kid*, kidney; *Int*, intestine. Bars, 500 μm.

thelium, but did not in the surrounding outer portion of the small intestine. It is noteworthy that the epithelium of pancreatic exocrine ducts and of small intestine were generated from the same endodermal epithelium, and they simultaneously expressed *MK* intensely although the surrounding mesenchymal tissues did not. A somewhat similar mode of *MK* gene expression was observed in the lung, another organ of endoderm origin; its epithelium intensely expressed *MK*, while another portion expressed *MK* to a considerably less degree (Fig. 3, *h* and *j*).

Restricted mode of *MK* expression was also observed in the pituitary gland (Fig. 4, *d* and *e*). Two ectodermal regions mutually interact with each other to form the pituitary gland (5). The oral plate ectoderm forms Rathke's pouch and becomes the anterior lobe of the pituitary gland. The brain ectoderm on the floor of the diencepharon forms an infundibular process and gives rise to the posterior lobe of the pituitary gland. *MK* transcript was detected intensely in the Rathke's pouch or anterior lobe of the pituitary gland. For the eye formation, mutual interaction between two ectoder-

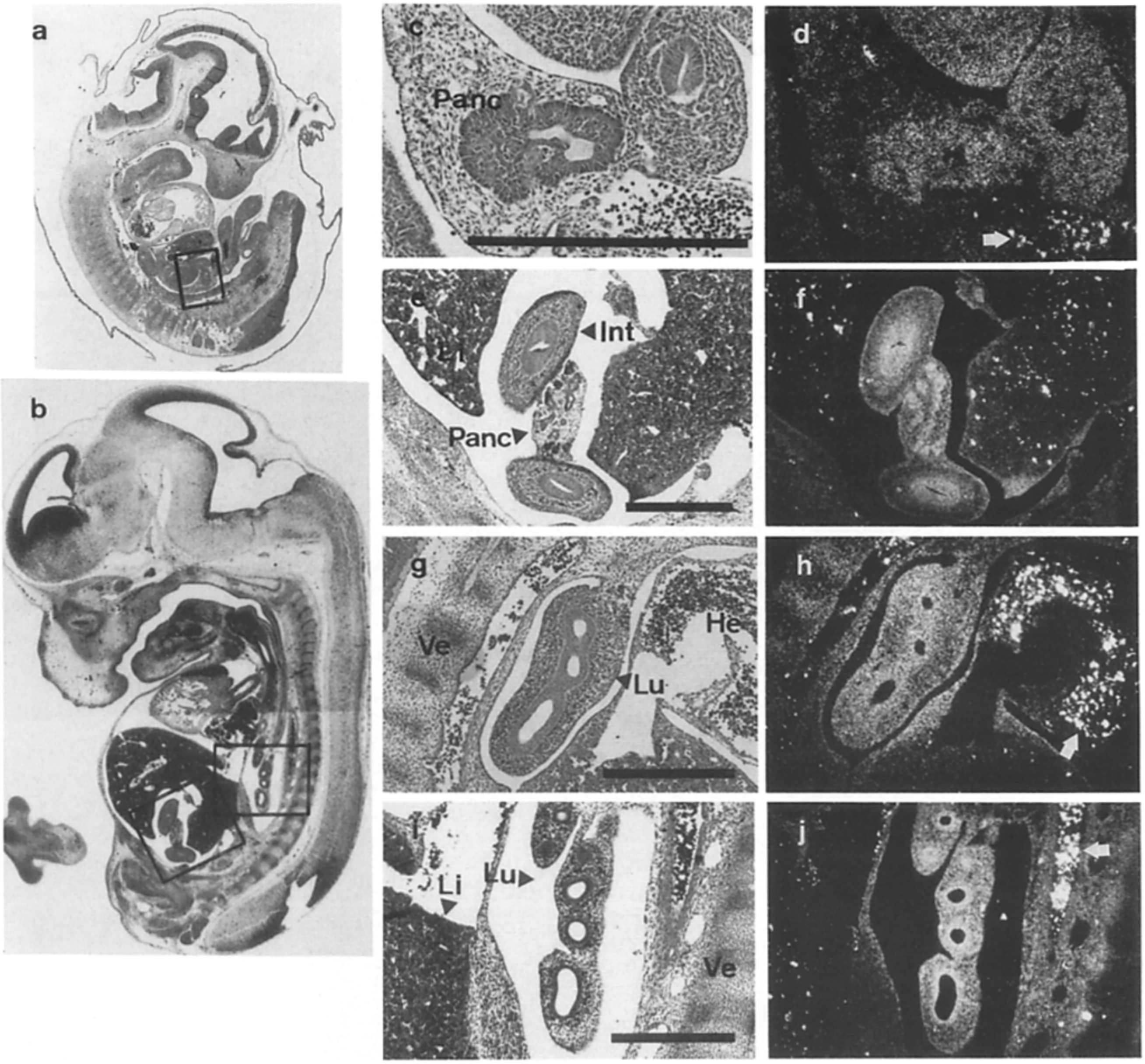


Figure 3. MK expression in the pancreas, the intestine, and the lung. Bright field (*a*, *b*, *c*, *e*, *g*, and *i*) and dark field (*d*, *f*, *h*, and *j*) photomicrographs of embryo sections stained with hematoxylin and eosin or hybridized with *MK* antisense probe. Sagittal planes are presented. *a*, *c*, *d*, *g*, and *h* are from an 11-d embryo, while *b*, *e*, *f*, *i*, and *j* are from a 13-d embryo. Squares in *a* and *b* are expanded in *c*, *e*, and *i*. Arrows indicate blood cells, and thus, nonspecific signals. The exposure time for *f* and *j* is 3 wk. *Panc*, pancreas; *Li*, liver; *Int*, intestine; *Lu*, lung; *He*, heart; *Ve*, vertebral column. Bars, 400 μm.

