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Developmental Analysis of the Cytomegalovirus Enhancer in Transgenic Animals†

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The major immediate-early promoter (MIEP) of human cytomegalovirus (HCMV) constitutes a primary genetic switch for viral activation. In this study, regulation of the enhancer-containing segment (nucleotides −670 to +54) of the HCMV MIEP attached to the *lacZ* reporter gene was examined in the developing embryos of transgenic mice to identify temporal and tissue-specific expression. We find that the transgene reporter is first detected as a dorsal stripe of expression in the neural folds of embryos at day 8.5 postcoitum (p.c.). A broad expression pattern is exhibited in embryos at day 9.5 p.c. This pattern becomes more restricted by day 10.5 p.c. as organogenesis progresses. By day 14.5 p.c., prominent expression is observed in a subpopulation of central nervous system cells and spinal ganglia, endothelial cells, muscle, skin, thyroid, parathyroid, kidney, lung, liver, and gut cells, and the pancreas and submandibular and pituitary glands. This distribution pattern is discussed in relation to human congenital HCMV infection. These results suggest that the transcriptional activity of the HCMV MIEP may determine in part, the ability of the virus to specifically target developing fetal tissues in utero.

Human cytomegalovirus (HCMV) infects most humans by adulthood and is a significant cause of morbidity and mortality for individuals in the clinical setting, particularly cancer patients, neonates, and patients with AIDS (18, 39). HCMV is also considered to be one of the leading causes of congenital infection in humans, affecting 40,000 children annually in the United States alone (15, 30). Approximately 20% of these children are born with some form of irreversible brain damage, resulting in various degrees of mental retardation and profound deafness (16, 36).

The host and viral factors that determine sites of infection and progression to disease in congenital HCMV infection are not known. For most DNA viruses, cell type (tissue) tropism is determined by three key types of virus-cell interaction. The initial tissue-selective interaction involves the attachment and penetration of cells by virus. HCMV is capable of entering many different cell types in vitro and in vivo (e.g., see references 25 and 29 and references therein), indicating that this initial interaction step is unlikely to be a major determinant for its tropism. The second type of virus-cell interaction, which is perhaps the most significant for HCMV, depends on appropriate levels of host-encoded transcription and/or replication factors (reviewed in references 12 and 26). In vivo, immune surveillance by the host provides the third determining tier of tropism. In this case, immune clearance would be expected to play a more prominent role in the adult than in the embryo. Thus, of these types of interactions, transcriptional regulation of the viral major immediate-early promoter (MIEP) constitutes a primary level by which this virus may determine tissue-specific expression and activation state in the host. Therefore, we are interested in the role that cellular and viral factors play in coordinating transcription from the MIEP. The MIEP is highly dependent on cellular transcription factors for activity and is composed of multiple domains, including promoter-proximal downstream elements, a highly complex enhancer domain, and various upstream modularity elements (12). The majority of these regulatory elements are clustered between nucleotide positions −670 and +54 which, for the most part, encompass the enhancer domain. Importantly, this enhancer-containing segment of the MIEP has recently been shown to be regulated in a tissue-specific fashion in adult transgenic mice, recapitulating in part the natural HCMV infection pattern in humans (4). Moreover, a recent transgenic study by Koedood et al. (23) using partial MIEP sequences located between nucleotides −525 and +13 [MIEP(−525/+13)] suggests a similar relationship may occur during fetal development. The transgenic mouse model, therefore, supports the prediction that optimal MIEP activity is a critical determinant in the outcome of a productive HCMV infection.

In this study, the MIEP transgenic mice serve as a paradigm for investigating the developmental expression of the MIEP. We present evidence that the MIEP enhancer-containing segment located between nucleotides −670 and +54 [MIEP (−670/+54)] confers both temporal and spatial control of expression on the MIEP during embryogenesis. These results complement and extend the recent MIEP transgenic embryo report by Koedood et al. (23). A comparison of our data with the spatiotemporal map developed by their MIEP(−525/+13) transgenic embryo system (23) reveals a number of new and significantly different findings that most likely relate to the

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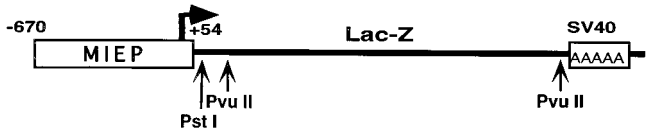


FIG. 1. Schematic of the construct used to generate MIEP-*lacZ* transgenic mouse lines. MIEP-*lacZ* contains a truncated version of the MIEP(−670/+54), lacking the modulator and NF1 regions, cloned upstream of the *lacZ* coding sequence. It also contains 238 nucleotides of simian virus 40 poly(A) sequence (nucleotides 2533 to 2770).

additional sequences located at nucleotide positions −670 to −525 and +13 to +54 of the MIEP used in this study. Taken together, the observed expression patterns show many parallels with tissues that are likely to be infected in utero in humans, suggesting that the MIEP enhancer domain is an important determinant in directing developmental expression of HCMV.

MATERIALS AND METHODS

Fixation and staining of whole embryos for β-galactosidase activity. The generation and identification of the transgenic lines used in this study are described elsewhere (4). Analysis of expression was performed on fetuses obtained by mating hemizygous transgenic animals with outbred CD-1 mice. By this means, each set of embryos produced contained approximately the expected proportion of transgenic (50%) and wild-type (50%) genotypes, thus providing internal controls to ensure that none of the staining was due to endogenous mammalian β-galactosidase. Pregnant females were sacrificed at various stages of pregnancy from 7.5 through 15.5 days postcoitum (p.c.). The middle of the day after identification of the fertilization plug was designated as day 0.5 of gestation.

Embryos were obtained by dissecting conceptuses directly from the decidua in cold phosphate-buffered saline (PBS) (2, 19); they were rapidly fixed in either 0.2% glutaraldehyde or 4% paraformaldehyde in PBS at 4°C for 15 to 90 min, rinsed, and then stained in X-Gal (5-bromo-4-chloro-3-indolyl-β-galactoside) at 37°C (13). In addition, day 10.5 and 11.5 p.c. embryos were stained in halogenated indolyl-β-galactoside (Bluo-gal) at 37°C for β-galactosidase activity. Depending on the stage of development, blue staining was apparent between 30 min and 6 h. Whole-mount embryos were photographed through a Wild-Leitz (M3Z) stereo microscope.

Histochemical analysis. Histochemical staining for β-galactosidase activity was performed on cryostat sections of (13.5 to 15.5 day p.c.) embryos stained with X-Gal. Briefly, the embryos (fixed in 4% paraformaldehyde and stored in 30% sucrose in PBS) were frozen in 30% sucrose. Twenty-micrometer-thick frozen sections were cut and poststained with X-Gal overnight at 4°C. Slides were rinsed in Tris (pH 7.5), counterstained briefly with eosin, and dehydrated through graded isopropyl alcohol. After brief immersion in xylene, the slides were coverslipped with permount and examined under a light microscope. Day 10.5 and 11.5 p.c. embryos stained with Bluo-gal were postfixed in 4% paraformaldehyde and dehydrated through graded ethanol solutions, cleared with methyl salicylate, and embedded in paraffin; then 6-μm-thick sections were cut. After being deparaffinized in xylene and hydrated in water, sections were counterstained briefly with eosin and dehydrated through graded isopropyl alcohol. After brief immersion in xylene, slides were coverslipped with permount and examined under a Ziess axioplan microscope and photographed using an Olympus photomicroscopy system.

