The rhythmic heart beat is regulated by a precisely timed sequence of pacemaking impulses that propagate through the cardiac conduction system (Tawara, 1906; Goldenberg and Rothberger, 1936; Bozler, 1942; Fig. 1A). Pacemaking impulses are periodically generated at the sinoatrial node and spread through atrial myocytes initiating contraction of both atrial chambers. The electrical excitation is then received by the atrioventricular (A V) node (Tawara, 1906) and A V -ring. After a slight delay, the impulse rapidly propagates along the A V bundle (His, 1893) and its branched limbs (Tawara, 1906), spreading into the working ventricular muscle through the Purkinje fiber network (Purkinje, 1845; Kolliker, 1902; Tawara, 1906).

Cells in the conduction system are distinguished from contractile myocytes by physiological, histological and molecular characteristics (reviewed in Mikawa and Fischman, 1996). In the chick heart, Purkinje fibers are identified as a cellular network that branches out from the AV-bundle, runs subendocardially, and penetrates into the myocardium along the arterial bed (Davies, 1930; Vassal-Adams, 1982; Lamers et al., 1991; Gourdie et al., 1995). The impulse-conducting cells exhibit a unique pattern of gene expression (reviewed in Schiaffino, 1997; Moorman et al., 1998). They upregulate a conduction cell-specific gap-junction protein (Gourdie et al., 1993; Delorme et al., 1995), and genes typically expressed in neuronal (Gorza et al., 1988, 1994; Gourdie et al., 1999) and skeletal muscle cells, such as slow twitch muscle myosin heavy chain (sMyHC) (Sartore et al., 1978; Gonzalez-Sanchez and Bader, 1985), atrial myosin heavy chain (AMyHC) (de Groot et al., 1987) and skeletal muscle myosin binding protein-H (Alyonycheva et al., 1997). Purkinje fibers also downregulate cardiac muscle specific myofibrillar proteins, such as cardiac myosin binding protein-C (cMyBP-C) (Gourdie et al., 1998), which are essential for normal heart muscle contractility (Watkins et al., 1995; Bonne et al., 1995).

This unique pattern of gene expression, particularly the
coexpression of neuronal and skeletal muscle genes, led to
speculations on the ontogeny of Purkinje fibers, being of either
neural or myocyte origin (reviewed in Mikawa and Fischman,
1996). The recent series of retroviral cell lineage studies in the
embryonic chick heart have shown that Purkinje fibers differen-
tiate from a subset of contractile myocytes at two
restricted sites, subendocardial and periarterial (Gourdie et al.,
1995; Cheng et al., 1999). The data suggest that Purkinje fiber
differentiation and its branching pattern may be regulated by
instructive cues from cells of the endocardium and developing
arteries (reviewed in Mikawa and Fischman, 1996; Mikawa,
1998; Gourdie et al., 1999). This idea has been supported by
our recent in ovo studies, in which inhibition of intramural
coronary arterial development in the embryonic heart results in
suppression of Purkinje fiber differentiation (Hyer et al., 1999).
Furthermore, ectopic placement of the coronary arterial bed
in the embryonic myocardium induces adjacent myocytes to
differentiate into Purkinje fibers (Hyer et al., 1999). This
inductive event appears to be a short-range paracrine
interaction, since Purkinje fiber differentiation is restricted to
a few myocyte layers juxtaposed to both bona fide and ectopic
vessels. A survey of vessel-associated paracrine factors
demonstrated that only endothelin (ET) (Yanagisawa et al.,
1988; Masaki et al., 1991; Yanagisawa, 1994) is capable of
inducing embryonic myocytes to differentiate into Purkinje
fibers in culture (Gourdie et al., 1998). These results also
suggested that an instructive cue of endothelial cell-derived
signal(s) may regulate Purkinje fiber differentiation and its
branching pattern. These data do not, however, explain how
Purkinje fiber recruitment is confined to subendocardial and
periarterial sites, excluding those juxtaposed to veins and
capillaries.