mal regions is also needed (5); one is the brain ectoderm (optic vesicle), and another is the head ectoderm which becomes the lens vesicle. The retina and the surrounding region intensely expressed *MK*, whereas the lens expressed it weakly (Fig. 4, *f* and *j*). The cornea differentiated from the lens epithelium expressed *MK* intensely (Fig. 4 *j*). Thus, as for the endodermal tissues mentioned above, the ectodermal tissues expressed *MK* with a specific fashion where proximate tissue interaction is prominent. Abundant *MK* transcripts were also detected in the brain ectoderm around the brain ventricles (Fig. 4, *k* and *l*).

In the mesodermal tissues, *MK* was also expressed in an interesting fashion. The caudal sclerotomic halves rapidly grow and become the major component of sclerotome at 11-d pc. *MK* was intensely expressed in these regions (Fig. 3 *h* and 5 *d*). On the 13-d pc, *MK* transcripts accumulated in the peripheral region of each vertebral body (Fig. 6 *f*).

Goulding and Pratt demonstrated that 13-cis-retinoic acid, a retinoic acid analogue, induced 8-d mouse embryos to develop anomalies, especially the size of the first and second pharyngeal arches is dramatically reduced (7). These regions are scheduled to form the upper and lower jaw construction and the middle ear. Thus, the homogeneous expression of *MK* in the upper and lower jaw (Fig. 5 *e*) is noteworthy considering the relationship of *MK* gene expression with retinoic acid.

