Cloning and Characterization of Ehox, a Novel Homeobox Gene Essential for Embryonic Stem Cell Differentiation*

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We report here the identification and characterization of a novel paired-like homeobox-containing gene (Ehox). This gene, identified in embryonic stem (ES) cells, is differentially expressed during in vitro ES cell differentiation. We have assessed Ehox function using the ES cell in vitro differentiation system. This has involved molecular and biological analyses of the effects of sense or antisense Ehox expression (using episomal vectors) on ES cell differentiation. Analysis of antisense Ehox-expressing cells indicates that they are unable to express marker genes associated with hematopoietic, endochondral, or cardiac differentiation following removal of leukemia inhibitory factor. In contrast, overexpression of Ehox using the sense construct accelerated the appearance of these differentiation markers. ES cell self-renewal and differentiation assays reveal that inhibition of Ehox activity results in the maintenance of a stem cell phenotype in limiting concentrations of leukemia inhibitory factor and the almost complete impairment of the cardiomyocyte differentiation capacity of these cells. We therefore conclude that Ehox is a novel homeobox-containing gene that is essential for the earliest stages of murine ES cell differentiation.

Murine embryonic stem (ES) cells are derived from the inner cell mass of the day 3.5 post coitus blastocyst and can be maintained in culture as a self-renewing totipotent population in the presence of the growth factor, leukemia inhibitory factor (LIF) (1, 2). These cells form the basis for much current murine transgenic and gene targeting technology (3) as they have the capacity to generate all tissues, including the germ line, when re-injected into the blastocyst (4, 5). ES cells can also differentiate in vitro into a wide range of cell types that are derived from all three embryological germ layers. These include hematopoietic lineages of mesodermal origin (6), neuronal cells of ectodermal origin (7), liver (8, 9) and pancreatic cell types derived from embryonic endoderm (10), as well as visceral and parietal extra-embryonic endoderm (11, 12). With the isolation of human ES cells (13, 14), it has been proposed that the in vitro differentiation capacity of these cells could be utilized for somatic cell therapy to treat a number of diseases (10, 15, 16). This technology, however, is severely limited by the heterogeneity of the differentiation process and by the limited molecular characterization of the system. A detailed analysis of the molecular mechanisms involved in the early differentiation steps may allow the development of lineage selection strategies (7) to generate therapeutic quantities of a specific cell type.

One of the most closely studied differentiation pathways using the ES cell differentiation system has been the commitment to, and the differentiation of, hematopoietic stem cells (17–20). We have recently described the in vitro differentiation of ES cells, cultured as embryoid bodies (EBs), and the time course of commitment of these cells to the hemopoietic lineage (21). Briefly, these studies have demonstrated that, following removal of LIF, components of the hemopoietic stem cell compartment incorporating both long term and short term repopulating stem cells are generated between days 3 and 5 of in vitro differentiation. Subsequently we have used subtractive hybridization to examine the genes expressed over this 2-day time frame (22), which we have now shown to be a valuable source of known and novel hemopoietic gene products.

The subtractive hybridization technology used in this study yields small cDNA fragments in the subtracted library and not full-length cDNA molecules (22). One of the small fragments in the subtracted library (clone name JB46) displayed homology with members of the homeobox-containing family of transcriptional regulators. The homeobox domain is a 60-amino acid DNA-binding motif, which is contained within the context of larger proteins that have diverse roles to play in development and in adult cell function (23). Many members of the homeobox gene families are key players acting as transcriptional master regulators during embryonic development and adult cell differentiation. Here we present the molecular and functional characterization of JB46, herein referred to as Ehox, which represents a highly divergent member of the paired-like homeobox gene family. We have used an episomal overexpression system to facilitate the functional analysis of Ehox in the ES cell differentiation system. Stem cell and differentiation assays as well as detailed real-time PCR analysis of sense Ehox and antisense Ehox-expressing ES cells suggest a fundamental role for this homeobox gene in regulating the initial stages of ES cell differentiation.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF265350 (Ehox).

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§ The abbreviations used are: ES, embryonic stem; LIF, leukemia inhibitory factor; EB, embryoid body; FCS, fetal calf serum; RT, reverse transcriptase; GFP, green fluorescent protein; TBST, Tris-buffered saline plus 0.1% Tween 20; GMEM, Glasgow’s minimal essential medium; EGFP, enhanced green fluorescent protein; IRES, internal ribosome entry site; EST, expressed sequence tag.

