Patterns of *Evi-1* expression in embryonic and adult tissues suggest that *Evi-1* plays an important regulatory role in mouse development*

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**Summary**

*Evi-1* is a putative protooncogene first identified as a common site of retroviral integration in murine myeloid leukemias. It encodes a 145 kDa nuclear DNA-binding protein that contains ten zinc-finger motifs separated into two domains, as well as an acidic domain. These features suggest that *Evi-1* encodes a transcriptional regulatory protein. In *Drosophila*, zinc-finger proteins such as *Kruppel* are involved in body plan patterning, and exhibit a spatially restricted pattern of expression in the embryo. To determine if *Evi-1* may be involved in morphogenetic processes in the mouse embryo, we have performed *in situ* hybridization and Northern blot analysis on embryonic and adult mouse tissues to delineate the spatial and temporal pattern of *Evi-1* expression. Our results show that *Evi-1* is expressed at high levels in a few tissues in the embryo and is widely expressed, albeit at generally low levels, in the adult. Regions that exhibit high-level expression in the embryo include: the urinary system and the Mullerian ducts; the bronchial epithelium of the lung; focal areas within the nasal cavities; the endocardial cushions and truncus swellings in the heart; and the developing limbs. Expression in the limb occurs at the highest levels from 9.5 to 12.5 days, is present in both hind and forelimbs, is absent at the apical ectodermal ridge, and does not appear to establish a gradient. This pattern of expression in the limb is reminiscent of other putative transcriptional factors such as *Hox-5.2* and retinoic acid receptor-gamma, consistent with the hypothesis that particular combinations or networks of transcriptional regulatory proteins are required for morphogenesis. Overall, these results suggest that *Evi-1* plays an important role in mouse development.

Key words: *Evi-1*, zinc-finger protein, mouse embryogenesis, transcription factors, protooncogenes, myeloid leukemogenesis.

**Introduction**

Investigations into the function of newly identified genes can proceed either by biochemical or genetic approaches. In *Drosophila* and yeast, the ability to create mutations with ease at cloned loci allows one to exploit the strength of genetics to determine gene function. In higher eukaryotes, however, the ability to create mutations in non-selectable genes is now in its infancy, so that the task of defining the function of a gene relies on other approaches, such as describing the spatial and temporal pattern of expression of the gene. Such a description can yield important clues about the role a gene plays throughout development and in the adult animal.

*Evi-1* (ecotropic viral integration site-1) was first identified as a common site of retroviral integration in virally induced myeloid tumors of AKXD mice (Mucenski *et al.* 1988). Subsequent cloning and sequencing of the cDNA corresponding to this locus (Morishita *et al.* 1988) showed that the gene encodes a polypeptide containing ten repeats with extensive homology to the zinc-finger domains first identified in the *Xenopus* transcription factor IIIA (Miller *et al.* 1985) and subsequently found in several other transcriptional regulatory factors. The predicted polypeptide also contains an acidic domain near the carboxy terminus, which is a feature present in several yeast transcription factors and may function as an activation domain for gene transcription (Struhl, 1987). These data suggest that *Evi-1* encodes a DNA-binding protein that acts to regulate mRNA transcription through interaction with one or more specific DNA sequence binding motifs. Indeed, recent studies have shown that *Evi-1* is localized to the nucleus (Matsugi *et al.* 1990), and *Evi-1* protein binds to DNA (Matsugi *et al.* 1990; Perkins, A. S., R. Fishel, N. A. Jenkins, N. G. Copeland, unpublished data). In myeloid leukemias,
the effect of retroviral insertion is to dramatically increase the transcription of Evi-1 (Morishita et al. 1988), suggesting that increased levels of the protein product contribute to the transformed phenotype. However, the exact role of Evi-1 in myeloid leukemogenesis and the putative target DNA sequences for Evi-1 binding and action are not known. The myeloid cell lines containing Evi-1 integrations are dependent on IL-3 for growth and are abnormal in their ability to terminally differentiate (Morishita et al. 1988). Thus, it has been proposed that expression of the Evi-1 gene interferes with normal differentiation of these cells (Morishita et al. 1990).