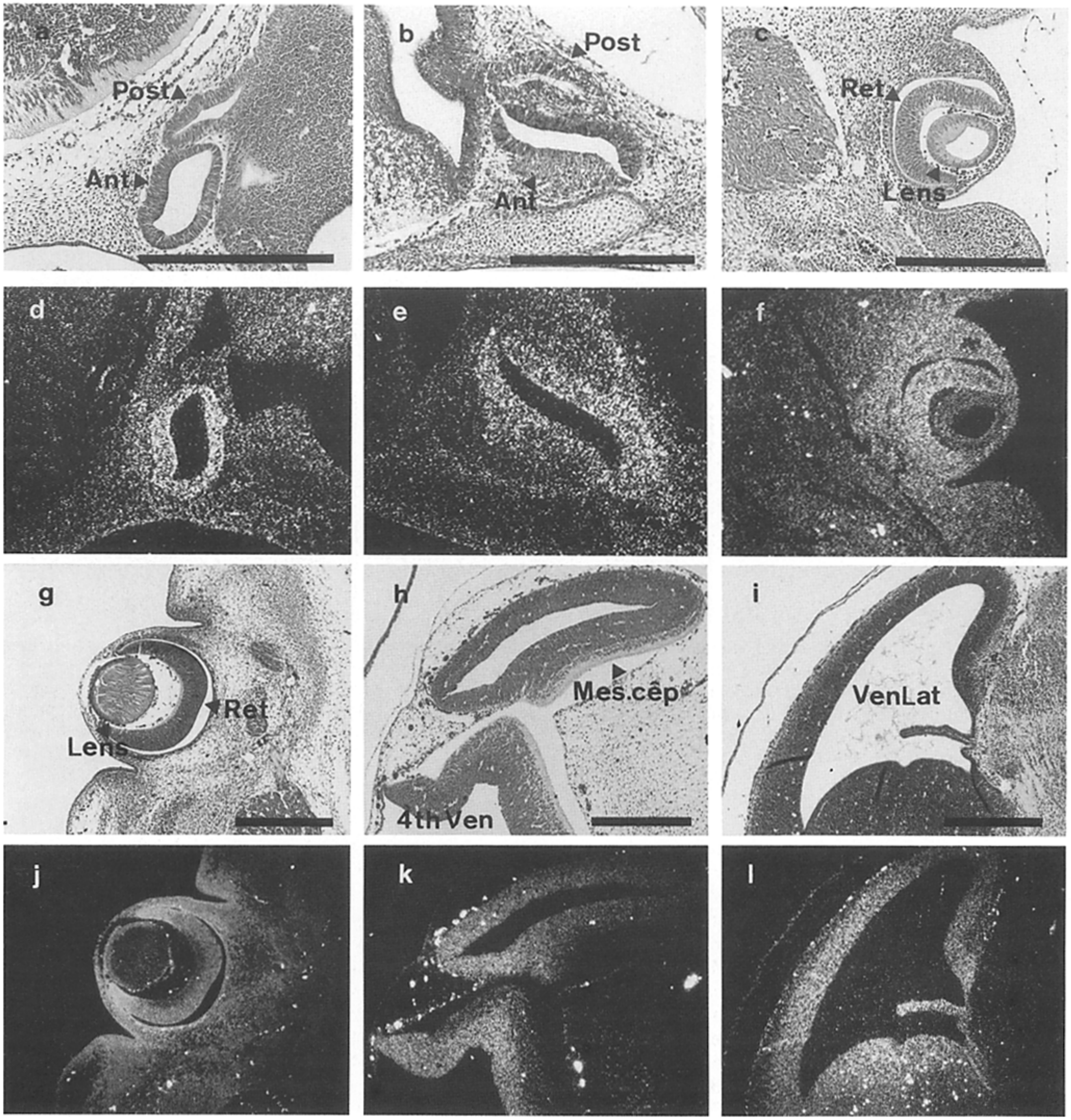


Figure 4. MK expression in the pituitary gland, around the eye and brain ventricles. *MK* antisense probe was hybridized to sections of an 11-d (*d*, *f*, and *k*) and a 13-d (*e*, *j*, and *l*) embryo. Dark field photomicrographs (*d*, *e*, *f*, *j*, *k*, and *l*) show the result, and bright field photomicrographs of the adjacent sections stained with hematoxylin and eosin are shown above the dark field photomicrographs (*a*, *b*, *c*, *g*, *h*, and *i*). Anterior (*Ant*) and posterior (*Post*) lobe of the pituitary gland are generated from the oral plate ectoderm and the brain ectoderm on the floor of the diencephalon, respectively. The exposure time for *j* is 3 wk; *Ret*, retina; *Mes cep*, mesencephalon; *4th Ven*, 4th ventricle; *Ven Lat*, lateral ventricule. Bars, 400 μm.

Limb formation has been extensively studied in the chick; the reciprocal induction between the apical ectoderm ridge and the mesenchyme, and the retinoic acid gradient required for the pattern formation of digits have been revealed in that system (5, 23). While the mechanism of the limb formation is poorly understood in the mouse, *MK* expression was almost uniform in the limb during 11–13 d pc, and no gradient of the expression was observed, except for the center of precartilage (the rudiment of limb bone) where the expression was poor (Fig. 5 *f*).