This paper is available on line at http://www.jbc.org/
**EXPERIMENTAL PROCEDURES**

**Cloning and Sequencing of Full-length Ehox—**To derive full-length *Ehox* cDNA clones, a day 5 EB cDNA library was generated using the Zap-Express kit (Stratagene, Amsterdam, The Netherlands) and all procedures were performed according to the instructions of the manufacturer. The hemopoietic potential of the day 5 EBs used to construct this library was verified as described previously (21, 22). The library was screened for *Ehox* cDNAs using a radiolabeled 484-bp probe, and again all procedures involved in the cloning of *Ehox* were carried out according to the instructions of the manufacturer. Following three rounds of library screening, positive clones were isolated and the inserts sequenced. The consensus full-length *Ehox* sequence was determined by examining the sequences of multiple Ehox clones.

**ES Cell Culture and Transfection—**ES cells were maintained in Glasgow’s minimal essential medium (GMEM) supplemented with 2-mercaptoethanol, nonessential amino acids, sodium bicarbonate, 10% fetal calf serum (FCS), and 100 units/ml recombinant LIF, on gelatinized tissue culture dishes (24). We used a subcloned of the E14tg2a cell line called E14T (kindly provided by A. Smith and I. Chambers) which expresses polyoma large T from an integrated pMGDNEO plasmid. E14T ES cells were “supertransfected” and 960 microfarads. Transfected E14T cells were selected in 2/11002 g/ml puromycin for 6 days, and then clones were pooled and maintained under selection throughout subsequent culture. Replicate transfection plates were stained with Leishman’s stain to quantify ES colony numbers. At least two separately derived transfected pools of cells were generated for all vectors.

**Generation of Differentiating ES Cell Populations for Gene Expression Analyses—**ES cells were supertransfected with vector, sense, or antisense *Ehox* constructs as outlined above and were maintained in the presence of 2 μg/ml puromycin throughout the experiments. Pools of transfected ES cells were allowed to form EBs by hanging-drop culture as described previously (21, 22). The EBs were harvested and differentiation initiated by removal of the LIF from the culture medium. EBs were harvested at daily intervals up to 6 days after initiation of differentiation and RNA prepared following lysis of the EB-derived cells in TRizol (Sigma). RNA was also prepared from undifferentiated ES cells as well as EBs obtained immediately following harvesting and prior to the initiation of differentiation.

**Northern Blotting—**20 μg of total RNA isolated from undifferentiated E14/T ES cells supertransfected with episomal plasmids to express *Ehox* sense, antisense, or empty vector was loaded per lane of a 2% formaldehyde, 1% agarose gel. Electrophoresis was done for 3 h at 80 V before blotting onto Hybond N nylon membranes. The blot was UV cross-linked and hybridized with a 20-mer labeled 700-bp *Ehox* cDNA probe overnight, and washed in 2x SSC, 0.1% SDS for 20 min at 65 °C before exposing to X-omat AR autoradiography film overnight and developing.

**Taqman “real-time” PCR Analysis of Marker Gene Expression Levels during ES Cell Differentiation—**Real-time PCR was carried out on the ABI PRISM® 7700 system (Applied Biosystems, Warrington, UK). Relative quantitation of gene expression levels was achieved using the standard curve method. The RNA used in the real-time PCR experiments was first DNase I-treated. Following the TaqMan® reverse transcriptase (RT) kit instructions the following reaction was set up with final concentrations of 1× TaqMan® RT buffer, 5.5 mm MgCl₂, 500 μM each dNTP, 1.25 μM random hexamers, 1.25 μM oligo(dT)₁₅, 0.4 units/μl RNase inhibitor, and 1.25 units/μl Multiscribe RT, RNA, and H₂O up to 5 μl. The reaction was incubated at 25 °C for 10 min and then at 48 °C for 30 min, and finally inactivated at 95 °C for 5 min. For the standard curve, 0.5 μl of each RNA dilution was added to the RT reaction; for the experimental samples, ~50 ng of DNA-treated RNA was added to each RT reaction. The cDNA product was stored at −20 °C until use. The primers and probes used in the real-time PCR analyses were obtained from MWG (Ebersberg, Germany) and were as shown below.

**Hprt**

Forward: 5′-GCTCGAGATGTCATGAAAGAGA-3′
Reverse: 5′-CGTCATCCATGGCCCTCGTG-3′
Probe: 5′-AAAGAACCAATGGACCCCTGTA-3′

**Cd34**

Forward: 5′-AAAGGGCAATGTGACCCCGA-3′
Reverse: 5′-ATGCGCGTTTCTGGAAGTG-3′
Probe: 5′-CAGGAGACGCGGACCCGCGA-3′

