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Cloning and characterization of a basic helix–loop–helix protein expressed in early mesoderm and the developing somites

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ABSTRACT **Basic helix–loop–helix (bHLH) heterodimer protein complexes regulate transcription of genes during the processes of differentiation and development. To study the molecular basis of early mesodermal differentiation, we sought to identify bHLH proteins from cells of mesodermal origin. By using an interaction cloning strategy with a radiolabeled recombinant bHLH protein, E12, a clone encoding a potential heterodimer partner was isolated from an endothelial cell library. This gene (bHLH-EC2) is most homologous to *Twist* but shares similarity within the bHLH domain with TAL1 and other members of this family. bHLH-EC2 is expressed in cultured endothelial cells and in embryonic stem cell, erythroleukemia, and muscle cell lines in a differentiation-dependent manner. *In situ* hybridization studies of mouse embryos reveal that bHLH-EC2 is expressed throughout the primitive mesoderm as early as 7.5 days postcoitum. Expression then becomes restricted to the paraxial mesoderm and to the dermamyotome of the developing somite. Expression of bHLH-EC2 in cells destined to become myoblasts thus predates expression of myogenic bHLH factors. bHLH-EC2 is expressed in early endothelial and hematopoietic cells *in vivo*, as shown by RNA studies of embryonic yolk sac and cultured cells derived from yolk sac explants. These findings suggest that bHLH-EC2 plays a role in the development of multiple cell types derived from the primitive mesoderm.**

The mesodermal germ layer in vertebrates is formed at gastrulation by inductive interactions (1–3). It is important to link the early molecular events of mesodermal induction with the determination of specific cell types, which ultimately constitute tissues derived from the mesoderm. This can be accomplished only through the study of genes expressed in mesodermal cells at this critical developmental period. Study of the genes regulating differentiation of the mesoderm in model systems such as *Drosophila* has indicated that transcription factors of the basic helix–loop–helix (bHLH) class have an important role in fundamental early processes such as dorsoventral patterning (4–6). Experiments in mammalian development concentrating on relatively late events, such as determination and differentiation of specific cell lineages, have also indicated that this class of transcription factors has a singularly important role in regulating the development of mesodermal tissues. Four transcription factors of the bHLH class have been shown to activate the muscle-specific program in cells in culture, and recent gene targeting experiments have provided some insight into the regulatory network that links these transcription factors (7–9). In addition, bHLH factors have been implicated in the differentiation of other mesodermally derived cell types, including those of hematopoietic lineage (10–12).

In the work described here, an interaction cloning strategy was used to identify bHLH factors involved in lineage commitment in additional cell types derived from the embryonic mesoderm. Because of our long-standing interest in endothelial cell biology, this cell type was chosen as the source for the cDNA libraries. The gene isolated in this fashion, bHLH-EC2, appears to be an early marker of the establishment of the mesodermal germ layer. It is expressed in cultured pluripotent embryonic stem (ES) cells, and expression increases immediately upon *in vitro* differentiation of these cells. At the earliest time point evaluated by *in situ* hybridization, 7.5 days postcoitum (d.p.c.), bHLH-EC2 is expressed throughout the mesoderm. Later, expression is restricted to precursor cells in the dermamyotome and the blood islands. These data suggest that bHLH-EC2 plays a role in early patterning of the mesoderm and subsequently may participate in the lineage determination of a number of cell types derived from the mesoderm.‖

MATERIALS AND METHODS

Cloning and Sequencing. A recombinant E12 fusion protein containing the bHLH domain of hamster shPAN-2 (amino acids 509–649, with mutations R554A and R556A) with a heart muscle kinase recognition sequence and the FLAG epitope was expressed and purified as described (12–14). E12 fusion protein was phosphorylated by heart muscle kinase in the presence of [γ-^{32}P]ATP and used to screen 1.5 × 10^6 plaques of both random- and oligo(dT)-primed human umbilical vein endothelial cell (HUVEC) λgt11 expression libraries (kindly provided by S. Orkin, Children's Hospital, Boston) (15). Denaturation and renaturation, hybridization, and washing conditions for the transfer membranes were performed as described (13). A 350-bp clone resulting from interaction cloning was subsequently radiolabeled by random priming and used to screen by DNA homology both the oligo(dT) HUVEC cDNA library and an 8.5-d.p.c. mouse embryo λgt10 cDNA library (kindly provided by Brigid Hogan, Vanderbilt University Cell Biology, Nashville, TN). Nucleotide sequences were obtained by a combination of dideoxynucleotide chain termination with Sequenase (United States Biochemical) and the Maxam and Gilbert techniques (16).

