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Human Cytomegalovirus (HCMV) Immediate-Early Enhancer/Promoter Specificity during Embryogenesis Defines Target Tissues of Congenital HCMV Infection

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Congenital human cytomegalovirus (HCMV) infection is a common cause of deafness and neurological disabilities. Many aspects of this prenatal infection, including which cell types are infected and how infection proceeds, are poorly understood. Transcription of HCMV immediate-early (IE) genes is required for expression of all other HCMV genes and is dependent on host cell transcription factors. Cell type-specific differences in levels of IE transcription are believed to underlie differences in infection permissivity. However, DNA transfection experiments have paradoxically suggested that the HCMV major IE enhancer/promoter is a broadly active transcriptional element with little cell type specificity. In contrast, we show here that expression of a *lacZ* gene driven by the HCMV major IE enhancer/promoter −524 to +13 segment is restricted in transgenic mouse embryos to sites that correlate with known sites of congenital HCMV infection in human fetuses. This finding suggests that the IE enhancer/promoter is a major determinant of HCMV infection sites in humans and that transcription factors responsible for its regulation are cell type-specifically conserved between humans and mice. The *lacZ* expression patterns of these transgenic embryos yield insight into congenital HCMV pathogenesis by providing a spatiotemporal map of the sets of vascular, neural, and epithelial cells that are likely targets of infection. These transgenic mice may constitute a useful model system for investigating IE enhancer/promoter regulation in vivo and for identifying factors that modulate active and latent HCMV infections in humans.

Human cytomegalovirus (HCMV) is widely distributed in all populations of the world (1, 5). Like other herpesviruses, HCMV can establish permanent residence in the host following primary infection and recurrent or persistent infections may occur. Despite these features, HCMV rarely causes symptomatic disease except in immunocompromised individuals and prenatally infected fetuses. In these cases, however, HCMV infections can be life threatening. Opportunistic HCMV infections in AIDS, cancer, and organ transplant patients are frequent causes of morbidity and mortality and are currently the subject of intense clinical investigation (5). In contrast, although congenital HCMV infection is the most common prenatal viral infection known in humans, the mechanisms of developmental damage by HCMV are relatively difficult to investigate and remain poorly understood.

Clinical surveys in North America and Europe suggest that ~1% of all newborns have been infected in utero by HCMV as a consequence of reactivated or primary maternal HCMV infection during pregnancy (56). Although the virus can apparently be transmitted to the fetus during active maternal infection at any time during pregnancy, severe birth defects due to HCMV have been mainly linked to primary maternal infection during the first trimester of pregnancy (56, 67). This observation suggests that the first-trimester embryo or fetus is most vulnerable and that preexisting maternal anti-HCMV antibody prevents virulent fetal infection in cases of reactivated maternal infection. The fetus can eventually mount an independent immune response which may be important in restricting mid- and late-gestation fetal infection; fetal anti-HCMV immunoglobulin M has been detected as early as week 22 of gestation (10). The outcome of congenital HCMV infection may also depend to some extent on host genetic factors and virus strain-specific differences in virulence (16). Among infants born with signs of congenital HCMV infection (including, minimally, anti-HCMV immunoglobulin M in newborn cord sera and/or virus in neonatal urine or tissue), approximately 10 to 15% have symptomatic developmental defects that can include sensorineural hearing loss, mental retardation, spasticity, seizures, and blindness (25, 26, 35, 44, 45, 47, 49, 57, 59, 67). For these reasons, congenital HCMV infection is a major public health problem. Animal cytomegaloviruses have been studied in an attempt to define relevant model systems for congenital and perinatal HCMV infections (3, 8, 16, 28). However, it has been unclear how closely these animal models correspond to human HCMV infections.

Studies of HCMV replication indicate that the virus has a narrow host range specificity, despite the fact that it can enter many cell types (4, 38, 60). Permissive host cell types must, as a first requirement, contain appropriate cellular transcription factors that bind to DNA sequences in the HCMV major immediate-early (IE) enhancer/promoter (hereafter referred to as the HCMV IE enhancer/promoter) to activate transcription of IE genes (reviewed in reference 22). For many nonpermissive human cell types, the block to viral replication entails an inability to produce IE transcripts (reviewed in reference 38). Proteins encoded by IE transcripts initiate the cascade of events which, in fully permissive cells, allows extensive expression of the viral genome and culminates in viral DNA replication and virion production (reviewed in references 38, 58, and 60). Cell type-specific differences in the levels of IE gene products are predicted to act as a switch mechanism in this process (22). However, somewhat paradoxically, dissection of IE viral sequences has failed to identify regulatory elements that confer

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highly cell type-specific transcription to reporter genes in cultured cells. Indeed, the major HCMV IE enhancer/promoter is widely reputed to be one of the strongest and most broadly active regulatory elements for directing transcription of heterologous genes in cell culture DNA transfection experiments (6, 17, 22, 58). In addition, the IE enhancer/promoter has been described as a "pan-active" transcriptional element for directing expression of heterologous genes in tissues of adult transgenic mice (52). These findings encouraged the notion that the IE enhancer/promoter is active in almost all cell types and that additional viral sequences are required to impose cell type-specific restrictions on IE gene expression.

In the report presented here, we contest this assumption and show that the activity of the HCMV IE enhancer/promoter segment from position −524 to +13 (segment −524 to +13) is inherently restricted in transgenic mouse embryos to sites that correlate strongly with known target tissues of congenital HCMV infection in human fetuses. This unexpected correlation provides strong evidence that cell type- and region-specific differences in activity of the IE enhancer/promoter play a fundamental role in defining HCMV tissue tropism and that transcription factors required for this regulation are highly conserved in mammalian embryos.

MATERIALS AND METHODS

Preparation of transgene DNA. Construction of pHCMV-IE-*lacZ*: the *Escherichia coli lacZ* gene was taken from pNL (19) as a 3-kb *Bam*HI-*Stu*I fragment and cloned into *Bam*HI-*Sac*I (blunt-ended) linearized vector pSCT (48). This *lacZ* gene is modified to encode β-galactosidase (β-Gal) with a nuclear localization signal from simian virus 40 large T antigen; this feature improves cellular resolution of β-Gal staining, particularly in the nervous system. The pSCT expression vector contains transcriptional regulatory sequences from position −524 to +13 of the HCMV major IE enhancer/promoter region and splice and polyadenylation signals from the rabbit β-globin gene (48). The *Pst*I-to-*Apa*I polylinker region of pBluescript (Stratagene) was cloned into an *Apa*LI site ~100 bp upstream of the HCMV IE enhancer/promoter to create convenient 5′ restriction sites for transgene excision. The transgene fragment for microinjection was isolated from *Xho*I-digested pHCMV-IE-*lacZ* DNA.

Generation of HCMV-IE-*lacZ* transgenic mice. Transgenic mice were produced by standard techniques essentially as described by Hogan et al. (27), using Swiss ICR outbred mice. Microinjection of DNA into pronuclei of one-celled fertilized mouse embryos was performed with an Olympus inverted microscope and Leitz micromanipulators. Injected 0.5-day-postcoitus (dpc) embryos were transferred to oviducts of pseudopregnant females and were either brought to term for the establishment of transgenic lines or were harvested at 10.5 dpc for direct analysis as F_0 embryos. Transgenic founders were identified by PCR of genomic DNA prepared from tail tips or extraembryonic tissues (11). PCR was performed with *lacZ*-specific primers (5′ primer, 5′-TTTCGCCAGCTGGCGTAATAGCGAA-3′; and 3′ primer, 5′-TAGATGGGCGCATCGTAACCGTGCAT-3′) for 30 cycles of 30 s at 95°C, 1 min at 59°C, and 1 min at 72°C. Transgenic founders identified by PCR were confirmed by Southern blotting of *Bam*HI-digested genomic DNA using a ^{32}P-labelled probe corresponding to the transgene *Xho*I fragment. Transgene copy numbers were quantitated by phosphorimage analysis of the hybridized filter by normalizing the 4.8-kb *Bam*HI band intensity (from head-to-tail tandem repeats) to the outer copy fragments (single-copy intensity) and comparison of these intensities to a standard curve of genomic equivalents of diluted *lacZ* plasmid mixed with nontransgenic mouse genomic DNA. From 54 F_0 mice, 5 transgenic founders which subsequently transmitted *lacZ* genes in Mendelian ratio to progeny were identified. Transgenic males of each line were mated to normal ICR females for analysis of *lacZ* expression in hemizygous transgenic embryos by β-Gal staining. Three of the five lines did not show detectable β-Gal staining in embryonic or adult tissues, suggesting integration site-dependent silencing of their transgenes. Two transgenic lines, HCMV-1 and HCMV-2, showed *lacZ* expression by β-Gal staining at all stages of embryogenesis analyzed (and in multiple adult tissues [not shown]). Additional founder embryos were generated for direct analysis at the 10.5-dpc stage following oviduct transfer. Of 26 F_0 embryos, 3 were found to be transgenic; of these, 2 were nonexpressing, while the 3rd embryo, HCMV-3, showed a staining pattern nearly identical in pattern and intensity to 10.5-dpc HCMV-1 embryos. Overall, HCMV-IE-*lacZ* transgenes were active in two of five transgenic lines and in one of three additional founder embryos obtained. *lacZ* expression patterns in the HCMV-1 and HCMV-2 lines were highly reproducible over multiple generations, although occasionally some HCMV-2 individuals produced slightly stronger or weaker patterns than usual (i.e., line HCMV-2 shows variable expressivity).

β-Gal staining of whole embryos and cryosections. Staged embryos derived from matings between transgenic males and nontransgenic females were removed from the uteri and dissected free of extraembryonic membranes in ice-cold phosphate-buffered saline (PBS) (27). For whole-mount β-Gal staining, embryos were fixed for 15 to 90 min (depending on stage) at 4°C in 4% paraformaldehyde freshly dissolved in wash solution (2 mM $MgCl_2$–0.02% Nonidet P-40 in PBS, pH 7.4) plus 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]. After fixation, embryos were washed three times for 20 min at 4°C in wash solution. The β-Gal stain solution contained 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.01% sodium deoxycholate, and 1 mg of Bluogal (GIBCO BRL) per ml in wash solution. Whole embryos were stained in light-tight vials for ~15 h at 30°C (unless noted otherwise) with gentle rocking and then were briefly soaked in wash solution before being photographed with a Wild stereomicroscope set-up with incidental fiber optic and bright-field base light sources. Embryos were postfixed for 6 to 20 h in fresh 4% paraformaldehyde in PBS (pH 7.4) and were dehydrated through graded ethanol solutions prior to paraffin embedding, sectioning, neutral red counterstaining, and mounting coverslips with DPX mounting medium (BDH Chemicals, Ltd). Photomicrography was done with a Zeiss Axiophot set-up. Penetration of fixative and stain in whole embryos becomes limiting after 11.5 dpc. Relative to sections of whole-mount-stained embryos, direct β-Gal staining of 12.5- and 13.5-dpc cryosections yielded higher resolution and stronger patterns and allowed better comparisons of *lacZ* expression levels in different tissues. For cryosectioning, embryos were harvested as described above, were fixed at 4°C for 45 to 60 min in 4% paraformaldehyde in PBS, and then were rinsed in PBS for 5 min and freeze-mounted in GSV 1 medium (SleeTechnik GmbH). Frozen sections 20 or 30 μm thick were cut on a cryostat, mounted on gelatin-coated slides, and air dried for 1 to 2 h at room temperature. Sections were encircled with an Immunopen (Calbiochem), and slides were fixed for 10 min at 4°C in 4% paraformaldehyde in PBS, were washed two times for 10 min in PBS, and then were placed horizontally in a humidified, light-tight box to be incubated for ~15 h at room temperature with ~100 μl of β-Gal stain solution per section. The slides were rinsed for 5 min in PBS, counterstained with neutral red, and mounted with coverslips.