**Scl**

Forward: 5′-CTTTTGAGCTCTACTGGGATAA-3′
Reverse: 5′-TACGGACCAATTGACCTTC-3′
Probe: 5′-GCTAGGGCGTGAGCAGACTGTG-3′

**β-globin**

Forward: 5′-TGACCCAGCCGCTTACTTGG-3′
Reverse: 5′-CCATGGGCTCTACTTTGTG-3′
Probe: 5′-AGACCTATCTCTGCTCTGCTATCATCGGTAAT-3′

**Gapdh**

Forward: 5′-GTCAAGACCGGCGCTCTCACT-3′
Reverse: 5′-CCATGGGGCCTCTACTTTGTG-3′
Probe: 5′-GACCTATCTCTGCTCTGCTATCATCGGTAAT-3′

**VE-cadherin**

Forward: 5′-GCTGCGGCAAAAGACCTGAGC-3′
Reverse: 5′-ACTGGTTCTGCGGATGGAGT-3′
Probe: 5′-AGGGUCAGCAGCAGCCGCGG-3′

**Fkh-1**

Forward: 5′-GA'TGACCGGCACGCTCTTTTG-3′
Reverse: 5′-CGAAAGACCACTACATGCTTCT-3′
Probe: 5′-CCCGGAAAACCATTTTTTGACAGATGATACACA-3′

**Nkx2.5**

Forward: 5′-TCCACGGCTAGGTCAGATCGT-3′
Reverse: 5′-AGTCTGTGTCCTGCCTGTG-3′
Probe: 5′-CCAGAACCCTGCTACAAAGTGGACGG-3′

**Oct-4**

Forward: 5′-GCAATCTCAGAGGGAACCTCT-3′
Reverse: 5′-TCTCCAACACTTCAACGGCATG-3′
Probe: 5′-TGAGCCCCTGTCGCCAGACGC-3′

The PCR reactions were carried out in a final volume of 25 μl in 96-well optical reaction plates. 12.5 μl of a 2 x TaqMan® Universal Master Mix was used together with 5 μl of cDNA from the RT step, the appropriate concentration of primer/probe, and the volume made up to 25 μl with H₂O. The plates were sealed using optical adhesive covers and run on the ABI 7700 sequence detection system following the recommended protocol. Briefly the plates were heated to 55 °C for 2 min and then held at 95 °C for 10 min to activate the AmpliTaq Gold. The PCR settings were: 95 °C for 15 s followed by an annealing/extension time of 1 min at 60 °C, and this was repeated for 40 cycles. For both the RT step and PCR step, positive displacement pipettes were used to ensure accurate aliquoting. Once the run was complete, the plate was analyzed with the sequence detection software and the control wells checked for contamination. Standard deviations were calculated using the method recommended by the supplier.

**COS Cell Co-transfection—**2 x 10⁵ COS 7 cells were plated in 35-mm dishes in 5 ml of GMEM supplemented with 10% FCS. Next day each culture was co-transfected with the *Ehox* expression vectors used in ES cell supertransfections. Cultures were transfected with a total of 3 μg of plasmid DNA using the FuGENE transfection reagent (Roche, Lewes, UK) according to the instructions of the manufacturer. Combinations of plasmids were made to transfec *Ehox* sense, antisense, or both sense and antisense in one culture. In addition all cultures received plasmids expressing EGFP as a transfection control. Transfected cells were allowed to grow for 3 days and examined for EGFP prior to processing for Western blotting.

**Antibody Generation and Western Blotting—**Polyclonal anti-*Ehox* antibodies were generated by immunizing rabbits with a chemically synthesized *Ehox* peptide representing a 24-amino acid region amino-
terminal to the homeobox region. The sequence of the peptide used was EKLPVEEPLRDVAQIKVDVQEPVQV. Both the peptide synthesis and the antibody generation were performed on a service basis by Invitrogen (Renfrewshire, UK). For Western blotting, COS cells were lysed on ice in 500 μl of sample buffer. Samples were incubated at 95 °C for 10 min and sonicated before 15 μl/ lane was separated on a 12.5% SDS-PAGE gel prior to semidry electrotransfer onto nitrocellulose. Blots were blocked for nonspecific binding by overnight incubation in 5% dried milk powder (Marvel) in 1× Tris-buffered saline, 0.1% Tween 20 (TBST) before hybridization for 1 h with a 1:5000 dilution of the anti-Ehox antibody in 5% milk/TBST. Following three 15-min washes in TBST, the blots were incubated for 1 h with a 1:2000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (SAPU, Scotland, UK) in 5% milk/TBST. Finally, blots were subjected to three 15-min washes in TBST, following which specific antibody binding was detected with ECL substrate and autoradiography. To control for loading, blots were stripped in 62 mM Tris, pH 6.8, 2% SDS, and 100 mM 2-mercaptoethanol at 50 °C for 45 min and washed with TBST before reprobing with a 1:200 dilution of anti-GFP antibody (Neomarkers, Suffolk, UK) in 5% milk/TBST and a 1:5000 dilution of a horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody essentially as described above. Again, detection was carried out using the ECL substrate.

