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Genetics

The Ets1 transcription factor is widely expressed during murine embryo development and is associated with mesodermal cells involved in morphogenetic processes such as organ formation

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ABSTRACT **The *Ets* family of genes encodes a class of transcription factors. *Ets1* is predominantly expressed in the lymphoid organs of neonatal and adult mice, whereas *Ets2* is expressed in every organ examined. In this study, we investigate the expression of *Ets1* and *Ets2* during murine embryonic development. Our data show that *Ets1* expression increases in embryos after implantation and during organogenesis such that it is expressed in all the organs of day-15 embryos studied. In later fetal stages, *Ets1* expression is predominant in the lymphoid tissues, brain, and organs that are undergoing branching morphogenesis (e.g., lung) but is dramatically reduced in other organs such as the stomach and intestine. In neonatal development, *Ets1* is expressed only in the lymphoid organs and brain. *In situ* hybridization analysis demonstrates that expression of *Ets1* occurs in mesenchymal cells of developing organs, in the nervous system, and in forming bone. Furthermore, expression of *Ets1* is upregulated in P19 cells induced to differentiate into mesoderm-like cells. *Ets2*, on the other hand, is expressed in differentiated and undifferentiated P19 and F9 cells and in all organs of embryonic, neonatal, and adult mice studied. These data suggest that *Ets1* plays an important role in mesodermal cells associated with morphogenetic processes such as organ formation and tissue modeling, whereas *Ets2* plays a more fundamental role in cells.**

The development of a single-cell zygote into an entire organism containing a variety of organs and differentiated cell types involves the coordinated expression of numerous genes. The mechanisms that govern the temporal and tissue-specific expression of many of these genes involve specific transcription factors (1–7). Several classes of transcription factors, including homeobox (3–5), leucine zipper (6), and helix–loop–helix proteins (7), have been shown to be critical in development. These different permutations of transcription factors are thought to play a major role in developing diverse pathways of morphogenesis. Thus, data on the expression patterns of transcription factors are invaluable to an understanding of both the putative biological role of such factors as well as the identification of downstream cellular target genes.

The Ets proteins bind a GGAA purine-rich core sequence found in the promoters or enhancers of various cellular and viral genes and can transactivate transcription from such promoter/enhancer elements. Examples include murine sarcoma virus long terminal repeat, stromelysin, urokinase plasminogen activator, *ETS1* by autoregulation, *TCRA*, and interleukin 2 (8–17). Although overexpression of *Ets1* and *Ets2* has been shown to transform murine fibroblasts (18, 19), the normal biological functions of members of the *Ets* family are as yet essentially unknown.

To study the functions of members of the *Ets* family, the expression patterns of various *Ets* genes have been carried out in a number of cell lines and organ systems (20–24). A varied pattern of expression for the different family members has been found. *Ets1*, for instance, is expressed in a temporal and tissue-specific manner—predominantly in the lymphoid organs of neonatal and adult mice (21). *Ets2*, on the other hand, is expressed in all organs of neonatal and adult mice studied and in a wide variety of cell lines investigated (21, 24). However, the expression patterns of *Ets1* and *Ets2* during murine embryonic development are unknown. In this study, we investigate the expression of *Ets1* and *Ets2* during murine embryogenesis and in F9, P19, and murine hematopoietic cell lines (of different lineages), so as to gain insight into the biological function of *Ets1* and *Ets2*.

MATERIALS AND METHODS

Mice (C57BL/6J × CBA)F_1 were mated overnight (vaginal plug = day 1). Mice were killed, organs were dissected out on the designated days, and individual organs from different animals were pooled.