In the 15-d embryo, *MK* gene expression was significantly detected in the kidney but not significantly in any other organs (Fig. 6, *h*, *j*, and *l*).

MK Expression Is Observed Continuously in the Kidney

RNA blot study has indicated that in the adult, the kidney expresses *MK* but other organs so far examined do not (11). Thus, we closely examined *MK* expression in the developing

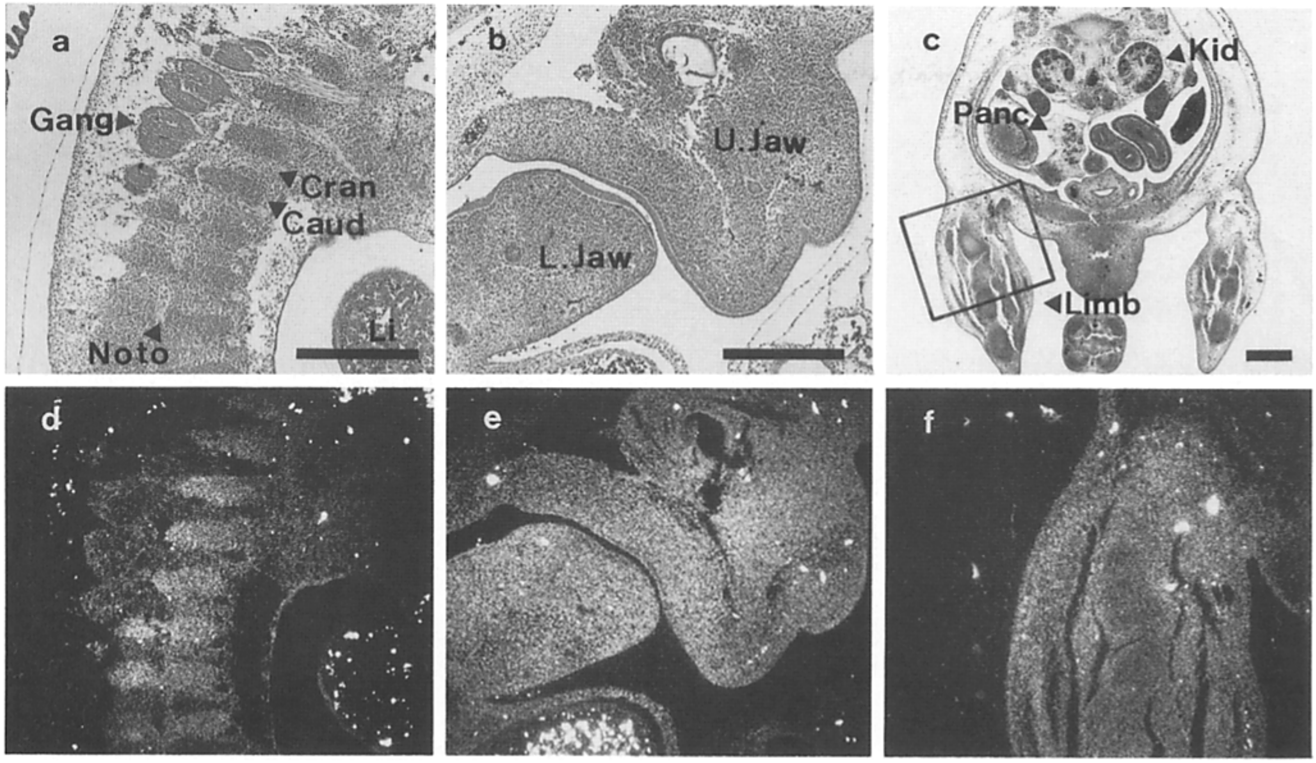


Figure 5. MK expression in the vertebral column, the upper and lower jaw, and the limb. *MK* antisense probe was hybridized to sections of an 11-d (*d* and *e*) and 13-d (*f*) embryo. Dark field micrographs (*d*, *e*, and *f*) show the result, and bright field photomicrographs of the adjacent sections stained with hematoxylin and eosin (*a*, *b*, and *c*) are shown above the dark field microphotographs. An 11-d embryo is presented with sagittal plane, and a 13-d embryo is shown by transverse section at the kidney level. The blood cells in the heart present nonspecific signals (*e*). *Gang*, ganglion; *Noto*, notochord; *Li*, liver; *Cran*, cranial sclerotomic half in the vertebral column; *Caud*, caudal sclerotomic half in the vertebral column; *Kid*, kidney; *Panc*, pancreas. Bars, 400 μm.

