Transcriptional enhancer factor 1 disruption by a retroviral gene trap leads to heart defects and embryonic lethality in mice

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We have used a retroviral gene trap in embryonic stem (ES) cells to derive a recessive embryonic lethal mouse strain, ROSA β -geo5. Mutant embryos display an enlarged pericardial cavity, brachycardia, a dilated fourth ventricle in the brain, and die between embryonic days 11 and 12. Whereas heart development in the mutant embryos is extensive, the ventricular wall is abnormally thin with a reduced number of trabeculae. Cloning of the trapped gene indicates that proviral insertion creates a null mutation in the transcriptional enhancer factor 1 (TEF-1) gene. Although transcription of a number of muscle-specific genes believed to be TEF-1 targets appears normal, the defect in cardiogenesis is likely attributable to diminished transcription of one or several cardiac-specific genes.

[Key Words: Transcription factor; TEF-1; cardiogenesis; gene trap]

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Targeted gene disruption in embryonic stem (ES) cells has become a prominent approach to study the role of genes critical in mammalian development, but it is limited to analyses of known genes. To identify and mutate new genes involved in vertebrate development, one potential strategy is insertional mutagenesis. To improve on the efficiency of this approach, we and others have devised gene trap approaches that rely on the ability of an endogenous promoter to drive the expression of a promoterless reporter gene, typically β -galactosidase (Gosler 1989; von Melchner and Ruley 1989; Friedrich and Soriano 1991; von Melchner et al. 1992). When the gene trap integrates into a transcriptional unit, the normal expression pattern of the tagged gene can be followed by expression of the reporter, and the insertion creates a mutation as well [for review, see Gridley 1991; Joyner et al. 1992].

Several groups have reported using gene traps in ES cells to screen for genes essential for mouse development (Friedrich and Soriano 1991; Skarnes et al. 1992; von Melchner et al. 1992). In a previous study from this laboratory, we used a retroviral gene trap vector, ROSA β -geo, in which a bifunctional β -galactosidase/neomycin phosphotransferase gene (β -geo) is used as a reporter (Friedrich and Soriano 1991). Whereas many mutant strains can be derived using gene traps, criteria to select which mutant to study need to be defined. One possibil-

ity is to select mutations based on the expression pattern of the trapped gene during development. However, targeted mutagenesis of some genes has shown that expression pattern is often a poor indicator of what phenotype might be expected. As a result, we have taken the approach of breeding gene trap insertions to homozygosity to identify developmental mutants, particularly those resulting in defects in early embryo development or organogenesis. Among 42 strains derived in our laboratory, 18 strains have a recessive embryonic lethal phenotype and 2 strains are recessive male sterile (Friedrich and Soriano 1991; unpubl.). In this study we report the characterization of the ROSA β -geo5 mutant in which the transcription enhancer factor 1 (TEF-1) gene has been disrupted.

Transcription factors play a vital role in basal level and tissue-specific regulation of gene expression, as well as in establishing cell differentiation during embryo development. TEF-1 was initially isolated from human HeLa cells and shown to bind specifically to the GT-IIC and *Sph* motifs in the SV40 enhancer. It is broadly expressed in many cell types but not in some hematopoietic cells (Xiao et al. 1991). TEF-1 belongs to a class of transcription factors that contains a DNA-binding motif, the TEA domain (Burglin 1991), in the amino-terminal end of these proteins. Included in this family are the *TEC1* gene from *Saccharomyces cerevisiae* (Laloux et al. 1990), the *AbaA* regulatory gene from *Aspergillus nidulans* (Mirabito et al. 1989), and the *scalloped* (*sd*) gene of *Drosophila melanogaster* (Campbell et al. 1992). These TEA

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domain-containing proteins regulate transcription of multiple genes. Both TEF-1 and TEC1 were isolated during analysis of enhancer elements (Laloux et al. 1990; Xiao et al. 1991). The *AbaA* gene controls development of the asexual apparatus of the filamentous fungus *A. nidulans*. It binds at *cis*-acting regulatory elements of several developmentally regulated genes and acts as a genetic switch to control development of the asexual spore (Andrianopoulos and Timberlake 1994). Mutation of the *sd* gene in *Drosophila* causes early embryonic lethality, implying that the *Drosophila* TEF-1 homolog plays an essential role in early development (Campbell et al. 1992). The diversity of functions displayed by these proteins suggests that TEA domain-containing proteins have numerous regulatory roles in development among different species.