F9 and P19 embryonal carcinoma cell lines were cultured in monolayers and induced to differentiate with retinoic acid (25). The following hematopoietic cell lines used were kindly provided by Don Metcalf and Alan Harris (Walter and Eliza Hall Institute, Melbourne, Australia), Wendy Cook (Department of Surgery, Melbourne University), and Sue Hasthorpe (Peter Maccallum Institute, Melbourne): IW32, F4N, FLSP, J2E, FDCP1, 416B, MI, 32D, WEHI 265, WEHI 2743, J774, PU-5, Fmp1.6, HC3, P815, W401.1, A8, AΨ7E, W413, W404.1, W112.1, VL3K7, and RB3.1 (MI, PU-5, and P815 were obtained from the American Type Culture Collection).

Northern blot analysis was carried out using guanidinium isothiocyanate as described (26–28). Hybridizations were carried out using the following probes: *Ets1*, a 1.8-kb murine cDNA fragment derived from the 3′ untranslated region of the gene; *Ets2*, the entire 3.4-kb murine cDNA (29). Probes were labeled with [^{32}P]dATP by random priming, hybridizations were performed overnight at 50°C, and filters were washed at 65°C in 1–0.1× SSPE as described (27).

In situ hybridization was carried out with radioactive (^{35}S-labeled) RNA probes synthesized *in vitro* using T7 and SP6 RNA polymerase to generate antisense and sense RNAs, respectively (30). Tissues were prepared from National Institutes of Health Swiss mouse embryos, fixed in 4% para-

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formaldehyde, and sectioned at a thickness of 7 μm (30). Hybridization was carried out for 18 h at 50°C and posthybridization washes initially were at 2× standard saline citrate (SSC) at 50°C and finally at 0.1× SSC as described (30). Tissues were counterstained with Giemsa or hematoxylin and eosin.

RESULTS

Expression of *Ets1* and *Ets2* During Murine Development. *Northern blot analysis.* *Ets1* is differentially expressed in the yolk sacs of mouse conceptuses (days 8–12). It is expressed at high levels on days 8–10, at detectable levels on day 11, and at low or undetectable levels on day 12 (Fig. 1*A*). This pattern of expression correlates with the presence of hematopoietic stem cells in the yolk sac. We are, however, unable to discriminate whether the expression of *Ets1* occurs in some or all of the different cell types found in the yolk sac (namely, undifferentiated mesenchymal cells, mesoderm cells, endothelial cells, and hematopoietic stem cells). *Ets2*, on the other hand, is expressed at low detectable levels in the yolk sac throughout this period (Fig. 1*A*).

Expression of *Ets1* is regulated in a temporal and tissue-specific manner during murine development in the embryo proper. *Ets1* expression is first detected by Northern blots of total embryonic RNA on day 8 of gestation. Thereafter, the levels of *Ets1* mRNA appear to increase in the embryo such