Cell Culture and Differentiation. HepG2 (hepatocarcinoma), A-172 (glioblastoma), HeLa (epithelial carcinoma), K562 and HEL (human erythroleukemia), JEG3 (choriocarcinoma), MOLT4 (T-cell leukemia), HUVECs and HSVSMCs (human saphenous vein smooth muscle cells) were obtained and cultured as described (17). MEL (murine eryth-

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Abbreviations: d.p.c., days postcoitum; ES cell, embryonic stem cell; bHLH, basic helix–loop–helix; HUVEC, human umbilical vein endothelial cell; LIF, leukemia inhibitory factor; HSVSMC, human saphenous vein smooth muscle cell.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. U08336).

roleukemia) cells were a gift of Stephen Brandt (Vanderbilt University Hematology, Nashville, TN) and were cultured and differentiated in the presence of dimethyl sulfoxide according to published methodology (11). C2C12 and G8 cells (embryonic myoblasts) were obtained from the American Type Culture Collection (ATCC) and differentiated by replacing 20% fetal calf serum with 5% horse serum. D3 ES cells were obtained from the ATCC and cultured as described in the presence of recombinant human leukemia inhibitory factor (LIF) (ESGRO; GIBCO/BRL) at 1000 units/ml (18). To induce differentiation, the ES cells were treated with trypsin and transferred to bacterial Petri dishes in medium lacking LIF (18). Cells from the murine yolk sac were derived as described but maintained in the presence of LIF instead of feeder layers (19).

RNA Expression Studies. Total RNA was prepared from cell lines, ES cells, and mouse organs by guanidinium isothiocyanate extraction and centrifugation through cesium chloride (16). Poly(A)$^+$ RNA was isolated from mouse embryos and yolk sacs with the Micro-fast track mRNA isolation kit (Invitrogen). For RNA blot experiments, 20 μg of total RNA was size fractionated, hybridized, and washed at high stringency as described (17). Integrity of the RNA and equal loading of the samples was verified for all blots by a second hybridization to an oligonucleotide complementary to 18S ribosomal RNA (ACGGTATCTGATCGTCTTCGAACC). For RNase protection analysis, the antisense transcript was transcribed from a 390-bp *Sac* I/*Bam*HI fragment of mouse cDNA in the presence of [^{32}P]UTP by T7 RNA polymerase. Approximately 100,000 cpm of the probe was hybridized to 15 μg of total RNA or to 1 μg of poly(A)$^+$ RNA at 48°C in 80% formamide for 12 hr. Protected fragments of 314 bp were analyzed on an 8% denaturing polyacrylamide gel. RNase protection of a mouse β-actin probe was performed as a control for every experiment.

***In Situ* Hybridization.** Sections were generated from paraformaldehyde-fixed, paraffin-embedded, timed mouse embryos according to established methodology or they were purchased from Novagen. Probes were generated by *in vitro* transcription of a 400-bp *Bam*HI/*Hin*dIII restriction fragment containing the most 3′ untranslated region of a mouse cDNA in pGEM 7Zf. The radioactive antisense complementary RNA was transcribed by T7 polymerase from plasmid linearized with *Bam*HI in the presence of 70 μCi of [α-^{33}P]UTP (1 Ci = 37 GBq). The opposite strand was transcribed by Sp6 polymerase from the *Hin*dIII-linearized plasmid. RNA probes were reduced to ≈100 bases by hydrolysis under alkaline conditions. All the sections were deproteinized, acetylated, hybridized, RNase treated, and washed by established methodology (16); 4×10^6 cpm of each complementary RNA was hybridized per cm^2 of sections. Exposure times varied from 7 to 26 days. In no case did the sense-strand control hybridizations show a specific signal above background.

RESULTS

Sequence Analysis of bHLH-EC2. The 975-bp sequence of the longest cDNA clone has an open reading frame that encodes a bHLH protein of 198 amino acids (Fig. 1*A*). bHLH-EC2 is homologous in its bHLH domain to the developmental factor *Twist* and to several members of a family of bHLH factors identified through their location at breakpoints of chromosomal translocations associated with T-cell leukemias (Fig. 1*B*) (20–23). TAL1 and Lyl1 have been implicated in the normal differentiation of hematopoietic cells (24). *Twist* is the only one of these factors that has homology to bHLH-EC2 outside of the bHLH domain, because of a glycine-rich region located in both proteins between the N terminus and the bHLH domain (25). There is minimal homology to the bHLH domains of the myogenic factors myoD and myogenin (Fig. 1*B*).