Human TEF-1 activates transcription through the SV40 and papillomavirus-16 enhancers (Davidson et al. 1988; Ishiji et al. 1992). Biochemical and immunological experiments indicate that TEF-1 is probably identical to M-CAT binding factor (MCBF; Farrance et al. 1992), which binds M-CAT sequence motifs (5'-CATTCCCT-3') present in a number of cardiac and skeletal muscle-specific genes, such as cardiac troponin C, T, and I, as well as myosins (Nikovits et al. 1986; Mar and Ordahl 1988, 1990; Mar et al. 1988; Thompson et al. 1991; Flink et al. 1992; Parmacek et al. 1992; Shimizu et al. 1992; Kariya et al. 1993). As the M-CAT motif has been implicated in regulation of these genes, TEF-1 may play a role in the regulation of M-CAT-dependent muscle-specific genes.

The ROSA β -geo5 strain characterized in this report, in which the TEF-1 gene has been disrupted, exhibits an embryonic lethal phenotype associated with heart dysfunction and homozygous embryos die between embryonic day 11 (E11) and E12 of gestation. Histological examination reveals an abnormally thin ventricle wall in the mutant embryo heart. However, cardiogenesis is initiated and expression of several potential TEF-1 targets appears to be normal. Thus, our analysis of the ROSA β -geo5 mice indicates that TEF-1 plays an essential role in the maturation stage of cardiogenesis.

Results

The trapped gene is broadly expressed

The ROSA β -geo5 mutant line was derived by infection of ES cells with the ROSA β -geo retroviral vector, as described previously (Friedrich and Soriano 1991), and will be referred to henceforth as ROSA5. Expression of the trapped gene in the ROSA5 strain was followed by X-gal staining of embryos at different stages of development. Before implantation, *lacZ* expression was observed in cleavage-stage embryos (Fig. 1A) and blastocysts in both the inner cell mass and the trophectoderm (Fig. 1B). At the primitive streak stage, X-gal staining was observed both in embryonic and extraembryonic compartments with higher expression in extraembryonic ectoderm (Fig. 1C). At E12, β -galactosidase staining was observed throughout the embryo (Fig. 1D). Histological sections

Figure 1. X-gal staining of heterozygous ROSA5 embryos. (A) Two-day cleavage stage; (B), 3-day blastocyst stage; (C) 7-day, primitive streak stage; (D) 12-day embryo. (ICM) Inner cell mass; (Te) trophectoderm. Wild-type embryos did not exhibit any background staining under the reaction conditions employed (data not shown).

showed β -galactosidase expression in all cells of the developing heart (data not shown). In the adult, staining was observed in kidney, lung, uterus, heart, and skeletal muscles. Other tissues, including brain, liver, thymus, or spleen, displayed lower levels of staining (data not shown). The connective tissues in certain organs, such as capsule of spleen and interlobular tissue of liver, were stained positive by X-gal, but lymphocytes did not exhibit β -galactosidase activity. Blood from these animals had no detectable β -galactosidase activity (data not shown). These findings indicate that the trapped gene in ROSA5 is expressed broadly, but not ubiquitously, during embryogenesis and in adult mice.