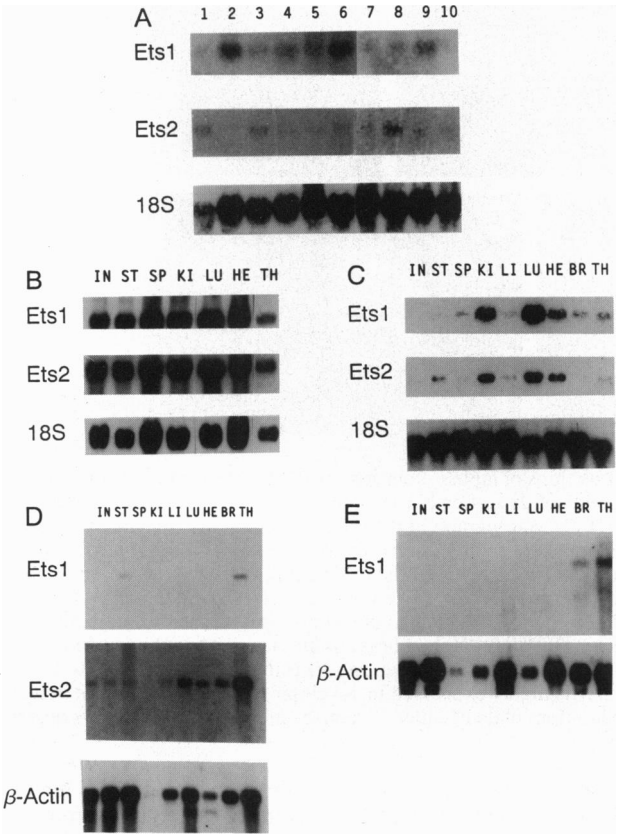

FIG. 1. Northern blot analysis of total RNA for *Ets1* and *Ets2* expression in the embryo proper and yolk sacs of mouse conceptuses (days 8–12) (*A*); in individual organs of day-15 embryos (*B*); day-16 embryos (*C*); adult [using poly(A)$^+$ RNA (*D*)]; and day-7 neonatal mice (*E*). Lanes: 1, 3, 5, 7, and 9, day-8, -9, -10, -11, and -12 conceptuses, respectively; 2, 4, 6, 8, and 10, day-8, -9, -10, -11, and -12 yolk sacs, respectively. IN, intestine; ST, stomach; SP, spleen; KI, kidney; LU, lung; LI, liver; HE, heart; BR, brain; and TH, thymus. Filters (embryonic tissues) were reprobed with an 18S ribosomal probe [β-actin expression may be regulated during embryonic development (31)] or with β-actin for neonatal and adult tissues.

that on day 15 of gestation *Ets1* is expressed at high levels in all of the individual organs examined (Fig. 1*B*), including the liver (data not shown). On day 16, however, a dramatic shift in the pattern of *Ets1* expression occurs; *Ets1* is no longer ubiquitously expressed at high levels (Fig. 1*C*). Rather, its high level of expression is maintained in some organs (e.g., lung), while in other organs (e.g., stomach and intestine) the levels of *Ets1* mRNAs are substantially reduced when compared to those of organs derived from day-15 fetuses. In day-7 neonatal mice, a further change in the pattern of *Ets1* expression occurs—expression now predominantly occurs in the lymphoid organs and in the brain (Fig. 1*E*). In adult mice, expression of *Ets1* is detected only in lymphoid organs and lung (at very low levels; Fig. 1*D*). It is also noteworthy that the levels of *Ets1* mRNA in adult tissues are very much lower than in embryonic tissues (and in neonatal tissues) since the detection of *Ets1* mRNA in adult tissues requires poly(A)$^+$ RNA, whereas in earlier stages total RNA is sufficient.

Levels of *Ets2* are also substantially higher in murine organs during embryonic development as compared to those from adult mice (Fig. 1 *B* and *D*). In adult mice, the thymus is the organ with the highest levels of *Ets2* mRNA (Fig. 1*D*). The major difference between expression of *Ets2* and *Ets1* is that expression of *Ets2* is still detected in all organs of the adult mouse studied, whereas expression of *Ets1* in adult mice occurs predominantly in the lymphoid organs.

In situ *hybridization analysis of* Ets1 *expression during development.* In light of our findings on the widespread expression of *Ets1* at specific times during murine embryo development (day 15) we carried out *in situ* hybridization analysis. Several interesting features of *Ets1* expression during embryogenesis were revealed. First, *Ets1* expression is detected in organs (Fig. 2 *A–C*) while they are being formed. *Ets1* expression is undetectable in organs such as the intestine in the late fetal to neonatal period when these have already been formed (Fig. 2*D*). Our data also demonstrate that *Ets1* is expressed in specific cells of developing organs. For example, in the developing intestine, the innermost endodermal cells are negative for *Ets1* expression while adjacent mesenchyme is positive (Fig. 2 *A–C*). Similarly, in the brain, *Ets1* expression is found in mesenchymal cells from day 9 onward, while ectodermal derivatives such as the wall of the brain and the retina are negative throughout the entire period we have studied (Fig. 2 *E–G*). However, organs such as the adrenal glands, which are mainly mesodermal in origin (being derived from the peritoneal mesothelium), and the thymus and spleen, which continue to express *Ets1* through to adult stages, show *Ets1* expression in all cells at relatively uniform levels (data not shown). Thus, these data strongly suggest that *Ets1* expression in the developing embryo is associated with the mesodermal cells during organ morphogenesis.