Costaining method to detect β-Gal activity and vWF in endothelial cells. Cryosections of 13.5-dpc transgenic and control embryos were prepared as described above. For costaining, the protocol of Mercer et al. (36) was followed, with minor modifications. Rabbit anti-human von Willebrand Factor (vWF) antibody (Sigma) diluted 1:1,000 was used as the primary antibody. Incubation with the primary antibody in β-Gal stain solution (lacking $MgCl_2$) was performed overnight at room temperature. The biotinylated secondary antibody and reagents for avidin-biotinylated horseradish peroxidase detection of the primary antibody were obtained from the Vectastain ABC kit (Vector Laboratories) and were used according to the manufacturers' specifications. Controls to confirm the anti-vWF specificity included omission of the primary antibody as well as use of other primary antibodies with different specificities.

RESULTS

Restricted activity of the HCMV IE enhancer/promoter during embryogenesis is conserved in three transgenic mouse lines. Two transgenic mouse lines (HCMV-1 and HCMV-2) and a third independent founder embryo (HCMV-3) carrying actively expressed *lacZ* reporter genes linked to the HCMV IE enhancer/promoter segment −524 to +13 were generated for analysis of the tissue-specific regulation of this viral segment during embryogenesis (see Materials and Methods). The HCMV-IE-*lacZ* DNA fragment used for microinjection into pronuclei of fertilized mouse eggs is depicted in Fig. 1. Southern blot analysis to estimate transgene genomic copy numbers in the three lines is shown in Fig. 2 (estimations: HCMV-1, 3 copies; HCMV-2, ~10 copies; and HCMV-3, 3 copies). Hemizygous transgenic embryos and wild-type littermates (controls) representing the transgenic lines HCMV-1 and HCMV-2 were assayed by β-Gal staining of whole embryos (8.25- to 12.5-dpc stages [Fig. 3 to 6]) and of cryosections (12.5 and 13.5 dpc [Fig. 6 and 7]). In addition, whole-mount stained embryos (including the independent founder embryo HCMV-3) were embedded in paraffin and sectioned for examination of internal structures (sections in Fig. 3 to 5).

All three transgenic lines revealed a conserved pattern of β-Gal staining primarily restricted to subsets of neural, epithelial, and vascular cell types. The 10.5-dpc founder embryo HCMV-3 (Fig. 4D, E, and H) showed a β-Gal pattern nearly identical to that of 10.5-dpc HCMV-1 embryos (Fig. 4A, F, G, and I) except for the lack of strong expression in the ventral

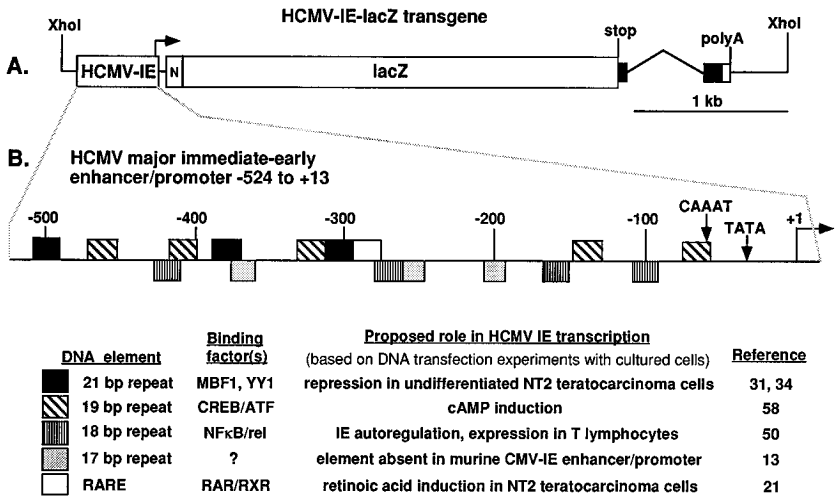


FIG. 1. HCMV-IE-*lacZ* transgene. (A) DNA fragment (transgene) used to generate transgenic mice by pronuclear microinjection of fertilized mouse eggs. N, nuclear localization signal from simian virus 40 large T antigen. Splice and polyadenylation signals are from the rabbit β-globin gene (46). (B) Expanded HCMV major IE promoter/enhancer region and DNA sequence elements implicated in its transcriptional control in cultured cells. cAMP, cyclic AMP; ?, unknown factor.

diencephalon of the brain. In contrast, and despite a higher transgene genomic copy number, HCMV-2 embryos yielded β-Gal patterns that were weaker than those of HCMV-1 embryos at all stages analyzed. In order to determine whether these differences were mainly quantitative or also qualitative, the β-Gal staining times for HCMV-1 embryos were systematically reduced for comparison with the weaker HCMV-2 patterns. These tests at 10.5 dpc and other stages not shown indicated that the sites of *lacZ* expression in the two lines were qualitatively very similar (for example, the β-Gal pattern in 10.5-dpc HCMV-1 embryos stained for 3 h was nearly identical to that of 10.5-dpc HCMV-2 embryos stained approximately five times longer [compare Fig. 4B and C]). One exception was that ventral diencephalon expression was still evident in HCMV-1 embryos, although it was not seen in HCMV-2 embryos (nor in HCMV-3). This site in HCMV-1 embryos may be due to ectopic activation, or alternatively, it might be a site of potential expression that is highly sensitive to repressive position effects. It is widely observed in transgenic studies that although tissue specificities of regulatory elements present on transgenes are often preserved in different chromosomal locations (i.e., different transgenic lines), transgene RNA levels may vary significantly from line to line without a clear correlation with the genomic copy number of the introduced gene (reviewed in references 43 and 68). Our data and those of others indicate that HCMV IE enhancer/promoter-driven transgenes are typically subject to this copy number-independent variation in expression level (18, 31, 52). Lower-level expression in the HCMV-2 line is characterized by consistently fewer positive cells relative to the HCMV-1 line in almost all the sites of expression in embryonic and adult tissues (latter not shown); in addition, the level of expression in each positive cell in HCMV-2 tissues is lower on average than that in HCMV-1 tissues. Recent analysis of transgenic flies suggests that tandem transgenes are prone to a mosaic inactivation phenomenon which resembles classical heterochromatin-induced position effect variegation (12). This inactivation effect increases with transgene copy number and may reflect an inherent heterochromatinization property associated with repeated DNA segments. Mosaic expression of transgenes also occurs in mice (46); circumvention of these effects may be dependent on inclusion of appropriate matrix attachment sites or other sequences that block heterochromatin formation.

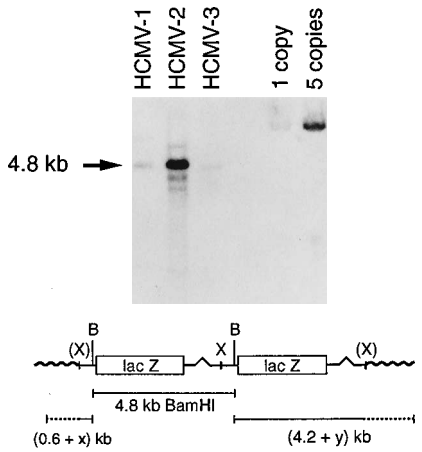


FIG. 2. Southern blot analysis of HCMV-IE-*lacZ* transgenes in three transgenic lines. Genomic DNA samples (10 μg) from HCMV-1 and HCMV-2 transgenic founder mice and from embryo HCMV-3 were digested with *Bam*HI (B). The *Xho*I (X) transgene fragment probe identifies a multicopy 4.8-kb *Bam*HI fragment (arrow) representing tandem head-to-tail transgenes and weaker single-copy fragments of unique size representing the 5′ and 3′ outermost transgenes in the set.

The HCMV-IE-*lacZ* transgenic lines studied here identify a conserved set of sites where the IE enhancer/promoter (segment −524 to +13) is active in 8.5- to 13.5-dpc mouse embryos. (The gestation period in mice is ~19 days; and 8.5- to 14-dpc mouse embryos correspond ontogenically to 3- to 8-week-old human embryos [29, 62]). These sites of activity can be divided into three classes on the basis of relative levels of β-Gal staining activity. Sites with high activity levels are easily detected in HCMV-2 embryos and also in HCMV-3 and in HCMV-1 embryos stained briefly. Presumably, cells at these embryonic sites have the best combination(s) of transcription factors and signal transduction components that activate the IE enhancer/promoter. The sites in this class include the dorsal neural tube in the vicinity of the otic pit (8.5 to 9.5 dpc) and the hindbrain roof and fourth ventricle choroid plexus (11.5 to 13.5 dpc); the endolymphatic sac and duct of the inner ear (10.5 to