ROSA5 mutants die at midgestation

A single-copy probe flanking the provirus insertion was isolated from a ROSA5 genomic library and used to distinguish the mutant and wild-type alleles on Southern blots (data not shown). No homozygous animals were found among 240 offspring from crosses between hetero-

zygous parents, indicating that the proviral integration causes embryonic lethality. To determine the time of lethality, embryos were genotyped using yolk sac DNA between E9.5 and E14.5 (Table 1). At E9.5, mutants were recovered in the expected Mendelian ratio and could not be distinguished from their wild-type or heterozygous littermates by size or gross morphology. Starting at E10.5, the yolk sacs of most of the homozygotes were pale, although the vascular system was clearly visible (Fig. 2A). The fourth ventricle of the brain was also enlarged (Fig. 2D). By E11.5, mutant embryos had dilated pericardial cavities (Fig. 2B) and a reduction of the heart rate to about half that of wild-type or heterozygous embryos. In most living mutants at this stage, pulsation of the heart was weak such that red blood cells were retained in the ventricle even after contraction. At E11.5, about half of the mutant embryos were necrotic or in the process of resorption. At E12.5, no viable homozygous mutant embryos were observed. These results indicate that proviral insertion in ROSA5 causes embryonic lethality between E11.5 and E12.5 of gestation.

Homozygous ROSA5 embryos display abnormalities in the heart

Homozygous embryos were dissected and prepared for histological sections. The defects found at E10.5 were tissue edema and an abnormality in the ventricle wall of the heart. In wild-type embryos, the heart had developed both atrial and ventricle chambers separated by the endocardial cushion tissue (Fig. 2E). Each chamber was lined with endocardium, consisting of myocardium and an endothelial layer, and the heart was surrounded by the pericardium. The trabeculation process, in which myoblast cells derived from pericardial mesoderm differentiate into myocytes, was initiated and extensive by E10.5. The interventricular foramen and septum were being formed. In contrast, in the mutant embryos, the ventricle wall was thinner and there were only one to two layers of trabeculae lining the wall, as compared with three to four layers in wild-type or heterozygous littermates (Fig. 2E,F). In addition, periodic-acid-Schiff's (PAS) positive staining "ghost" particles were observed underlying endocardial cells in the mutant, possibly representing degenerating myocytes (Fig. 2G,H). No other defects were detected in the mutant embryos by histo-

logical examination of other tissues, including the liver, somites, and nervous system. No histological abnormality was observed in brain except for the dilation of the fourth ventricle (data not shown).

To examine the subcellular structures of differentiating myoblasts, transverse sections of heart ventricles from E11.5 homozygous mutant embryos and control embryos were examined by transmission electron microscopy. Myofibers appeared normal with alternate A and I banding patterns, and Z lines were also observed (Fig. 3A–D). In the longitudinal axis, orderly arrangements of actin filaments around the large myosin filaments were observed in cross section through the A bands (Fig. 3E,F). Mitochondria appeared normal and desmosomes and fascia adherens were also present (data not shown). Thus, the mutation in ROSA5 does not lead to inherent defects in the formation of myofibrils in embryonic myocardial cells.

The trapped gene in the ROSA5 mutant encodes TEF-1

To identify the disrupted gene in the ROSA5 mutant, a cDNA library was constructed from liver RNA of ROSA5 using a *lacZ* reverse primer. The library was screened with another *lacZ* primer adjacent to the splice acceptor site. Among five clones characterized, the longest one contained 410 bp of cDNA sequence 5' of the splice acceptor site. Comparison of the cDNA sequence with the GenBank data base indicated that it shared 70% nucleotide identity with the 5'-untranslated region of the human TEF-1. A 260-bp DNA fragment from this cDNA clone was used to screen a randomly primed ES cell cDNA library, and two cDNA fragments with 2.4- and 1.4-kb inserts were sequenced. The cDNA sequence has an open reading frame encoding a protein of 426 amino acids (Fig. 4) that shares 421 identical amino acids with the human TEF-1 protein (Xiao et al. 1991). The AUU codon at nucleotide 463 rather than an AUG codon at position 517 is presumed to be the site of translation initiation of mouse TEF-1 as the human TEF-1 gene uses an AUU codon exclusively as an initiator. The TEA domain, which has DNA-binding activity, is identical between mouse and human TEF-1. About 1.4-kb of mouse TEF-1 cDNA has been cloned independently (Blatt and DePamphilis 1993) and encodes a protein almost identical to that shown in Figure 4 except for two amino acid differences at the 5' end.