It should be noted that the textual description of the expression patterns at various stages of development is an account of all of our observations. Photographs have been selected to illustrate the points made in the text.

RESULTS

Embryonic expression of the HCMV MIEP enhancer-containing transgene. A preliminary test for transgene expression was performed by staining day 10.5 p.c. embryos, produced from three previously characterized transgenic lines, for β-galactosidase activity (4). A schematic of the transgene construct is shown in Fig. 1. Animals from each line produced litters containing the expected 50% positive for the transgene, with essentially identical and very specific patterns of expression. Expression patterns were confirmed at all developmental stages in the embryos of the three independent lines.

Transgene expression was first detected in day 8.5 p.c. head fold-stage embryos (Fig. 2A and B). This expression coincided with the end of gastrulation and the beginning of neuralation. *lacZ* stain was observed mainly at the site of formation of the head folds, demarcating the neural plates. This indicates a rapid onset of transgene expression in late gastrulating embryos, coinciding with formation of the neural plate.

FIG. 2. Whole-mount staining of embryos with (A, C, E, G, and I) or without (B, D, F, H, and J) the MIEP-*lacZ* transgene. (A and B) Day 8.5 p.c. embryos. Magnification, ×20. (C and D) Day 9.5 p.c. embryos. Magnification, ×15 and ×16, respectively. (E and F) Day 11.5 p.c. embryos. Magnification, ×10 and ×8, respectively. (G and H) Day 13.5 p.c. embryos. Magnification, ×6.5. (I and J) Day 15.5 p.c. embryos. Magnification, ×6.5.

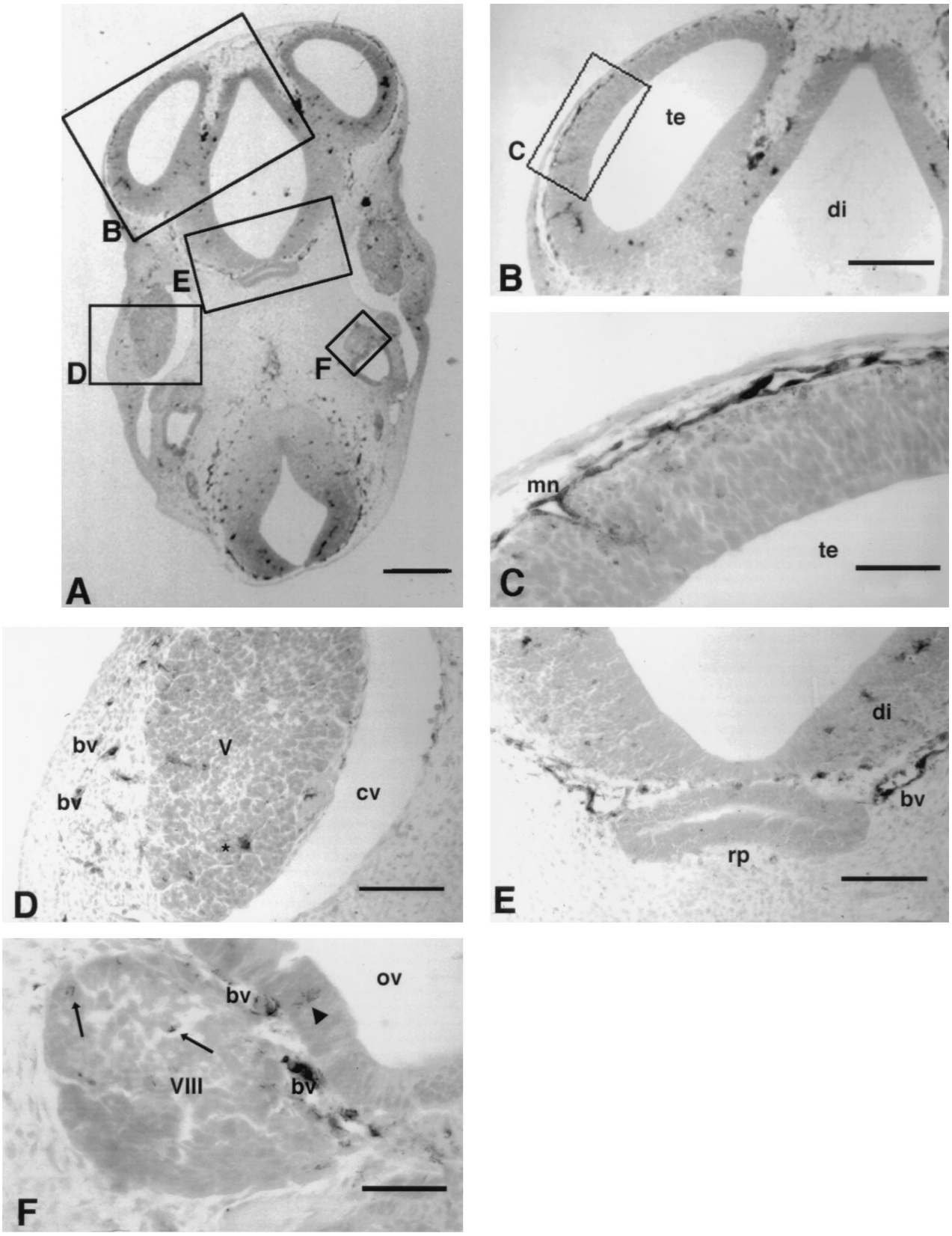


FIG. 3. Day 10.5 p.c. embryo. (A) Cross-section through the fore and hindbrain of a 10.5 day p.c. transgenic embryo. Enclosed areas are shown enlarged in the respective panels. Bar, 500 μm. (B) Enlargement of area B, showing β-galactosidase-positive cells in the telencephalon (te) and diencephalon (di). Bar, 200 μm. (C) Enlargement of area C, showing positive meningial cells (mn) near the telencephalon (te) and some faint staining near the pial surface of the telencephalon. Bar, 50 μm. (D) Enlargement of area D, showing trigeminal ganglion (V) and adjacent anterior cardinal vein (cv), with positive nerve cells (*) and positive endothelial cells in small blood vessels (bv). Bar, 100 μm. (E) Enlargement of area E, showing Rathke's pouch (rp) negative with positive blood vessels (bv) surrounding the diencephalon (di) as well as positive cells in the diencephalon. Bar, 100 μm. (F) Enlargement of area F, showing the otic vesicle (ov) and vestibulocochlear ganglion (VIII), with β-galactosidase-positive cells (arrows). Arrowhead indicates positive cells in the inner ear epithelium. Bar, 50 μm.

By day 9.5 p.c., a broad staining pattern was observed in the olfactory placode, branchial arches (first and second), all blood vessels, somites, the maxillary process, cardiohepatic eminence, and forelimb buds (Fig. 2C and D). Staining was also observed in the central nervous system and in various sensory ganglia, including the trigeminal, vestibulocochlear, nodose, and dorsal root ganglia. The broad expression pattern indicates a possible activation of transgene transcription in somatic and neural tissues.

By days 10.5 and 11.5 p.c., expression of the transgene became more restricted and began to show sharp tissue-specific variation as organogenesis progressed. Strong staining was observed in the region of the fourth ventricle of the hindbrain, as well as in the somites, maxillary process, and blood vessels, especially the branchial arch artery originating from the heart (Fig. 2, compare panels E and F).

