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Original Article

HMG-17, a chromosomal non-histone protein, shows developmental regulation during organogenesis

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ABSTRACT We used the differential hybridization technique for isolating developmentally regulated genes from the mouse metanephric kidney. In this screening, we identified the cDNA encoding high-mobility-group protein 17 (HMG-17), a chromosomal non-histone protein which modulates the conformation of transcriptionally active chromatin. Using Northern blot analysis, the HMG-17 mRNA was strongly expressed during embryogenesis and downregulated in various adult murine organs. At the histological level, the transcript localized to differentiating tissue regions and was apparently downregulated in mature structures indicating that HMG-17 expression is linked to cell differentiation. HMG-17 can thus be regarded as a general marker for tissues or cells undergoing differentiation during organogenesis.

KEY WORDS: *proliferation, cell differentiation, high-mobility-group proteins, differential hybridization*

Introduction

The developing kidney offers an excellent model system for cloning differentially expressed genes involved in the epithelial-mesenchymal interactions that guide the development and in the epithelial cell differentiation that follows these interactions. The development of the mouse metanephric kidney starts on embryonic day 11 when an epithelial ureter bud bulges from the Wolffian duct and invades the metanephrogenic mesenchyme. The subsequent events depend on reciprocal inductive interactions between these two tissue types. The mesenchymal cells surrounding the ureter bud induce the bud to branch, and these branches eventually form the collecting duct system of the kidney. In response to signals from the ureter tree, the mesenchymal cells undergo mesenchyme-to-epithelium transition: They aggregate and, through several intermediate stages, form the epithelium of the nephrons, the secretory units of the kidney (Saxén, 1987).

Expression of various types of molecules, including growth factors and their receptors, signaling molecules, second messengers, transcription factors and various intra- and extracellular structural proteins has been reported at different timepoints throughout the induction, cell differentiation and morphogenesis of the kidney (Bard *et al.*, 1994,1996; Lechner and Dressler, 1997; Vainio and Müller, 1997). Molecules essential for early kidney development include Wilms tumor suppressor gene 1 (*WT-1*) (Kreidberg *et al.*, 1993), the glial-cell-line-derived neurotrophic factor (*GDNF*) and its tyrosine kinase receptor *c-Ret* (Durbec *et al.*, 1996; Treanor *et al.*, 1996). *WT-1* and GDNF are expressed in the metanephric mesenchyme at the onset of kidney development (Pritchard-Jones *et al.*, 1990; Armstrong *et al.*, 1992; Hellmich *et al.*, 1996) , and *c-Ret* is abundant in the epithelial ureter bud (Pachnis *et al.*, 1993; Avantaggiato *et al.*, 1994). The knock-out mice of these molecules show a similar phenotype: The kidneys are either totally missing or severely abnormal, leading to death during embryogenesis or soon after birth (Kreidberg *et al.*, 1993; Schuchardt *et al.*, 1994,1996; Moore *et al.*, 1996; Pichel *et al.*, 1996; Sanchez *et al.*, 1996). Some molecules, e.g., the transcription factor *Pax-2*, are required only for a certain phase of kidney development whereafter they are downregulated. In transgenic mice, which constantly express *Pax-2*, the nephrons develop abnormally (Dressler *et al.*, 1990, 1993; Dressler and Douglass, 1992).

Several methods have been employed for cloning developmentally regulated genes (Maser and Calvet, 1995; Wan *et al.*, 1996). These include differential cDNA library screening

Abbreviations used in this paper: BrdU, bromodeoxyuridine; EDTA, ethylenediaminetetraacetic acid; EST, expressed sequence tag; FCS, foetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDNF, glial-cell-line-derived neurotrophic factor; HMG-17, high-mobility-group protein 17; MEM, Eagle's Minimal Essential Medium; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS, sodium dodecyl sulphate; WT-1, Wilms tumor suppressor gene 1.

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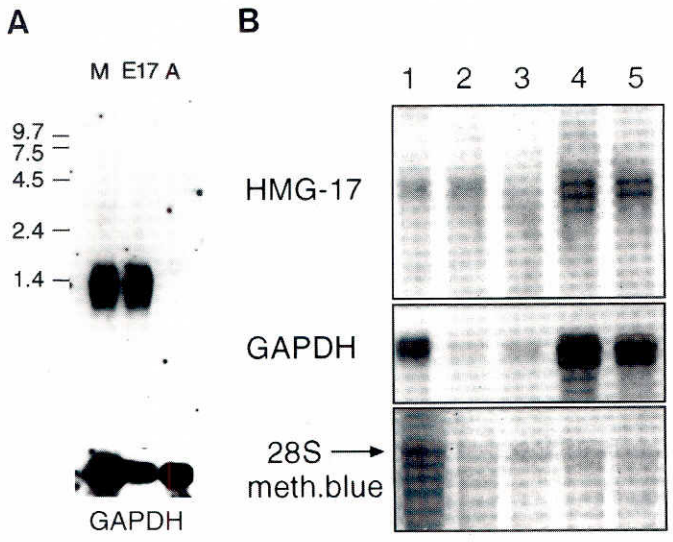