Reverse transcription-polymerase chain reaction (RT-PCR) was used to determine whether the disruption of TEF-1 in ROSA5 creates a null mutation. E9.5 embryos derived from heterozygous crosses were used to isolate RNA, and DNA fragments representing the wild-type (570 bp) or mutant (258 bp) alleles were amplified by RT-PCR using two different pairs of primers. As shown in Figure 5A, only the 570-bp DNA fragments were amplified in wild-type embryos (lanes 5,6,9,10), both the 570- and the 258-bp products were produced in heterozygous embryos (lanes 1–4,7,8,13,14), and only the 258-bp fragment was amplified in homozygous mutant embryos (lanes 11,12). The absence of the wild-type frag-

Table 1. Genotype of ROSA5 embryos

Age (days)	Wild type	Heterozygote	Homozygote
9.5	9	29	12
10.5	15	35	14
11.5	15	24	12
12.5	13	27	7
14.5	2	5	1

Embryos were isolated at different stages of gestation and genotyped by Southern blots of yolk sac DNA using a genomic flanking probe.

Figure 2. Phenotype of the ROSA5 mutant embryos. (A) E11.5 mutant and wild-type embryos were dissected from the uterus, and the decidua was removed to expose embryos with the yolk sac intact. The arrow indicates a homozygous mutant embryo. Note the paleness of the yolk sac. (B) The yolk sac was removed to display the enlarged pericardial cavity and the dilated hindbrain of the mutant embryo. Another pair of wild-type E11.5 embryos (C) and ROSA5 mutant E11.5 embryos (D) are shown to display dilation of the fourth ventricle in the brain prior to the enlargement of pericardial cavity. Histological analysis of heart tissue from heterozygous and homozygous ROSA5 embryos (E–H). Transverse sections of heart from heterozygous (E,G) and mutant (F,H) embryos are shown. Similar planes of sections are shown in E and F and in G and H. Note the thin ventricle wall (F) and the PAS-positive particles underlying endocardial cells (H) indicated by arrows. (EC) Endocardial cushion; (IVS) interventricular septum; (LV) left ventricle; (RV) right ventricle. Magnification, (E,F) 59 $\times$ (G,H) 236 $\times$.

ment in the mutant embryos indicates that the gene trap insertion prevents the production of 5' exons of TEF-1.

To address the possibility that splicing bypasses the reporter gene to create aberrant TEF-1 messages, another set of primers was used to determine whether TEF-1 exons 3' to the reporter gene are still produced. A pair of TEF-1 primers were designed to amplify a 366-bp fragment at the 3' end of TEF-1 cDNA, spanning the translation termination codon and the 3'-untranslated region. RT-PCR reactions with this set of primers failed to detect TEF-1 message from homozygous mutant ROSA5 embryos (Fig. 5B, lanes 7–10). Thus, TEF-1 messages 3' to the insertion site of the gene trap vector are not produced. Taken together, these RT-PCR analyses indicate that the gene trap insertion in ROSA5 creates a null allele of the *TEF-1* gene.

Potential targets of TEF-1 are not down-regulated in ROSA5 mutants

TEF-1 is a transcription factor that has been proposed to be involved in the regulation of several cardiac muscle-specific genes (see Discussion). Genes such as cardiac troponin T (cTnT; Mar and Ordahl 1988), cardiac troponin C (cTnC; Parmacek et al. 1992), cardiac troponin I (cTnI; Nikovits et al. 1986), and myosin heavy chains (Thompson et al. 1991; Flink et al. 1992; Shimizu et al. 1992; Kariya et al. 1993) contain TEF-1 binding sites within their regulatory regions. Thus, it is conceivable that the phenotype observed in the ROSA5 mutants might be attributable to abnormal regulation of one or more of these potential TEF-1-regulated genes.