Examination of intact stained embryos at days 13.5 through 15.5 p.c. revealed specific surface staining in the skin, blood vessels, vibrissa rudiment (whiskers), and around the eye and auditory opening (Fig. 2, compare panels G with H and I with J, respectively). We conclude from these results that the MIEP of HCMV is functional during embryogenesis. Moreover, these observations indicate that transcriptional activation of the MIEP first occurs during the process of establishing the basic body plan, after which expression becomes restricted to specific sites of tissue differentiation.

To identify more precisely the spatial distribution of transgene expression during organogenesis, serial sections of fetuses from days 10.5 through 14.5 p.c. were prepared and examined for *lacZ* expression.

Days 10.5 to 11.5 p.c. embryos. Day 10.5 p.c. embryo sections showed transgene expression restricted to specific cell types throughout the head and body regions. Frontal sections showed strong staining of cells in the diencephalon of the brain (Fig. 3A and B; Fig. 4G). Immediately outside the brain, intense staining was developed in the meningial cell layer at the diencephalon and telencephalon regions (Fig. 3C). Note, in Fig. 4G very few meningial cells appear positive; however, examination of serial sections of this embryo clearly showed meningial staining (data not shown), indicating a restricted subpopulation of cells in the meninges that express the transgene. Weaker staining was observed near the pial surface of the telencephalon (Fig. 3C). Transgene expression was also detected in a subpopulation of nerve cells in the developing brain and spinal cord as well as in the trigeminal ganglion (Fig. 3D), vestibulocochlear ganglion (Fig. 3F), and dorsal root ganglia (Fig. 5C). In addition, there was a striking layer of expression in the region of the developing cerebellum corresponding to the choroid plexus anlagen (Fig. 6A and B; day 14.5 p.c.). Otic vesicles associated with the third branchial arch and sclera of the eye were also positive.

Prominent *lacZ* activity was also observed in the body of day 10.5 and 11.5 p.c. embryos. Staining was observed in the somites, in part, colocalized to the myotomal compartment which harbors the myogenic precursor cells. Consistent with this observation subpopulations of skeletal muscle cells were stained in day 13.5 and 15.5 p.c. embryos (data not shown). Proliferating germinal matrix cells and neuronal cells of the spinal cord also exhibited transgene expression (Fig. 4A to C; Fig. 5). Embryo capillary and arterial endothelial cells were heavily stained, including the aorta and the carotid and pulmonary arteries (Fig. 4D, E, H, and I; Fig. 3; Fig. 5B and D to F) and placental vascular endothelial cells (Fig. 7). Other tissues which showed a subpopulation of cells positive for expression in day 10.5 and 11.5 p.c. embryos were the germinal ridges, intracoetonic cavity, unidentified blood cells, and the heart region, including the truncus, epicardium, and myocardium.

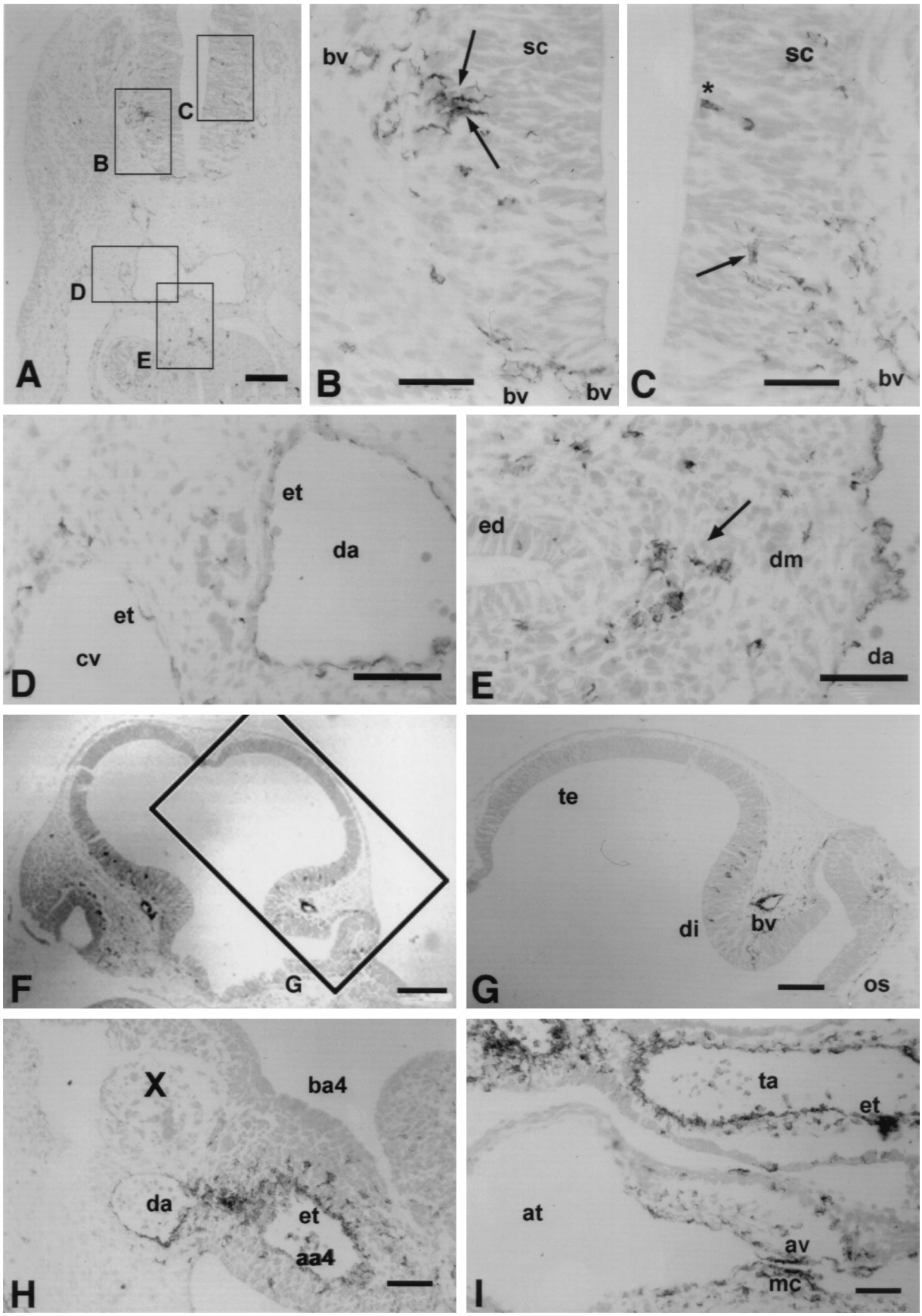
Day 14.5 p.c. embryos. By day 14.5 p.c., epithelial cells have already begun to differentiate (such as the epithelia located in the oral cavity, the esophagus, wall of the stomach, whisker follicles, and glandular epithelium) and the first ossification centers have appeared. Significant transgene expression was detected in the olfactory and buccal epithelium (Fig. 6C and D), the lining of the tongue (as well as striated muscle of the tongue) (Fig. 6E), the tooth primordium (Fig. 6F), the lining of the stomach and epithelium of the whisker follicles (data not shown), pulmonary epithelial cells (Fig. 6G), and chondrocytes in the ribs (Fig. 6I).