ET was originally identified as a potent vasoconstrictor
(Yanagisawa et al., 1988) secreted by endothelial cells in a
shear stress-dependent manner (Yoshizumi et al., 1989). Three
ET isopeptides, ET-1, ET-2 and ET-3 (Inoue et al., 1989), have been
identified. They bind to two seven-membrane-spanning G
protein-coupled receptors, endothelin receptor A (ET A R) and
endothelin receptor B (ET B R) (Arai et al., 1990; Sakurai et al.,
1990). The biosynthesis of ET occurs through two steps
of proteolytic processing, resulting in the production of
biologically active peptides. Preproendothelin (preproET), a
nascent polypeptide of approximately 200 amino acid residues,
is first translated and then digested by furin-like peptidases into
a biologically inactive intermediate (bigET). BigET is then
proteolytically activated by the highly specific ET converting
enzyme (ECE), a member of the type II membrane-bound
metalloprotease family (Xu et al., 1994; Emoto and
Yanagisawa, 1995). Of the three ET isopeptides, ECE catalyzes
the conversion of bigET-1 to ET-1 with greatest efficiency
(Emoto and Yanagisawa, 1995). The expression patterns of ET,
ET A R and ECE-1 in the developing chick heart and the
potential role of ET-signaling in Purkinje fiber differentiation
in vivo. Data are presented demonstrating that while embryonic
myocytes ubiquitously express ET A R, they are not induced by
preproET-1 in the absence of ECE-1. However, ECE-1 is
expressed predominantly in endocardial and arterial
endothelial cells along which bona fide Purkinje fiber
recruitment takes place. When preproET-1 and ECE-1 are
ectopically coexpressed in the embryonic myocardium after
retroviral injection, cardiac myocytes are induced to express
Purkinje fiber markers in vivo. These results suggest that
embryonic myocytes are competent to respond to ET-signal in
vivo, and that expression of ECE-1 plays a key role in defining
an active site of ET signaling in the heart.

MATERIALS AND METHODS

Cloning of chicken ECE-1 cDNA and northern blots

An NcoI fragment of bovine ECE-1 (Xu et al., 1994) was labeled with
32P by random priming and then used for screening an embryonic day
7 (E7) chicken cDNA library. From approximately 1x107 phage
plaques, four positive clones were isolated and subcloned into the
EcoRI site of pBluescriptII vector (Stratagene). The clones were
sequenced in both directions, using the dyeoxynucleotide sequencing
technique. The sequence data revealed that all clones overlapping and
that the longest clone, BS4E9, encoded the full-length ECE-1 cDNA.
The chicken ECE-1 cDNA sequence has been deposited with the
EMBL/GenBank/DDJB database and assigned the accession number
AF230274. RNA was extracted from frozen hearts with Trizol reagent
(Gibco BRL). Total RNA (10 μg per lane) was separated in a
formaldehyde/1.2% agarose gel, transferred to a Hybond-N
membrane (Amersham), and hybridized with a random-primed 32P-
labeled Ac4 fragment of chicken ECE-1 cDNA.

RNA probes
cDNAs of Cs42 (Beyer, 1990), cMyBP-C (Yasuda et al., 1995), ET A R
and ETE R (Kempf et al., 1998) were synthesized from E10 and E16
chicken heart RNA by RT-PCR. The human ET-1 cDNA (Yanagisawa
et al., 1988) was amplified by PCR from pCIXZppET (see below).
The primers used are as follows: forward 5'-GTCGCCGCCCAACAGG-
TAGAAA-3' and reverse 5'-GTCGCCACGGGCTGAACTT-3' for
Cs42; forward 5'-ACCGACTGGTTGCTTTTGGG-3' and reverse
5'-TTTCACTGGAAGGCCAAC-3' for cMyBP-C; forward 5'-TAA-
ACTGCAGTTGGCAGAGG-3' and reverse 5'-GGATCGATT-
GAACGAGGACGAC-3' for ET A R; forward 5'-AACATTTCTTTAC-
CCGACCT-3' and reverse 5'-AGCAAATAAAAAGGCTACTCT-3'
for ET B R; forward 5'-TGGCTCGAGATCTCTCTAAAG-3' and reverse
5'-TGTTAA TGCCAA TGTAGTCCA T-3'