To test this hypothesis, Western blot and RT-PCR

Discussion

In this report we demonstrate that a mutation of the *TEF-1* gene by a retroviral gene trap results in a recessive lethal phenotype. ROSA5 mutant embryos die between E11 and E12 of gestation and show an enlarged pericardial cavity, brachycardia, and a dilated fourth ventricle

TEF-1 and heart development

TEF-1 was initially isolated from HeLa cells based on its ability to bind specifically and cooperatively to the GT-IIC and *SphI* motifs in the SV40 enhancer (Xiao et al. 1991). It is broadly expressed in many cell types, but not in some hematopoietic cells, consistent with the lack of β -galactosidase expression that we have observed in lymphocytes and in the blood. Characterization of the ROSA5 mutant demonstrates that TEF-1 plays an essential role in cardiac muscle development in mammals. The homozygous mutants die between E11 and E12 of gestation, a critical period for cardiogenesis. The heart is the first organ to appear during organogenesis, and rapid growth of embryos requires efficient removal of metab-

60	GGAGATTCGGCTGCGGAGATTCATTCGTTCTCTGCTCATTCGGACAGATTCCTGATCGCTCGGG	66
61	CGCGCGCGGCGCCCGACGAGCGAGCGAGCTCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG	132
123	GCACTTGGCAAGCTCGTTTCCAGCTCGCGGGAGCTCGCGGGCTCGCGGGCGCGCGCGCGCGCGCGCG	198
137	CGCGCGCGGCGAGCGAGCTCGTGGAGCTGACCGGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG	264
245	CGCGCGCGCTCGCTCGCTCGCGCGCGCGCGCGCGCGCGCTCGCGCGCGCGCGCGCGCGCGCGCGCGCG	330
331	CAGGAGTACACCGCTGGAGAGTACGCGGAGGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG	396
337	CGGCTGCGTGCTGTTTGTCTTCTTCAAGGCTCGCGCGCGCGCGCGCGCTCGCTTGGAAAAACCAACCGCGCAA	461
462	ATTGACCGCGAGCGAGTGGAGCGCGAGGAGGAGCGAGCGCTCGCGAAAAAGTGAAGAGGTGAGCGACTCG	527
	1E P S S W S G H * S I A E N M R H C S D	
528	CGAGATTAAGCGATGTGACACAGCGCGGAGGCGCTCGGAGTCTGTATATGACGAGCATTTCCAG	593
	A D K P I D N D A E G V S W S I D E S F Q	
594	GAGCGCTCGGCTATCTATCGCGCGCTGGGAGGAGAAAATCTATTCACAGCAAGCGAGCAAAATG	659
	E A L A I Y P F C G R G R K I L S D E G K M	
660	TATGCTGAAGAAAGAAATGATGACCGCAAGTACATCAAACTGAGGACGGGAAGAACAGGACAGGAGAG	725
	Y T P N H L I A R Y I K L R T G G K T R T R T	
726	CAAGTCTGATGTCACATTCAGGTTCTTGGCAGAAGGAASATTCGTGATTTCATTCACAGCTGAAG	791
	T S H S I D A R R K R S D P H S K L K	
732	GATGACATGTCACAGGACAGGCGCTCGACGACATGCGTCCGATGCTACGACCGCAAGTCTGCTCG	857