kidney. The metanephros, which is the rudiment of the kidney, appears on the 11-d pc. The ureteric buds extend from the Wolffian duct and become surrounded by condensed metanephrogenic mesenchyme (5). *MK* transcript accumulated uniformly in both the ureteric bud and the metanephrogenic mesenchyme (Fig. 6 *d*). Thus, *MK* is already expressed at the onset of the kidney formation. The kidney continued to express *MK*, but its localization became patch-like along with the progress of embryogenesis (Fig. 6, *e*, *f*, and *h*). Longer exposure of in situ hybridization demonstrated that *MK* transcripts accumulated in the epithelial tissue of the kidney in the 13-d embryo (Fig. 6 *f*).

RNA slot blot analysis was carried out to confirm some of the data obtained by in situ hybridization. Both the neonatal and adult kidney expressed *MK*, while other organs, especially the pituitary gland and the small intestine which expressed *MK* at 11–13-d pc and ceased to express by the 15-d pc had no or hardly detectable *MK* RNA in the adult (Fig. 7). It may be noted that the neonatal kidney expressed more *MK* RNA than the adult kidney. Finally, the absence of *MK* transcript in the brain, spleen, and testis of the adult mice was shown previously by RNA slot blot analysis (11).

Discussion

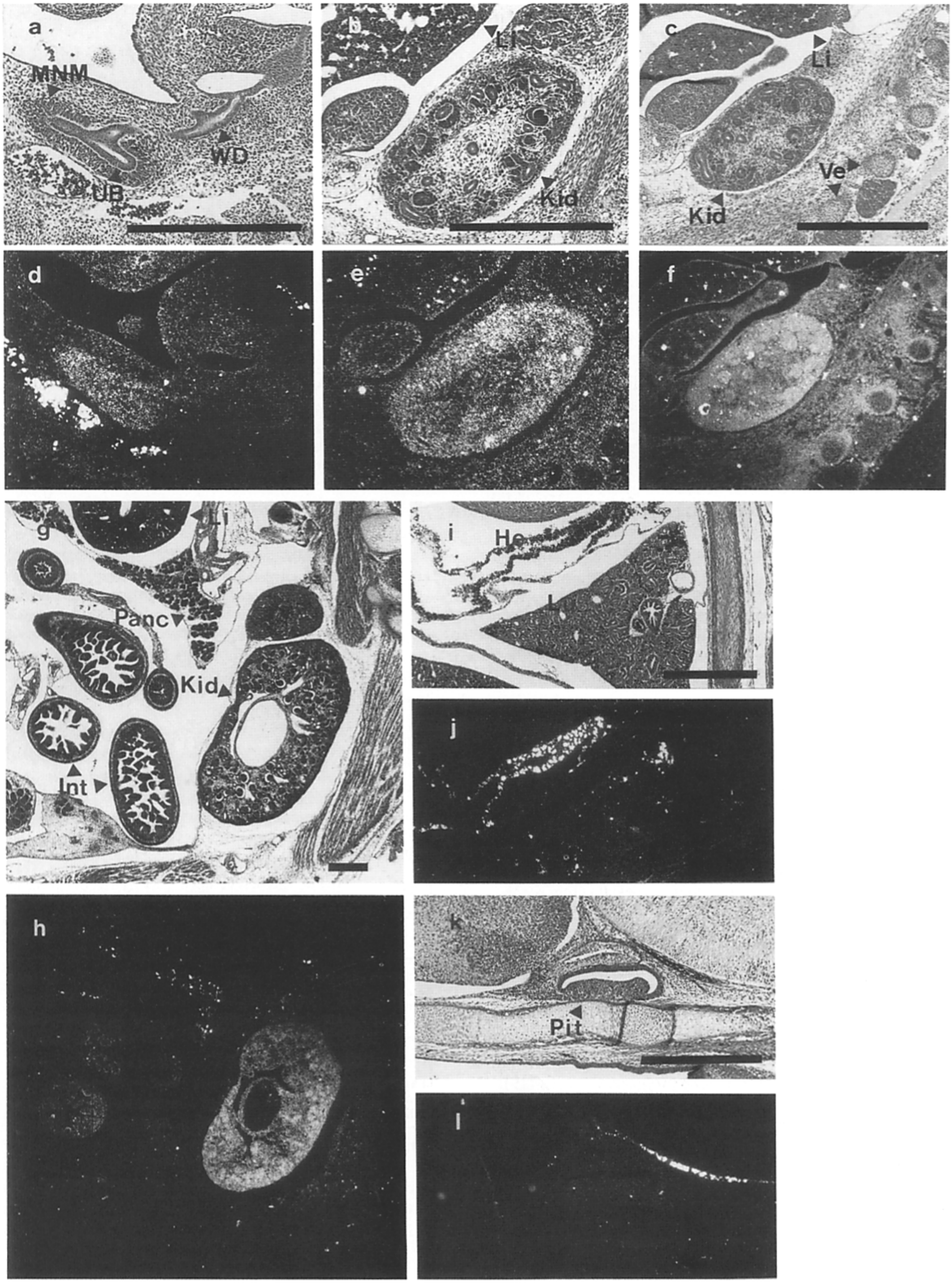
The mode of *MK* expression during mouse embryogenesis has been clarified as the result of the present investigation. *MK* expression becomes detectable in the entire embryoblast of the 7-d embryo. The wide spread expression is also observed in the 9-d embryo. In the 11–13-d embryo, the site expressing *MK* becomes progressively restricted, and in the 15-d embryo only the kidney expresses it. The progressive decrease of *MK* expression observed in the midgestation embryo is generally consistent with the result of RNA blot analysis in our previous study (11). The gradual restriction of the expression to the kidney also agrees with the result of the previous study. While *MK* RNA was scarcely detectable in the 13-d embryo by RNA blot, several sites in the embryo clearly expressed *MK* RNA as the result of in situ hybridization.