In Vitro Differentiation of Cardiomyocytes—EBs were generated in 10-μl hanging drop cultures containing 300 cells in GMEM, 10% FCS, 100 unit/ml LIF, and 2 μg/ml puromycin. After 7 days, medium was removed and colonies were stained for alkaline phosphatase activity according to the instructions of the manufacturer (AP leukocyte kit; Sigma, Poole, Dorset, UK). Colonies were examined microscopically for intensity of red stain (stem cell) (26) and morphology and scored as either a stem cell or a differentiated colony. Two independently derived pools were assayed three times for each transfection. Subsequent analysis was from 5 × 10³ transfected cells plated per 35-mm dish in duplicate and analyzed after 5 days in culture.

RESULTS

A Clone from an ES Cell Subtracted Library Encodes a Fragment of a Novel Divergent Homeobox Gene—Analysis of cDNA clones from a previously generated ES cell-derived subtracted library (22) revealed one clone, JB46, that displayed homology with members of the homeobox family of transcriptional regulators. This homology is limited and is evident at the nucleotide level. The full-length cDNA for JB46 was isolated from a day 5 EB full-length cDNA library (22). JB46 was expressed in undifferentiated ES cells and that this low expression of Ehox in the adult thymus, with the only other sites of significant expression being the skin and testes (Fig. 2b). Intriguingly, despite the preferential expression in the thymus, other lymphoid-associated organs, such as the lymph nodes and Peyer’s patches, do not reveal significant expression of Ehox.

Thus, Ehox is expressed at varying levels in both undifferentiated and differentiating ES cells, as well as in adult tissues, indicating potential roles for this divergent homeobox-containing gene in both developmental and adult contexts.

The Use of an Episomal Overexpression Strategy to Investigate the Impact of Altered Ehox Expression on ES Cell Function—One advantage of using the in vitro differentiation of ES cells to identify novel genes is that this system can also be used to analyze the functional roles for identified genes. We have used an episomal expression system (28, 29) to characterize the impact of alterations in Ehox expression levels on ES cell function and differentiation. This system involves transfection of an episomal expression construct containing a polynoma virus origin of replication into E14/T ES cells, a subclone of E14tg2a, which stably express the polynoma large T antigen. The interaction of the large T antigen with the polynoma origin of replication allows efficient episomal propagation of incoming plasmids. This system has high transfection efficiencies (supertransfection), and high levels of expression can be achieved and maintained. In addition, problems associated with ectopic gene expression in ES cells, such as site-specific silencing of integrated transgenes, are circumvented by supertransfection. Large numbers of clones are generated by this transfection system, thus allowing pools of clones to be isolated and phenotypically characterized, thereby avoiding interclonal variation.

We have used this system to investigate the functional role of Ehox in ES cell differentiation using sense and antisense Ehox expression constructs. Prior to initiating the episomal transfection experiments, we sought to identify an effective antisense construct that was capable of neutralizing Ehox in transfected cells. We have tested two antisense cDNA constructs, the first containing a 220-bp antisense fragment specific for the 5’-non-homeobox-encoding region of the gene and the second containing the full-length Ehox antisense fragment (Fig. 3a). As ES cells do not express detectable levels of EHOX protein (see below), the effectiveness of these antisense cDNAs was tested...
in COS7 cells co-transfected with Ehox and either one or other of the antisense constructs. EGFP encoding vectors were also included in the co-transfections to allow comparison of relative transfection efficiencies between experiments. Western blot analysis of the COS7 cell lysates revealed that both antisense constructs were effective at reducing EHOX protein expression, although the full-length Ehox antisense construct was most effective, resulting in almost complete neutralization of EHOX protein expression (Fig. 3b). This neutralization of expression was seen with both wild type protein and a MYC-tagged full-length protein. Given the apparently greater ability of the full-length antisense construct to neutralize EHOX protein expression, this construct has been used in the functional studies reported below.

Phenotypic Effects of Sense or Antisense Ehox Expression in ES Cells—To assess the function of Ehox, we generated episo-
mal expression constructs carrying sense or antisense Ehox cDNA driven by the ubiquitous CAG promoter. In a number of ES cell transfection experiments, large numbers of clones were generated with the control vector or antisense Ehox-expressing vector (Table I). In contrast, under directly comparable conditions, a markedly lower (150-fold) number of Ehox sense-expressing ES cell clones was generated. Northern blot analysis of the pools of supertransfected cells indicates high levels of expression of the Ehox antisense mRNA but lower levels of sense expression in the limited number of sense transfectants obtained (Fig. 3c). Endogenous Ehox expression was undetectable, using Northern blotting, in wild type or vector control ES cells. EHOX protein was detectable by Western blotting of extracts from sense transfectants but, in keeping with the absence of detectable message, was not seen in the vector control cells (Fig. 3c). It is important to note that, although high level expression of sense Ehox was difficult to attain in the ES cell transfectants, the cells that do express Ehox message and protein at the levels shown in Fig. 3c display a normal phenotype and demonstrate no detectable differences in growth or survival compared with wild type or vector control ES cells. Therefore, it is clear that, although high level expression of Ehox is apparently incompatible with either survival of ES cells or with the maintenance of an undifferentiated phenotype, moderate expression levels can be attained with no obvious deleterious effects on measurable cellular parameters such as cell growth and survival.