By day 14.5 p.c., Rathke's pouch has fused with the diencephalic infundibulum, forming the anterior pituitary anlagen from its epithelium. Although the Rathke's pouch in day 11.5 p.c. embryos was negative for transgene expression (Fig. 3E), strong staining of cells was observed in the intermediate and anterior pituitary lobes (Fig. 6D) at day 14.5 p.c. Strong transgene expression was also observed in all three layers of skin, the peridermal, epidermal, and dermal layers (data not shown). Other organ systems showing intensely stained subpopulations of cells included the pancreas (Fig. 6P), lung (Fig. 6G), skeletal muscle (data not shown), liver (Fig. 6O), kidney (Fig. 6N), blood cells (Fig. 6K), thyroid and parathyroid glands (Fig. 6H), and adrenal glands (Fig. 6M).

A summary of transgene expression in differentiating organs at different developmental stages is shown in Table 1.

Transgene expression in extraembryonic structures. Although expression was not observed in day 7.5 p.c. primitive streak stage embryos, strong transgene expression was observed at this time in cells corresponding to the precursor placental cells of the ectoplacental cone (Fig. 7B). As embryonic development progresses, cells of the ectoplacental cone become associated with the chorion, allantois, and endometrium to form the placenta. At these later stages of development, the placenta was negative for staining; however, the umbilical vein endothelium was highly positive for transgene expression. (Fig. 7A). Other extraembryonic tissues showing strong expression were the yolk sac (Fig. 7D), which regulates the fluid volume of the amniotic cavity and serves as a nutritive organ for the developing embryo, and a subpopulation of cells in the amnion compartment (Fig. 7C), which provides thermal and physical insulation for the fetus and causes the progressive uterine distension necessary for normal uterine, placental, and fetal development. Evidence of transgene activity at these sites suggests that HCMV could potentially interfere with normal

FIG. 4. Day 10.5 p.c. embryo. (A) Cross-section of 10.5 day p.c. transgenic embryo sectioned at the dorsal aorta region. Boxes indicate areas enlarged in panels B through E, respectively. Bar, 120 μm. (B) Spinal cord (sc) with β-galactosidase-positive staining in the neural motor column (arrows), as well as staining of small blood vessels (bv) outside of the central nervous system. Bar, 50 μm. (C) Spinal cord (sc), β-galactosidase-positive neuronal cell (arrow), proliferating germinal matrix cells (*), and endothelial cells of a small blood vessel (bv). Bar, 50 μm. (D) Portions of the dorsal aorta (da) and cardinal vein (cv), showing β-galactosidase-positive endothelial cells (et). Bar, 50 μm. (E) Portions of the dorsal aorta (da) and dorsal mesentery (dm) with β-galactosidase-positive endoderm cells (ed) and possible neuronal cells (arrows). Bar, 50 μm. (F) Cross-section through the head region. Box indicates approximate area enlarged in panel G. Bar, 250 μm. (G) β-galactosidase-positive cells in the diencephalon (di) but not in the telencephalon (te). Positive endothelial staining of blood vessels (bv). os, optic stalk. Bar, 120μm. (H) Cross-section through the fourth branchial arch (ba4). Note the positive endothelial cells (et) in the aortic arch 4 (aa4) and dorsal aorta (da). Nodose ganglion of the vagus nerve (×) is negative. Bar, 50 μm. (I) Positive endothelial cells (et) in the heart truncus arteriosis (ta) and atrium (at) and myocardial cells (mc) in the atrioventricular canal (av). Bar, 50μm.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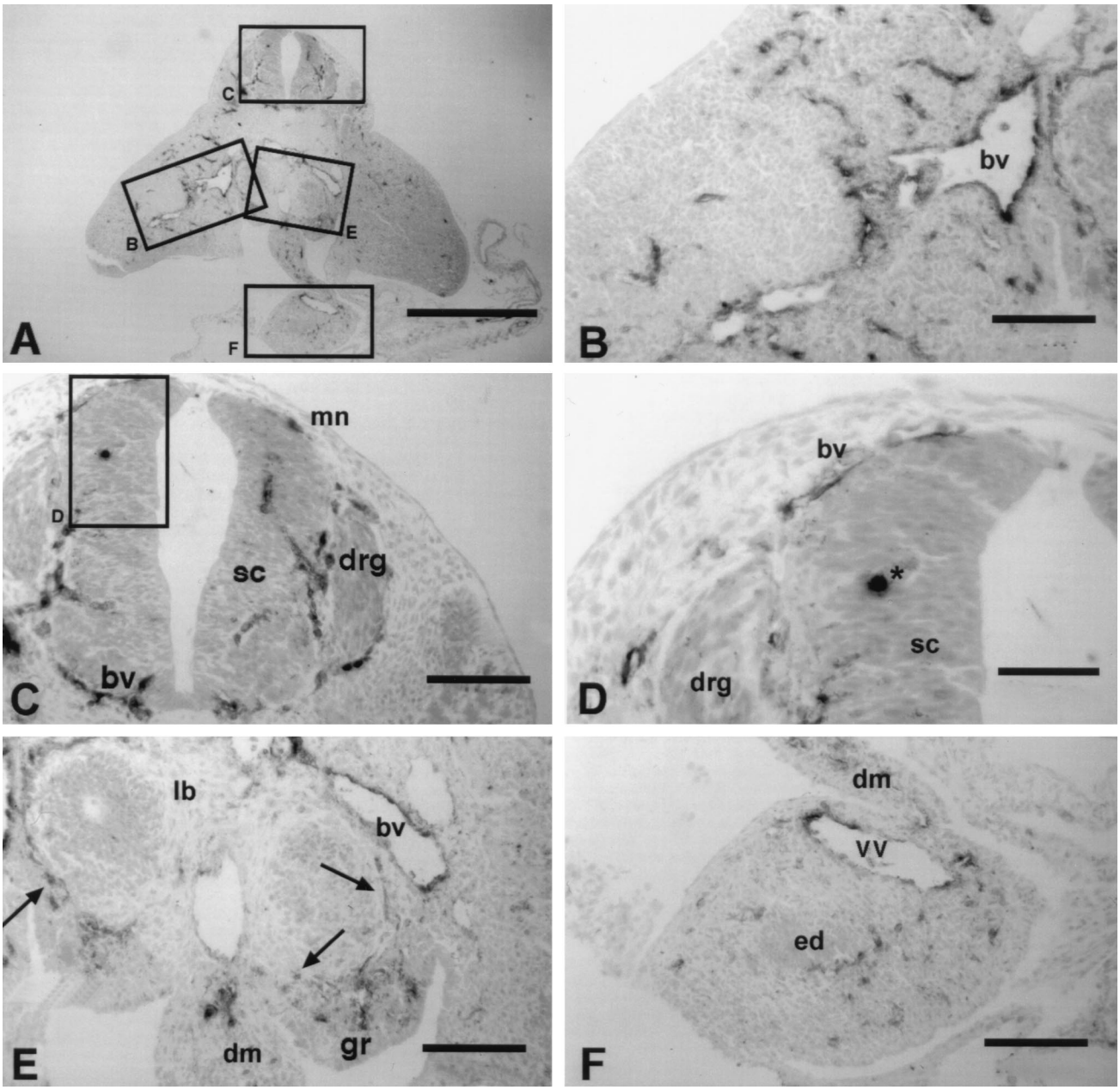