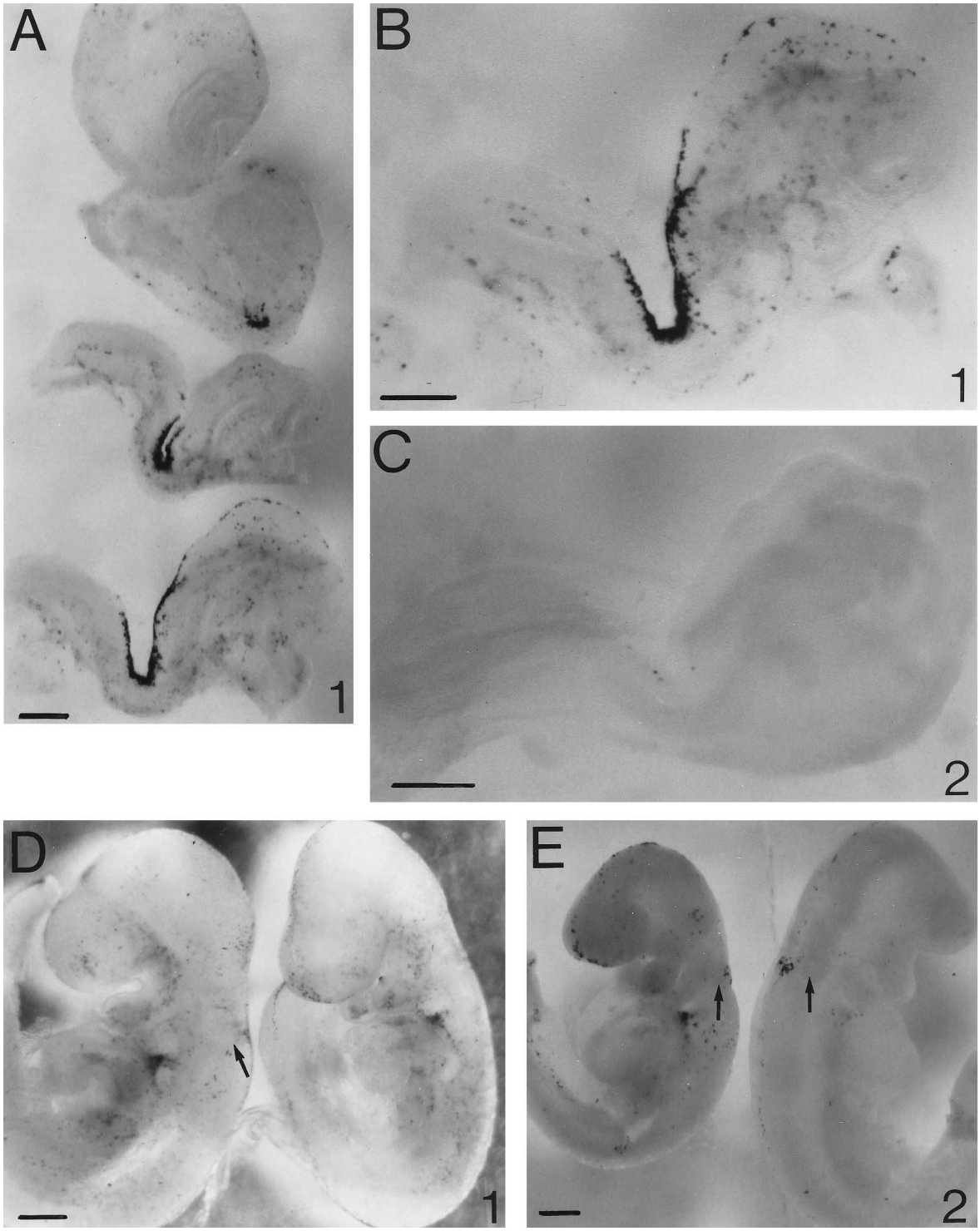
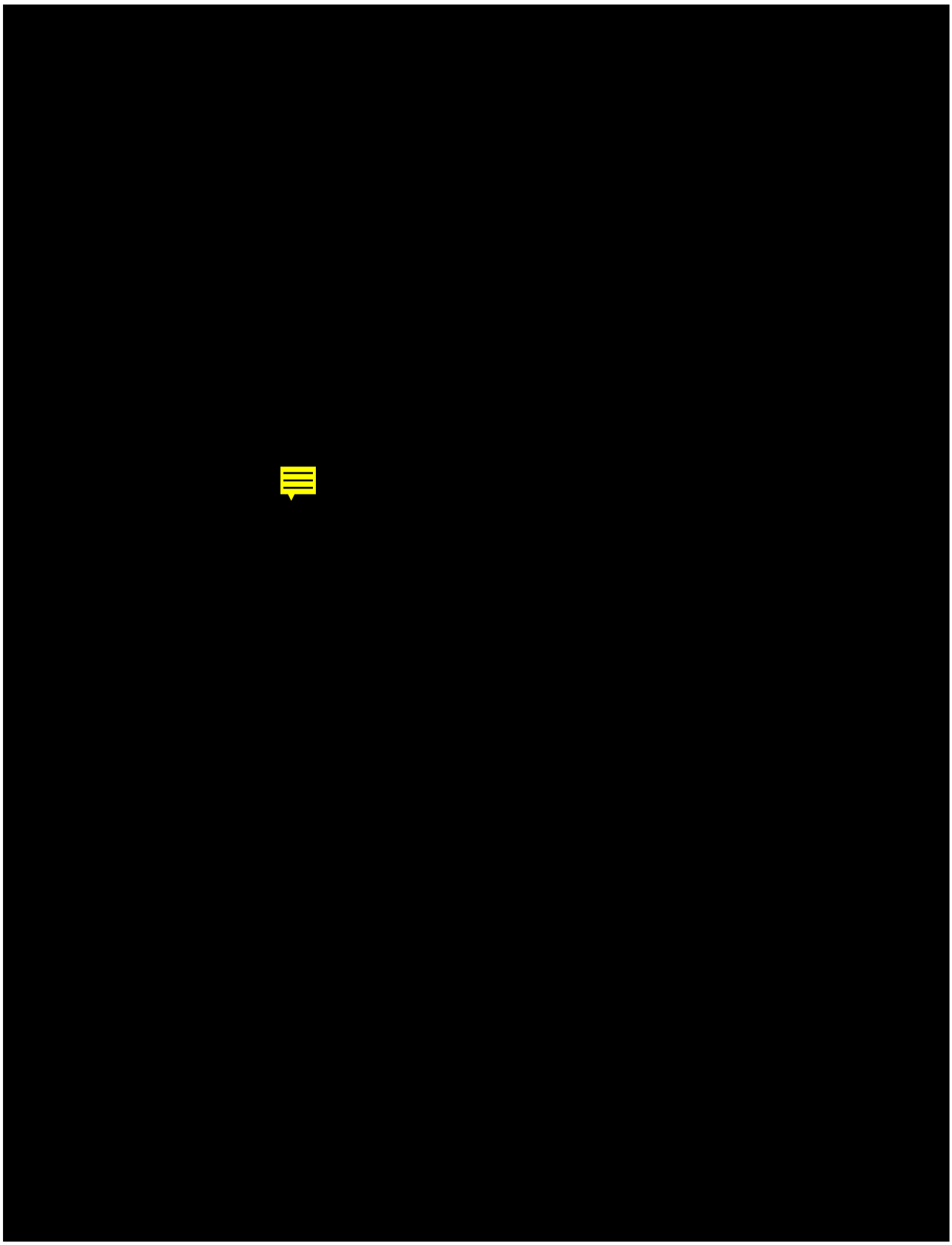


FIG. 3. *lacZ* expression in 8- to 9.5-dpc embryos. Note that for every panel shown in Fig. 3 to 6 the transgenic line number of the β-Gal-stained embryo is noted in the lower righthand corner (HCMV-1 as 1, HCMV-2 as 2, and HCMV-3 as 3) and a 300-μm scale bar is shown in the lower lefthand corner. (A to C) Embryos at 8 to 8.5 dpc, with rostral or head ends facing to the right and ventral surfaces down. For 9.5-dpc embryos (D and E), arrows indicate the approximate position of the otic vesicle (primordia of the inner ear) just lateral to the hindbrain (at approximately rhombomere 5 level). (A) HCMV-1 embryos 8 to 8.5 dpc (5 to 12 somites). The yolk sac and amnion have been dissected from the two older (two lowest) embryos. (B) HCMV-1 embryo at 8.5 dpc. (C) HCMV-2 embryo at 8.5 dpc. (D) Two 9.5-dpc HCMV-1 embryos (littermates) presenting side and side-front views. The dorsal side is to the right, and the head end is up. (E) Two 9.5-dpc HCMV-2 embryos (littermates) showing variable expressivity of this line. The more advanced embryo (right) shows the typical β-Gal staining pattern for HCMV-2; the less typical pattern of the lefthand embryo closely resembles the HCMV-1 pattern seen in panel D.



13.5 dpc) and parts of the saccule, utricle, cochlea, and semicircular canals (12.5 to 13.5 dpc); the nasal placode, nasal cavity, and olfactory epithelia (9.5 to 13.5 dpc) and oropharyngeal epithelia next to primary palate (13.5 dpc); follicles of vibrissae and tactile hair follicles (11.5 to 13.5 dpc); the third to sixth branchial arch arteries and carotid and pulmonary arteries and the arch of the aorta as well as some lung bud mesenchyme (9.5 to 13.5 dpc); the dorsal aorta, aortic sac, and truncus arteriosus (9.5 to 13.5 dpc); and umbilical and chorionic plate blood vessels (10.5 to 13.5 dpc).

Sites with moderate to low activity levels are those that are only barely detected in HCMV-2 embryos and in HCMV-1 embryos stained briefly (but easily detected with longer staining of HCMV-1 embryos). Presumably, one or more factors required for activity are limiting in these cell types relative to those in the high-level activity sites (or are more abundant in the case of possible repressors). The sites with moderate-to-low activity levels include the rest of the dorsal neural tube (8.5 to 9.25 dpc); the dorsal midline and roof of the third ventricle (10.5 dpc); the choroid plexus of the third and lateral ventricles (12.5 to 13.5 dpc); rare, scattered cells throughout the central nervous system (CNS), with more in the ventral CNS posterior to the hindbrain (10.5 to 13.5 dpc); the facial-acoustic ganglia complex (gVII-VIII) (11.5 to 12.5 dpc) and vestibulocochlear ganglia (gVIIIv, c) (13.5 dpc); scattered cells in other peripheral nervous system (PNS) ganglia (11.5 to 13.5 dpc); cranial and spinal nerves and branches and sympathetic trunk (mainly consistent with pre-Schwann cells) (10.5 to 13.5 dpc); segmental ectoderm patterns including dorsal stripes, lateral body spots, and mammary gland primordia (11.5 to 13.5 dpc); esophageal epithelium, trachea, and tooth primordia (13.5 dpc); perioptic vascular plexus, hyaloid plexus, optic stalk, pigment layer of the retina, perioptic mesenchyme, and cornea (11.5 to 13.5 dpc); neovasculature sprouting from the dorsal aorta (10.5 to 11.5 dpc) and CNS vascular plexus (10.5 to 13.5 dpc); the atrioventricular endocardial cushion and valves (10.5 to 13.5 dpc); the anterior pituitary (12.5 to 13.5 dpc); pancreatic and nephric primordia (12.5 to 13.5 dpc); and rare cells in liver, adrenal, and urogenital sites (12.5 to 13.5 dpc).

The diencephalic thalamus region constitutes a third class unique to HCMV-1 embryos. The β-Gal patterns that demonstrate these expression sites are described below. The correlations between sites of IE enhancer/promoter activity and known sites of congenital HCMV infection in human fetuses are presented in Discussion.

HCMV IE enhancer/promoter activity in the dorsal neural tube of 8- to 9.5-dpc embryos. The *lacZ* expression pattern in 8-dpc HCMV-1 embryos consisted of rare, scattered cells in the yolk sac and precardiac mesoderm (top embryo in Fig. 3A and higher-magnification ventral views not shown). Coincident with initiation of neural tube closure, a group of cells with strong β-Gal activity was seen in the dorsal neural tube at the site of closure (Fig. 3A, second embryo from top). Between 8.25 and 8.5 dpc, this expression expanded into a dorsal stripe of irregular outline extending from the caudal neuropore to the hindbrain and then more sparsely into the open neural folds of the head (Fig. 3A and B). The stripe was several cells deep and wide at its broadest midaxial point and included both surface ectoderm and neuroepithelium (sections not shown). In 8.5-dpc HCMV-2 embryos (Fig. 3C), *lacZ* expression was detected in only a few cells in the dorsal neural tube. Their axial level corresponded to that where the most expressing cells were seen in HCMV-1 embryos (i.e., caudal hindbrain and upper spinal cord).

In 9-dpc HCMV-1 embryos (not shown), β-Gal-positive cells were seen along the dorsal axis and scattered dorsolaterally over the caudal hindbrain between the otic vesicles (inner ear primordia). At 9.5-dpc (Fig. 3D and sections not shown), positive cells were detected in HCMV-1 embryos in the third branchial arch artery, heart outflow tract, dorsal aorta, rare scattered cells in head and upper-trunk mesenchyme, and a sparse track of cells from the branchial arch 3 region caudally along the midgut wall and dorsal mesentery. Expression was also detected in vitelline blood vessels, nasal placode, and limited portions of the yolk sac and allantois. As seen at 8.5 dpc, 9- to 9.5-dpc HCMV-2 embryos exhibited levels of expression reduced relative to those of HCMV-1 embryos; most showed only a few positive cells limited to the dorsal area adjacent to and slightly posterior to the otic placodes (approximate level of hindbrain rhombomere 5) (Fig. 3E and sections not shown). Again, this location corresponded to the axial level with the most expressing cells in HCMV-1 embryos. Occasionally, a 9.5-dpc HCMV-2 embryo with a stronger β-Gal pattern that nearly approximated the HCMV-1 pattern was obtained, revealing the high degree of qualitative similarity between the two lines (Fig. 3, compare the lefthand HCMV-2 embryo in panel E to the HCMV-1 embryos in panel D).