To assess the effects of variations in Ehox expression on ES cell differentiation in vitro, pools of sense and antisense Ehox-expressing and empty vector-containing ES cells (hereafter referred to as control ES cells) were allowed to form EBs and then to differentiate in the absence of LIF for 6 days. RNA was prepared from undifferentiated ES cells and from differentiating EBs at daily intervals over the 6 days of in vitro differentiation. The multilineage differentiation potential of the ES cells was analyzed using real-time PCR assessment of quantitative alterations in lineage-specific gene expression levels and the phenotypic consequences of Ehox sense or antisense expression defined on this molecular basis. This analysis has the advantage of being more rigorously quantitative than some of the available bioassays and can be used to measure emergence of gene expression patterns that precede the development of assayable lineage-specific cells, or that on their own are not able to define a cellular phenotype. The marker genes used in this study include hemopoietic, endothelial, cardiac, undifferentiated ES cell and housekeeping genes. Levels of both Gapdh and Hprt were unaltered throughout the differentiation of the

TABLE I

| Construct                                | No. of puromycin-resistant colonies |
|------------------------------------------|------------------------------------|
| CAG puro (control)                       | 1938 ± 200                         |
| CAG/sense ehox/IRES puro                 | 13 ± 6                             |
| CAG/antisense ehox/IRES/puro             | 2156 ± 521                         |

Fig. 2. Ehox is expressed during ES differentiation and in a subset of adult tissues. a, real-time PCR analysis of Ehox expression during in vitro differentiation of embryoid bodies. Expression levels are normalized to the levels detected in undifferentiated ES cells. b, real-time PCR analysis of Ehox expression in a range of adult murine tissues. Expression levels are reported relative to the basal expression seen in the adult murine heart.

Fig. 3. Full-length antisense Ehox constructs efficiently neutralize Ehox protein expression in co-transfected COS7 cells. a, diagrammatic representation of the two constructs generated and tested for antisense activity. b, Western blot analysis of the effects of the antisense constructs on EHOX expression in cotransfected cells. The positive and negative controls represent extracts from COS7 cells transfected with either Ehox constructs alone (positive control) or with empty vector (negative control). c, Northern (left) and Western blot (right) analyses of expression of Ehox sense or antisense in transfected ES cell pools. Northern blots were normalized with respect to levels of the 18 S ribosomal band (indicated). WT, wild type; AS, antisense; V, vector; S, sense.
control ES cells or the ES cells expressing the Ehox sense or antisense constructs and displayed very similar absolute levels in each of the three cell types. For this reason, the majority of the real-time data has been normalized to the endogenous Hprt levels, seen in undifferentiated vector control ES cells, to allow for quantitative comparison between lines. β-globin is the sole exception to this method of data reporting, and the reasons for this are given below.

To analyze the impact of alterations in Ehox expression on the emergence of primitive hemopoietic cells, we have studied the temporal and quantitative patterns of emergence of Scl (30) and Cds34 (31). As shown in Fig. 4a, levels of Scl expression are seen to rise above undifferentiated levels by day 5 and to be further increased by day 6 of differentiation in the control ES cells. The overexpression of sense Ehox in the ES cells markedly speeds up the increase in Scl expression levels, with enhanced levels being detectable by day 3 and expression peaking at day 4. Thus, although there are no significant effects on the levels of Scl expressed, the expression is detectable 2 days earlier in the Ehox-sense-expressing ES cells compared with the control ES cells, suggesting a role for Ehox in acceleration of differentiation in vitro. Intriguingly, in the antisense Ehox-expressing cells, expression of Scl did not rise above the very low background levels seen in the undifferentiated ES cells at any time point studied. Similarly, with Cds34, levels were seen to rise above background at day 6 in the control ES cells, whereas in the Ehox-sense-expressing ES cells this expression was detectable 1 day earlier at day 5 of differentiation. Again, there are no significant differences in the levels of Cds34 expression between the control and Ehox-expressing ES cells. As with Scl, in the antisense-expressing cells, expression of Cds34 did not rise above background levels seen in undifferentiated ES cells at any time point studied. Thus, analysis of these two genes suggest that expression of sense Ehox accelerates the emergence of markers of hemopoietic stem cell activity and, in contrast, expression of antisense Ehox delays or blocks enhanced expression of these genes over the time frame studied.