Cell-Specific Expression of bHLH-EC2. The expression of bHLH-EC2 was determined in a variety of human cell lines by RNA blot analysis (Fig. 2*A*). Hybridization of the radiolabeled bHLH-EC2 probe was performed under conditions of high stringency and revealed a 1-kb message in the HUVEC RNA sample. This result provided evidence that the 975-bp sequence of the longest human clone represented most of the bHLH-EC2 transcript. A prominent signal in the RNA from HEL cells, and a weak signal in the RNA from K562 cells, indicates that cultured erythroleukemia cell lines also express

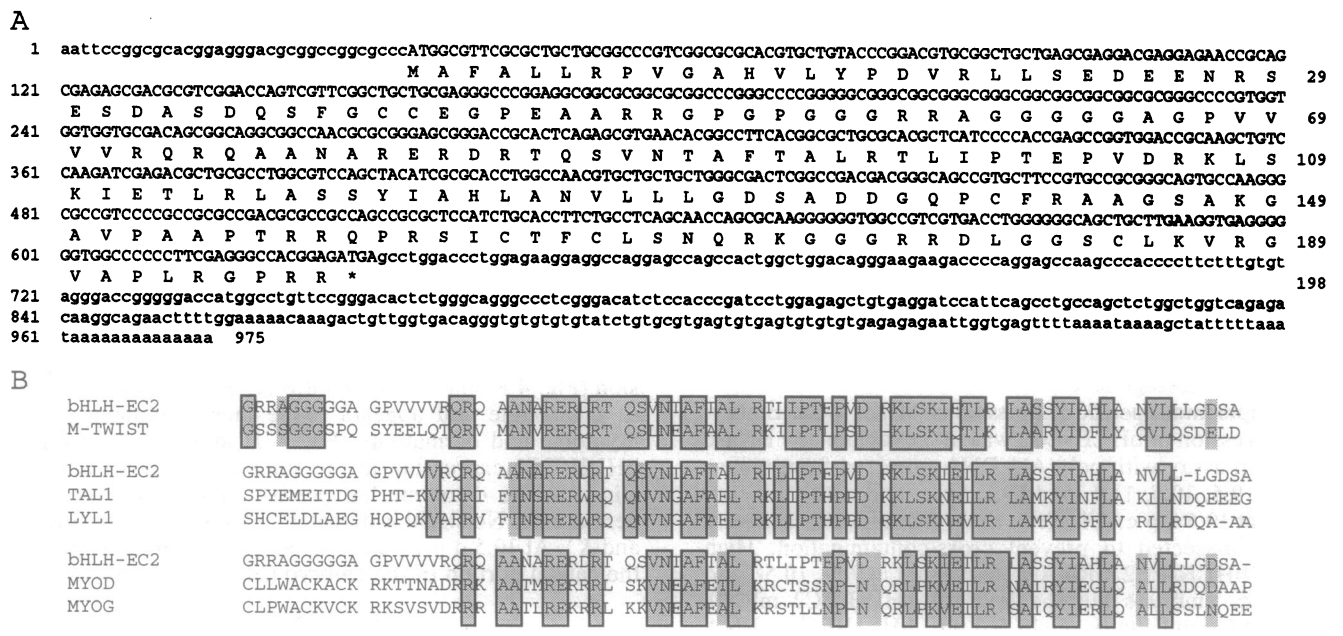

FIG. 1. (*A*) Primary nucleotide sequence and deduced amino acid sequence of human bHLH-EC2. Base pair number is indicated on the left, and amino acid number is indicated on the right. Asterisk denotes translation stop codon. (*B*) Separate comparisons of bHLH-EC2 (amino acids 56–134) with mouse Twist (M-TWIST), leukemia-associated bHLH factors (TAL1 and LYL1), and myogenic bHLH factors (MYOD and MYOG).