The second interesting feature emanating from the *in situ* hybridization experiments is expression of *Ets1* in the nervous system. On day 9, the mesencephalic neural crest cells around the neural pore express high levels of *Ets1* (data not shown) in a manner analogous to that in the chicken embryo (32). In later-stage embryos (days 14 and 15), the *Ets1* gene is expressed at very high levels in the gray matter of the spinal cord, nervous tissue within the cerebral cortex (Fig. 2 *H* and *I*), and cells of the brain surrounding the fourth ventricle (Fig. 2*J*).

The third interesting finding is the expression of *Ets1* in developing bone during murine embryogenesis. *Ets1* mRNA is detected in vertebrae, tail, developing limbs, and other sites where bone development occurs (Fig. 2 *K–N*).

***Ets1* and *Ets2* Expression in F9 and P19 Embryonal Carcinoma Cell Lines.** To examine the expression pattern of *Ets1* and *Ets2* during differentiation, we compared their expression in two different embryonal carcinoma cell lines, F9 and

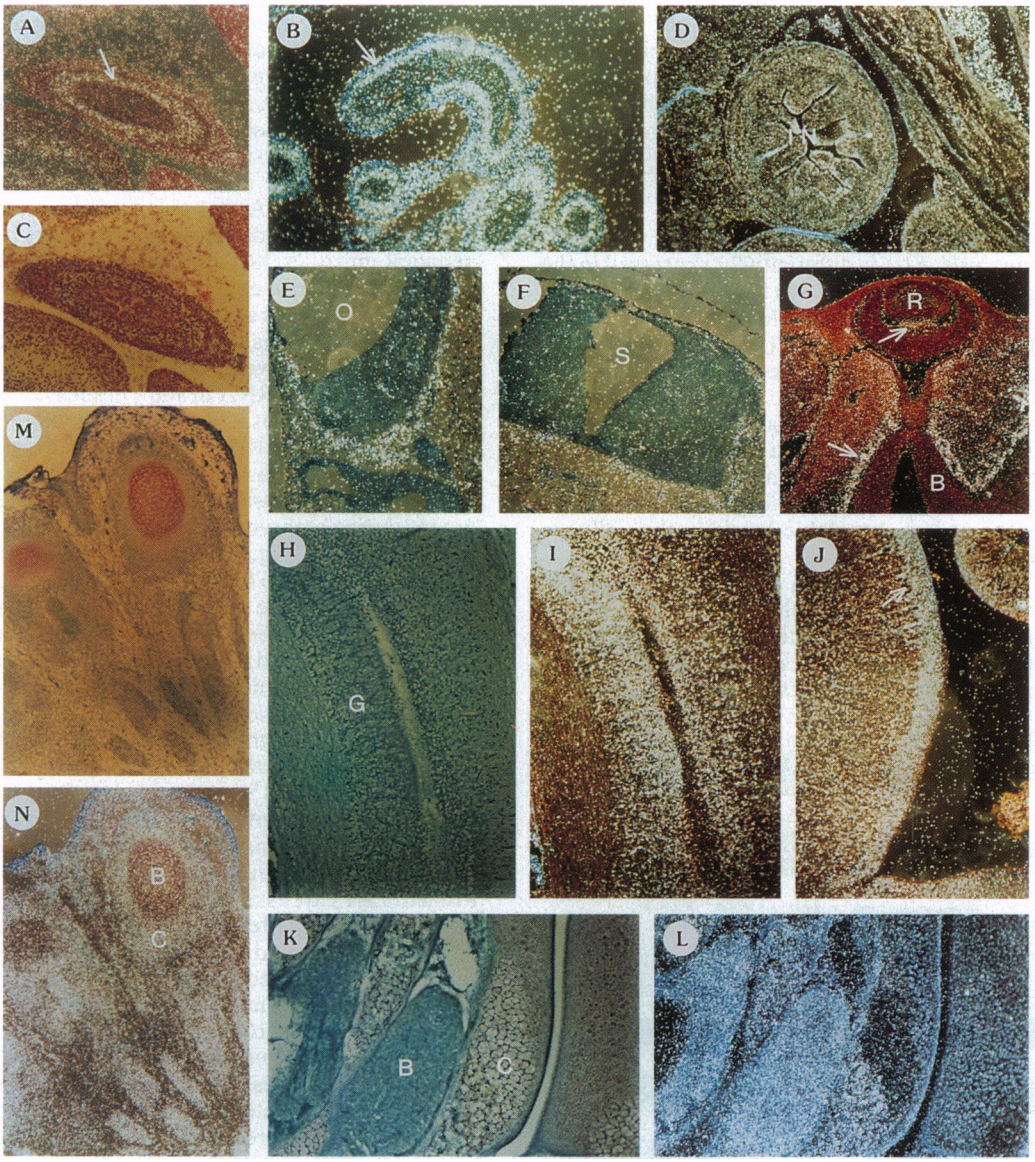

FIG. 2. Expression of *Ets1* detected by *in situ* hybridization to parasagittal sections of mouse embryos. (*A–D*) Expression of *Ets1* in the small (*A*) [dark-field (DF) micrograph] and small and large (*B*) (DF micrograph) intestine of day-14 embryos. Expression occurs in mesenchymal cells and is absent in intestinal epithelium. *C* is a light-field (LF) micrograph of *A*. (*D*) DF micrograph of the large intestine (IN) from a day-16 mouse embryo, which is negative for *Ets1* expression. (*E–G*) Expression in mesenchymal cells surrounding the otic vesicle (O) (*E*), spinal canal (S) (*F*), and mesenchymal cells immediately adjacent to brain (B) (*G*). Expression is also detected in the optic nerve (arrows) but not in the brain (B) and retina (R) (*G*). (*E* and *F*) DF micrographs of day-10 embryos. (*G*) DF micrograph of day-14 embryo. (*H–J*) Expression of *Ets1* in nerve tissue. (*H* and *I*) LF (*H*) and DF (*I*) micrographs from the lumbar region of the spinal cord of day-15 embryos. Gray matter (G) adjacent to the central canal is positive for *Ets1* expression, whereas white matter is negative. (*J*) DF micrograph of brain tissue surrounding the fourth ventricle in day-15 embryos. Cerebral cortex is positive for *Ets1* expression, whereas white matter is negative. (*K–N*) Expression of *Ets1* in developing bone. (*K* and *L*) LF (*K*) and DF (*L*) micrographs of vertebral bone sections. *Ets1* is highly expressed in developing bone (B) and is not detected in cartilage (C). (*M* and *N*) LF (*M*) and DF (*N*) micrographs of sections of the hindfoot plate of a day-14 mouse embryo. Cartilage (B) is negative for *Ets1* expression, whereas the forming bones (C) are positive. (×10.)

P19, especially since these cell lines differentiate into different lineages. F9 cells grown in a monolayer and treated with 1 μM retinoic acid differentiate into endodermal cells (25), whereas P19 cells under similar conditions differentiate into mesoderm-like cells (25). Our data (Fig. 3) show that *Ets1* expression is not detected in undifferentiated F9 and P19 cells. However, *Ets1* expression is induced in differentiated P19 cells but not in differentiated F9 cells. These results therefore show that endoderm-like cells do not express *Ets1*, whereas mesoderm-like cells do. In contrast, the *Ets2* gene is expressed in F9 and P19 cells irrespective of differentiation status.