FIG. 5. Day 11.5 p.c. embryos. (A) Cross-section at the thoracic (abdominal) region of a 11.5 day p.c. transgenic embryo. Enclosed areas are shown enlarged in the respective panels. Bar, 500 μm. (B) Enlargement of area B, showing a plexus of blood vessels (bv) in the limb, with positive endothelial cells. Bar, 100 μm. (C) Enlargement of area C, showing spinal cord (sc) with β-galactosidase-positive cells in blood vessels (bv), meninges (mn), and dorsal root ganglion (drg). Bar, 100 μm. (D) Enlargement of area D, showing a portion of the spinal cord (sc) with a positive neuronal cell (*) and positive neuronal cells in blood vessels (bv). drg, dorsal root ganglia. Bar, 50 μm. (E) Enlargement of area E, showing positive endothelial cells in blood vessels (bv) and mesenchymal cells of the dorsal mesentery (dm) but lacking positive cells in the lung bud (lb). gr, germinal ridge. Bar, 100 μm. (F) Enlargement of area F, showing β-galactosidase-positive endoderm cells (ed) at the level of the forelimb bud, positive cells in the dorsal mesentery (dm), and endothelial cells in the vitellin vein (vv). Bar, 100 μm.

fetal development by disrupting the function of these extraembryonic structures.

DISCUSSION

We report here the expression pattern of a *lacZ* transgene under the control of the HCMV enhancer-containing domain [MIEP(−670/+54)] during mouse embryogenesis from days 7.5 to 15.5 p.c.. The developmental pattern of expression mediated by the MIEP-*lacZ* transgene suggests that the enhancer domain is involved in determining spatiotemporal control of MIEP expression during early morphogenic events and in the differentiation of various organ systems.

HCMV has been detected in a variety of organs, including brain, spinal cord, peripheral ganglia, eye, ear, alimentary tract, lung, liver, pancreas, kidney, heart, muscle, blood, endothelium, gonads, pituitary, salivary, adrenal, and thyroid glands, bone, tooth (epithelium), and skin (5, 14, 17, 18, 21, 22, 30, 31, 36, 37). HCMV infection in these various organ systems is predominantly associated with epithelium-derived cells, al-

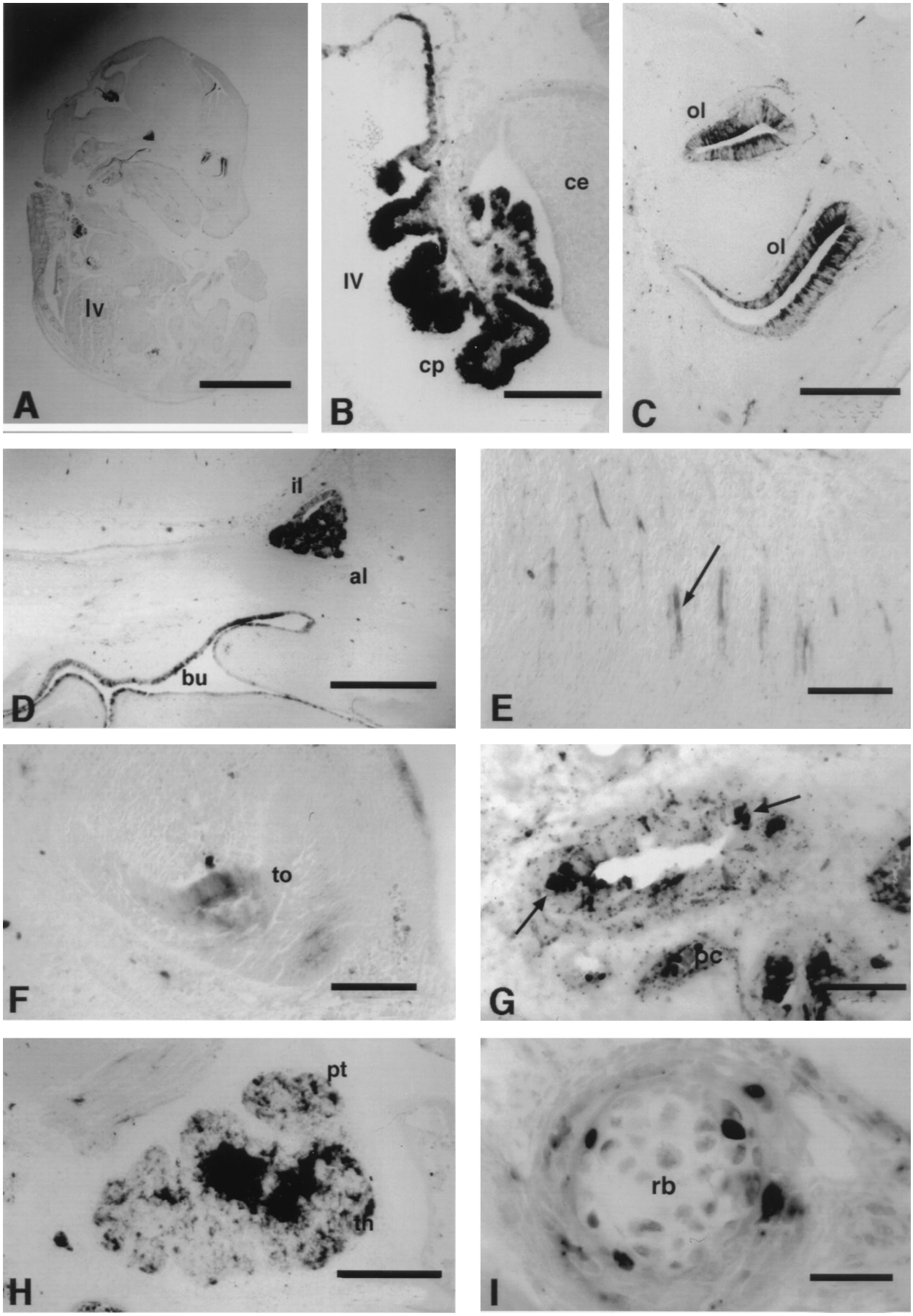
though muscle, bone, blood, and endothelium may also be infected. In parallel, we find that MIEP-driven expression in transgenic embryos overlaps with these sites of natural infection and, in particular, coincides with differentiating epithelium. For example, in day 14.5 p.c. embryos, differentiated epithelial cells show strong *lacZ* transgene expression (i.e., oral epithelia, olfactory epithelia, stomach wall, tooth primordia, whisker follicles, and glandular epithelium). Similarly, in the histiogenesis of the anterior pituitary derived from Rathke's pouch epithelium, MIEP-driven *lacZ* expression, which is highly expressed in the anterior pituitary, is undetectable prior to differentiation and proliferation of the epithelia of Rathke's pouch.