Fig. 1. Northern blot analysis of HMG-17 expression. (A) *Expression of HMG-17 during kidney development. Lanes: metanephrogenic mesenchyme (M), embryonic day-17 kidney (E17) and adult kidney (A). The film was exposed at -70°C for four days. HMG-17 is expressed at a high level in the undifferentiated metanephrogenic mesenchyme and the 17-day embryonic kidney; in the adult kidney the transcript is not discernible. GAPDH probing was performed to control the intactness of the RNA.* **(B)** *Expression of HMG-17 in different adult mouse organs. Lanes: 1, kidney; 2, lung; 3, intestine; 4, heart; 5, brain. To detect a signal in the weakly positive organs, the film was exposed at -70°C for 14 days which is an overexposure compared to Figure 1A. HMG-17 is detected at a low level in heart and brain and hardly at all in kidney, lung and intestine. To show the amounts of RNA loaded, the filter was stained with methylene blue. The intensity of the ribosomal 28S RNA is roughly similar in the different lanes, except in lane 1 (kidney), in which the amount loaded is higher.*

(Sargent, 1987; Almendral *et al.*, 1988), subtractive cloning (Travis and Sutcliffe, 1988; Rubenstein *et al.*, 1990; Barila *et al.*, 1994), and differential display (Liang and Pardee, 1992; Liang *et al.*, 1993; Kretzler *et al.*, 1996). In all of these techniques, differentially expressed genes are identified based on different mRNA levels in different cell types or in the same cell type in altered conditions. In this way, it is possible to recognize changes in gene expression occurring, e.g., upon a pathological process or at different stages of organ development. Cell-type specific cDNA:s have also been identified by text searches of the expressed sequence tag (EST) databases (Fujita *et al.*, 1998).

We chose the differential screening technique for identifying genes which are induced or downregulated during kidney organogenesis. In the kidney model system, it is feasible to separate the epithelial and mesenchymal cell lineages from each other before differentiation occurs. We used here a probe prepared from isolated mesenchymes that represent the undifferentiated tissue, in combination with probes prepared from kidneys at various stages of development. As compared to screenings using whole kidney tissue material (Kretzler *et al.*, 1996), this approach offers a better chance for identifying genes that are involved in the mesenchyme-to-epithelium transition. One of the sequences identified in our differential screening analysis was high-mobility-group protein 17 (HMG-17). We show that HMG-17 is downregulated during organogenesis and that its expression is coupled to cell differentiation rather than to proliferation during development.

Results

HMG-17 is downregulated during organogenesis

A cDNA library prepared from 17-day embryonic mouse kidney was screened for genes expressed differentially at different developmental stages of the kidney. The cDNAs were characterized by determining their nucleotide sequence and by confirming the differential expression of the potentially interesting ones by Northern blotting using total RNA derived from undifferentiated metanephric mesenchymes and embryonic day-17 and adult kidneys. In this way, the cDNA encoding HMG-17 was identified as one displaying clear developmental regulation: the HMG-17 mRNA of 1.4 kb was expressed in the undifferentiated metanephrogenic mesenchyme and the 17-day embryonic kidney and then downregulated during adulthood (Fig. 1A). Northern blot analysis of adult tissues revealed, however, a low level of HMG-17 expression in heart and brain; hardly any signal was detectable in kidney, lung and intestine (Fig. 1B).

Localization of the HMG-17 transcript in the 14-day embryo

In situ hybridization of 14-day whole embryo sections revealed wide HMG-17 expression although the levels of the mRNA in different organs varied (Fig. 2A-D). Hybridization with the sense control probe gave no signal (Fig. 2E-F). In the liver, the transcript concentrated in the differentiating hepatocytes whereas the hematopoietic cells showed hardly any signal (Fig. 2A-B). In the intestine and pancreas both the epithelial and mesenchymal tissue components were positive for HMG-17 (Fig. 2A-B). Also in the heart the transcript was evenly distributed (Fig. 2C-D). In the lung, the bronchial and respiratory systems start to develop on embryonic day 14 (Ten Have-Opbroek, 1991). At this stage HMG-17 was abundant both in the bronchial branches and the mesenchymal cells with the exception of the mesenchymal cells surrounding the central bronchioles which were negative (Fig. 2C-D).

Localization of the HMG-17 transcript in embryonic day-17 organs

On embryonic day 17, the expression of HMG-17 in several organs was more restricted than on embryonic day 14. In the kidney, the transcript was abundant in the cortical collecting ducts and in the nephronogenic region, which contains undifferentiated mesenchymal cells and differentiating epithelial structures, such as comma- and S-shaped bodies (Fig. 3A-B). In the more mature structures of the nephron, the proximal tubules and glomeruli, as well as in the stromal mesenchyme and the epithelial cells of the pelvis, HMG-17 was already downregulated.

In the heart, the myocardium of both the ventricles and the atria expressed HMG-17 (Fig. 3C-D). The endocardium was also positive. This was well presented in the atrioventricular valve, where the endocardial cells showed a strong signal. The myocardium of the valve, which contains more dense connective tissue than cardiac muscle, was only weakly labeled (Fig. 3C-D).