In situ hybridization
Fertilized chicken eggs were incubated at 38°C and staged according
to the number of days of incubation or, more precisely, according to
Hamburger and Hamilton (1951). Whole embryos or hearts were fixed
in 4% paraformaldehyde/PBS and processed as described for whole-
mount or frozen section in situ hybridization (Henrique et al., 1995). In brief, whole-mount in situ hybridization was carried out by fixing embryos and hearts with paraformaldehyde, then treating with proteinase K for 20 minutes. Depending on the stage and size of the samples, concentrations of proteinase K were adjusted as follows: 10 μg/ml for E1-E4 embryos and E7-E10 hearts, 70 μg/ml for E15-18 hearts. After postfixation with 4% formaldehyde and 0.1% glutaraldehyde in PBS, samples were rinsed with PBS, preincubated with the hybridization mixture for 1 hour at 65°C, and reacted overnight at 65°C with 0.1-0.5 μg/ml DIG-labeled RNA probes. After 65°C washes and blocking, the samples were incubated overnight at 4°C with alkaline phosphatase-conjugated anti-DIG antibody (1:2000 dilution, Boehringer). After washing to remove unbound antibodies, samples were stained with the NBT/BCIP mixture at room temperature until color developed. Some stained embryonic hearts were further processed for histochemistry, as described (Mikawa et al., 1992; Gourdie et al., 1995). For frozen-section in situ hybridization, proteinase K treatment was omitted. After overnight hybridization, sections were treated with RNase A.

Retroviral vectors, cell culture and virus infection

The HinCl fragment of bovine ECE-1 cDNA (Xu et al., 1994) was ligated with XbaI linkers and cloned into the XbaI site of the spleen necrosis virus (SNV)-based expression vector, pCXIZ (Mikawa, 1995; Mima et al., 1995). The resulting plasmid was designated as pCXIZECE. pCXIZEppET was constructed by inserting a full coding region of the human preproET-1 cDNA (Yanagisawa et al., 1988) into the Smal site of pCXIZ. The BglII-EcoRI fragment of the human preproET-1 cDNA was ligated into the BamHI-EcoRI site of the ClaI2Nco adapter vector (Hughes et al., 1987). This plasmid was digested with ClaI, and the fragment containing a full coding region of preproET-1 was subcloned into the ClaI site of pRCASBP(A) (Fekete and Cepko, 1993). The resulting plasmid was termed RCASppET. Propagation and concentration of SNV-based vectors were described elsewhere (Mikawa et al., 1992, 1996; Fischman and Mikawa, 1996). Production of RCAS-based viruses was performed as described (Morgan and Fekete, 1996). Ventricular segments were isolated from the E3 heart tube, infected with viruses, cultured in DMEM containing 10% FBS, 1% chick embryonic extract, penicillin and streptomycin. Myocytes were prepared from ventricular segments of E3 hearts, maintained in monolayer, and either transfected with a mixture of DNA and LipofectAMIN plus (Gibco BRL) according to the manufacturer’s recommendations or infected with viruses. 3.5 days later, cells were fixed with 70% ethanol for 10 minutes at −20°C. In vivo injection of embryonic hearts was described elsewhere (Mikawa et al., 1992; Mima et al., 1995; Fischman and Mikawa, 1997).

Histochemistry and immunohistochemistry

Embryos and hearts were removed and fixed by immersion overnight in 2% paraformaldehyde/PBS at 4°C. Virus-infected hearts were further processed for staining with X-gal, red gal (Research Organics) and/or alkaline phosphatase in whole mounts as described (manufacturer’s recommendation). Immunohistochemistry was carried out either in whole mount or on frozen sections. Ventricular segments were fixed with 70% ethanol, treated with 0.1% Triton X-100 in PBS, and processed for whole-mount immunostaining (Han et al., 1992). Frozen sections of ethanol-fixed hearts were prepared as described (Gourdie et al., 1995). Primary antibodies used in the present study are ALD58 against sMyHC (Gonzalez-Sanchez and Bader, 1985), MF20 against sarcomeric MyHC (Bader et al., 1982), anti-β-Gal (5 prime 3 prime Co), EAP3 against EAP300 (Gourdie et al., 1995), 3C2 against viral gag proteins (Potts et al., 1987), the guinea pig-318 anti-Connexin 40/42 (Gourdie et al., 1998), and anti-AmyHC antibody (Sanders et al., 1984). Immunofluorescent and immunoperoxidase labeling was undertaken, according to methods detailed previously (Gourdie et al., 1995, 1998; Hyer et al., 1999).