On the basis of these observations, we propose that the MIEP-*lacZ* transgenic mouse system may be a useful animal model system for dissecting viral tropism and developmental expression of HCMV as determined by cellular transcription factors interacting with the viral promoter. Further, this model system may also be valuable in providing insight into the pathogenesis of congenital HCMV infection. For example, HCMV

TABLE 1. Expression driven by MIEP (−670/+54) compared with that of MIEP(−525/+13) of Koedood et al. (23) in transgenic mice at different embryonic stages

Organ and cell type	Transgene expression at day p.c.											
	8.5			10.5			11.5			14.5		
	#1[a]	#2[a]	Reference 23	#1	#2	Reference 23	#1	#2	Reference 23	#1	#2	Reference 23[b]
Primary germinal layer												
Ectodermal cells	+	+	+	NA[c]	NA	NA	NA	NA	NA	NA	NA	NA
Endodermal cells	−	−	−	NA	NA	NA	NA	NA	NA	NA	NA	NA
Mesodermal cells	−	−	−	NA	NA	NA	NA	NA	NA	NA	NA	NA
Nervous tissue												
Neurons of the spinal cord	ND[d]	ND	ND	+	+	+	+	+	+	+	+	+
Diencephalon	ND	ND	ND	+	+	+	+	+	+	−	−	NI[e]
Retina	ND	ND	ND	+	+	+	+	+	+	−	−	+
Dorsal root ganglia	ND	ND	ND	+	+	+	+	+	+	+	+	+
Meninges	ND	ND	ND	+	+	+	+	+	+	−	−	−
Telencephalon	ND	ND	ND	−	−	−	+	+	−	−	−	−
Inner ear epithelium	ND	ND	ND	+	+	+	+	+	+	+	+	+
Trigeminal ganglia	ND	ND	ND	+	+	+	+	+	+	+	+	+
Vestibulocochlear ganglion	ND	ND	ND	+	+	+	+	+	+	+	+	+
Hindbrain	ND	ND	ND	+	+	+	+	+	+	NA	NA	NA
Olfactory epithelium	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+
Choroid plexus	ND	ND	ND	ND	ND	ND	+	+	+	+	+	+
Connective tissue												
Lung mesenchymal cells	ND	ND	ND	+	+	+	+	+	+	+	+	+
Heart region	ND	ND	ND	+	+	+	+	+	+	+	+	+
Blood cells	ND	ND	ND	+	+	NI	+	+	NI	+	−	NI
Dorsal mesentery	ND	ND	ND	+	+	+	+	+	+	+	+	+
Esophagus	ND	ND	ND	ND	ND	ND	+	+	+	+	+	+
Tongue	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	−
Rib chondrocytes	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	NI
Epidermis	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+
Striated muscle	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	−
Eye sclera	ND	ND	ND	+	+	+	+	+	+	+	+	+
Endocrine tissue												
Thyroid	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	−
Parathyroid	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	−
Pancreas	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+
Submandibular gland	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	−
Rathke's pouch	ND	ND	ND	−	−	NI	−	−	NI	NA	NA	NA
Pituitary	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+
Other tissues												
Adrenal	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+
Kidney	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+
Liver	ND	ND	ND	+	+		+	+	+	+	+	+
Pharynx epithelium	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+
Tooth primordia	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+
Connective tissue, maxillary	ND	ND	ND	+	+	+	+	+	+	NA	NA	NA
Branchial arches	ND	ND	ND	+	+	+	+	+	+	NA	NA	NA
Endothelial cells	—[f]	—	—	+	+	+	+	+	+	+	+	+
Mesothelial cells	—	—	—	+	+	+	+	+	+	+	+	+

[a] Two independent transgenic mouse lines.
[b] Results in this column represent tissues positive at day 13.5 p.c. in reference 23.
[c] NA, not applicable at this stage.
[d] ND, tissues not developed at this stage.
[e] NI, not identified.
[f] —, embryos examined in situ only.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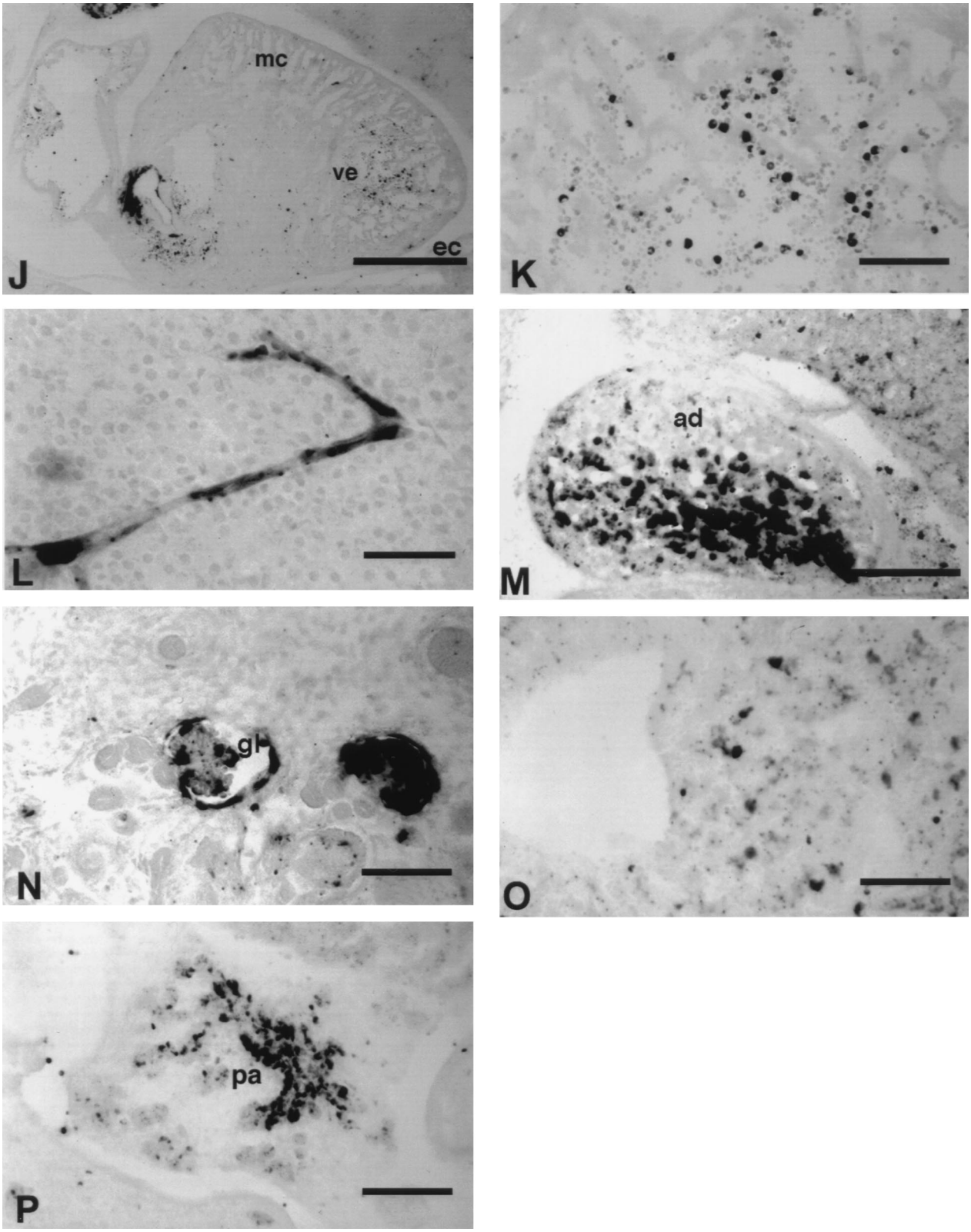