From the mode of *MK* gene expression, we can speculate its function. First of all, the *MK* gene is expressed transiently in all cell lineages (Fig. 8). Thus, it is reasonable to think that *MK* gene product is generally involved in some process required for cell differentiation. Furthermore, the transient expression mode indicates that *MK* is not a housekeeping gene nor presents the phenotypes of terminally differentiated cells.

The restricted mode of *MK* expression in the 11–13-d embryo needs special attention, and the following four points are worthy of mention.

(*a*) The area expressing *MK* can be generally considered to be in the intermediate stage of differentiation. For example, in the endodermal epithelium of the pancreas, pancreas-specific enzymes are not produced significantly at the period. 3–5 d later when *MK* expression ceases, the level of the enzyme activity increases ~1,000-fold (21, 27).

(*b*) As has been mentioned, some of the area intensely ex-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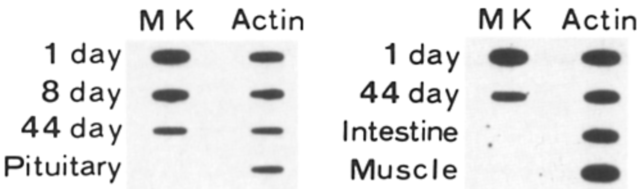

Figure 7. *MK* expression in postnatal periods. RNAs from the kidney (1, 8, and 44 d after birth), the pituitary gland, the muscle, and the intestine (from the adult mice) were blotted to a nitrocellulose filter that was then hybridized with ^{32}P-labeled *MK* cDNA or β-actin probe. Each spot contains about 5 μg RNA, and the exposure time is 24 h.