In this report we describe results from Northern and in situ hybridization analysis of Evi-1 expression in adult and embryonic mouse tissues. These data reveal a spatially and temporally restricted pattern of Evi-1 expression that overlaps that of several other putative transcription factors, such as Hox-5.2, Hox-5.3 (Dollé and Duboule, 1989; Oliver et al. 1989), Hox-7 (Robert et al. 1989; Hill et al. 1989) and retinoic acid receptor γ (RARγ) (Dollé et al. 1989; Ruberte et al. 1990), suggesting that Evi-1 plays a regulatory role during mouse embryogenesis.

Materials and methods

RNA preparation and analysis

Six week old C57BL/6J female mice were killed and trimmed organs were frozen immediately in liquid nitrogen. RNA was prepared as described by Chomczynski and Sacchi (1987). Briefly, tissues were homogenized with a Polytron homogenizer in RNAzol (2 ml per 100 mg tissue), which is a mixture of guanidinium isothiocyanate and phenol (Cinna/Biotecx). The mixture was extracted with chloroform (0.2 ml per 2 ml homogenate), and the aqueous phase was precipitated with an equal volume of isopropanol. Polyadenylated RNA was purified by affinity chromatography using oligo(dG)·poly(dC)·cellulose (Collaborative Research), as described (Aviv and Leder, 1972). The yield of RNA recovered from the column ranged from 1% to 6%. Fractionation of polyadenylated RNA on agarose gels was performed as described by McMaster and Carmichael (1977) (Fig. 1, left panel), or by Perkins et al. (1983) (Fig. 1, right panel). Either 0.5−5 μg (Fig. 1, left panel) or 8−12 μg (Fig. 1, right panel) of RNA were analyzed per lane. RNA was then transferred to nylon membranes (Zetabind; Cuno, Inc.), as described (Thomas, 1980), and hybridized to 32P-labeled probes for Evi-1, which were prepared using a Multiprime labeling kit (Amersham). Hybridizations were performed as described by Church and Gilbert (1984) for Fig. 1, left panel or by Wahl et al. (1979) for Fig. 1, right panel, except that dextran sulfate was omitted from the prehybridization and hybridization buffers. The filter for Fig. 1, left panel, was washed twice (15 min each) in 0.51 of buffer A (0.5% bovine serum albumin, 1 mM EDTA, 40 mM phosphate buffer, pH 7.2, and 5% sodium dodecyl sulfate (SDS)) and three times (15 min each) in 0.33 of buffer B (1 mM EDTA, 40 mM phosphate buffer, pH 7.2, 1% SDS) at 65°C. The filter for Fig. 1, right panel was washed in a final buffer containing 0.1×SSC and 0.1% SDS at 55°C. The filters were then exposed to film for 72 h (Fig. 1, left panel) or 24 h (Fig. 1, right panel) with two intensifying screens at −70°C.

Preparation of probes for in situ hybridizations

The 454 bp BglII–HindIII fragment of p58.2−1 (Morishita et al. 1988), containing the 5' end of the Evi-1 cDNA was subcloned into BamHI–HindIII digested pBluescript KS(−) (Stratagene) using standard techniques. Antisense 32P-labeled Evi-1 probe was synthesized from this template using T3 polymerase with a RNA Transcription Kit (Stratagene). The probe was purified as described by Sassoon et al. (1988). The hybridization probe used for Figs 2H and 3D was labeled with 32P by nick translation as described (Rigby et al. 1977) using a 1.2 kb SpHl fragment of p58.2−1 which spans a region of Evi-1 cDNA essentially devoid of zinc fingers.

In situ hybridizations

C57BL/6j mice were mated, and pregnant females were killed at various days thereafter. Embryos were dissected, fixed in phosphate-buffered 4% paraformaldehyde, and processed as described (Sassoon et al. 1988). For in situ hybridization of tissue sections, the technique described by Wilkinson et al. (1987), as modified by Sassoon et al. (1988) was employed for analysis of embryos. In situ hybridizations of adult uterus and kidney (Figs 2H and 3D) were performed as described by Haase et al. (1982).