In the lung, marked developmental changes occur after embryonic day 16 (Ten Have-Opbroek, 1991). The bronchial and respiratory systems develop further and formation of the alveolar ducts and terminal alveolar sacs begins. The ciliated columnar epithelial cells of the terminal bronchioles and low columnar or cuboidal epithelial cells of the respiratory bronchioles were strongly positive for the HMG-17 mRNA (Fig. 3E-G). When the bronchioles

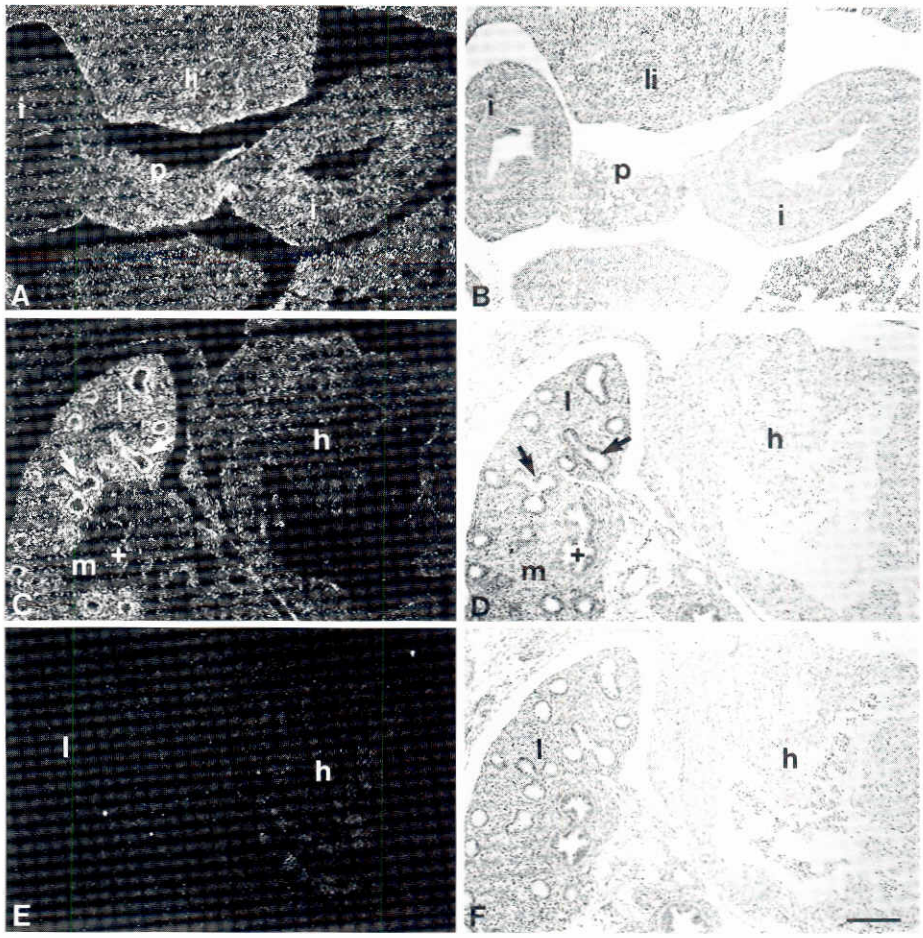

Fig. 2. Localization of the HMG-17 transcript in day 14 mouse embryos. *In situ hybridization with HMG-17 antisense (A-D) and sense (E-F) probes. A,C and E are darkfield images and B,D and F the corresponding brightfield images.* **(A-B)** *The liver (li), intestine (i) and pancreas (p) show evenly distributed HMG-17 signal.* **(C-D)** *In the lung (l), HMG-17 is abundant in the mesenchymal cells and branches of the bronchioles (arrowhead). Mesenchymal cells (m) surrounding the central branch of the bronchiole (+) have ceased to express HMG-17. In the heart (h) HMG-17 is expressed homogeneously.* **(E-F)** *A section hybridized with the sense probe shows no signal. (l), lung; (h), heart. Bar, 200 μm.*

continue as alveolar ducts and alveolar sacs, the epithelial cell morphology changes to a flattened one (Fig. 3H-I), which is better suited for the exchance of gases taking place in the alveoli after birth. These flattened cells showed hardly any HMG-17 expression. Adjacent alveolar sacs are separated by the interalveolar septum, which consists of supporting connective tissue and three main types of cells, viz., type I cells, type II cells and endothelial cells. Type I cells or flattened surface epithelial cells line all alveolar spaces. Type II cells are able to proliferate; they can also transform to type I cells and thus are the main source of renewed cells lining alveoli (Ten Have-Opbroek, 1981,1991). The interalveolar septa showed weak HMG-17 expression except in the periphery of the lung, i.e., in the region of alveolization, where the transcript was abundant.

In the embryonic day-17 intestine, the HMG-17 transcript concentrated in the developing crypts between the villi and the underlying muscular and connective tissue layers (Fig. 4A-B). In some villi, the HMG-17 signal extended a few cell layers along the villus whereas the tips were consistently negative. The cells of the villi differentiate from stem cells located in the crypts. While differentiating, these cells migrate along the villus and finally bud off from the tip (Gordon and Hermiston, 1994). The expression of HMG-17 correlated well with this differentiation gradient.