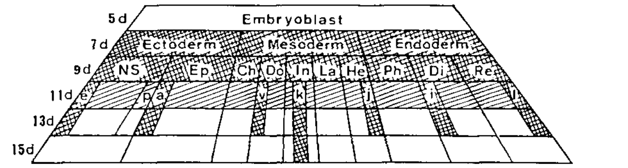

Figure 8. Temporal and spatial pattern of *MK* expression during mouse embryogenesis. The mode of *MK* expression visualized by in situ hybridization is summarized. Chordamesoderm (*Ch*) produces the notochord. Dorsal mesoderm (*Do*) produces bone, muscles, cartilage, and dermis. Intermediate mesoderm (*In*) produces the urogenital system. Lateral mesoderm (*La*) produces heart, blood vessels, and blood cells. Head mesoderm (*He*) produces muscles of the face (4). The box corresponding to the intermediate mesoderm in 11-d embryo consists of the genital ridge, the mesonephros, and the metanephros. Thus, the box *K* in 11-d represents metanophros. *NS*, nervous system; *Ep*, epidermis; *Ph*, pharynx; *Di*, digestive tube; *Re*, respiratory tube; *e*, neural ectoderm around the eye and brain ventricle; *p*, posterior lobe of pituitary gland; *a*, anterior lobe of pituitary gland; *v*, limbs and caudal sclerotomic half of vertebral column; *k*, kidney; *j*, upper and lower jaw; *i*, intestine and pancreas; *l*, lung. ▦, *MK* is intensely expressed. ▨, *MK* expression is moderate.

pressing *MK* gene is the site where secondary embryonic induction takes place.

(*c*) When two or more tissues interact to form an organ, *MK* is intensely expressed in the epithelial tissue; the epithelium is derived either from the ectoderm (the anterior lobe of the pituitary gland and the retina), from the endoderm (the epithelium of the lung, the pancreas, and the intestine), or from the mesoderm (the epithelium of the kidney). Therefore, *MK* gene product may be involved in generation of epithelial tissues or their interaction with other tissues.

(*d*) Mode of *MK* expression in embryonic mesoderm may also be worth comment. The somites differentiate into the sclerotome, myotome, and dermatome between 11-d to 13-d pc. The caudal half of one sclerotome grows rapidly, fuses with the cranial half of the sclerotome immediately caudal to it, and eventually forms a vertebral body. Thus, each vertebral body originates from two adjacent somites. *MK* expression in the caudal half of each sclerotome and then in the peripheral region of each vertebral body suggests that *MK* not only plays some organizational role in the morphogenesis of the vertebra, but it has some additional role in the subsequent chondrification. This possible role in the remodeling sequence of the embryonic mesoderm may also explain *MK* expression in the limb and the upper and lower jaw.

The molecular nature of *MK* transcript is also helpful in considering its function. A polypeptide defined by *MK* gene is rich in basic amino acids and cysteine. Results recently obtained in our laboratory suggest that *MK* polypeptide is a secretory one (M. Tomomura, K. Kadomatsu, S. Matsubara, and T. Muramatsu, submitted for publication). These molecular features of the predicted *MK* polypeptide is reminiscent of a growth factor. The importance of growth factors in the control of development has been well documented recently. Notably, members of the TGF-β family play critical roles in mesoderm induction in *Xenopus* embryos (12, 26) and in the formation of dorsal structures in *Drosophila* embryos (19). As to the expression in the kidney, there is some possibility that *MK* polypeptide acts as a hormone secreted by the kidney to stimulate differentiation of certain cells in later stages and adulthood, just like the case of erythropoietin (10, 13, 15). We are currently studying whether *MK* polypeptide works as a growth factorlike substance.

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Figure 6. *MK* expression in the kidney. Bright field (*a*, *b*, *c*, *g*, *i*, and *k*) and dark field (*d*, *e*, *f*, *h*, *j*, and *l*) photomicrographs of embryo sections stained with hematoxylin and eosin or hybridized with *MK* antisense probe. *a* and *d* are from an 11-d embryo; *b*, *c*, *e*, and *f* are from a 13-d embryo; and *g*, *h*, *i*, *j*, *k*, and *l* are from a 15-d embryo. On 11-d pc the ureteric bud (*UB*) extends from the Wolffian duct (*WD*) and comes in contact with the metanephrogenic mesenchyme (*MNM*) to form metanephros, the rudiment of adult kidney. Note that positive signals were not detected in the intestine, the pancreas, the lung, nor the pituitary gland in the 15-d embryo (*h*, *i*, and *l*). The exposure time for *f* is 3 wk. *Kid*, kidney; *Li*, liver; *Int*, intestine; *Panc*, pancreas; *Ve*, vertebral body. Bars, 500 μm.

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