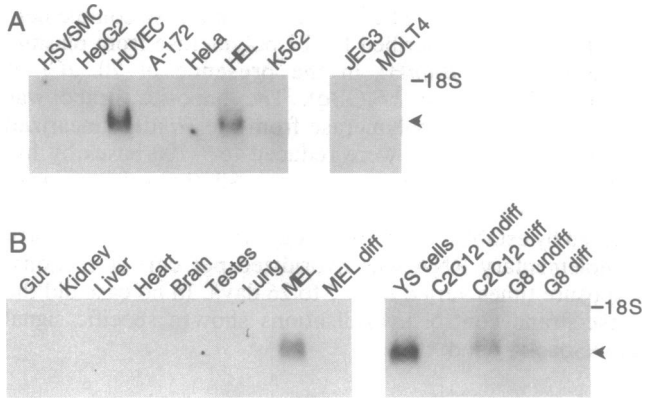

FIG. 2. Northern analysis of the distribution of bHLH-EC2 mRNA from human cell lines (*A*) and mouse organs and cell lines (*B*). (*A*) Transcript of ≈1 kb was visualized in RNA from HUVECs and in two human erythroleukemia cell lines, HEL and K562. No hybridization was detected in HSVSMCs, HepG2 (hepatocarcinoma), A-172 (glioblastoma), HeLa (epithelial carcinoma), JEG3 (choriocarcinoma), or MOLT4 (T-cell leukemia). (*B*) A similar sized transcript was detected in MEL cells and in murine embryonic yolk sac (YS) cells. C2C12 and G8 murine myoblast lines had low levels of bHLH-EC2 mRNA at baseline (detectable on original autoradiograph). Differentiated C2C12 cells had a significant increase in message levels, while G8 cells showed a decline in message level with differentiation.

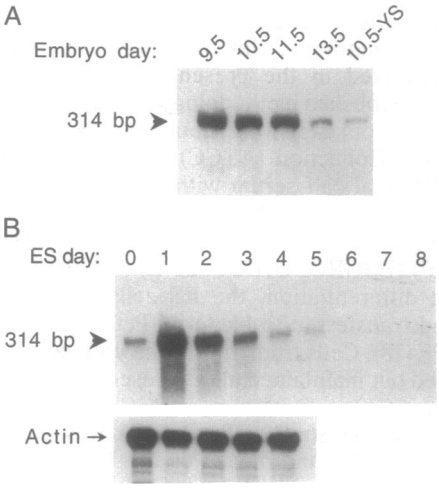

FIG. 3. RNase protection analysis of the expression of bHLH-EC2 during mouse embryonic development (*A*) and *in vitro* differentiation of ES cells (*B*). Poly(A)$^+$ RNA was extracted from embryos collected at 9.5, 10.5, 11.5, and 13.5 d.p.c. and from embryonic yolk sacs at 10.5 d.p.c. (10.5-YS). Protection of the 314-bp bHLH-EC2 transcript reflects expression of bHLH-EC2 before differentiation (ES day 0) and during the first 5 days of differentiation. RNAs from days 0–4 were analyzed separately with a β-actin probe to verify the integrity and quantitation of the RNA samples during the time course of bHLH-EC2 expression.

bHLH-EC2. Human cells HSVSMCs, HepG2, A-172, HeLa, and MOLT4 did not show hybridization to the bHLH-EC2 probe.

To characterize further the expression pattern of the bHLH-EC2 gene, RNA blot analyses were performed on mouse tissues and established cell lines (Fig. 2*B*). The partial mouse cDNA used as a probe in these studies does not contain the bHLH domain, thus making it unlikely to cross-hybridize with transcripts from other genes on the basis of homology in the bHLH domain. There was no bHLH-EC2 message detected in organs from adult mice, including small intestine (gut), kidney, liver, heart, brain, testes, and lung. The mouse erythroleukemia line MEL expressed bHLH-EC2 at a high level under normal culture conditions but did not express bHLH-EC2 after dimethyl sulfoxide-induced differentiation. To investigate the possibility of expression of bHLH-EC2 in early cells of the endothelial and hematopoietic lineages in the blood islands, RNA of cultured yolk sac cells was studied. These cells were derived from 8-d.p.c. embryonic yolk sac and maintained in the presence of LIF in an attempt to maintain the embryonic phenotype (19). These cells had the highest level of bHLH-EC2 mRNA, as shown by the strong signal on the Northern blot. Two myoblast cell lines, C2C12 and G8, appeared to have low levels of bHLH-EC2 mRNA in the myoblast state. Differentiation of C2C12 resulted in an increase in the level of bHLH-EC2 message, while differentiation of G8 cells resulted in a decrease in bHLH-EC2 message level.