Neovasculature is a major site of IE enhancer/promoter activity at 10.5 dpc. In 10.5-dpc embryos (Fig. 4), *lacZ* expression was expanded relative to earlier expression and was most striking in vascular and cardiovascular sites. Expressing cells were continuous along the heart outflow tract (aortic sac and truncus arteriosus) and into the branchial arch arteries and dorsal aortae (see the HCMV-1 embryo stained for 3 h [Fig. 4B] and the HCMV-2 embryo stained for ~15 h [Fig. 4C]). Of these sites, the major (third, fourth, and sixth) branchial arteries contribute to the carotid arteries, the arch of the aorta, and the pulmonary arteries, respectively, while the outflow tract gives rise to the aortic and pulmonary trunks and valves. Neovasculature sprouting from the dorsal aortae showed striking expression (including intersegmental arteries [Fig. 4I] and vasculature invading the CNS and head region, in particular, the CNS vascular plexus [Fig. 4G and H] and perioptic vascular plexus [Fig. 4E]). Other sites of expression included the atrioventricular endocardial cushion (mitral and tricuspid valve primordia [Fig. 4G]), ventral region of the upper spinal cord (Fig. 4G and H), ventral diencephalon (HCMV-1 [Fig. 4A and B]), roof of the third ventricle and overlying ectoderm (Fig. 4A and midbrain and diencephalon sections not shown), nasal placode, some lung bud mesenchyme, rare scattered cells in the dorsal mesentery, the vitelline venous plexus, the epidermis, and a subset of cells in the endolymphatic diverticular

FIG. 4. *lacZ* expression in 10.5-dpc embryos. In all panels except B, embryos were stained for β-Gal activity for ~15 h at 30°C. The approximate planes of the transverse sections (panels E to I) are indicated in the whole-embryo photos (panels A, B, and D) by lines labelled with the appropriate panel letter. (A) Two HCMV-1 littermates. (B) HCMV-1 embryo stained for 3 h to show only the sites of stronger *lacZ* expression. (C) HCMV-2 embryo. (D) HCMV-3 embryo (founder). (E) Head section of HCMV-3 showing expression in the internal carotid artery (ica) and perioptic vascular plexus and anastomosis (pva) in the vicinity of the optic cup (op). tel, telencephalon lateral ventricle. (F) HCMV-1 embryo section showing expressing cells in the endolymphatic diverticular appendage (ov/e) on the dorsal aspect of the otic vesicle adjacent to the hindbrain (hb). (G) HCMV-1 embryo section showing expression in dorsal aortae (da) and vasculature sprouting towards the CNS, heart atrioventricular endocardial cushion (ec), ventral CNS, and vascular plexus of the CNS near the hindbrain-spinal cord junction. t, trachea. (H) HCMV-3 embryo section showing expression in dorsal aortae (da), spinal cord (sc), and vasculature sprouting into the CNS. drg, dorsal root ganglion. (I) Section through lower trunk of HCMV-1 embryo showing the midline dorsal aorta (mda) and sprouting intersegmental arteries (isa). The scale and numbering are explained in the legend for Fig. 3.

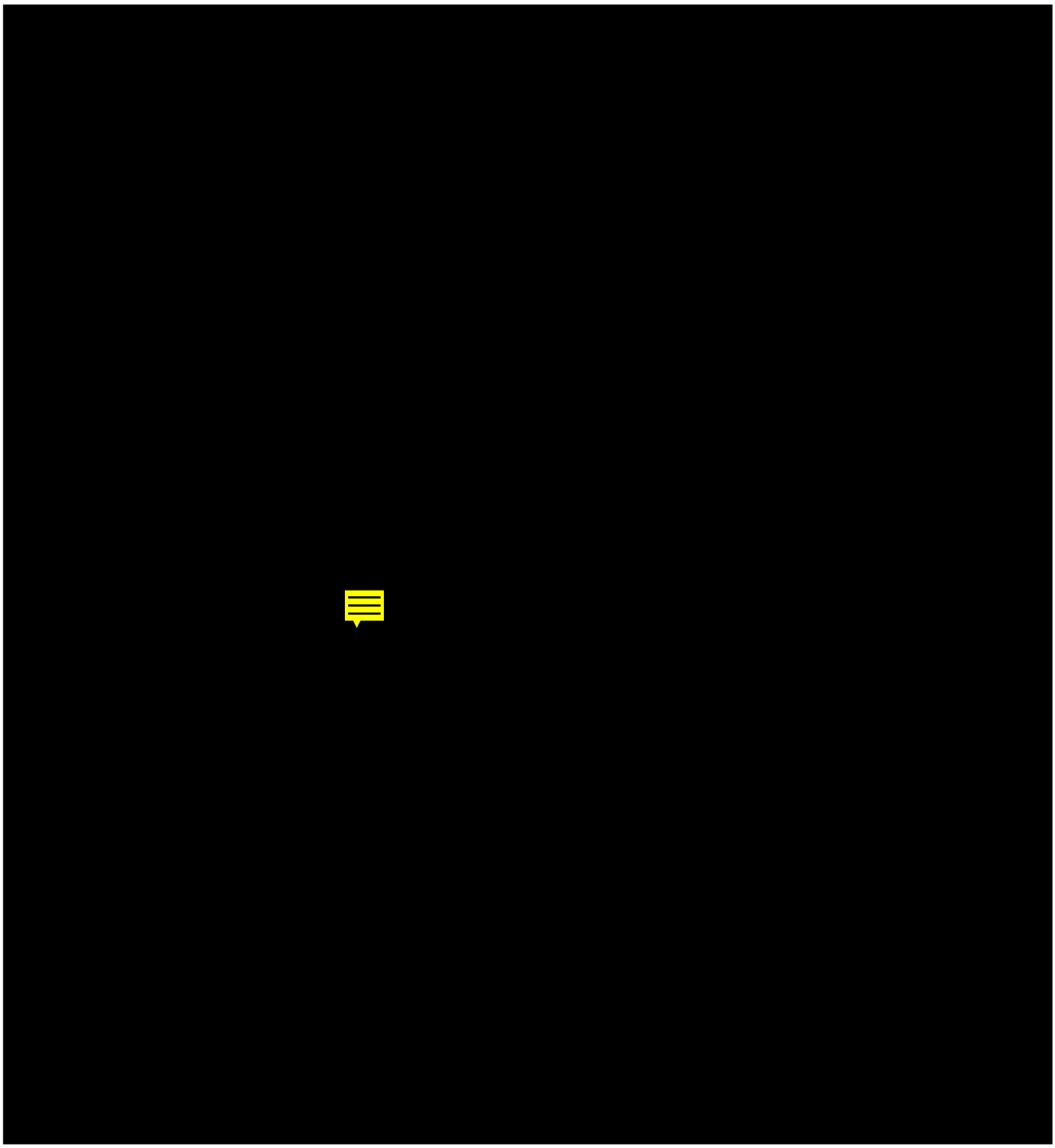


FIG. 5. *lacZ* expression in 11.5-dpc embryos. (A) β-Gal-stained HCMV-1 embryos at 11.5 and 12 dpc. Dashed lines show the approximate planes of HCMV-1 transverse sections in panels C and D. (B) β-Gal-stained 11.5-dpc HCMV-2 transgenic and nontransgenic littermates, showing transgene expression in hindbrain roof (hr), endolymphatic sac and duct (e) of the inner ear, and yolk sac (ys). (C) Head section (right half) showing expression in endolymphatic sac, dorsal part of the otic vesicle (ov), facio-acoustic ganglion complex (gVII-VIII), and parts of the eye (hyaloid plexus [hy] and perioptic mesenchyme [p]) and trigeminal ganglion (gV). (D) Section showing expression in endolymphatic sacs and ependymal roof of the hindbrain. (E) HCMV-1 placenta and yolk sac. cp, chorionic plate on the inner side of the placenta. The outer portion of the placenta (maternal decidual tissue) is nontransgenic. (F) Closer view of placental chorionic plate blood vessels (embryonic tissue) shown in panel E. The scale and numbering are explained in the legend for Fig. 3.

appendage (Fig. 4F) (primordia of the inner ear endolymphatic sac) of the otic vesicle.

Neural and epithelial sites of activity in 11.5- to 13.5-dpc embryos. After 10.5 dpc, neural and epithelial sites superseded vasculature as the major sites of HCMV IE enhancer/promoter activity. The greatest activity in 11.5- to 13.5-dpc embryos (Fig. 5 to 7) occurred in the caudal hindbrain roof and choroid plexus of the fourth ventricle, inner ear endolymphatic sac,