RESULTS

Early and late markers of Purkinje fiber differentiation

In the present study, the ventricular myocardium was probed for induction of specific markers that could distinguish conduction cells from contractile myocytes. In this way, ectopic Purkinje fiber differentiation induced by activated ET-signaling in the embryonic heart could be detected. The markers used included neurofilament (EAP300), connexin 42 (Cx42), atrial myosin heavy chain (AMyHC) and slow twitch skeletal muscle myosin heavy chain (sMyHC). In the mature heart (Fig. 1B-E), these markers are coexpressed in the Purkinje fiber network, while no or undetectable levels of expression of these markers were seen in beating myocytes. Our initial survey of ectopic Purkinje fiber differentiation in the mature heart revealed a complex distribution of authentic Purkinje fibers, particularly the intramural cells, that might complicate our ability to differentiate marker-positive cells as either ectopically induced or bona fide Purkinje fibers. To avoid this potential complexity in interpreting experimental results, embryonic stages were examined in which the ventricular myocardium had developed few or no differentiated intramural Purkinje fibers. By E19 (Fig. 1F-I), robust expression of all markers was already evident in developing Purkinje fibers, in both subendocardial and periarterial cell populations. In contrast, in E13 hearts, all markers were virtually undetectable in ventricular myocytes (Fig. 1J-M), with a few exceptions: Cx42 expression was robust in endothelial cells and just above detectable levels in a myocyte subpopulation juxtaposed to developing larger vessels (Fig. 1L). A weak signal of EAP300 was also observed in a myocyte subpopulation around larger vessels (Fig. 1M). Thus, in the E13 ventricular myocardium, expression of AMyHC and sMyHC was absent and Cx42 and EAP300 expression was restricted to a minor population of myocytes adjacent to a few, developing larger vessels. These results demonstrated that ectopic, precocious conversion of myocytes into Purkinje fibers could be studied in the nonperivascular and nonsubendocardial regions of the E13 myocardium by probing for the induction of late markers such as sMyHC and AMyHC. Early markers, Cx42 and EAP300, served in the present study as probes for assuring that induced, late marker-positive cells also coexpressed the early marker genes.

Expression of preproET alone is not sufficient to convert embryonic myocytes to Purkinje fibers in vivo

In culture, conversion of contractile myocytes into conducting cells can be monitored by probing for upregulation of Purkinje fiber-specific gene products as shown in Fig. 1 and for downregulation of a myocyte-specific myofibrillar protein, such as a myosin-binding protein, cMyBF-C (Gourdie et al., 1995). Every third serial section from control and infected hearts was first stained with an antibody against gag, or hybridized with antisense probe for human preproET-1 to identify cells expressing exogenous preproET-1. Sections positive for gag or human preproET-1 served as a reference for staining of adjacent sections with antibodies specific for Purkinje fiber markers.
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Fig. 1. Expression pattern of early and late Purkinje fiber markers in developing chick hearts. (A) Schematic illustration of the cardiac conduction system. Ao, aorta; at, atrium; avn, atrioventricular node; avr, atrioventricular ring; bb, bundle branch; sepf, subendocardial Purkinje fibers; ipf, intramural Purkinje fibers. Cryosections through adult chicken heart (B–E), E19 embryonic heart (F–I) and E13 embryonic heart (J–M) were immunolabeled (green signals) with ALD58 against sMyHC (B,F,J), anti-AMyHC antibody (C,G,K), anti-Cx42 antibody (D,H,L) and EAP3 against EAP300 (E,LM). Double immunostaining (B) with MF20 (red signal) demonstrates sarcomeric MyHC in both Purkinje fibers and ordinary myocytes. Single arrows, marker-positive Purkinje fibers; double arrows, endothelial cells; asterisks, lumen of arteries.