***Ets1* and *Ets2* Expression in Hematopoietic Cell Lines.** These experiments were carried out to gain further insight into the putative role of *Ets1* and *Ets2* in hematopoiesis. Our data show that expression of *Ets1* occurred in cell lines from only some lineages of the hematopoietic system. The vast majority of nonlymphoid cell lines investigated did not express the *Ets1* gene at detectable levels (Fig. 4*A*). In the odd instances in which expression was detected, the levels of mRNA were low (Fig. 4*A*, lane 4). *Ets1* expression was, however, detected at relatively high levels in one of three murine mast cell lines investigated (Fig. 4*A*). In contrast to the nonlymphoid cell lines, all B-, T-, and plasmacytoma cell lines investigated expressed *Ets1* at very high levels (Fig. 4*B*). *Ets1* expression occurred in B cells at similar levels irrespective of differentiation status (Fig. 4*B*). Furthermore, the expression of *Ets1* in T-cell lines was independent of the CD4/CD8 status of

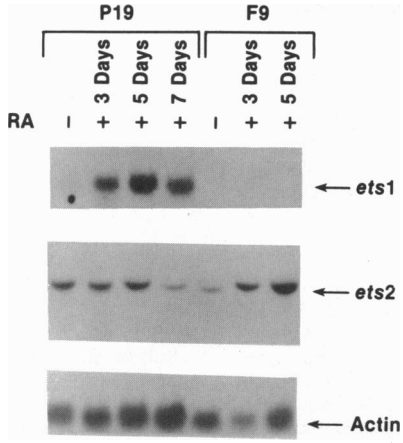

FIG. 3. Expression of *Ets1* and *Ets2* in undifferentiated and differentiated F9 and P19 cells by Northern blot analysis. *Ets1* is not expressed in F9 cells irrespective of differentiation status. *Ets1* expression is upregulated in differentiated P19 cells. β-Actin is used as a control.

specific cell lines. Thus, expression of *Ets1* is restricted predominantly to lymphoid lineages in the murine hematopoietic cell lines investigated, consistent with data on the expression of *Ets1* in adult and neonatal organs. *Ets2*, on the other hand, was expressed at similar levels in the vast majority of murine hematopoietic cell lines studied (Fig. 4).

DISCUSSION

Our data on expression of *Ets1* during murine embryonic development are interesting for several reasons. Currently, it is thought, based on findings (21) in neonatal and adult mice, that expression of *Ets1* occurs predominantly in the lymphoid