FIG. 6. Day 14.5 p.c. embryo. (A) Longitudinal section of day 14.5 p.c. MIEP transgenic mouse embryo. lv, liver. Bar, 1 mm. (B) Choroid plexus (cp) of the 4th ventricle (IV) and adjacent cerebellum (ce), showing highly β-galactosidase-positive cells in the choroid plexus. Bar, 250 μm. (C) β-galactosidase-positive olfactory epithelium (ol). Bar, 500 μm. (D) Positive cells in the intermediate lobe (il) and anterior lobe (al) of the pituitary gland and the buccal epithelial lining (bu). Bar, 500μm. (E) Tongue, with β-galactosidase-positive striated muscle (arrow). Bar, 100 μm. (F) Lower jaw, with β-galactosidase-positive tooth primordium (to). Bar, 100 μm. (G) β-Galactosidase-positive pulmonary epithelial cells (arrows) and pulmonary capillaries (pc). Bar, 100μm. (H) Highly positive cells in the thyroid (th) and parathyroid (pt). Bar, 10 μm. (I) Positive chondrocytes in the rib (rb). Bar, 50 μm. (J) Positive blood cells in the ventricle (ve) of the heart. Note the absence of β-galactosidase activity in the epicardium (ec) and myocardium (mc). Bar, 500 μm. (K) Enlargement of ventricular area from panel J, showing positive blood cells within the ventricle. Bar, 100 μm. (L) Positive endothelial cells lining the brain capillary. Bar, 50 μm. (M) Positive cells in the adrenal gland (ad). Bar, 250 μm. (N) β-Galactosidase-positive cells lining the glomeruli (gl) in the kidney. Bar, 100 μm. (O) Positive hepatocytes in the liver. Bar, 100 μm. (P) β-Galactosidase-positive cells in the pancreas (pa). Bar, 100 μm.

FIG. 7. Extraembryonic structures. (A) Positive cells lining the umbilical vein (uv) from a 10.5 day p.c. transgenic embryo; note that the placenta (pl) is negative. Bar, 250 μm. (B) β-Galactosidase-positive precursor placental cells in the ectoplacental cone (epc) of a 7.5 day p.c. transgenic embryo. Note that the embryo (em) and decidua (de) are negative. Bar, 50 μm. (C) Day 15.4 p.c. transgenic embryo surrounded by amnion. Note the sporatic positive cells in the amnion. Bar, 2 mm. (D) Yolk sac dissected from a day 14.5 p.c. transgenic mouse embryo. Note cells positive for β-galactosidase expression. Bar, 1 mm.

microgyria is a common feature in the brain pathology of congenitally infected human infants (7) and has been postulated to result from insufficiency of cerebral blood supply rather than direct viral disturbance of neurogenesis or gliogenesis (9). The potency of transgene expression in brain vasculature suggests a role for HCMV in perturbing angiogenesis and blood-brain barrier function, thus providing a causative link between HCMV and ischemia. Another example is provided by the pattern of transgene expression in extraembryonic tissues, which suggests two potential modes of HCMV infection during fetal development. One mode is suggested by *lacZ* expression in precursor placental cells. These cells may serve as a vehicle for replicating virus to pass from the endometrium, infect the umbilical endothelium, and disseminate to the embryo (3). MIEP expression in the amnion indicates another possible mode of transmission. Here, free virus or infected amniotic cells may shed into the amniotic fluid as the developing fetus begins to imbibe surrounding fluid.

The observation of MIEP-*lacZ* expression in subpopulations of cells in the various differentiated organ systems is particularly intriguing. For example, only a subset of muscle cells within the tongue and chrondrocytes in the rib were found to express *lacZ* without any obvious difference from their nonexpressing neighbors. It is likely that prior developmental events limit which cells permit MIEP activation. At one level, epigenetic control via methylation of DNA or via position effect variegation may lead to cell-specific restriction. Alternatively, this restriction may operate by providing a cellular repressor to the MIEP (35) or by limiting the population of cells which express a positive transcription factor (12). In addition, extracellular signals that regulate transcription factor activity could affect one or more of these processes. In this connection, we note that the pattern of transgene expression overlaps with many sites in the embryo that express the RAR and RXR subfamilies and which likely use retinoic acid for their growth and differentiation (10, 32, 33). In particular, the differentiation of epithelial cells (such as those in the developing teeth, whisker follicles, lung, salivary glands, skin, chondrocytes, and oral cavity) strongly correlates with expression of the gamma subtype retinoic acid receptor (33) and is consistent with the notion that HCMV may opportunistically use the retinoid signaling pathway for activation (1, 11). Indeed, the MIEP contains multiple signal-regulated elements, including an array of NF-κB, ATF, AP-1, and retinoid receptor (RAR/RXR) binding sites (6, 11, 20, 28, 34, 38), which likely accounts for the apparent promiscuous activity of the promoter in transient transfection assays because of an elevated activation state under conditions of in vitro cell culture. However, in vivo it is possible that cell-specific combinatorial interactions among these sites may modify transcription factor activity and integrate signal inputs from different signaling pathways, thereby affecting whether a signal-regulated transcription factor, in a given cell, activates or represses transcription. Moreover, the presence of signal-regulated elements in the MIEP is likely to lead to restrictions that may also exist at levels other than transcription. These restrictions might operate within a signal transduction pathway.

Another possible mechanism for restricted expression within defined cell populations may involve migratory components with different origins. For instance, neuronal cells of the trigeminal ganglion are derived from two distinct migratory components which originate from the epibranchial placodes and

neural crest cells in the day 8.5 to 9.0 p.c. embryo (8). Indeed, MIEP expression is apparent in neural crest-derived cells at day 8.5 p.c., but not in epibranchial placodes, and expression is highly restricted to a specific subpopulation of trigeminal ganglion cells in day 10.5 embryos, perhaps signifying a cell lineage marker for those cells. All these possibilities and perhaps others are likely to determine the final level of tissue-specific MIEP expression. The transgenic model provides a means to test these possibilities, which will be the subject of future investigations.

In an earlier report (24), it was shown that an HCMV MIEP segment from nucleotides −302 to +72 was tightly regulated in a cell-specific manner in embryos from transgenic mice. The study by Kothary et al. (24) indicates that the −302 to +72 segment of the MIEP is sufficient for initial activation at day 8.0 to 8.5 p.c. and later on for directing expression in the spinal and sensory ganglia, choroid plexus, muscle, and a subset of endothelial cells. More recently, a similar study by Koedood et al. (23) was reported in which an enhancer-containing segment (nucleotides −525 to +13) of the MIEP was investigated for embryonic expression. In line with our results, these investigators detected a similar expression pattern (for a comparison between the two studies see Table 1). Although all sites of expression detected by Koedood et al. are positive for the MIEP(−670/+54) transgene, significant differences involving new sites of expression are noted in the present study. These marked exceptions include the thyroid and parathyroid glands, salivary glands, and striated muscle in day 14.5 p.c. embryos, indicating that additional sequences outside the major enhancer segment (i.e., those sequences located from nucleotides −670 to −525 and +13 to +54) may contribute to tissue-specific expression. We infer, based on a comparative analysis of our results with those of Kothary et al. (24) and Koedood et al. (23), that the −670 to −525 segment is required for efficient glandular expression (in particular, thyroid, parathyroid, and salivary glands), whereas the +13 to +54 segment is important for expression in muscle. Note, the two previous studies did not examine expression in blood cells, chondrocytes, and, most importantly, in extraembryonic structures. Overall, our findings together with those of Koedood et al. (23) strongly indicate that the HCMV MIEP is a critical control point for determining viral tropism in vivo. In the embryo, two phases of transgene expression are exhibited. The first phase occurs during body plan formation which subsequently becomes highly restricted in the second phase upon differentiation of various organ systems. How other regions surrounding the promoter, in particular the modulator region (27, 35), affect promoter activity remains open. However, the promoter segment studied here is sufficient for recapitulating more fully the natural infection pattern by HCMV in neonates.