Fig. 2. Myocytes are converted into Purkinje fibers by mature ET-1 but not by preproET-1. (A–C) Double immunostaining of embryonic myocytes with anti-MyBP-C (red signal) and anti-sMyHC-antibody (green signal) before (A) and after (B,C) exposure to ET peptide (10^{-7} M) for 3 days (B) and 5 days (C) in culture. (D) Whole-mount X-gal staining of E4 tubular heart infected at E3 with SNV-based virus encoding preproET and β-gal. (E,F) Sections from E18 heart infected with CXIZppET virus were stained with ALD58 (purple, arrows in E) and X-gal (blue, asterisk in F), identifying ALD58-positive authentic periarterial Purkinje fibers (arrows in E) but no ALD58-signal in and around myocytes infected with CXIZppET virus (asterisk in F). (G–J) Whole-mount staining of ventricular segments for alkaline phosphatase (purple signal in G) and sMyHC (green signal in H–J) after 5 days in culture following RCASAP virus infection (G), RCASppET virus infection (H), exposure to 10^{-7} M ET (I) and untreated (J). ot, outflow tract; v, ventricle.

1998). Under our culture conditions, the phenotypic conversion was induced within 3 days of exposure to ET-1 (Fig. 2A–C). In order to test whether ET-1 could induce embryonic myocytes to convert their phenotype from contractile to conducting cells in vivo, a recombinant retrovirus (CXIZppET) encoding preproET-1 and a reporter gene, β-galactosidase (β-gal), was introduced into the presumptive ventricular wall at E3. Infected hearts were then examined at various stages after infection for ectopic Purkinje fiber differentiation. By E4, expression of the transgenes was already evident (Fig. 2D). In no case, however, did we find ectopic expression of Purkinje fiber markers, either in infected or adjacent myocytes in the ventricle (Fig. 2E). At 15 days postinfection a time when expression of late markers is robust in authentic Purkinje fibers (Fig. 2F), Purkinje fiber markers were still absent in and around the virally infected cells. To test dose effects, myocytes of the ventricular segment of E3 heart were infected with RCASppET virus in vitro (Fig. 2G–J). Despite massive viral infection (Fig. 2G), no marker expression was detected (Fig. 2H), while robust expression was induced by adding mature ET-1 peptide (Fig. 2I). These results strongly suggest that embryonic myocytes are capable of responding to mature ET-1, but unable to convert mature ET-1 from preproET-1. Indeed, the sandwich-enzyme immunoassay (Suzuki et al., 1989) of culture medium from infected myocytes revealed that approx. 1 ng of bigET-1 was secreted from 10^6 myocytes per day, but no processed active ET-1 was detected (data not shown). These results led to the hypothesis that ECE-1, the enzyme converting bigET-1 into mature ET-1, may be absent from embryonic myocytes, and that localization of ECE-1 may be a key mechanism for defining the site of Purkinje fiber differentiation within the embryonic heart.

Chicken ECE-1 cDNA
To examine the expression pattern of ECE-1 during heart development, chicken ECE cDNA was cloned and used for in situ hybridization analysis. A 3.7 kb clone (BS4E9) was isolated, containing a 2259 nucleotide open reading frame, along with 5' and 3' untranslated regions. The deduced amino acid sequence of the BS4E9 clone consists of 752 amino acid

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residues with a calculated molecular mass of approximately 85 kDa. A database search and comparison revealed that the chicken ECE sequence maintains all of the structural characteristics of the ECE family. The enzyme contains a short intracellular domain, a single transmembrane domain, a long extracellular domain and no signal peptide sequence. There is 100% homology with mammalian ECE-1 in the zinc-binding region within the extracellular catalytic domain. Over 80% homology was identified between the BS4E9 clone and mammalian ECE-1, while about 60% homology was found with ECE-2 (data not shown). Based on these data, the isolated cDNA clone BS4E9 was designated a chicken ECE-1.