	C Q T A K D K A L O H M A A M S S A Q I V S	
758	GCTCATGCGCATCCACAGCAAGCTGGGCGTGGATTCGACGCGCCCACTTCGCGGGGGGGTCTCG	923
	A T A I H N K L G L P G I A P I R P T F P G G * P	
924	GGGTTCTGGCGCTGGGATGATGACAGGAGCAAGCGAGGCTCTACAGAGCGCTCAAGCGCTTGTGTG	989
	G F X F G M I Q T G Q G P G S S Q D V K P F V	
991	CAGCGAGCTTACCGCATCGACGAGGAGCTACAGCGCCCATTCACAGGTTTGGAGCTACGTGACGCC	1055
	C Q A Y F I Q P A V T A P I F G F E P T * A	
1086	CGACCGCTCGAGTCTGCTCGCGACGCGCGATGCCATTGGCAACAAACCAAGCTCGCGCTCGGTGGA	1121
	P A P S V P A W Q G R S I T T X L R L V E	
1122	TGCTCGCGCTTGTGAGACAGGACAGAGAGCCAGCACTGTACACAAACCAACCTCTCTGTCGACATC	1187
	F S A F L E Q Q R D P D S Y N K H L F V H I	
1188	GGGCACTGCAACCACTCTTACAGTGAACGCTGTGCTCGAATCTGTGGACATTCGTCGAGATATATGAC	1253
	G H A N H S V S D P L L E S V D I R Q I Y D	
1292	AAATTCCTGAAAAGAGAGTGGCTTGAAGAGCTGTGTGGAAGAGGCCCTCAAAAGCGCTCTCTTC	1319
	K F F E K K G L K L F G K G P N A N P F	
1320	CTCTGCAAAATCTGCGCGGCACTTAACTGCAATATCAAGACAGCGCGGGGCGCTTATGAGTGTG	1385
	L Y Z F W A D A N C I O D D A G A P Y G V	
1386	AGGAGTCAGTATGAGAGTTTGAGAACATGACAGTACTCGTTCTGCACCAAAAGTGTGCTCTTTGGG	1451
	S * O Y E S S E N M T V T C S T K V C S F G	
1492	AAACAAATATGAAAAGATGAGACGAGAGATTCGAGGAGTGGAGATGGTGAGTCTGCTGTACCGA	1517
	K Q V V E K V E T E Y A F R E N G R F V R Y	
1518	ATAAACCGCTGGCAATCTGTGATATATGATCAACTTATCTACCAAGCTCAAAACCGCTTACCAAG	1583
	I N P S P H C E Y M I N F I K H L K H L E	
1584	AAATATGATGACAGACTTTTGGAAACCTTCAACATATCTGTTGGTGTGAACCAAGGAGGTACAC	1649
	K Y M N S N S V L E N P T I L L V T V N R T D	
1682	CAGAAAGCTCTGCTTGCATGGCTCTGTGATTTGAAGTCTGCAATAGCAAGCAAGGACGAGGACGAC	1715
	C E T L L C M A C V F E V S N S E H G A Q H	
1714	CAATCTACAGCGCTTGTGAGGAGCTGAACCTGGTATTATATAGAGAGATTTGTGATATACACA	1781
	H I Y E L V K D	
1882	CGAGCTTGGCGACGACAGCTTCGATATCGAAGCAGTGAAGCTCCAGCGCGACGAGGCT	1847
1892	TCACTCTGGCGCTCGAGTGGCTCGACCTTGACCTCTGATCCTGTTCGAGTGAACATTTT	1913
1914	TGCTATCTTGTGATGATATGATCTGTAGCTGTGAAGTGTGATGACAGTTT	1979
1981	TCGACACCGCAAGCTTCTGGGAAGAGTGAATCTTACGACGCTAAGCTCTCTCGACGGCT	2045
1984	GTTCCTCTTGTGTAGAAAATATGACGACAGGAGATGGCCACGGGGCGATGATCGCTCTTATAC	2111
2112	AAATGATATGCTGTTTGTGTTTGTGTTTTCAGCAATAAAATCTTGT	2177
2178	AAATCTTAAATGAAATGTAAATAGTACAGGAAACATTAAGATCTTTPAGACATATCTTTCAGAT	2243
2244	AAATAGTGAGGAATATGAGAAAGTGAAATTCACATTAATTTTTTAAATAAGAAATCTTCTTC	2309
2312	TTTTTAAACGAAATGTAACTATAGCTCTTAAGACATGCTTGGTGTGAACGGTAAATCTCGAGGG	2375
2376	GGGCGCTTGGCGTCAAGGCTTT	2439