Developmental-Specific Expression of bHLH-EC2. Embryonic expression of bHLH-EC2 was first evaluated by RNase protection assay with poly(A)$^+$ RNA prepared from mouse embryos 9.5, 10.5, 11.5, and 13.5 d.p.c. and with yolk sacs isolated at 10.5 d.p.c. (Fig. 3*A*). Embryos and yolk sacs were carefully dissected to prevent cross-contamination. High levels of bHLH-EC2 mRNA were present in 9.5-, 10.5-, and 11.5-d.p.c. embryos but the level of bHLH-EC2 mRNA diminished significantly by 13.5 d.p.c. bHLH-EC2 mRNA was expressed in the embryonic yolk sac at the single time point investigated.

To investigate the expression of bHLH-EC2 in the early embryo, we used an *in vitro* ES cell differentiation model. Pluripotent ES cells, when removed from feeder layers or other sources of LIF, differentiate into a variety of cell types, including skeletal and cardiac muscle cells, hematopoietic cells, and endothelial cells (18). RNA isolated from D3 ES cells pre- and postdifferentiation was examined by an RNase protection assay (Fig. 3*B*). ES cells expressed bHLH-EC2 at a low level in the undifferentiated state and expression markedly increased with differentiation. The induction of bHLH-EC2 expression declined by day 4 and there was no detectable expression after this time. A mouse β-actin probe was used to verify that this pattern of induction was not due to differences in RNA amount or integrity. Expression of bHLH-EC2 in the pluripotent ES cells and its immediate induction by differentiation suggest that bHLH-EC2 may be expressed very early in mouse development.

A detailed spatial and temporal analysis of bHLH-EC2 expression was obtained by *in situ* hybridization of sections of embryos between 7.5 and 11.5 d.p.c. (Fig. 4). Expression of bHLH-EC2 was detected at the earliest stage that was analyzed, 7.5 d.p.c., and was found to be restricted to the intraembryonic mesoderm (Fig. 4 *A–C*). There is no signal associated with the membranes or the maternal tissue included in the sections at this stage. Also, there is no expression in the neuroectoderm or the forming notochord. At 8.0–8.5 d.p.c., bHLH-EC2 is expressed in the condensing paraxial mesoderm and the early somite (Fig. 4 *D–G*). At this stage, there is evidence of a rostral–caudal pattern of expression (Fig. 4 *F* and *G*). By 9.5 d.p.c., expression is confined to the somite and appears to be most prominent in the myotome and dermatome (Fig. 4 *H* and *I*). At 10 d.p.c., bHLH-EC2 expression in the somite appears to have declined in the myotome but persists at a high level in the dermatome. No signal is detected in the sclerotome (Fig. 4 *J* and *K*). At 10.5 d.p.c., expression is seen only in somites in the caudal portion of the embryo (Fig. 4 *L* and *M*).

DISCUSSION

Cloning and nucleotide sequence analysis indicates that bHLH-EC2 is a newly discovered member of the bHLH family of transcription factors. By virtue of the cloning strategy and the presence of a basic region, it seems likely