In the liver, the HMG-17 mRNA was abundant in the differentiating hepatocytes whereas the haematopoietic cells contained hardly any transcript (Fig. 4C-D). Cells surrounding the hepatic and central veins were positive. Some of these small groups of cells resembled ductal structures.

At this stage of development the spleen contains both developing hematopoietic and lymphatic tissue. The latter concentrates in the periphery of the organ and around the large vessels. The HMG-17 mRNA mainly localized to the lymphatic tissue whereas the haematopoietic cells showed hardly any signal (Fig. 4E-F).

In embryonic day-17 testis the HMG-17 probe hybridized to the developing tunica albuginea and the condensing mesenchymal cells underlying it (Fig. 4G-H). Expression was also observed in the developing interstitium, which at this stage consists of differentiating mesenchymal and Leydig cells, still indistinguishable from each other. The seminipherous tubules were also positive.

Proliferation during organogenesis

To find out whether HMG-17 expression correlates with proliferation, we labeled embryonic day-14 and day-17 embryos *in utero* with bromodeoxyuridine (BrdU). In the embryonic day-14 lung both the epithelial and mesenchymal tissue components were positive for BrdU (Fig. 5A). In the liver (Fig. 5B) and the heart (not shown), proliferating cells were also present all over the organ. In the intestine the epithelial cells were at this stage dividing slightly more actively than the cells of the muscular layer (not shown). Also, in the pancreas the epithelial branches contained more dividing cells than the surrounding mesenchymal tissue (Fig. 5B). In the lung on embryonic day 17 the cells of the