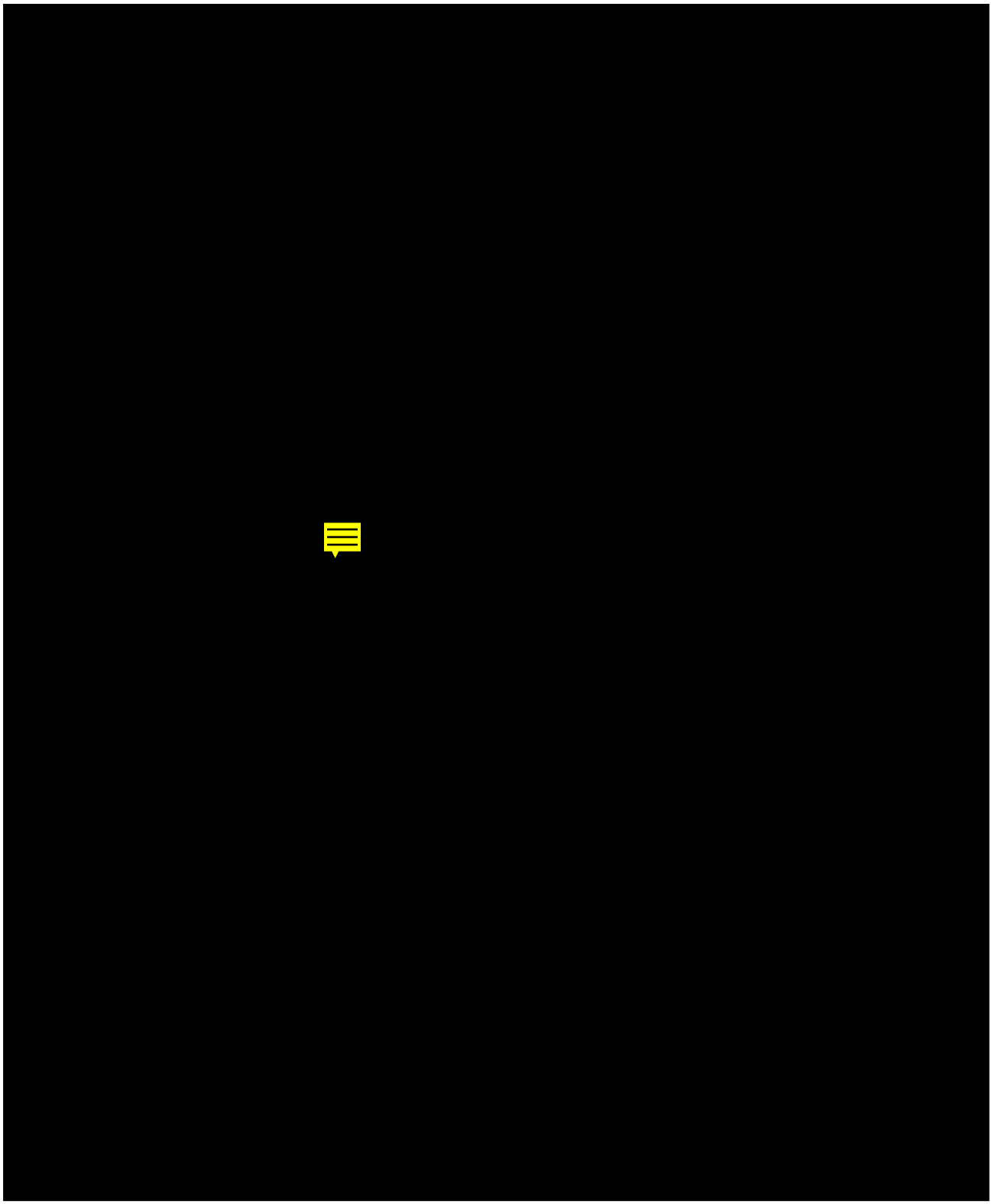


FIG. 6. *lacZ* expression in 12.5-dpc embryos. (A) β-Gal-stained HCMV-1 embryo. (B) β-Gal-stained HCMV-2 embryo. The dashed line indicates the plane of head dissection shown in panel I. (C to H) Cryosections stained directly for β-Gal activity. All the sections are frontal sections from 12.5-dpc HCMV-1 embryos. (C) Lung buds (lb), pulmonary (pa) and carotid arteries, and vagal trunk. (D) Vestibular and cochlear ganglia (gVIIIv-c) and saccule of inner ear. (E) Eye showing expression in optic stalk (os), hyaloid plexus (hy), future pigmented layer of the retina (pr), and maxillary division of the trigeminal nerve (V). (F) Outflow region of the heart showing expression in aortic (a) and pulmonary (p) trunk origins and left ventricle (lv) above the liver and posthepatic inferior vena cava. (G) Expression in the anterior pituitary (ap) and remnant of the connecting stalk to the roof of oropharynx, right and left internal carotid arteries (ica), trigeminal ganglion (gV) and maxillary division of trigeminal nerve (V), and lingual arteries in the tongue. (H) Section at level of the hindlimbs showing expression in cells overlying spinal cord (sc) and dorsal root ganglia (drg), in spinal nerves, sympathetic trunks, and lumbosacral plexus (lsp). (I) Head of HCMV-2 embryo (oriented nose up) with lower jaw and caudal hindbrain removed to view the roof of the oropharynx and fourth ventricle of the brain, showing expression in the endolymphatic sac (e), hindbrain roof (hr), whisker pits, and other sites. The scale and numbering are explained in the legend for Fig. 3.

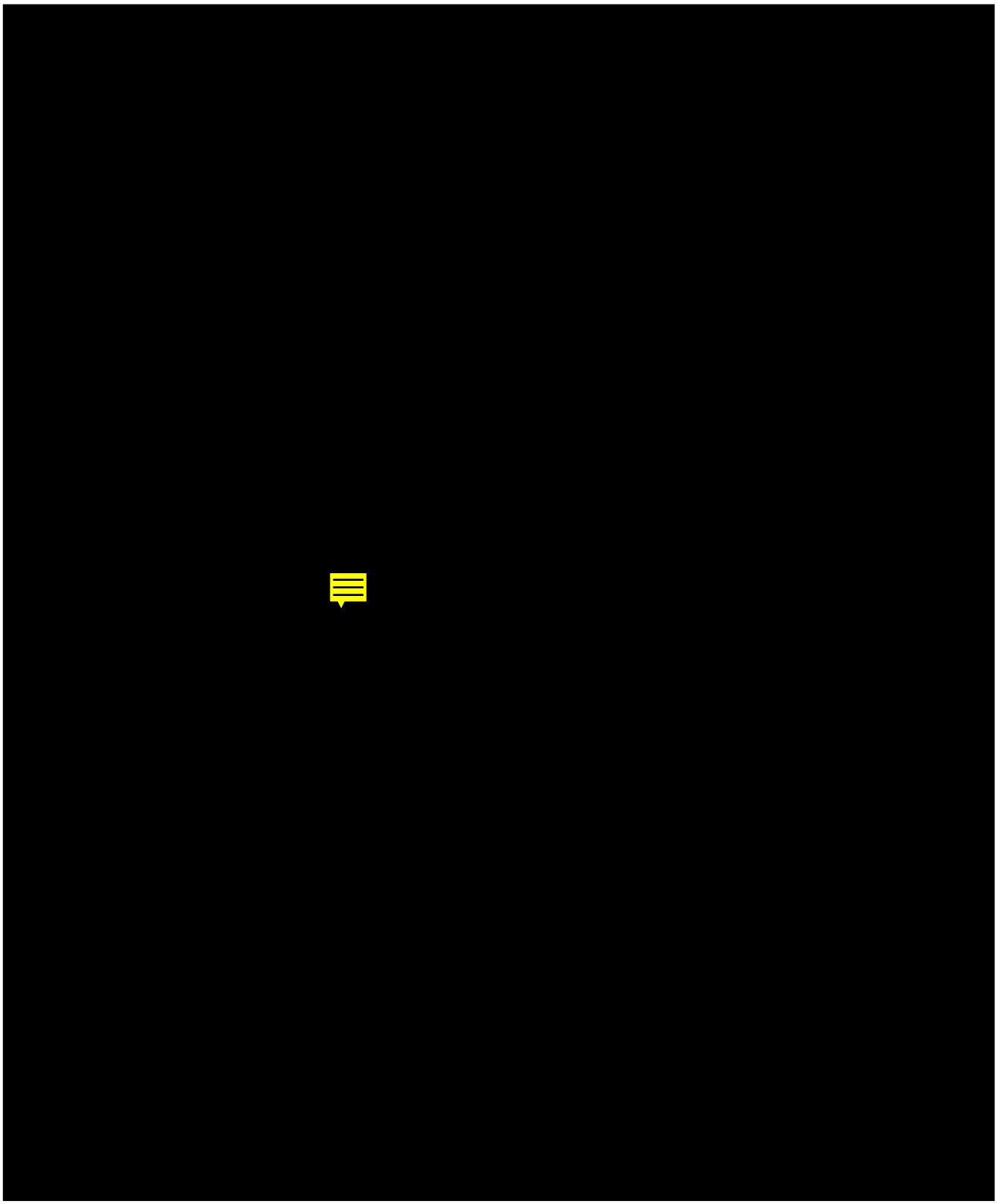


FIG. 7. *lacZ* expression in 13.5-dpc embryos. Cryosections are stained directly for β-Gal activity (A to F) or for β-Gal activity and anti-vWF immunoreactivity (G and H); all are sagittal sections from HCMV-1 embryos. The dorsal aspect is on the left in panels A to C, G, and H and on the right in panels D to F. (A) Expression in fourth ventricle ependyma and choroid plexus, subependymal region of hindbrain floor, inner ear saccule, cranial ganglia (eighth; weaker in the seventh and fifth), cranial and spinal nerves, and whisker pits. (B) Expression in choroid plexus of fourth and lateral ventricles, ependymal and subependymal sites in hindbrain floor, cranial and spinal nerves, nasal cavity and olfactory epithelium, scattered cells in the CNS, outflow region of heart, and weakly in the submandibular gland. (C) Expression in fourth ventricle choroid plexus, diencephalon (thalamus), anterior pituitary, nasal cavity and olfactory epithelium, esophagus, trachea, aortic and pulmonary trunks, nephric and urogenital sites, and weakly in the hepatic venous plexus and midgut dorsal mesentery. (D) Expression in fourth ventricle choroid plexus

nasal cavity, follicles of vibrissae (whisker pits), and tactile hair follicle primordia. Significant expression also occurred in choroid plexus regions of the third and lateral ventricles, hindbrain wall ependyma, ventral diencephalic thalamus (HCMV-1), and rare scattered cells throughout many parts of the brain (Fig. 7A to C). Strong expression in the PNS occurred along nerves in a pattern consistent with that of pre-Schwann cells (for example, the trigeminal nerve [Fig. 6G and 7G], spinal nerves and lumbosacral plexus [Fig. 6H], and cranial and spinal nerves [Fig. 7A, B, and H]). Except at their perimeters and nerve roots, most PNS ganglia showed only rare positive cells (e.g., the trigeminal ganglion [Fig. 6G and 7G] and dorsal root ganglia [Fig. 6H]). Exceptions were the facial-acoustic ganglia complex (gVII-VIII at 11.5 dpc [Fig. 5C]) and the vestibular and cochlear ganglia in the inner ear (subdivisions of gVIII after 12.5 dpc [Fig. 6D]), where positive cells were densely distributed.

In addition to the endolymphatic sac and duct (Fig. 5B to D; Fig. 6A, B, and I; and Fig. 7D), parts of the semicircular canals, saccule-utricle, and cochlea also showed IE enhancer/promoter activity (Fig. 7D and E). Other epithelial sites of expression included portions of the oropharynx (Fig. 7F), esophagus, and trachea (Fig. 7C). Activity in whisker pits and tactile hair follicle primordia coincided with related activity in other epidermal spots arrayed along the body, beginning at 11.5 dpc. These sites included segmental dorsal stripes over the spinal cord and dorsal root ganglia (Fig. 5A; Fig. 6A, B, and H), two parallel rows of lateral body spots (Fig. 5A, 12-dpc embryo) in register with spinal nerves, and mammary gland primordia (Fig. 5A, reduced set of large ventralmost spots of the 12-dpc embryo). The disposition of the two lateral rows suggests they are mammalian counterparts of epidermal placodes, which in fish and aquatic amphibians contribute to the lateral line vestibular system (7). This mechanosensory system relies on sensory hair cells considered to be related to vertebrate inner ear sensory hair cells.

Vascular sites of *lacZ* expression after 11.5 dpc included the placental chorionic plate blood vessels (Fig. 5E and F), pulmonary and carotid arteries (Fig. 6C and G), aortic and pulmonary trunks (Fig. 6F and 7C), and hyaloid plexus of the eye (Fig. 5C and 6E). Additional parts of the eye with activity included the optic stalk and optic nerve, retinal pigmented epithelial layer, corneal ectoderm, perioptic mesenchyme, and the eyelid margin (Fig. 5C and 6E). *lacZ* expression also occurred in the anterior pituitary (Fig. 6G and 7C), tooth primordia (not shown), and pancreatic and nephric primordia; and rare, scattered cells were seen in the liver, dorsal mesentery, hepatic venous plexus, and adrenal and urogenital primordia (Fig. 7C).

IE enhancer/promoter activity in a subset of endothelial cells at 13.5 dpc. HCMV IE enhancer/promoter activity in some blood vessel endothelial cells was confirmed by a double-staining analysis in which β-Gal activity and anti-vWF immunoreactivity were coassayed on 13.5-dpc embryo cryosections (Fig. 7G and H). Nuclear β-Gal activity and cytoplasmic staining for vWF, an endothelial cell cytoplasmic marker, were colocalized in a subset of endothelial cells. In addition, some β-Gal-positive nuclei that were not strictly associated with vWF-immunostaining were seen along the perimeters of blood vessels (sections not shown). This last subset could represent pericytes (smooth muscle precursor cells); however, whether pericytes and endothelial cells are truly distinct during embryonic neovascularization is still a matter of dispute (53). The majority of β-Gal-stained nuclei at 13.5 dpc were not in blood vessels but rather at epithelial and CNS sites and along nerves. In Fig. 7G and H, β-Gal-stained nerves (trigeminal nerve [panel G] and cervical spinal nerve [panel H]) are shown adjacent to double-stained blood vessels. This perineural nuclear staining pattern is consistent with activity in neuroglial, pre-Schwann cells.