Results

Northern analysis of Evi-1 expression in adult mouse tissues

RNA was extracted from several adult mouse organs, poly (A) selected and analyzed on Northern blots using either the full-length Evi-1 cDNA (Fig. 1, left panel) or a 1.2 kb SpHl fragment (bp 1108 to 2365; Morishita et al. 1988) that is essentially free of zinc-finger sequences (Fig. 1, right panel) as probes. Neither of these probes shows any cross-hybridization with other loci on Southern blots (data not shown). Evi-1 expression is found in several adult tissues (Fig. 1). To compare the relative level of Evi-1 expression in different tissues, we quantitated the relative amount of β-actin transcript in each lane by rehybridizing the Northern blots shown in Fig. 1 with a β-actin probe (Fig. 1, bottom panel). Based on these data, we estimate that high levels of Evi-1 transcripts are present in ovary and kidney, while lower levels are present in lung, uterus and heart. We have also detected Evi-1 transcripts in other adult mouse tissues by Northern analysis, including liver, intestine, thymus, spleen, stomach and brain (data not shown).

Two distinct transcripts were detected with the full-length Evi-1 probe (Fig. 1). A 7.5 kb transcript is predominant in lung, whereas a 6.2 kb RNA is detected in CP1 embryonic stem cells and ovary. Whether the different sized transcripts originate through alternative usage of 5' transcriptional initiation sites, splicing signals, or polyadenylation signals is not known.

In situ hybridization for Evi-1 expression during mouse embryogenesis

Evi-1 expression in the mouse embryonic stem cell line, CP1 (Fig. 1), indicates that Evi-1 is expressed during early embryonic development. To determine the spatial extent of Evi-1 expression during later time points in
Evi-1 expression in mouse development

Evi-1 expression in mouse development rapidly developing in the lumbar region. Mesonephric tubules extend medially from the mesonephric duct, which develops caudally towards the urogenital sinus. Evi-1 transcripts were detected throughout the course of the mesonephric duct, as well as in mesonephric tubules (Fig. 2A,B). At 12.5 days p.c., the mesonephros has developed an array of tubules within the urogenital ridge. Evi-1 expression is high within the mesonephric tubules (Fig. 2C,D), as well as within the Wolffian duct which is the caudal portion of the mesonephric duct (Fig. 3A,B). A distinct ring of expression was seen in the mesenchyme surrounding the more caudal end of the Wolffian duct, a region where the seminal vesicle and prostate will develop in the male.

At 14.5 days p.c., the metanephros is well-formed, and appears as an ovoid structure in the pelvic region, but then shifts cranially over the ensuing days to its final position in the abdominal retroperitoneum. Evi-1 expression is very high in the tubules of the metanephros at 14.5 days p.c. (Fig. 2E,F). No significant expression was detected in the developing adrenal gland (data not shown) or testis (Fig. 2E,F).

Evi-1 is also expressed at significant levels in the adult kidney (Fig. 1). In situ hybridization of sections of adult kidney using a nick-translated DNA probe for full-length Evi-1 in the tubules throughout the cortex and external medulla (Fig. 2G,H). No expression was detected in the glomeruli.

Evi-1 expression in the female genital system

The Mullerian duct develops as an invagination of the coelomic epithelium on the ventrolateral side of the urogenital ridge and can be seen in cross-section in Fig. 3A, ventral to the Wolffian duct. Evi-1 expression was seen in the Mullerian duct at 12.5 days p.c. (Fig. 3B). In females, the Mullerian ducts fuse to form the uterus and the upper third of the vagina. By Northern analysis, we have evidence of Evi-1 expression in the adult uterus (Fig. 1). In situ hybridization of frozen sections of adult uterus with a nick-translated DNA probe for Evi-1 revealed a high level of Evi-1 expression in the tubules throughout the cortex and external medulla (Fig. 2G,H). No expression was detected in the glomeruli.