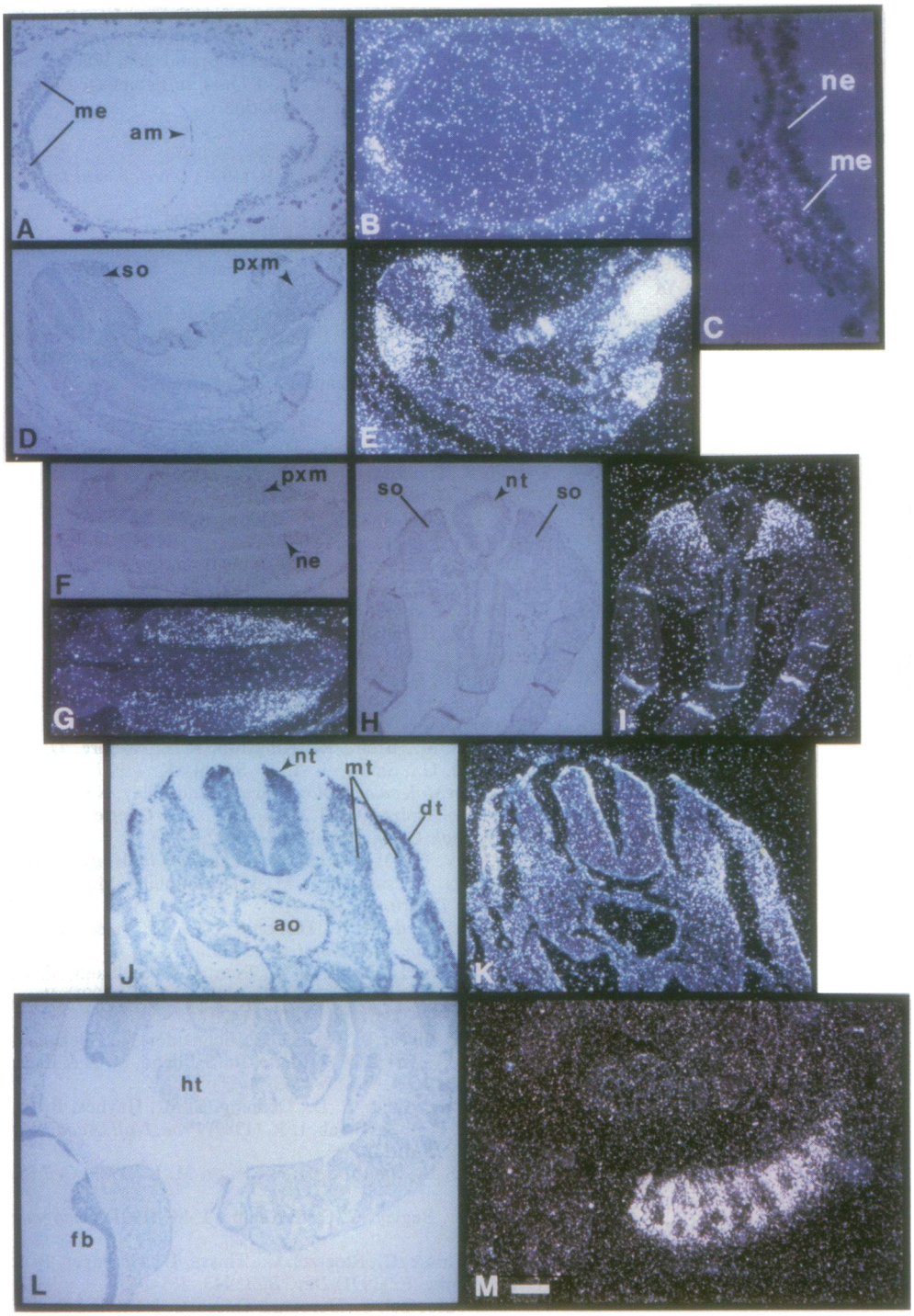

FIG. 4. *In situ* hybridization analysis of the pattern of expression of bHLH-EC2 during embryonic development (7.5–10.5 d.p.c.). (*A* and *B*) bHLH-EC2 is expressed only in the intraembryonic mesoderm at 7.5 d.p.c. (*C*) Enlargement of section adjacent to the one in *A* and *B*, with hybridization signal over mesodermal but not neuroectodermal cells. (*D* and *E*) Transverse section of turning embryo at 8.5–9 d.p.c., showing expression of bHLH-EC2 in forming rostral somites and condensing caudal paraxial mesoderm. (*F* and *G*) Section of dorsal embryo and hindbrain at 8.5 d.p.c., revealing expression in paraxial mesoderm. (*H* and *I*) Transverse section through the caudal region of a 9.5-d.p.c. embryo with labeling of the dermamyotome. (*J* and *K*) At 10.5 d.p.c., bHLH-EC2 expression has declined in the myotome but persists at a high level in the dermatome and is absent from the sclerotome. (*L* and *M*) Parasagittal section of a 10.5-d.p.c. embryo, showing a rostral–caudal pattern of expression. me, Mesoderm; am, amnion; so, somite; pxm, paraxial mesoderm; ne, neuroepithelium; nt, neural tube; mt, myotome; dt, dermatome; ao, dorsal aorta; ht, heart; fb, forebrain. (*A*, *B*, and *D–K*, ×8; *C*, ×40; *L* and *M*, ×4; bar = 200 μm.)

that this factor heterodimerizes with members of the E12 family of ubiquitous bHLH factors and functions as a positive transcriptional regulator. This possibility is supported by *in vitro* binding studies, which reveal that recombinant bHLH-EC2 protein can bind cognate DNA sequences only when combined with E12 or other ubiquitous bHLH factors (E.E.Q., unpublished data).