DISCUSSION

HCMV IE enhancer/promoter specificity during mouse embryogenesis identifies known sites of congenital HCMV infection in humans. When removed from the context of the human cytomegalovirus genome, the ~500-bp enhancer/promoter region of the HCMV IE gene is one of the strongest and most broadly active elements known for driving transcription of heterologous genes in cell culture DNA transfection experiments. In contrast, transcription of IE genes is stringently regulated in the setting of the HCMV genome. Thus, it has been assumed that the HCMV IE enhancer/promoter segment can activate but not restrict IE gene transcription and that additional viral regulatory sequences must be required to block the activity of this element in cell types that are nonpermissive for HCMV infection.

Contrary to this assumption, we show here that the HCMV IE enhancer/promoter segment −524 to +13 is highly region and cell type specific in transgenic mouse embryos. Furthermore, the restricted sites of HCMV IE enhancer/promoter activity in the mouse embryo correlate strikingly with known sites of congenital HCMV infection in human fetuses (as discussed in more detail below). There is substantial reason to believe that the specificity of the IE enhancer/promoter conserved in these three transgenic mouse lines is a valid representation of the specificity of this element in human tissues. First, faithful cell type-specific regulation of human gene promoters in transgenic mice is widely documented (43) and reflects the high degree of evolutionary conservation of transcription factors. Second, it is highly unlikely that the correlation between sites of IE enhancer/promoter activity and known sites of congenital HCMV infection is a fortuitous coincidence. Rather, the correlation provides strong evidence that, in the absence of specific immunity, the course of congenital infection in the human fetus is probably largely dependent on how and when the virus gains access to the subset of fetal cell types that are competent to activate the IE enhancer/promoter.

Birth defects most commonly associated with congenital HCMV infection are listed in Table 1. Information about the cell type specificity of congenital HCMV infection has relied, until recent times, mainly on histological identification of cytomegalic cells (enlarged infected cells with viral inclusion bodies) in tissue sections from infants and fetuses that had died of cytomegalic inclusion disease (CID; an often fatal syndrome

(cp) and inner ear sites, including the endolymphatic duct (e), posterior semicircular canal (just below e), cochlea (c), and cranial nerve branches (below c). (E) Expression in a subset of epithelial cells in the inner ear saccule. (F) Nose region showing expression in the olfactory epithelium (oe) and oropharyngeal epithelium adjacent to the primary palate (p). t, tongue. (G and H) Cryosections double stained for β-Gal activity and anti-vWF immunoreactivity. Cells with β-Gal activity have blue nuclei; immunostained endothelial cells have brown cytoplasmic particles. (G) Trigeminal ganglia (gV) and nerve (nV) (ophthalmic branch) adjacent to double-stained blood vessel (bv) just ventral to the brain. (H) Double-stained blood vessel adjacent to cervical spinal nerve (n). Blood cells are visible as white orbs in the vessel. The scale is as explained in the legend for Fig. 3.

TABLE 1. Developmental defects most often associated with congenital HCMV infection in humans[a]

Defect	Frequency (%)[b]		Infected cell types implicated[c]	Reference(s)
	Observed	Control		
Abnormal cerebrospinal fluid[d]	17 (3/18)	NA	NA	47
Sensorineural hearing loss (moderate to profound)	13 (22/168)	1 (2/186)	CY: inner ear endolymphatic compartment nonsensory epithelial cells. IH: CY, gVIIIc, organ of Conti	25, 49, 57, 67[b]; 9, 57[c]
Abnormal cranial X-ray or CT scan[e]	9 (5/53)	0 (0/37)	NA	59, 67
Mental retardation with neuromuscular defects[f] (usually with brain abnormalities noted at birth)	3 (6/175)	0 (0/64)	Brain CY: endothelial and perivascular cells, neural and ependymal cells	25, 47, 49, 59[b]; 26, 69[c]
Vision defects (optic atrophy, chorioretinitis, corneal opacity, blindness)	2 (4/180)	0 (0/163)	CY: endothelial cells of retina and choroid; optic nerve	25, 49, 57, 59, 67[b]; 41[c]
Heart or pulmonary defects[g]	1 (2/143)	NA	NA	25, 35, 49, 59
Stillbirth	2 (1/53)	NA	NA	25, 45
Infant mortality, with immediate cause of death	3 (4/159)	NA	NA	25, 47, 49, 59
Unspecified, at 2 days	(1/53)	NA	NA	25
Bronchopulmonary dysplasia, at 4 mo	(1/64)	NA	NA	49
Bronchiolitis and pneumonitis, at 4 mo	(1/64)	NA	CY: alveolar epithelia and capillaries, free cells in bronchi and alveoli	49[b]; 26, 69[c]
Respiratory arrest at 8 mo	(1/24)	NA	NA	59

[a] The incidence of congenital HCMV infection in the United States is estimated to be ~0.5 to 2% of all infants born (reviewed in reference 1); ~5% of congenital HCMV cases involve severe disseminated infection (CID); and defects often are found in combination in infants with CID.

[b] Data from clinical patient studies cited. Screening criteria for congenital infection include anti-HCMV immunoglobulin M in umbilical cord sera and/or HCMV in neonate urine. Defect frequencies of uninfected controls are given in the adjacent column. Observed, number of infected infants with the defect relative to the total number of infected infants tested. Control, number of infected infants with the defect relative to uninfected control infants tested. NA, data not available.

[c] Data from neonatal autopsy reports cited. CY, identification of characteristic cytomegalic cells (enlarged infected cells with viral inclusion bodies). IH, immunohistochemical identification of HCMV antigens (a more-sensitive indicator than cytomegaly). NA, data not available. gVIIIc, cochlear ganglion.

[d] Elevated cell count and/or protein content; symptoms of breakdown in blood-brain barrier.

[e] CT, computed tomographic scan. Brain abnormalities include periventricular diffuse radiolucencies (low-density areas); long-term effects are not yet known.

[f] Seizures, spastic di- or quadriplegia, and hypotonia. Brain abnormalities include microcephaly, periventricular calcifications, microgyria, hydrocephaly, immature cerebral cortex, and others.

[g] Pulmonary and mitral valve stenosis, atrial and ventricular septal defects, tetralogy of Fallot, bronchopulmonary dysplasia, and others.

of disseminated, multiorgan HCMV infection which occurs in ~5% of congenital HCMV infection cases) (2, 9, 10, 26, 65, 69). It is sometimes difficult to conclusively identify the specific cell types which have become cytomegalic, since cell type-specific markers and morphology can be grossly altered (63). This problem is compounded in CID, in which the architecture of infected tissue is frequently damaged by inflammation and necrosis and in which infection may be progressive with early targets being obliterated or no longer apparent. In addition, more recent immunohistochemical and in situ hybridization studies have shown that not all HCMV-infected cells demonstrate cytomegalic features (30, 40, 63). The HCMV-IE-*lacZ* transgenic mice described here provide an independent means of assessing potential infection targets (on the basis of their competence to activate the IE enhancer/promoter) in normal tissue where cell type-specific markers and morphology are undisturbed.

HCMV IE enhancer/promoter activity in neovasculature. HCMV can be passed to the fetus by placental infection during periods of maternal viremia (56); however, the specific cell types involved have not been clearly defined. Our analysis identifies a potential vascular route into the fetus that is demarcated by IE enhancer/promoter activity in placental and umbilical blood vessel endothelia (Fig. 5E and F). This possibility is consistent with the known ability of HCMV to infect endothelial cells (33, 55, 65) and is supported by several case studies of congenitally infected fetuses or newborns in which cytomegalic cells were observed to be abundant in umbilical blood vessels (69) and in endothelial cells of placental villi (2).

Vasculitis is a common feature in HCMV-infected organs in adults and children with CID, and proliferating capillary endothelial cells were first noted as a conspicuous site of infection in a 1958 case study of a 10-year-old child with fatal CID (65). On the basis of adult CID histopathology, it has been proposed that HCMV disseminates via seeding of organs by infected endothelial cells (40, 66). In vitro studies indicate that primary endothelial cells and arterial smooth muscle cells can support HCMV replication for extended periods in the absence of overt cytopathy (33, 55, 64). Also, HCMV infection can result in anchorage independence of cultured endothelial cells (55), suggesting that infected endothelial cells may enter the circulation to disseminate virus. HCMV can be transmitted by blood products from healthy seropositive donors, and it has been proposed that latently infected peripheral blood mononuclear cells are the principal carriers of HCMV in blood (54, 61). However, a recent study of adults with active HCMV infections showed that only a small subset of circulating blood cells were fully permissive for expression of HCMV IE, early, and late antigens. These were endothelial cells that had apparently entered the circulation during occult vascular infection (22). Twice as big as lymphocytes, these cells expressed some but not all endothelial cell markers and no lymphocyte or myelomonocytic markers. They were present in 10 of 14 patients with diverse HCMV infections. It is known that cells of this size (35- to 45-μm diameter) can become trapped in pulmonary capillaries (14). Thus, Grefte et al. (23) have proposed that circulating, infected endothelial cells could contribute to the high rate of mortality by pneumonitis observed for congenital, perinatal, and adult HCMV infections.

In view of these studies documenting HCMV infection of endothelial and arterial smooth muscle cells, it is significant that the IE enhancer/promoter is highly active in the neovas-

culature of the mouse embryo. Our data suggest that proliferating neovasculature may be the primary route of viral entry and dissemination during virulent first-trimester HCMV infection. The neonatal mortality rate for congenital HCMV is ~3%; pneumonitis and respiratory and bronchopulmonary defects are most frequently cited as the immediate causes of death. The fetal pulmonary trunk (Fig. 6F) and pulmonary arteries (Fig. 6C) are major, persistent sites of IE enhancer/promoter activity that may be important infection targets leading to pneumonitis and bronchopulmonary defects. IE enhancer/promoter activity in aortic and pulmonary trunks and mitral and tricuspid valve primordia identifies potential infection sites that are consistent with known cardiovascular birth defects linked with HCMV (24, 35). Since it has been noted that cultured endothelial cells and arterial smooth muscle cells can support HCMV replication for prolonged periods with limited cytopathy (55, 64), it is possible that fetal cardiovascular sites may be more frequently infected than the birth defect statistics in Table 1 suggest.

Congenital deafness and neurological defects caused by HCMV. The fourth ventricle choroid plexus (major production site of cerebrospinal fluid) and the adjacent endolymphatic duct of the primordial inner ear are the two sites of strongest IE enhancer/promoter activity in 11.5- to 13.5-dpc mouse embryos. Thus, it is highly significant that the most common birth abnormalities linked to congenital HCMV are abnormal cerebrospinal fluid (17%) (47) and sensorineural hearing loss (13%) (25, 49, 57, 67). Our data indicate that nonsensory epithelial cells of the endolymphatic sac and duct may be the principal targets of infection leading to hearing defects. Parts of the inner ear semicircular canals, the utricle (data not shown), the saccule, the cochlea, and the vestibular and cochlear ganglia also show activity. These sites are consistent with those identified by studies of autopsied infants with CID in which cytomegalic cells and HCMV antigens were noted in nonsensory endolymphatic epithelia (epithelia of the endolymphatic sac and duct, cochlea, and other inner ear chambers are continuous) (9, 39) and in the cochlear ganglion (57).

The choroid plexus consists of specialized ependymal cells (brain ventricle epithelia) uniquely apposed to the highly vascularized pia mater surrounding the brain. The choroid plexus and inner ear endolymphatic sac are believed to be related because of their developmental proximity and their roles in maintaining cerebrospinal fluid and inner ear endolymph fluid, respectively. The high frequency of infants with congenital HCMV infection having abnormal cerebrospinal fluid (47) suggests the possibility that infection of the choroid plexus is a predisposition for infection in other CNS sites and in the inner ear.