To examine whether the effects of alterations in Ehox levels seen with the stem cell genes were also seen with erythroid genes, many of which are co-expressed with the stem cell genes over the day 3/day 5 time frame, we have studied the patterns of emergence of β-globin (32) and Gata1 (33). In the results shown for β-globin, the data are normalized to day 6 control cell β-globin levels, as β-globin was undetectable in undifferentiated ES cells and could not, therefore, be expressed as a ratio of Hprt expression at this time. Similar to the results seen for Scl and Cds34, both β-globin and Gata1 transcript levels initially rise above background levels after 6 days of differentiation in control cells (Fig. 4b). The overexpression of Ehox in the ES cells accelerates the emergence of both these erythroid genes by ~2 days, although, in contrast to Gata1, the levels of β-globin expression are lower in the sense transfectants than in the control transfected cells. Again, as seen with the Scl and Cds34, the antisense cDNA appeared to block in-
increased expression of both erythroid genes within the time course analyzed. The antisense-expressing cells showed no increase in Gata1 expression over that seen in undifferentiated ES cells, whereas with β-globin, expression was undetectable at any time point during the differentiation of antisense-expressing ES cells. Even in the undifferentiated ES cells, β-globin was undetectable. Thus again, examination of these erythroid genes further supports the hypothesis that overexpression of Ehox accelerates the process of hemopoietic differentiation and that expression of the antisense slows this process down or, alternatively, completely blocks it.

The Effects of Ehox Sense and Antisense Are Not Specific to Hemopoiesis—To test the possibility that the effects of alterations in Ehox expression may also be evident in non-hemopoietic lineages, we have examined the emergence of gene markers of the endothelial and cardiac lineages during the differentiation of the transduced ES cells. Expression of the endothelial markers Flk1 (34) and VE-cadherin (35) begins to increase above background levels seen in undifferentiated ES cells following 6 days of differentiation of the control ES cells (Fig. 4c). In contrast, expression of Ehox sense cDNA accelerates the appearance of both genes by 4 days for Flk1 and 2 days for VE-cadherin. In addition to the earlier induction time of these two genes, expression of sense Ehox increases the levels of expression compared with that seen with the control ES cells at day 6. Comparable with the results observed with hemopoietic genes, in the ES cells expressing the Ehox antisense construct, expression of both endothelial marker genes does not rise above the background levels seen in undifferentiated ES cells at any time point studied. Similar results were observed with the cardiac marker gene Nkx2.5 (36), expression of which does not rise above background levels in the control cells at any point during the time course studied. This is in keeping with the longer time required for the emergence of cardiac cells in this in vitro differentiating system. The sense Ehox ES cells, in contrast, show marked elevation of Nkx2.5 expression after 4 days of differentiation. As with the control cells, the antisense-expressing ES cells show no rises in Nkx2.5 expression over the levels seen in undifferentiated control ES cells. Thus, these data demonstrate that the acceleration of differentiation and the block in differentiation seen with the Ehox sense- and antisense-expressing ES cells, respectively, are not specific to the hemopoietic lineage and are also observed in both the endothelial and cardiac lineages.

Expression of Antisense Ehox Maintains the Stem Cell Phenotype of ES Cells in the Absence of LIF—To examine whether the effects of the Ehox sense and antisense constructs are mediated at the level of ES cell commitment, we have analyzed their effects on the expression levels of Oct4 (37), a gene expressed preferentially in undifferentiated ES cells. As shown in Fig. 4d, expression of Oct4 was reduced to less than 20% of its control levels by day 6 of differentiation of the control ES cells. In keeping with the apparent “speeding up” of the differentiation of ES cells overexpressing Ehox, Oct4 expression is reduced to basal levels by day 3 of differentiation. Again in keeping with the observed effects on molecular markers of ES cell differentiation, expression of Oct4 does not alter significantly over the differentiation period in the ES cells expressing antisense Ehox, indicating that these cells are remaining in a relatively undifferentiated state.