Evi-1 expression in the limb bud

At 9.5 days p.c., when the forelimb bud emerges and is composed of morphologically undifferentiated mesoderm, Evi-1 expression was clearly seen throughout the non-ectodermal cells of the limb (Fig. 4B). The pattern of Evi-1 expression in the limb is homogeneous and the apical ectodermal ridge (AER) is negative. Evi-1 expression was not observed in the somites (Fig. 2A,B; Fig. 4A,B). At 12.5 days p.c., when precartilaginous blastema are forming, homogeneous Evi-1 expression was observed in the limb (Fig. 4D), similar to that
Fig. 2. *Evi-1* expression in the developing and adult urinary system. *In situ* hybridization of anti-sense 35S-labeled RNA *Evi-1* probe to embryonic (A through F) tissues and *in situ* hybridization of 35S-labeled DNA probe to adult (G and H) tissues. Left-hand panels show bright-field illumination; right-hand panels show the identical field under dark-field illumination. (A and B) Frontal section through a 9.5 days p.c. embryo, showing the somites (s), coelomic cavity (c), mesonephric duct (m), and the mesonephric tubules (t). (C and D) Parasagittal section through a 12.5 days p.c. embryo, showing the mesonephric duct (m), genital ridge (g), mesonephric tubules (t), the caudal end of the lung (l) and prevertebrae (pv). (E and F) Frontal section through a 14.5 days p.c. embryo, showing the liver (unlabelled), metanephros (mt), and testis (te). (G and H) Adult kidney, showing the cortex (co) and external medulla (em). Magnification: A and B, 140x; C and D, 56x; E and F, 17.5x; G and H, 56x.

observed at 9.5 days p.c. The expression of *Evi-1* is not confined to the limb bud proper, but extends into the lateral mesoderm in the torso, in areas destined to form the musculoskeleton of the limb girdle. By 14.5 days p.c., the cartilage is well-formed but not yet ossified, and the digits are separated, *Evi-1* expression is limited to the perichondrium of the limb cartilage, both in the forelimb (Fig. 4F) and the footplate (Fig. 4H). In the latter, *Evi-1* expression was seen in the interdigital region.

The sclerotome-derived prevertebral condensations also exhibited a low but significant level of *Evi-1* expression at both 12.5 (Fig. 2D) and 14.5 days p.c. (Fig. 5B).

**Evi-1 expression in the heart**

At 12.5 days p.c., the ventricles are partially separated into left and right, and the atria and ventricles are distinct. The truncus arteriosus, the single outflow vessel at this stage, is being divided by rapidly growing opposing ridges of mesenchyme arising on the right superior wall and the left inferior wall of the vessel. These ridges, termed the truncal (more distally located) and conal (more proximally located) ridges, are composed of loose mesenchyme similar in histological appearance to the endocardial cushions, which contrib-

Fig. 3. *Evi-1* expression in the female genital system detected by *in situ* hybridization. (A and B) Bright-field and corresponding dark-field view of a frontal section through a 12.5 days p.c. embryo, hybridized with 35S-labeled RNA probe for *Evi-1*, showing the urogenital ridges. Abbreviations: coelomic cavity (c), Mullerian ducts (m), Wolffian duct (w) and intestinal epithelium (i). (C and D) Bright-field and corresponding dark-field view of adult uterus, hybridized with 35S-labeled DNA probe for *Evi-1*, showing the endometrial epithelium and surrounding stroma and glands. Magnification: 150x.
Fig. 4. Evi-1 expression in the limb buds. In situ hybridization of mouse embryos using anti-sense 35S-labeled Evi-1 probe. (A and B) Upper left panel diagrams a 9.5 days p.c. embryo, indicating the plane of section used for panels A and B. These sections show the forelimb bud (fl), somites (s), the neural tube (nt) and the apical ectodermal ridge (aer). (C and D) Frontal section of a 12.5 days p.c. embryo, showing the posterior half, including the hind limb (hl). (E and F) Cross section through the forelimb bud of a 14.5 days p.c. embryo, with centrally located cartilage anlage of the bones. The lower panel shows a dark-field view of the same field, with hybridization to the perichondrium. (G and H) Section through the footplate of a 14.5 days p.c. embryo. Magnification: A and B, 38×; C and D, 24×; E and F, 60×; G and H, 24×.