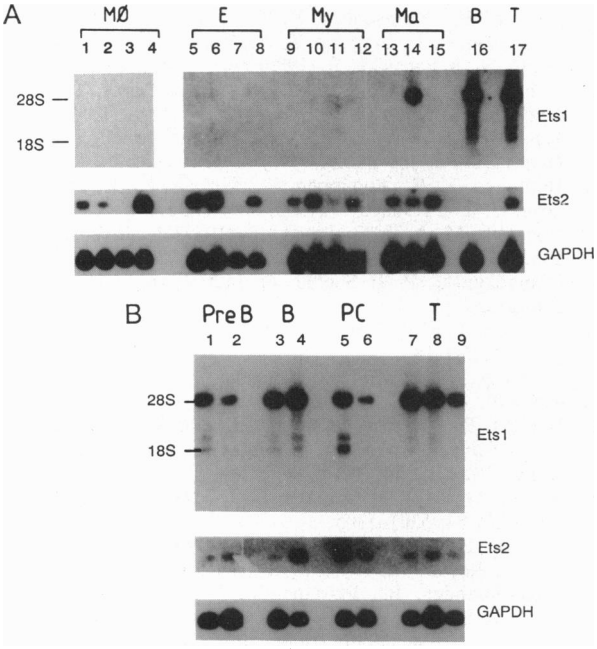

FIG. 4. Expression of *Ets1* and *Ets2* in murine hematopoietic cell lines. (*A*) Poly(A)$^+$ Northern blot of macrophage (lanes 1–4), erythroid (lanes 5–8), myeloid (lanes 9–12), mast (lanes 13–15), B- (lane 16), and T- (lane 17) cell lines. Lanes: 1, W265; 2, W274; 3, J774; 4, PU5; 5, F4N; 6, FLSP; 7, IW32; 8, J2E; 9, MI; 10, 416B; 11, FDCP1; 12, 32D; 13, Fmp1.6; 14, HC3; 15, P815; 16, AΨ7E; 17, VL3K7. (*B*) Poly(A)$^+$ Northern blot of various B-, T-, and plasmacytoma cell lines. Lanes: 1, W401.1; 2, A8; 3, W413; 4, W404.1; 5, P3; 6, MPC11; 7, W112.1; 8, VL3K7; 9, RB3.1. Filters were reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

organs. In this study, we demonstrate that expression of *Ets1* is much more widespread during murine embryonic development as compared to neonatal and adult stages. Our data show that expression of *Ets1* increases in murine embryos after implantation and occurs in organs during organogenesis. In later fetal stages, *Ets1* expression is predominant in the lymphoid tissues, brains, and organs that are being remodeled and/or undergoing branching morphogenesis (33, 34) (e.g., kidney, lung, and salivary gland; data not shown for salivary gland) but is dramatically reduced in other organs such as stomach and intestine. In neonatal development, *Ets1* is expressed only in lymphoid organs and brain; while in adult organs the gene is only expressed in lymphoid organs. *Ets1* is also highly expressed in bone during bone formation and/or modeling. *In situ* hybridization analysis demonstrates that *Ets1* expression is limited to mesoderm, its derivatives, and some neuronal tissue. These findings provide insight into the biological role of the *Ets1* gene.

Ets1 expression during murine embryo development is associated with morphogenesis and/or tissue modeling. These morphogenetic processes, including branching morphogenesis, involve the degradation and remodeling of the extracellular matrix (33). The enzymes necessary for degradation and remodeling of the extracellular matrix include the matrix-degrading metalloproteinases and the plasminogen activators (33). *Ets1* is known to regulate transcription of matrix-degrading metalloproteinases such as stromelysin (11) and urokinase plasminogen activator (12). Thus, it may be that the function of the *Ets1* transcription factor during these morphogenetic processes is to regulate transcription of the genes involved in extracellular matrix degradation and remodeling.

Expression of *Ets1* in developing organs occurs in a specific subset of cells. Our data demonstrate that *Ets1* is expressed at high levels in cells of the mesodermal lineage. Furthermore, we find in several instances that the cells in closest contact with the ectodermal cells express *Ets1* at very high levels, perhaps indicating that this transcription factor is involved in polarization of the mesenchyme. Expression of *Ets1* in mesodermal lineages is further supported by our embryonal carcinoma cell experiments. We find that *Ets1* expression occurs in cells induced to differentiate into mesoderm-like cells and that expression is not detected in cells induced to differentiate into extraembryonic endoderm, an epithelial-like cell. Furthermore, we do not detect the expression of *Ets1* in epithelial cells (with the exception of endothelial cells, which are mesodermal in origin) and in the endodermal derivatives of the developing embryo. Thus, these data show that *Ets1* expression occurs in mesodermal cells of organs in the developing mouse embryo.

The suggestion that *Ets1* expression is strongly associated with mesodermal cells is also consistent with, and gives insight into, the data of *Ets1* expression in the chick (32). *Ets1* has been shown to be expressed in endothelial cells (32) and is strongly associated with formation of immature blood vessels (10, 32) in chicken embryos and in tissues from aborted human fetuses. Our data on the expression of *Ets1* in differentiated P19 embryonal carcinoma cells suggest that the expression of *Ets1* cannot be attributed solely to blood vessel formation or specific expression of this gene in endothelial cells *per se*. Rather, it appears from our data on *Ets1* expression during embryogenesis and in embryonal carcinoma cells that expression of *Ets1* generally occurs in mesodermal lineages or cells, of which endothelial cells are a part. Thus, expression of *Ets1* in mesodermal cells of organs in developing embryos may also occur in other animal species.