Mental retardation, neuromuscular disorders, and seizures are usually linked defects that are observed in ~3% of infants with congenital HCMV infection. These symptoms often occur in combination with sensorineural hearing and vision defects. The spatiotemporal progression of IE enhancer/promoter activity in the mouse embryo from invasive neovasculature to CNS vascular plexus to hindbrain roof and choroid plexus and then into subependymal brain regions outlines a first-trimester infection route that is consistent with the pattern of CNS damage frequently seen in human infants with CID (26, 45, 69). This striking pattern of IE enhancer/promoter activity during vascularization of the embryonic CNS (Fig. 4G and H) demarcates a potential path for HCMV into the brain prior to maturation of the blood-brain barrier. HCMV encephalitis in adults, such as occurs in ~20% of patients with AIDS, has significant features in common with congenital HCMV CNS infections. It has been proposed that adult HCMV encephalitis may proceed by two routes (66). Most frequently, the pathology suggests that infection of endothelial cells causes a breach in the blood-brain barrier and hematogenous seeding of infected endothelial cells along brain microvasculature. Somewhat less often, HCMV appears to disseminate via the brain ventricular surfaces, suggesting that infection may spread via cerebrospinal fluid from the choroid plexus. Infected cell types identified in cases of adult HCMV encephalitis include endothelial cells, ependymal and choroid plexus epithelial cells, and subependymal neurons and astrocytes (reviewed in reference 15). Analyses of IE enhancer/promoter activity in neonatal and adult brains of our transgenic mice are highly consistent with these cell types and with the above-mentioned models of HCMV CNS infections (17a).

IE enhancer/promoter activity in embryonic nerves and perineural vasculature (Fig. 6H and 7H) suggests that PNS infections are also responsible for some neurological and respiratory birth defects due to congenital HCMV. HCMV PNS infections are known to occur in patients with AIDS and have been noted in cases of infant CID (26). It has been proposed that in adult HCMV polyneuritis a breakdown in the blood-nerve barrier caused by HCMV-infected endothelial cells allows the virus to infect Schwann cells, resulting in chronically inflamed peripheral nerve roots (15, 66). Consistent with this theory, Schwann cells proliferate and become permissive for HCMV replication following nerve injury in vitro (15).

Other sites of congenital HCMV infection. Sites of IE enhancer/promoter activity in the embryo that correlate with other congenital infection sites seen in infants with CID can only be briefly mentioned here. Activity in hyaloid plexus endothelial cells and the pigmented layer epithelium of the retina identifies key HCMV infection sites pertinent to chorioretinitis and blindness. These, as well as the perioptic vascular plexus, optic stalk, and other nerves innervating the eye, are potential infection targets relevant to the congenital optic atrophy that often accompanies CNS infection (57). Additional correlative sites include the kidney, pancreas, and anterior pituitary (2, 10, 26, 69); these sites of infection have rarely been linked to specific congenital damage. Transient hepatosplenomegaly, limited almost exclusively to infants with CID, appears to be due to cytomegalic cells in bile duct and capillary epithelium in the liver and in sinusoids and blood vessels of spleen trabeculae (26, 69). In survivors of infant CID, these infection sites resolve, leaving only CNS and sensory defects as sites of permanent damage. In our analysis, liver parenchyma showed only scattered positive cells at 13.5 dpc; and the spleen primordium was not a site of significant expression up to 13.5 dpc. The activity level in adult spleen and liver was also very low; however, both sites showed substantial activity in neonatal mice (unpublished data). These findings suggest that liver and spleen may be mainly susceptible to infection between late gestation and the neonatal period, a suggestion which is consistent with clinical observations (5). Two other symptoms frequently associated with CID, petechial hemorrhages and thrombocytopenia, are most likely due to vasculitis caused by widespread endothelial cell infection (26, 65, 69).

Regulation of the HCMV IE enhancer/promoter in vivo versus in cultured cells. Two groups have previously reported that the HCMV IE enhancer/promoter was pan-active and universally expressed in adult transgenic mice (18, 52). Unfortunately, these conclusions were misleading oversimplifications of the data presented in both studies. In these reports, transgene expression levels varied substantially between different adult tissues (by up to 10,000-fold in one transgenic line and up to 1,000- and 100-fold in the two other lines in one of the studies [18]). Furthermore, there was little or no analysis of the

cell type specificity of expression within tissues. Other researchers have since reported more restricted activity of the HCMV IE enhancer/promoter in adult transgenic mice (37). Analysis of postnatal and adult mice of our lines indicate that the activity of the IE enhancer/promoter segment −524 to +13 varies significantly in a tissue- and age-dependent manner and is distinctly cell type restricted in tissues where it is active (unpublished data).

In a study by Kothary et al. (32), a truncated segment −304 to +72 HCMV IE promoter driving *lacZ* gene expression was reported to show unusual cell type specificity in transgenic mouse embryos. The starting rationale of that work was not related to IE regulation, and the authors did not identify or discuss the possibility of correlation between IE enhancer/promoter specificity and congenital HCMV infection specificity. By comparing our data with those of Kothary et al., we can conclude that the IE enhancer segment −524 to −304 region contains positive elements required for activity in many congenital infection sites (inner ear, eye, choroid plexus, heart, pancreas, and anterior pituitary) and probably also negative elements that reduce activity in most PNS ganglia. Both constructs showed expression in midgestation embryos in the dorsal neural tube, blood vessels, hair follicle primordia, olfactory epithelium, and pre-Schwann cells, indicating that sequences between −304 and +13 are sufficient for expression in these sites.

Why do transgenic mice reveal an inherent cell type specificity of the HCMV IE enhancer/promoter while, for the most part, DNA transfection experiments with cultured cells do not? Unrestricted proliferation and direct access to high concentrations of serum factors as occurs in culture are not typical conditions for most cells in vivo. It may be significant that the infection strategies of HCMV include an ability to cause and exploit situations of vascular damage and tissue repair (15, 65). Perhaps, the activity of the IE enhancer/promoter in DNA transfection experiments represents a strategical viral response to cues that in vivo signal conditions suitable for viral opportunism. It has been noted that the HCMV IE enhancer/promoter can be down-regulated in some cultured cell types when it is stably integrated in the cellular genome, suggesting that the method of transient DNA transfection is itself partly responsible for promiscuous IE enhancer/promoter activity. In one study, the IE enhancer/promoter was repressed following stable integration but was inducible by heat shock or inhibition of protein synthesis (20). These data are consistent with negative regulation mediated by a labile repressor. In other studies, integrated copies of IE enhancer/promoter-driven reporter genes were initially active in murine primary fibroblasts but were repressed within 2 weeks of transplantation into mouse dermis (42, 51).

In contrast to most cultured cells, undifferentiated human NT2/D1 embryonal carcinoma cells show relatively weak IE enhancer/promoter activity in transient transfection experiments (reviewed in reference 22). Two DNA-binding factors, MBF-1 and YY-1, which recognize several 21-bp repeats between positions −300 and −524 have been implicated in this repression (31, 34) (Fig. 1). However, we note that segment −300 to −524 is not required to establish or maintain repression of the IE promoter in the majority of cells where it is inactive in 8.5- to 13.5-dpc mouse embryos (lack of this segment in the transgenic construct used by Kothary et al. [32] mainly resulted in loss rather than gain of expression sites relative to our transgenic construct).

In conclusion, we have shown that the HCMV IE enhancer/promoter (segment −524 to +13) is a highly cell type specific regulatory element in transgenic mice. Sites of activity during mouse embryogenesis correspond to known targets of congenital HCMV infection in humans, strongly supporting the idea that this viral regulatory element is a fundamental determinant of HCMV tissue tropism. In addition to providing important insights into congenital infection, the transgenic mouse lines reported here may be useful in further studies of IE enhancer/promoter regulation in vivo to identify signals that modulate active and latent HCMV infections at other stages of human life.