Evi-1 expression in the respiratory system
We first observed Evi-1 transcripts in the developing lung at 12.5 days p.c., when the lung is a rapidly developing set of bronchi generated by dichotomous division (Fig. 5B). It appears that both the tracheal and bronchial epithelia contain Evi-1 transcripts. The surrounding mesenchyme and the visceral pleura appeared negative, as did the adjacent esophagus and the epithelium of the oral cavity, indicating that Evi-1 expression is highly restricted within these foregut-derived structures. At 14.5 days p.c. (Fig. 5D), the lung is the organ with the most intense signal from the Evi-1 probe. The labeling continues to outline the developing trachea, bronchi and bronchioles. In the adult lung the presence of Evi-1 transcripts was clearly demonstrated by Northern blot analysis (Fig. 1). Thus, like the kidney, Evi-1 expression continues through the embryonic and fetal stages into the adult lung. Of note, as shown in Fig. 5B, Evi-1 expression was also seen at 12.5 days p.c. in two derivatives of the visceral arches: the mandible, located below the tongue, and the hyoid cartilage, in the area ventral to the pharynx.

Evi-1 expression in the developing nasal cavity
At 12.5 days p.c., while the nasal chambers are deepening, a medial invagination of the nasal epithelium develops bilaterally which will form the vomeromedia1 organ of Jacobson (Theiler, 1989; Arey, 1966). This organ is innervated by fibers from the first cranial nerve and serves as an additional taste sensor in rodents (Arey, 1966). As shown in Fig. 6, a high level of Evi-1 expression was detected in the epithelial layer in this invagination, which is undergoing rapid proliferation and invasion into the underlying mesenchyme. In addition, there is a very localized region of mesenchyme lateral to each nasal cavity that exhibited a significant level of staining with Evi-1 probe. This labeling was found at a fold in the lateral wall of the nasal chamber, and may coincide with the formation of conchae.
Fig. 5. *Evi-1* expression in the developing lung and heart. (A and B) Parasagittal section through a 12.5 days p.c. embryo, showing the tongue (to), truncus arteriosus (ta) and conus (c) of the heart (h), esophagus (e), prevertebrae (pv), and the lung (l). (C and D). Parasagittal section of a 14.5 days p.c. embryo, showing the lung (l) and the heart (h). Magnification: A and B, 38×; C and D, 20×.

Fig. 6. *Evi-1* expression in the nasal cavities. Frontal section through the head of a 12.5 days p.c. embryo, showing the nasal cavities (n.c.). Magnification: 50×.

Discussion

We have examined the extent of *Evi-1* expression in adult and embryonic tissues using Northern blot analysis and *in situ* hybridization. The results indicate that *Evi-1* has a unique spatial and temporal pattern of expression in the developing mouse embryo, and suggest that *Evi-1* plays an important role in key morphogenetic events in several tissues. From our *in situ* hybridization data, it appears that high levels of *Evi-1* expression are restricted in development to a few regions such as kidney, lung, uterus, heart and nasal passages; it is possible, however, that a low level of *Evi-1* expression occurs throughout the embryo since
exposed grains of emulsion are present over almost all organs. Certainly this is true in the adult, where Northern blot analyses revealed Evi-1 expression in several tissues (Fig. 1; data not shown).

Interestingly, our analysis revealed that the pattern of Evi-1 expression overlaps significantly with that of other putative or proven transcriptional regulatory proteins. In the limb, Evi-1 expression overlaps with Hox-1.1 (Mahon et al. 1988), Hox-5.2 and Hox-5.3 (Dollé et al. 1989), and Hox-7 (Hill et al. 1989; Robert et al. 1989) as well as the retinoic acid receptors α, β, and γ (Dollé et al. 1989). Evi-1 is expressed throughout the development of the urinary system, exclusively in the epithelial ducts and tubules.

Analysis of embryos revealed that Evi-1 is expressed in the surrounding mesenchyme. Our studies on Evi-1 suggest several specific developmental processes in which Evi-1 may play an important role, such as renal and pulmonary organogenesis and limb development. These possibilities can be probed genetically in transgenic mice by attempting to create dominant-acting gain-of-function mutations by directing high levels of Evi-1 expression to inappropriate tissues or mutant forms of Evi-1 to tissues such as the kidney, lung, or limb, where Evi-1 is normally expressed. Recent experiments of this type have helped to delineate the role of Hox-1.1 in development (Kessel et al. 1990). Also, loss-of-function mutations generated by homologous recombination may provide important clues into the function of Evi-1 in mouse development.

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References


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