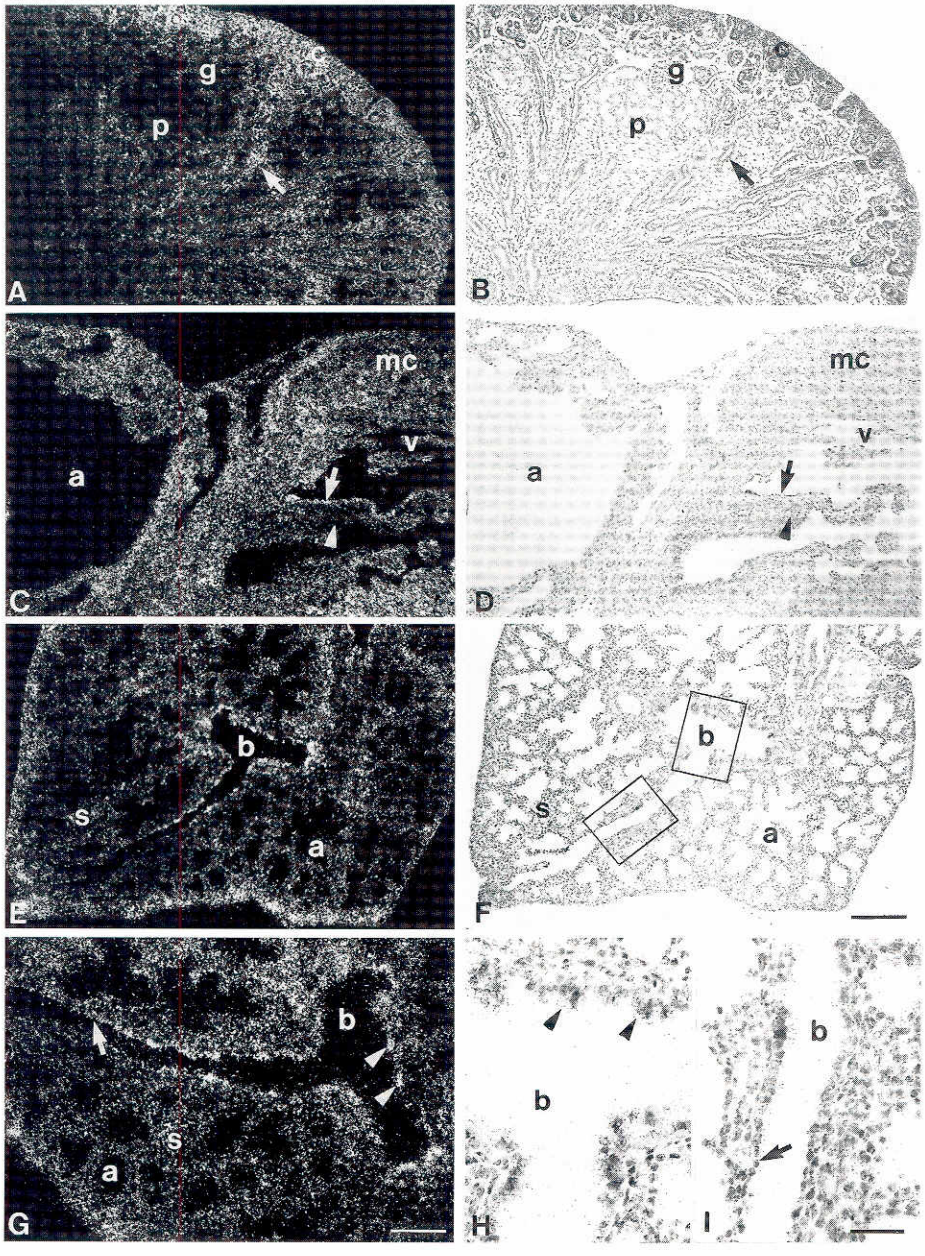

Fig. 3. Localization of the HMG-17 transcript in embryonic day-17 organs. *A,C,E and G are darkfield images, B,D and F brightfield images of A,C and E. Higher magnifications of F: The upper rectangle is shown in H and the lower one in I.* **(A** *and* **B)** *The HMG-17 mRNA is abundant in the peripheral collecting ducts (arrow) and cortical region (c) of the kidney, containing undifferentiated mesenchyme, condensing mesenchymal cells and early stages of differentiating epithelia. The transcript is downregulated in more mature structures, such as proximal tubules (p) and glomeruli (g).* **(C** *and* **D)** *In the heart, the HMG-17 mRNA is evenly expressed in ventricular (v) and atrial (a) myocardium (mc) and endocardium; the endocardial cells lining the atrioventricular valve are marked with an arrow. The myocardium of the valve, indicated by an arrowhead, shows less signal.* **(E-I)** *In the lung, HMG-17 is abundant in the periphery of the organ and in the developing bronchioles (b). Expression declines in the region (arrow in Figures G and I) where the cuboidal cells of the bronchioles abruptly change into flattened epithelial cells lining the alveolar sacs. Arrowheads in G and H indicate ciliated columnar epithelial cells lining the bronchioles, expressing HMG-17 at a high level. The cells between them, showing hardly any signal, are either nonciliated Clara-cells or small alveoli. Cells of the interalveolar septa (s) between alveoli (a) are weakly positive. Bar, 200 μm in A-F; 100 μm in G and 50 μm in H-I.*

interalveolar septum, evidently the developing type II cells (Ten Have-Opbroek, 1981,1991), were proliferating most actively, especially around the newly forming alveoli (Fig. 5C). The epithelial cells of the developing bronchial system showed very little BrdU signal; these cells were, however, strongly positive for the HMG-17 mRNA (Fig. 3E-I). In the intestine, strong BrdU labeling indicating rapid cell division was detected in the crypts between the villi and also in the underlying muscle and connective tissue layers (Fig. 5D). These cells and, differing from the region of BrdU-positivity, often also a few cells extending along the villus were positive for the HMG-17 transcript (Fig. 4A-B). In the heart and liver, proliferation was observed almost uniformly throughout the organs (not shown). In comparison, the HMG-17 signal in the liver concentrated in the hepatocytes and cells surrounding the hepatic and central veins (Fig. 4C-D).

Discussion

Differential expression of HMG-17

In this study we utilized the differential hybridization technique to isolate developmentally regulated cDNAs from the mouse kidney. HMG-17 was identified as a clone expressed at a high level during embryonic kidney development and downregulated in the adult organ. HMG-17 is a chromosomal non-histone protein, which has previously been shown to function in generating or maintaining the chromatin conformation of transcriptionally active genes (Albanese and Weintraub, 1980; Weisbrod *et al.*, 1980; Schröter and Bode, 1982; Weisbrod, 1982). HMG-17 binds to core histones and arranges chromatin into a more open conformation, thereby facilitating the access of the transcriptional machinery to the target sequence; it also increases the turnover of the transcriptionally

active templates (Trieschmann *et al.*, 1995a,b; Tremethick and Hyman, 1996). Considering the important regulatory function of HMG-17, little is known about its role in cell proliferation or in differentiation and development, processes involving major changes in gene expression.

HMG-17 concentrates in differentiating cells during organogenesis

Organogenesis is characterized by strictly controlled up- and downregulation of genes, some of which are expressed in a wide range of tissues and cell types, while others are active in very limited regions or specific cellular populations. The function of HMG-17 in regulating the structure of transcriptionally active genes suggests that it could be expressed in actively differentiating parts of the embryonic organs. This led us to study the HMG-17 mRNA localization in various organs at different developmental stages. The stages chosen for the analysis were embryonic day 14, when most of the organs undergo active differentiation, and embryonic day 17, when both mature and still differentiating structures are present.

In situ hybridization of 14-day embryos revealed that the HMG-17 transcript was widely expressed in the embryo, although the expression level varied between and within different organs. For example, the heart showed a rather weak signal as compared to the lung. In the lung the bronchial branches and mesenchymal cells in the periphery exhibited abundant HMG-17 expression whereas the mesenchymal cells surrounding the more central branches of the bronchioles showed weak or negligible expression.

At a later developmental stage, on embryonic day 17, the differences were more dramatic. In some organs, such as the kidney, lung and intestine, it is possible to distinguish actively differentiating regions from mature structures, which renders these organs particularly suitable for studying the correlation between cell differentiation, proliferation and expression of a specific molecule. In the kidney, HMG-17 was abundant in the cortical collecting ducts and the nephronogenic region whereas the more mature structures had already ceased to express HMG-17. Also the lung is differentiating actively at this stage, especially the bronchial and alveolar systems, which were positive for HMG-17. In the intestine HMG-17 expression concentrated in the crypts harboring the stem cells, which divide and differentiate into epithelial cells, as well as the underlying muscular and connective tissue layers. In the embryonic day-17 liver HMG-17 localized rather to differentiating hepatocytes and small ductal type structures than to hematopoietic cells. The heart showed fairly ubiquitous signal throughout the organ. In the spleen the signal concentrated in the lymphatic cells. On embryonic day 17 the testis is differentiating actively. This holds true both for the interstitium and the seminipherous tubules, both of which were found to express HMG-17.