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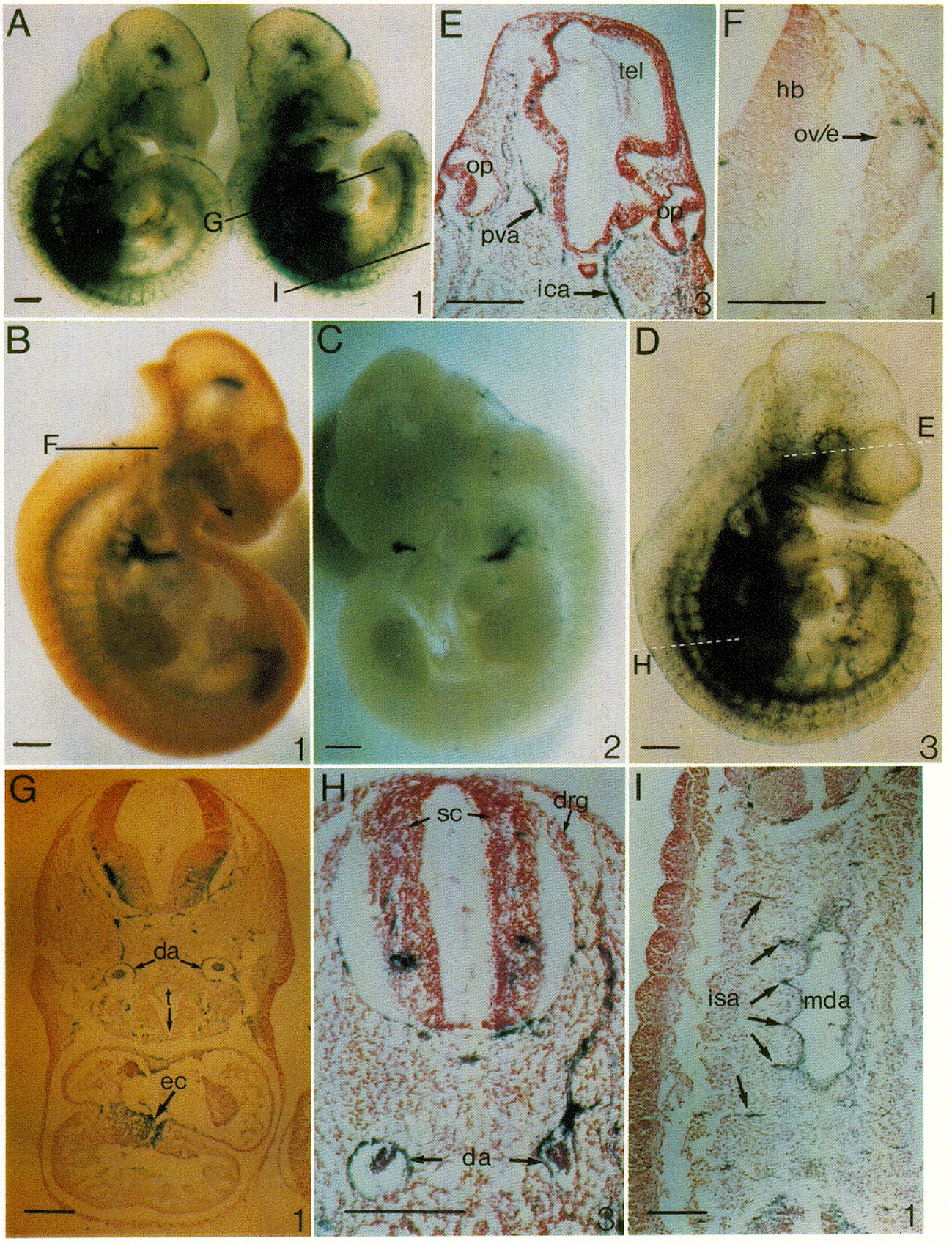
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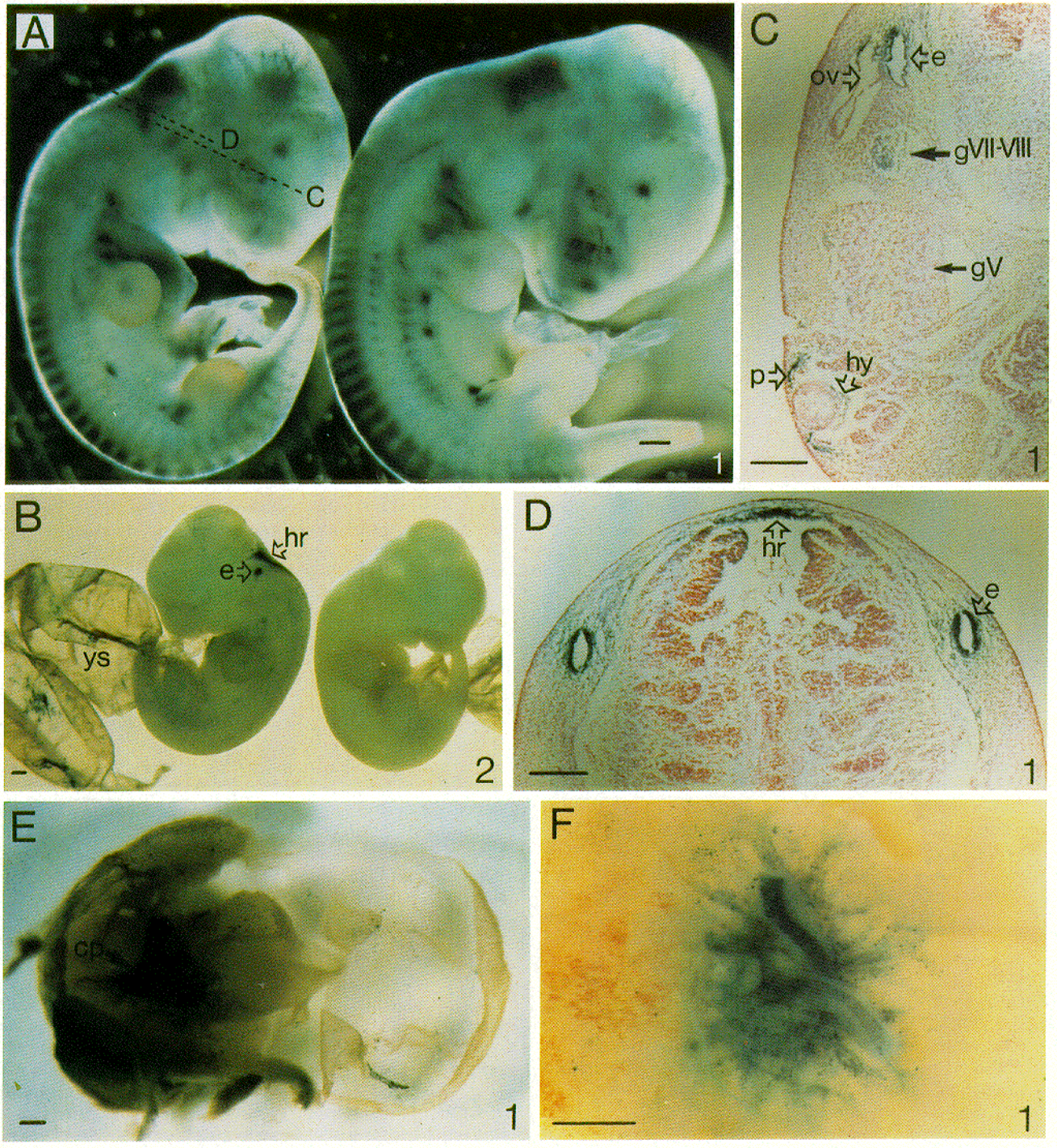
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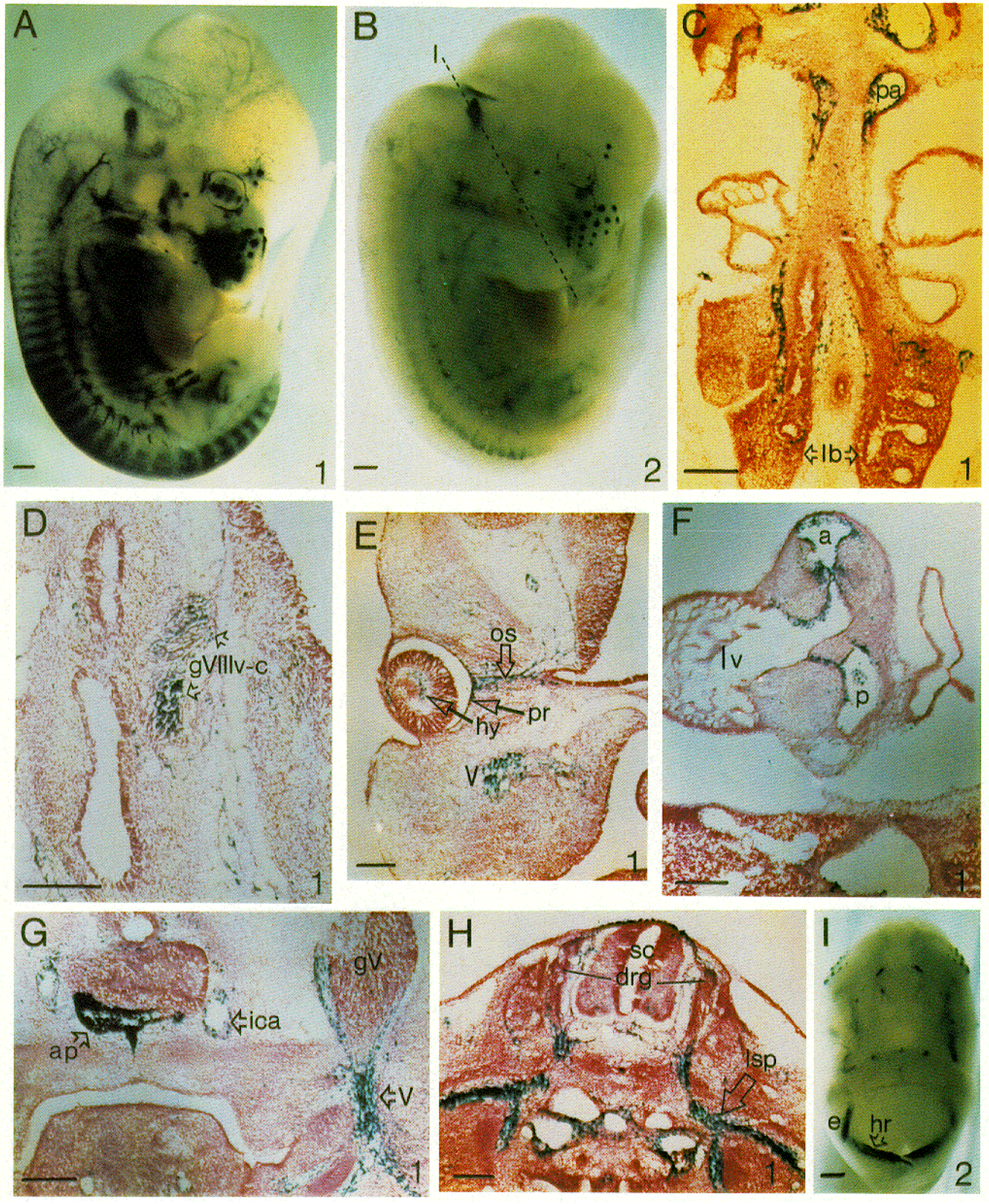
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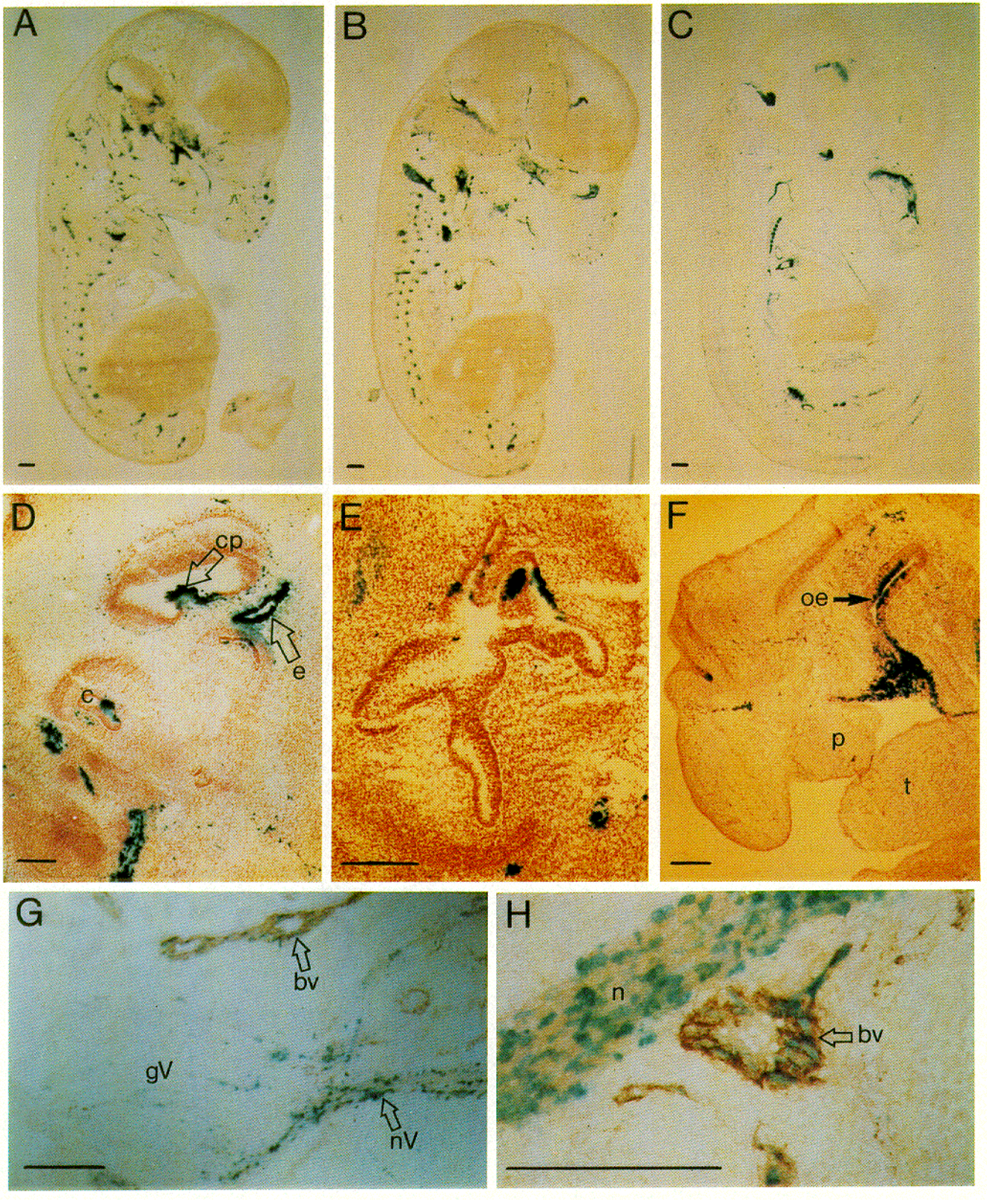
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