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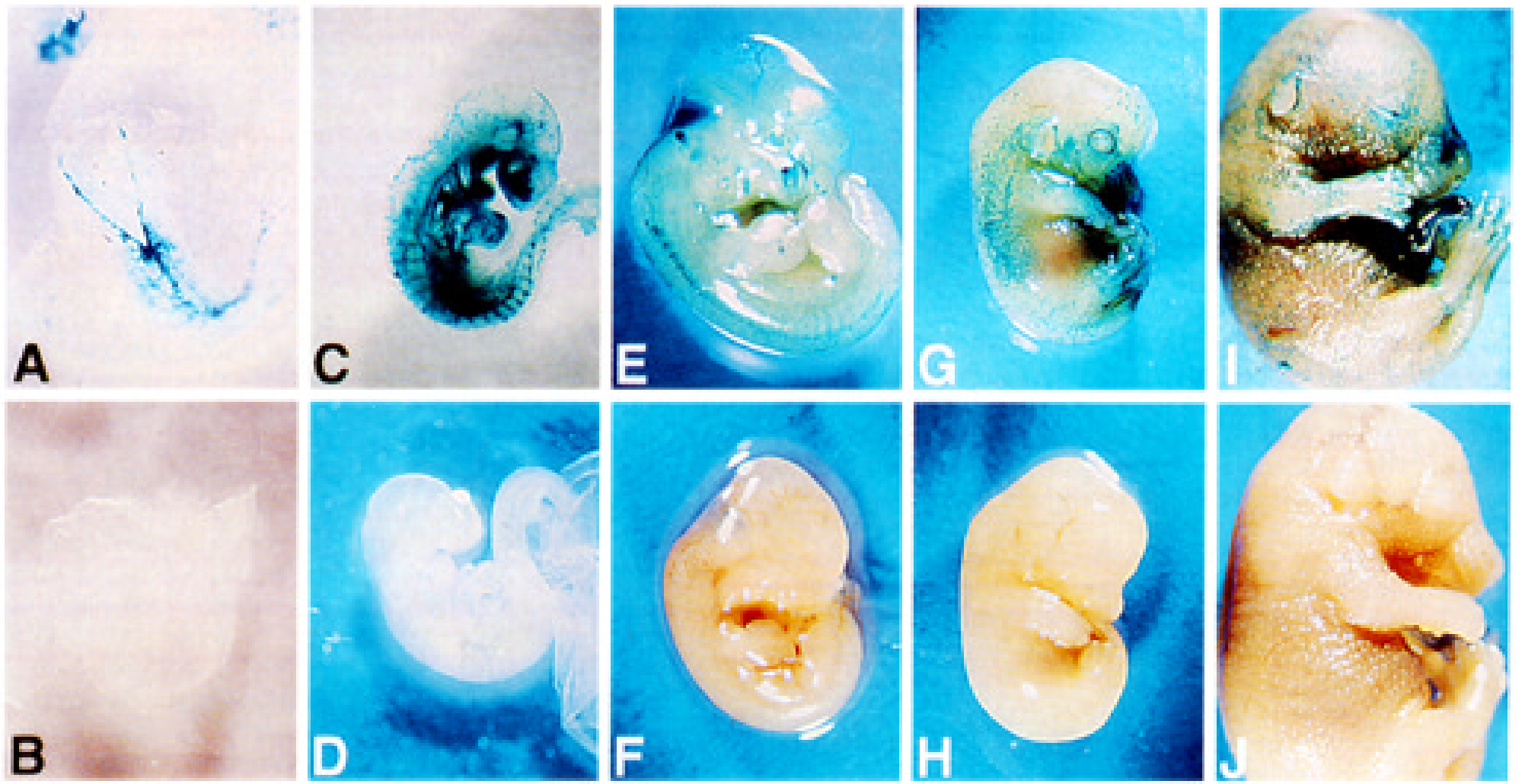
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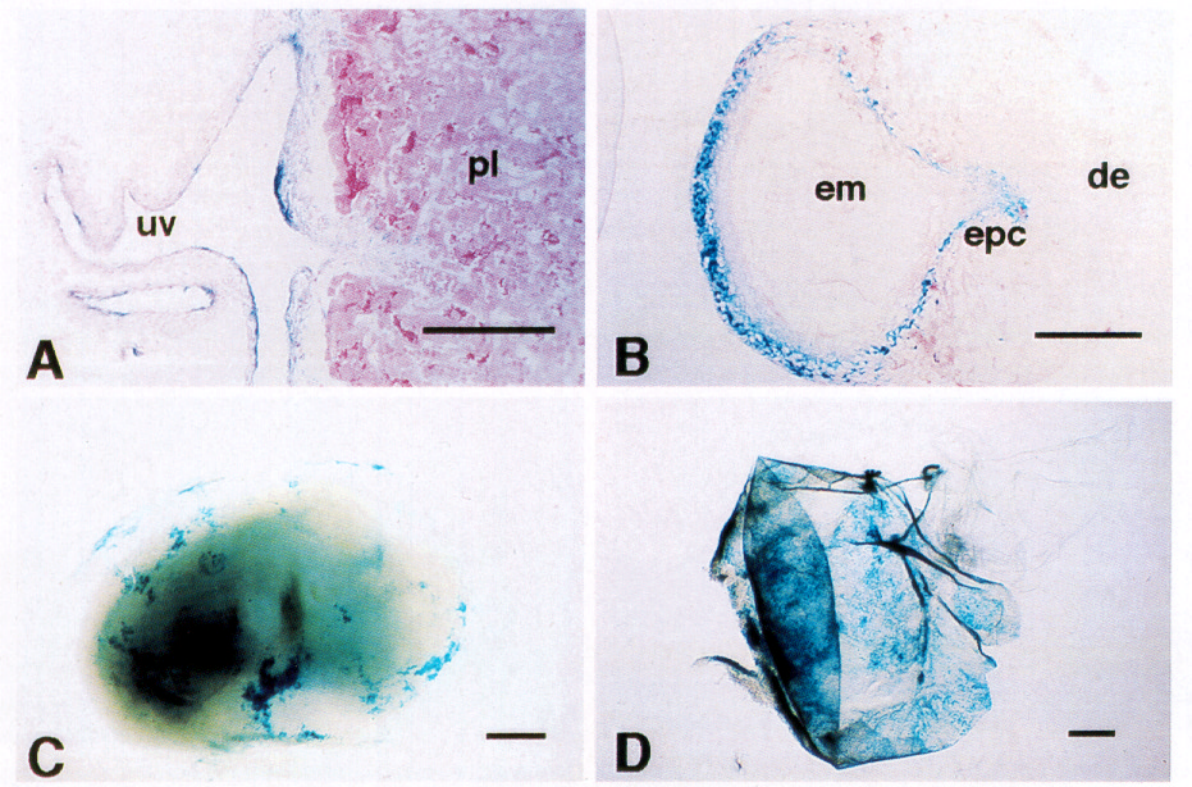
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