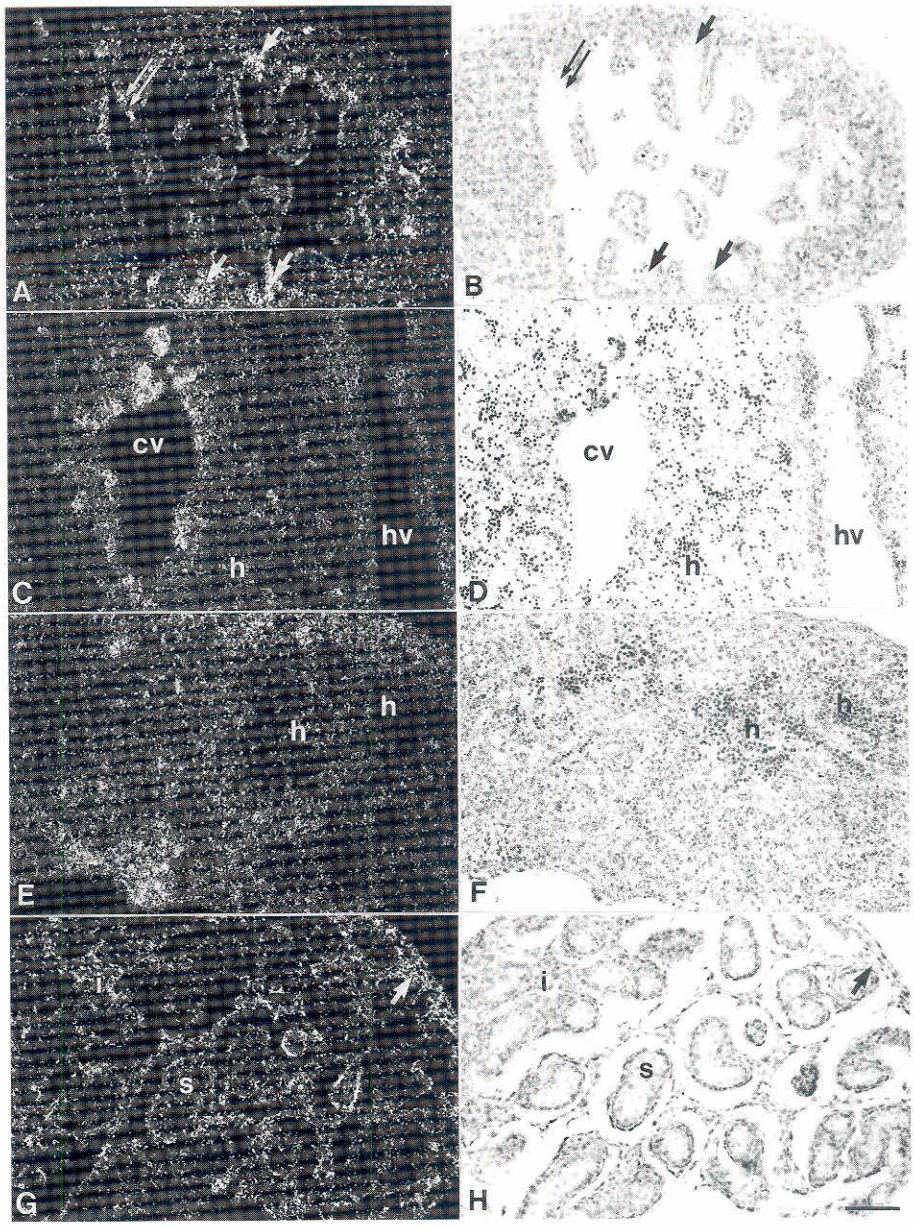

Fig. 4. Localization of the HMG-17 transcript in embryonic day-17 organs. *A,C,E and G are darkfield images and B,D,F and H the corresponding brightfield images.* **(A-B)** *In the intestine, HMG-17 is expressed in the developing crypts between the villi (arrow) and in the underlying muscular and connective tissue layers. The HMG-17 signal often extends a few cell layers along the villus (double arrow).* **(C-D)** *In the liver, the HMG-17 transcript concentrates in the differentiating hepatocytes, whereas the haematopoietic cells (h) show hardly any signal. Cells surrounding the hepatic vein (hv) are weakly positive. Groups of cells around the central vein (cv), some appearing as ductal structures, are strongly positive.* **(E-F)** *In the spleen the HMG-17 transcript concentrates in the lymphatic cells; the hematopoietic cells (h) show hardly any signal.* **(G-H)** *In the testis, the seminipherous tubules (s), the developing interstitium (i) as well as the developing tunica albuginea (arrow) and the underlying condensing mesenchymal cells are all positive for HMG-17. Bar, 100 μm.*

These data suggest that HMG-17 is expressed predominantly in cell types which are undergoing active cell differentiation. This