ECE-1 expression pattern in the embryonic heart

Northern blot analysis of mRNAs from embryonic chick hearts with ECE-1 cDNA as a probe revealed the existence of one transcript throughout heart development (Fig. 3A). The chicken ECE message was 4.7 kb, the same size as mammalian ECE-1 mRNA (Xu et al., 1994). Using the antisense transcript of this clone as a probe, the expression pattern of chick ECE-1 during embryogenesis was examined by whole-mount in situ hybridization. At E1, ECE-1 expression was detected in the neural fold and head process, but no detectable signal was seen in the cardiogenic mesoderm (Fig. 3B). At E2, ECE-1 expression was evident in the brain, neural tube and intestinal port, but was not yet detectable in the tubular heart (Fig. 3C,D). At E3, an ECE-1 signal first became detectable in the heart region, including the outflow tract and branchial arches (Fig. 3E,F). A weak signal also appeared in a subpopulation of the endocardium, when the color reaction was extended much longer. At E7, preferential expression of ECE-1 in the heart was seen in the endocardium of cardiac cushions (Fig. 3G). By E10, ECE-1 signal became robust in the atrioventricular valves (Fig. 3H-J). At this stage, a faint signal of ECE-1 first appeared in the myocardium as a network in both the septal and free walls, in addition to a subpopulation of endocardium (Fig. 3H-J). The ECE-1 positive network extended further within the myocardium as embryos developed (Fig. 3K-M).

Association of ECE-1 expression and Purkinje fiber differentiation

The unique pattern of ECE-1 expression, both intramural and endocardial, was in striking contrast to the broad expression of the ETA R receptor throughout the ventricular myocardium (Fig. 4A-F). The timing and location of the intramural ECE-1 expression was quite similar to the pattern of coronary artery development (Rychter and Ostadal, 1971; Mikawa and Gourdie, 1996), suggesting that the ECE-1 may occur preferentially in cells of the developing arteries. This idea was tested by examining paraffin sections of hearts which were preprocessed for whole-mount in situ hybridization followed by an extended color reaction. Histological examination of these sections revealed that, along with the endocardial endothelia, ECE-1 was highly expressed in arterial endothelial cells of larger arteries such as the descending arteries of the interventricular septum (Fig. 4G). The level of ECE-1 mRNA was much lower in smaller arteries and arterioles, and not above background levels in either myocytes or venous beds (Fig. 4H-J). These results clearly demonstrate that ECE-1 expression in the ventricular myocardium occurs predominantly in the endocardium and developing coronary arteries, both of which are the known sites for recruitment of adjacent myocytes into impulse-conducting Purkinje fibers (Gourdie et al., 1995; Cheng et al., 1999). Indeed, whole-mount in situ hybridization with a probe for Cx42, an upregulated Purkinje fiber marker (Gourdie et al., 1998), demonstrated that Cx42 exhibits the same distribution and localization profile as seen in ECE-1 expression (Fig. 5A-D).
An inverse staining pattern was found for cMyBP-C (Fig. 5E,F), a myocyte-specific myofibrillar protein whose expression disappears in cells differentiated into Purkinje fibers (Gourdie et al., 1998). This spatial association between ECE-1 expression and the site of Purkinje fiber recruitment was further confirmed by in situ hybridization analyses on sister sections. Again, ECE-1 was found to be expressed predominantly in endothelial cells of developing arteries (Fig. 5G). Cx42 was expressed in both endothelial cells and developing Purkinje fibers (Fig. 5H), as seen by immunohistochemistry (Fig. 1). In contrast, the cMyBP-C signal was missing in both of these cells as well as in vascular smooth muscle cells (Fig. 5I). cMyBP-C, however, was highly positive in surrounding, unrecruited contractile myocytes. These data demonstrate that expression of ECE-1 in the ventricular wall of embryonic hearts occurs predominantly at the regions where Purkinje fiber recruitment takes place.