Because of similarity in the bHLH domain, the presence of a glycine-rich region, and early mesodermal expression, this bHLH factor appears most similar to the developmental factor *twist*. *twist* was initially characterized in *Drosophila* (*twi*) as one of the first zygotic genes necessary for mesoderm formation (26). *twi* homologs have now been cloned and their expression patterns characterized in *Xenopus* and mouse (*Twist*) (25, 27). Comparison of published *in situ* data for mouse *Twist* with that presented here for bHLH-EC2 reveals that these two genes have nonoverlapping patterns of expression (25). While bHLH-EC2 is initially expressed throughout the mesoderm in the late primitive streak embryo, by the time mouse *Twist* expression begins bHLH-EC2 has localized to the paraxial mesoderm and the dermamyotome of the developing somite. Mouse *Twist* expression begins after evolution of the mesoderm into somites and is expressed in the sclerotome where bHLH-EC2 expression is absent. In addition, mouse *Twist* is expressed in the lateral mesoderm and mesodermally derived tissues in the head, while bHLH-EC2 expression remains restricted to the somites. Expression of these homologous bHLH factors thus appears to subdivide the mesoderm, suggesting that they may serve a similar developmental function in different cell lineages derived from the mesodermal germ layer.

RNA blot experiments indicate that cultured endothelial cells and hematopoietic cells express bHLH-EC2. Despite experiments in embryos spanning embryonic days 7.5–11.5, there was no evidence by *in situ* hybridization of specific labeling of endothelial cells associated with vascular structures or hematopoietic cells found in the blood islands. While expression in cultured cells of these lineages might reflect an *in vitro* artifact, it remains a distinct possibility that bHLH-EC2 is expressed during early development in these cell types. The observed decrease in bHLH-EC2 expression in the MEL cell line is consistent with such a hypothesis. Evidence for expression of bHLH-EC2 in one or both of these cell types *in vivo* was derived from detection of bHLH-EC2 mRNA in murine yolk sac and in cultured yolk sac cells. While cultured yolk sac cells have been derived as a potential source of multipotent hematopoietic precursor cells, such cells may also have endothelial potential (19). It has been postulated that both hematopoietic and endothelial cell precursors derived from a single common progenitor, the hemangioblast (28). Such a cell would be found in small numbers in the blood islands and might not be detected by *in situ* hybridization experiments. In addition, early endothelial cells, angioblasts, may also escape detection by this method in the developing embryo. For instance, it is now well accepted that angioblasts are present in the early somite (29). It would be impossible to distinguish angioblasts from cells of other lineages in the dermamyotome, where all cells appear to express bHLH-EC2. More sensitive experiments, such as immunohistochemistry, will be required to investigate the intriguing possibility that bHLH-EC2 is expressed during an early phase of hematopoietic and endothelial cell differentiation.

bHLH-EC2 mRNA is detected in mesodermal cells that are fated to become myoblasts and is expressed in cultured myoblast lines. While this gene is a member of the same class of transcription factors as the myogenic regulatory factors, it does not share the conserved myogenic domain and is thus unlikely to directly specify the muscle cell program (30). Since expression of bHLH-EC2 precedes expression of the earliest myogenic factor myf5, it may have a role in developmental processes upstream of the myogenic factors. While recent gene targeting studies have provided great insights into the interplay between the four myogenic factors (myf5, MyoD, myogenin, MRF4), there is currently little information linking the early processes of mesodermal induction to their expression (7–9). bHLH-EC2 might be involved in such processes through a number of possible roles. bHLH-EC2 may function to maintain an embryonic noncommitted state until appropriate differentiation signals are provided in the somite. Alternatively, bHLH-EC2 alone or in combination with mouse *Twist* may provide positional cues for subdivision within the somite. Such a function would be similar to the role of *twi* in demarcating the ventral mesoderm in *Drosophila* (4). Alternatively, bHLH-EC2 may serve a function similar to the proneural genes of *Drosophila*, endowing cells of the dermamyotome with the potential for developing into myoblasts (31). These issues need to be addressed through genetic manipulation in cell culture model systems as well as in the mouse. It will also be important to clone and characterize the homologs of this factor and investigate their role in mesodermal development in model systems such as *Xenopus* and *Drosophila*. Data indicating that *twi* has a role in the development of adult muscle in *Drosophila* suggests that it will be particularly interesting to investigate the role of bHLH-EC2 in muscle development in this model system (32).

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