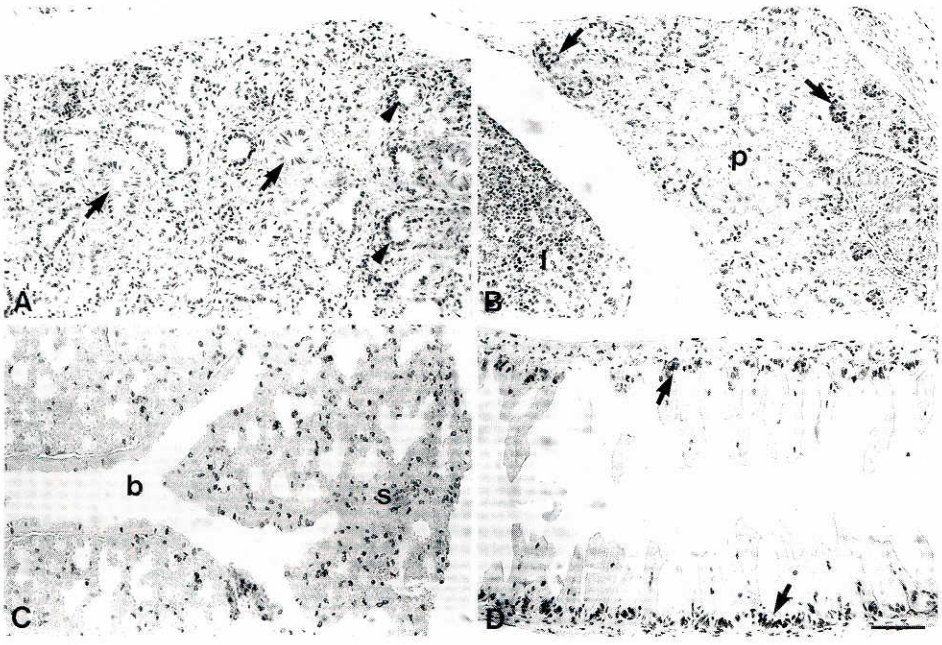

Fig. 5. BrdU-labeling in embryonic day-14 (A-B) and day-17 (C-D) organs. (A) *In the embryonic day-14 lung both epithelial and mesenchymal cells proliferate actively. Terminal bronchioles with columnar epithelial cells are indicated with an arrow and secondary or tertiary branches of the bronchioles, consisting of cuboidal epithelial cells, with an arrowhead.* **(B)** *The liver (l) contains dividing cells throughout the organ. In the pancreas (p) there are frequent proliferating cells in the developing epithelial branches (arrow).* **(C)** *In the embryonic day-17 lung the dividing cells concentrate to the interalveolar septa (s) in the periphery of the organ; the cells lining the bronchioles (b) show little proliferative activity.* **(D)** *In the intestine, cells are proliferating rapidly in the crypts between the villi (arrow) and in the underlying muscular and connective tissue layers. Bar, 80 μm.*

accords with the suggested function of HMG-17 in regulating the structure of transcriptionally active chromatin (Albanese and Weintraub, 1980; Weisbrod *et al.*, 1980; Schröter and Bode, 1982; Weisbrod, 1982). When the tissues reach a mature state during embryonic development or adulthood, the expression of HMG-17 is downregulated.

Relationship between HMG-17 expression and proliferation

During embryonic development, cell differentiation and division typically proceed simultaneously. To see if HMG-17 expression correlates with proliferation, we labeled various organs by BrdU. In the embryonic day-14 tissues, active cell division was taking place virtually ubiquitously. On the embryonic day-17, however, comparison of HMG-17 expression and BrdU-positivity yielded interesting results. In some tissues, such as the intestine, proliferation and HMG-17 expression were seen in the same cell types, such as the intestinal crypt cells which both divide actively and differentiate. Importantly, HMG-17 expression extended higher up along the villus-crypt axis than dividing cells. In the lung, abundant expression of HMG-17 similarly included structures displaying very low BrdU incorporation. In the liver, proliferating cells were present throughout the organ whereas HMG-17 concentrated only to a limited cell population. These observations thus suggest that HMG-17 expression is coupled to cell differentiation rather than to proliferation. The results are in accordance with the earlier studies performed on cultured cells (Pash *et al.*, 1990; Shakoori *et al.*, 1993). HMG-17, suggested to function in regulating the structure of transcriptionally active chromatin (Albanese and Weintraub, 1980; Weisbrod *et al.*, 1980; Schröter and Bode, 1982; Weisbrod, 1982), can thus be regarded as a general marker for regions undergoing cell differentiation in developing organs.

Materials and Methods

Tissue material

Organs were dissected from (CBAxNMRI)F1 mouse embryos and NMRI adult males (testis) or females (other organs). The day of the appearance of the vaginal plug was designated as embryonic day 0. For isolating undifferentiated metanephrogenic mesenchymes, embryonic day-11 kidney rudiments were treated with 2.25% pancreatin, 0.75% trypsin in Tyrode's solution at room temperature for 20 seconds whereafter the mesenchymes were mechanically separated from the ureter buds. The mesenchymes were let to recover in Eagle's Minimal Essential Medium (MEM) supplemented with 10% foetal calf serum (FCS) at 37°C for a few hours.