**In vivo induction of Purkinje fiber differentiation by coexpression of preproET-1 and ECE-1**

The unique localization of ECE-1 mRNA strongly suggests that production of mature ET may occur in a spatially restricted manner in the developing myocardium. If so, the spatially regulated production of ET may play a role in defining which myocyte population undergoes Purkinje fiber differentiation, all of which equally express ET\(_A\) receptor. This possibility was tested by creating an ectopic site of preproET-1 and ECE-1 in the developing ventricular myocardium. This was accomplished by infection with two different types of
recombinant virus (Fig. 6A). One was a replication-competent, RCAS-based vector engineered to express preproET-1 (RCASET virus). The second was a replication-defective, SNV-based vector designed to coexpress ECE-1 with β-gal (CXIZECE virus). To distinguish transgenes from an endogenous gene expression, and to detect the ectopic sites of exogenous gene expression, mammalian versions of preproET-1 and ECE-1 cDNAs were inserted into the viruses. Expression of the exogenous genes from the viral vectors was assured at both transcriptional and translational levels by first introducing these vectors into myocytes in culture, followed by subsequent northern and western blot analyses (data not shown). An RCAS-based vector encoding alkaline phosphate (RCASAP) and an SNV-based vector encoding β-gal (CXIZ) were used as controls. Biological activities of RCASppET and CXIZECE vectors were tested by probing for the induction of Purkinje fiber markers in cultured myocytes (Fig. 6B). Expression of preproET with control β-gal alone did not show any activation of Purkinje fiber marker expression in myocytes (Fig. 6B), as seen in Fig. 2. In contrast, coexpression of ECE-1 with preproET-1 gave rise to activated markers at levels comparable to those seen after the addition of mature ET-1 (Fig. 6B). These results demonstrate definitively that coexpression of ECE-1 and preproET-1 is sufficient for converting embryonic myocytes into Purkinje fibers in vivo.

These viral vectors were microinjected into the presumptive ventricular myocardium at E3. The resulting hearts were then examined at E13, a stage when authentic periarterial Purkinje fibers were rarely observed in the myocardial wall (Fig. 1). To minimize potential embryonic lethality caused by ectopic ET-1 production, only 1–2·10^2 virions of the recombinant vectors were introduced per heart, thus restricting the colocalization of exogenous ECE-1 and preproET-1 to small areas within the myocardium. Under these conditions, some embryos were allowed to develop until hatching. The hatchlings showed no detectable cardiac abnormalities when compared to control embryos. Hearts coinfected with control viruses, CXIZ and RCASAP, exhibited only a small population of β-gal positive (CXIZ infected) cells intermingled within a widespread region positive for alkaline phosphatase (RCASAP infected) (Fig. 6C,D). In situ hybridization of cryosections of hearts that were coinfected with RCASppET and CXIZECE viruses...
as ET, to initiate Purkinje fiber differentiation in vivo. These results strongly suggest that embryonic colocalization of exogenous preproET-1 and ECE-1, but not by conducting Purkinje fibers. This inductive response can be induced to express a set of markers specific for impulse-differentiated, contractile embryonic myocytes can be induced by localized production of mature ET, which requires coexpression of preproET and ECE-1 (Fig. 7). This model is further supported by our earlier cell lineage studies that cells of the cardiac conduction system are recruited locally from differentiated and beating myocytes (Gourdie et al., 1995; Cheng et al., 1999; reviewed in Mikawa and Fischman, 1996; Mikawa, 1998; Gourdie et al., 1999), and that the local conversion of embryonic myocytes into Purkinje fibers depends upon vessel-derived paracrine signal(s) in vivo (Hyer et al., 1999).