Another interesting finding from our data on *Ets1* expression in murine embryos is the expression of the gene in the developing nervous system. Our Northern blot data demon-

strate that *Ets1* is expressed in the brains of mice during embryonic and neonatal development. *In situ* hybridization analysis demonstrates that the meninges and periventricular cells express *Ets1* at very high levels. Furthermore, *Ets1* expression also occurs in the nerve tissue of the cerebral cortex and in the gray matter of the spinal cord of developing embryos. In both these instances, the white matter is negative for *Ets1* expression. We detect relatively high levels of *Ets1* mRNA in the brains of neonatal mice, thus suggestive of an important role for *Ets1* in the nervous system since in mice the nervous system continues to develop during the neonatal period (35). It has previously been reported (36) that the *ETS* protooncogenes are expressed in astrocytes of the human cortex. However, that study could not discriminate between *Ets1* and *Ets2* expression, since a polyclonal antibody raised against a region of v-*ets* with homology to both *Ets1* and *Ets2* was used. Data of *Ets1* expression in migrating neural crest cells (32) and in nerve tissue of developing embryos may also be relevant to understanding the molecular genetics of neuronal tumors. It has been shown (37) that *Ets1* is expressed in cells of peripheral embryonal neuroectodermal tumors (specifically, neuroepithelioma and neuroblastoma). Thus, the finding that *Ets1* is expressed in developing nervous system may be related to expression of the gene in neuronal tumors.

We have also found *Ets1* expression in the yolk sac while the hematopoietic stem cells are found in that particular tissue during development. In addition, Vandenbunder *et al.* (32) found that in the chicken embryo early hematopoietic foci express *Ets1*, whereas later hematopoietic foci express *Myb*. They proposed that this alternative expression of *Myb* and *Ets1* in hematopoietic cells may be due to a similar function being carried by these two protooncogenes at different times of ontogeny. It may be that *Ets1* is involved in transcriptional regulation of other genes that play a role in early hematopoiesis. We (16) have previously shown that the Ets1 protein binds sequences found in the promoter of the granulocyte colony-stimulating factor gene, a growth factor required for hematopoiesis. Furthermore, in adult tissues and in hematopoietic cell lines, *Ets1* is highly expressed in lymphoid organs and in B- and T-cell lines, respectively, thus suggesting a role for *Ets1* in these particular hematopoietic lineages. Nevertheless, the definition of the putative role that *Ets1* plays in hematopoiesis, and indeed in the lymphoid lineage, awaits further experimentation.

Unlike the relatively restricted pattern of *Ets1* expression, *Ets2* continues to be widely expressed in the organs of adult mice. Indeed, *Ets2* is also found to be widely expressed in various lineages of hematopoietic cell lines and in F9 and P19 cells, irrespective of the differentiation status. The relatively widespread expression of *Ets2* may be suggestive of a fundamental role for this gene. Such a role may be in mitosis of cells. This idea is supported by the finding that expression of *Ets2* is necessary for completion of meiosis in *Xenopus* oocytes (38). Many of the factors involved in meiosis are also involved in mitosis. Thus, these data suggest different functions for the *Ets1* and *Ets2* transcription factors.

In conclusion, our data suggest that *Ets1* expression is strongly associated with mesodermal cells in organs undergoing morphogenetic processes and that the *Ets1* transcription factor has an important role in murine embryo development, whereas *Ets2* has a more fundamental role in cells. The use of homologous recombination for the generation of mice that have a null mutation for *Ets1* and *Ets2* would be useful for establishing the function of these transcription factors in murine embryo development.

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