Taken together, these data suggest that the antisense Ehox blocks ES cell differentiation. We therefore sought to substantiate this hypothesis using biological assays. Undifferentiated ES cell colonies are identifiable morphologically and by the expression of alkaline phosphatase (26). We have therefore examined the impact of Ehox antisense expression on the self-renewal/differentiation of ES cells in vitro as measured by these two parameters. Initially, this has involved a study of the sensitivity of the undifferentiated status of the ES cells to alterations in LIF concentration. In the presence of 100 units/ml LIF, the antisense and control ES cells form undifferentiated colonies that are morphologically indistinguishable (Fig. 5a). However, the antisense-expressing ES cells displayed a markedly different sensitivity to LIF withdrawal to that seen with the control cells (Fig. 5b). Thus, even at 1 unit/ml LIF, 49% of the antisense-expressing ES colonies displayed a stem cell phenotype compared with only 20% of the control ES cell colonies. This difference is even more evident in the absence of LIF with only 4% of the control ES cell colonies displaying a stem cell phenotype compared with 36% of the antisense-expressing colonies. Typical colonies generated by the different ES cell populations maintained in the presence of 1 unit/ml LIF.
Ehox and ES Cell Differentiation

are shown in Fig. 5c. Control ES cells and ES cells overexpressing Ehox give rise to morphologically differentiated colonies in 1 unit/ml LIF, which show little alkaline phosphatase staining. In contrast, under the same conditions, antisense-expressing cells generated small compact colonies and displayed an alkaline phosphatase-positive undifferentiated phenotype (Fig. 5c). These colonies, however, were smaller than the stem cell colonies seen in the antisense transfectants in 100 units/ml LIF (Fig. 5a). This reduction in colony size is also seen in the few morphologically identifiable stem cell colonies generated by control transfected cells in 1 unit/ml LIF, suggesting that, although Ehox antisense does inhibit differentiation of ES cells, it does not completely compensate for the absence of LIF. The limited number of colonies that did not stain for alkaline phosphatase observed in the antisense ES cell cultures in the absence of LIF were not typical of differentiated ES cell colonies, as they did not form the diffuse morphologically distinct colonies seen with the control cells even in the absence of LIF (Fig. 5c). This suggests that the presence of the antisense transcripts markedly inhibits ES cell differentiation and that the differentiated colonies that are produced are atypical. Thus, these dose-response data further strengthen the argument that ES cells expressing antisense Ehox have a compromised capacity for differentiation and can maintain a stem cell phenotype even in limiting LIF concentrations. These observations therefore provide further evidence of a fundamental role for Ehox in early ES cell differentiation.

Antisense Ehox Blocks Cardiomyocyte Differentiation of ES Cells—To further investigate the impact of variations in Ehox expression on ES cell differentiation, we examined the competence of Ehox sense- and antisense-expressing ES cells for cardiomyocyte differentiation (12). EBs derived from ES cell transfectants were cultured in suspension for 5 days in the absence of LIF before plating onto gelatinized dishes. Beating cardiomyocytes were counted daily over the following 10 days in culture (Fig. 6). In four of five experiments, no beating cardiomyocytes were generated in the antisense Ehox-expressing cells. In the fifth experiment, 1 of 24 EBs differentiated into small regions of beating cardiomyocytes on the final day of observations. In contrast to this lack of differentiation in antisense Ehox-expressing cells, 38% of Ehox sense-expressing EBs and 19% of control EBs were beating by day 10. These data further support the conclusion that antisense Ehox inhibits ES cell differentiation and suggests that the few differentiated ES cell colonies that are observed in the antisense-expressing ES cells in the absence of LIF (Fig. 5) are compromised in their ability to develop into functional mature cell types.

DISCUSSION

Murine embryonic stem cells are derived from the day 3.5 blastocyst and are maintained as a totipotent self-renewing population of cells by culturing in the presence of the peptide growth factor LIF. Upon removal of LIF, ES cells undergo apparently spontaneous in vitro differentiation with commitment to a range of embryological lineages. The system is therefore useful in the analysis and characterization of molecular pathways involved in a range of differentiation processes. Approaches such as gene trapping and differential expression analyses have been used to capitalize on the in vitro differentiation potential of ES cells (38–40). Although there have been some significant developments from these analyses, one of the major limitations in the current use of ES cells in addressing issues relating to lineage-specific differentiation is the inability to exclusively generate large numbers of a specific cell type in culture. Lineage-specific selection strategies using mouse ES cells have been used successfully to circumvent this problem (7), but clearly it would be advantageous to be able to direct differentiation exclusively along specific pathways. The present inability to do this is likely to hamper progress in ES cell-based cell replacement therapy, which has been proposed for human diseases such as Parkinson’s disease and diabetes (10, 15, 16). The detailed characterization of the molecular pathways involved in early differentiation processes would clearly aid in the understanding and subsequent manipulation of the ES cell differentiation system.