We have shown previously that embryonic myocytes can be induced in culture by mature ET-1 to upregulate Purkinje fiber-specific genes and to downregulate myocyte-specific genes (Gourdie et al., 1998). The in vitro data, however, do not explain how a vessel-derived diffusible factor, such as ET, confines its activity so precisely to just a few cell layers of myocytes. It has also been unclear why myocytes adjacent to veins are not recruited into the conduction cell lineage. There may be mechanisms that neutralize the inductive activity of ET in myocyte populations distal to arteries or those adjacent to venous beds. Alternatively, the present data suggest that this restricted patterning may result from more developmentally regulated processes. ECE-1, which converts bigET into mature ET, is expressed predominantly in endocardial and arterial endothelial cells, but is undetectable in venous endothelia. Since ETA receptor, the predominant isoform of ETRs in the heart (Clouthier et al., 1998), is ubiquitously expressed throughout the myocardium, restricted expression of ECE-1 among cardiac endothelial cell populations may be a determinant in localizing activation of an ET-signal for Purkinje fiber recruitment within the myocardium. In support of this concept, a series of endothelin-related gene disruption studies have demonstrated that while bigET diffusion is widespread, mature ET acts only locally (Baynash et al., 1994; Hosoda et al., 1994; Yanagisawa, 1994; Clouthier et al., 1998; Yanagisawa et al., 1998). Indeed, our present study demonstrates that within a preproET virus-infected myocyte population, cells that are induced to express Purkinje fiber markers are restricted to myocytes infected with ECE virus and those adjacent to the infected cells.

Two ECE isozymes, ECE-1 and ECE-2, both of which catalyze the conversion of bigET to ET, have been identified in mammals (Xu et al., 1994; Emoto and Yanagisawa, 1995). Since we examined only ECE-1 in the present study, the potential role of ECE-2 remains to be determined; however, while ECE-1 is active at neutral pH and coupled with the paracrine secretion of ET (Xu et al., 1994), ECE-2 is active at acidic pH, indicating a lysosomal, scavenger function (Emoto and Yanagisawa, 1995). It is likely, therefore, that in the embryonic myocardium the paracrine production of mature ET is primarily regulated by ECE-1, rather than ECE-2. This idea is further supported by our results that introduction of exogenous preproET-1 alone into a larger area of the myocardium does not induce any myocyte to express Purkinje fiber markers. Thus, any ECE-2 present in the embryonic heart would appear to be insufficient for converting myocytes to Purkinje fiber recruitment, while ETA receptor is broadly expressed throughout the myocardium. Thus, all the data presented are consistent with a model in which recruitment sites of Purkinje fiber differentiation within ETA receptor-positive myocyte populations are defined, at least partly, by localized production of mature ET, which requires coexpression of preproET and ECE-1 (Fig. 7). The present study demonstrates for the first time that viruses or those singly infected with either RCASppET or demonstrated a widespread area of preproET-1 expression in the ventricular myocardium (Fig. 6F). Immunostaining with anti-β-gal antibody on consecutive sister sections showed a subpopulation of cells infected with CXIZECE virus within the large preproET-expression sector (Fig. 6F). Double immunostaining of sister sections revealed that CXIZECE-infected myocytes and adjacent myocytes were positive for Purkinje fiber markers, such as AMyHC (Fig. 6G), sMyHC (Fig. 6H) and Cx42 (Fig. 6I, J), with frequencies of approx. 80%, approx. 40% and 100% per infected site, respectively (Fig. 6O). Thus, there was higher probability in inducing Cx42 (an early differentiation marker), than AMyHC and sMyHC (late markers). In no case did we find ectopic induction of Purkinje fiber markers in regions coinfected with control viruses or those singly infected with either RCASppET or CXIZECE (Fig. 6K–O). These results suggest that embryonic myocytes are competent to respond to exogenous preproET-1 and ECE-1 and can express Purkinje fiber-specific markers precociously and ectopically within the embryonic heart. This inductive response of myocytes appeared to be dependent on the colocalization of preproET-1 and ECE-1 and not on either expression alone.

**DISCUSSION**

The present study demonstrates for the first time that differentiated, contractile embryonic myocytes can be induced in vivo to express a set of markers specific for impulse-conducting Purkinje fibers. This inductive response can be triggered in the embryonic heart by coexpression and colocalization of exogenous preproET-1 and ECE-1, but not by either alone. These results strongly suggest that embryonic myocytes are competent to respond to an inductive signal, such as ET, to initiate Purkinje fiber differentiation in vivo. Furthermore, we show that endogenous ECE-1 expression in the ventricular wall is restricted predominantly to the sites of
In vivo induction of Purkinje fiber differentiation

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