Differential hybridization

The library used for the hybridization was prepared from 17-day embryonic mouse kidney. The cDNA first strand was synthesized from 150 μg of total RNA by using an oligo-dT-primer and AMV-RT (Life Science Inc.) for reverse transcription. Second strand synthesis and cDNA ligation with the lambda arms were performed according to the Stratagene lambda ZAP-cDNA Synthesis protocol. The Giga Pack Gold Kit (Stratagene) was used for packaging the library into phage particles. The unamplified cDNA library was plated on the *E. coli* strain PLK-F'. Duplicate filters of each dish were hybridized with [α-^{32}P]dCTP-labeled first strand cDNA probes representing embryonic day 11 uninduced metanephrogenic mesenchyme or 17-day embryonic kidney. The filter hybridized with the 17-day kidney probe was stripped and reprobed with an adult kidney cDNA probe. The probes were made from five micrograms of total RNA labeled by reverse transcription in the presence of [α-32P]dCTP (essentially as in the first strand cDNA synthesis). Hybridization was performed in 5xSSC (NaCl/NaCit buffer), 0.02% sodium dodecyl sulphate (SDS), 0.1% N-lauroylsarcosine, 1.5% DIG blocking reagent (Boehringer Mannheim) containing the labeled probes at the concentration 0.22x106 cpm/ml at 68° for 48 h. The filters were washed twice for 30 min in 0.1xSSC, 0.5% SDS at 60°C and exposed on Agfa X-ray films with intensifying screens at -80°C for 22 h. The clones that showed differential expression were isolated, and the inserts were recovered in pBluescript SK(-) by the Stratagene *in vivo* excision protocol. The cDNAs were analyzed by dideoxy DNA sequencing using the T7 Sequencing Kit (Pharmacia) and the expression patterns of the potentially interesting cDNAs were confirmed by Northern blot analysis.

RNA isolation and Northern blot

Total RNA of mouse embryonic day-11 undifferentiated metanephrogenic mesenchymes, 17-day embryonic kidney and different adult mouse organs was isolated by the guanidinium isothiocyanate method (Sambrook *et al.*, 1989). The RNAs were separated in 1.6% agarose-formaldehyde

gels and blotted onto Gene Screen Plus™ membrane (Dupont Co., Wilmington, DE). The probes were labeled with [α-^{32}P]dCTP by random priming or polymerase chain reaction (PCR) and hybridized in 50% formamide, 5xSSC, 5x Denhardt's solution, 0.5% SDS at 42°C for 18 h. The filters were washed in 0.5xSSC, 0.5% SDS at 55°C and exposed on Amersham X-ray film at -70°C for four or fourteen days. The blots were also hybridized with rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA to verify that the RNAs were intact. Methylene blue staining was used to visualize the RNA on the filters.

In situ hybridization

In situ hybridization was performed essentially as described (Lütcke *et al.*, 1993). The pBluescript SK(-) carrying the 900-bp HMG-17 cDNA was linearized either with EcoRI or XhoI for the production of antisense or sense probes, respectively. The single-stranded RNA probes were labeled with [35S]UTPαS (Amersham) by the SP6/T7 run off- transcription method (Riboprobe II Core System, Promega). Embryonic day-14 whole embryos or embryonic day-17 organs were fixed with 4% paraformaldehyde and embedded in paraffin. Five-micrometer sections were hybridized with the antisense and sense probes in parallel in 60% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 8.0), 5 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0), 10% dextran sulphate (Mw 500 000), 1xDenhardt's solution, 0.5 mg/ml torula yeast RNA and 0.1 M dithiothreitol (DTT) at 52°C for 15-20 h. After hybridization the sections were washed in high stringency conditions (50% deionized formamide, 2xSSC, 30 mM DTT; 65°C) twice for 30 min, dipped in Kodak NTB-2 autoradiography emulsion and exposed at 4°C for seven days.

BrdU-labeling and immunohistology

NMRI females on the 14th or 17th day of pregnancy were injected intraperitoneally with BrdU (Amersham). After two and a half hours the animals were sacrificed, whole 14-day embryos or dissected organs of 17-day embryos were fixed in 3.5% paraformaldehyde and embedded in paraffin. Five-micrometer sections were deparaffinized, rehydrated in descending series of ethanol, and the endogenous peroxidase blocked by treating the sections with 0.5% hydrogen peroxide (H_2O_2) in methanol for 30 min. To increase the permeability and denature the DNA the sections were incubated in 0.1% Triton X-100 in phosphate-buffered saline (PBS) at room temperature for 15 min and thereafter in 2 N HCl at 37°C for one hour (isolated embryonic day-17 organs) or in 0.25% trypsin followed by 4 M HCl at 37°C for 15 min each (day-14 whole embryos). HCl was neutralized by several washes with 0.1 M sodium borate buffer, pH 8.5. After rinsing with PBS the sections were incubated with 6 μg/ml of anti-BrdU antibody (Boehringer) diluted in 5% FCS, 0.5% saponin in PBS at 4°C overnight. Detection was carried out using the Vectastain ABC Peroxidase Mouse IgG kit (Vector Laboratories, Inc.) according to the manufacturer's instructions.

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