Here we report the characterization of a novel homeobox-containing protein that appears to be essential for the processes of ES cell differentiation in vitro. Ehox is a highly divergent homeobox gene, and the limited homology shown with other members of the homeobox gene family is restricted to the homeobox region itself. The closest homologues are members of the paired and paired-like homeobox protein subfamilies; however, a number of characteristics of the Ehox sequence indicate that it is a paired-like protein rather than a paired family member (23). For example, Ehox does not contain a paired domain and lacks the serine residue at position 50 of the homeodomain that is a hallmark of the paired family of proteins. In addition, the presence of a lysine residue in place of this serine and an intronic region, between the coding sequences for amino acids 46 and 47, affiliate Ehox specifically with the paired-like protein family.

We have opted for the use of an episomal expression system to characterize the phenotypic consequences of alterations in Ehox expression in ES cells. Intriguingly, we have been unable to generate large numbers of ES cell clones overexpressing Ehox. The few ES cell clones that were obtained, and that showed overexpression of Ehox (albeit lower transcript levels than were achievable with antisense constructs), grew normally as undifferentiated ES cells in the presence of LIF, but there was a marked acceleration in the appearance of specific differentiation markers when they were induced to differentiate into embryoid bodies in the absence of LIF. When Ehox activity is inhibited by expression of antisense constructs, undifferentiated ES cells survive in the presence of LIF and grow in a manner comparable to that of wild type ES cells, indicating
that Ehox is not essential for ES cell maintenance under these conditions. However, in limiting concentrations of LIF, there is a persistence of undifferentiated ES cell colonies, when Ehox activity is inhibited, compared with control ES cells. Although the undifferentiated status of the ES cells is maintained in the antisense transfectants following LIF depletion, the small size of the colonies suggests that antisense Ehox does not completely compensate for the absence of LIF. Perhaps more importantly, no colonies with the characteristic differentiated phenotype were observed in cells expressing the antisense Ehox construct.

Together, the data presented support the conclusion that Ehox is essential for ES cell differentiation. However, it is not the only factor that has been implicated in regulation of ES cell differentiation. Clearly, LIF is important in maintaining totipotency as is the absolute level of the transcription factor Oct4 (43–45). Whether and how Ehox interacts with these known regulators of ES cell totipotency is unclear. As mentioned above, it is apparent from Fig. 5c that, although the undifferentiated status of the ES cells is maintained in the antisense transfectants following LIF depletion, the small size of the colonies suggests that antisense Ehox does not completely compensate for the absence of LIF. Additionally, although in the Ehox-expressing clones studied here, and in wild type ES cells, the effects of LIF and Oct4 are dominant over the differentiation-inducing effects of Ehox, appropriate increases in Ehox expression can lead to dominance over the suppressive effects of LIF and Oct4 (see below). In many ways, the effects of enhanced Ehox expression in inducing ES cell differentiation are reminiscent of those seen with Oct4. However, preliminary micro-array analyses using control and Ehox-expressing ES cells did not reveal any significant alterations in Oct4 expression in the Ehox transfectants. This, along with the lack of trophoblast differentiation in the antisense Ehox-expressing cells, suggests that Ehox and Oct4 regulate ES cell differentiation through alternative mechanisms.

So, in what manner might elevated Ehox levels induce ES cell cell differentiation? Clearly, given the observed impact of alterations in Ehox expression on a wide range of differentiation pathways, it is likely that Ehox mediates its regulatory effects at an early stage of the differentiation process. If Ehox worked to directly trigger differentiation upon removal of LIF, it might be assumed that, although the time course of differentiation would be unaltered, there would be an increase in the expression levels of markers of differentiation. In this respect, it is interesting to note from the PCR analyses outlined in Fig. 4 that Ehox does not in general appear to increase the amount of lineage-specific transcripts produced but accelerates their appearance. One other possibility, therefore, is that the acceleration of the differentiation process is brought about by Ehox reducing ES cell cycle time although this seems unlikely, as ES cells overexpressing Ehox display doubling times that are indistinguishable from wild type or antisense-expressing cells (data not shown). However, this evidence of a lack of impact of Ehox expression on cell cycle time in undifferentiated ES cells does not mean that there may not be effects on ES cell cycle time following removal of LIF and during the subsequent differentiation steps. This possibility is currently under investigation. It is also possible that Ehox primes the embryoid bodies for differentiation by regulating the expression of key genes essential for the earliest stages of this process. Overexpression of Ehox may therefore lead to a more fertile ES cell/embryoid body environment for differentiation, and expression of the antisense may consequently block the differentiation process. Detailed micro-array analyses are currently under way to examine these issues.

In summary, the data presented suggest a role for Ehox, a novel paired-like homeobox gene in the regulation of ES cell differentiation. Furthermore, these studies emphasize the value of the use of epimodal expression systems with in vitro ES cell differentiation assays in analyzing the function of genes for which ablation or overexpression would be incompatible with in vivo analyses.

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Cloning and Characterization of Ehox, a Novel Homeobox Gene Essential for Embryonic Stem Cell Differentiation
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