Figure 4. Nucleotide and predicted amino acid sequences of mouse TEF-1 cDNA. Sequences were derived from two independent cDNA clones. The slash at nucleotide 407 indicates where TEF-1 is fused to the ROSA β -geo reporter gene in the ROSA5 chimeric transcript. The TEA domain is underlined. Amino acids indicated by an asterisk (*) differ between human and mouse TEF-1.

gions of a number of cardiac- and skeletal muscle-specific genes, such as cTnT (Mar and Ordahl 1988), cTnC (Parmacek et al. 1992), cTnI (Nikovits et al. 1986), and myosins (Thompson et al. 1991; Flink et al. 1992; Shimizu et al. 1992; Kariya et al. 1993). In vitro transfection experiments suggest that the M-CAT motif and its binding factor, MCBF, are involved in the regulation of these genes.

The known activity of TEF-1 in muscle-specific gene regulation and the cardiac phenotype observed in TEF-1 mutant embryos suggest that the gene trap insertion may lead to changes in the expression pattern of one or more potential TEF-1 targets. However, expression of four such targets does not appear to be affected. Furthermore, somites and limb buds of the mutant embryos appear to develop normally, indicating that skeletal muscle development is not affected. There are several possible explanations for these observations. First, TEF-1 may be critical for the expression of another gene that we have not tested. Second, the presence of a binding site for a transcription factor in the regulatory region of a gene does not necessarily mean that expression of the gene is critically dependent on the factor. For example, an MCBF-binding M-CAT motif is present in the skeletal actin promoter (Mar and Ordahl 1990), but it can be mutated without affecting the activity of the promoter in transfection assays (Chow and Schwartz 1990). Third, some of the potential targets of TEF-1 may be down-regulated in a limited number of heart cells. We would have missed this by examining expression in the entire embryos because of the difficulty in obtaining enough protein from heart of individual E10 embryos. It is postulated that the *trans*-activation function of TEF-1 is most likely mediated by a limiting, cell type-specific intermediary factor (Xiao et al. 1991; Ishiji et al. 1992). It is possible that some TEF-1 targets may be down-regulated in certain cells of the mutant heart because of the presence of a putative cardiac-specific intermediary factor that modulates the activity of TEF-1. Isolation of cell lines from mutant embryos would enable us to perform a biochemical analysis in search of the intermediary factors required for TEF-1 activity and to identify unknown TEF-1 targets.

Does TEF-1 have other physiological functions besides its role in cardiogenesis? TEF-1 expression is found in most tissues and *lacZ* staining in ROSA5 heterozygotes confirms that TEF-1 is present in early, cleavage-stage embryos. TEF-1-binding sites were shown to be able to specifically enhance gene expression in ES cells and mouse cleavage-stage embryos, suggesting that TEF-1 has the capacity to activate gene expression during early stages of embryo development (M'elin et al. 1993). The observation that TEF-1 mutant embryos survive until E11 of gestation without obvious defects except in the heart suggests that the function of TEF-1 at earlier stages may be functionally compensated by other genes. Interestingly, several isoforms of TEF-1 have been isolated from chicken (Stewart et al. 1994). It remains to be seen whether different isoforms of TEF-1 exist in mouse and, if they do, whether they have unique or overlapping

functions in vivo. Another interesting question to be addressed is whether other functions of TEF-1 at late stages of development are masked by embryo lethality at E12. Rescuing the heart phenotype may unveil additional functions of TEF-1 during late stages of embryogenesis.

Materials and methods

Derivation of mutant mice

The ROSA5 mutant was derived by infection of ES cells with the retroviral gene trap vector ROSA β -geo as described previously (Friedrich and Soriano 1991). According to the rules of the Mouse Nomenclature Committee, this strain will be designated TgR(ROSA5)5Sor.

Histology

Mouse embryos were dissected in PBS and fixed in Bouin's solution for 6 hr at room temperature. They were then gradually dehydrated in ethanol, cleared with HistoClear, and embedded in paraplast. Sections were cut at 6–8 μ m using a Reichert 2030 microtome. Sections of embryos were stained either with hematoxylin and eosin, or PAS stain.

Electron microscopy

E10 embryos were fixed in half-strength Karnovsky's fixative, rinsed in buffer, and postfixed in 2% collodion-buffered osmium tetroxide. They were dehydrated in graded ethanol and propyleneoxide and embedded in Polybed 812. Sections of 80–90 nm were cut and stained with saturated aqueous uranyl acetate and Millonig's lead stain. They were examined and photographed with a JEOL 100-SX electron microscope operating at 80 kV.

Construction and screening of cDNA library

The TEF-1/ β -geo fusion mRNA sequences were isolated from a β -geo primed cDNA library. The cDNA library was constructed with poly(A)⁺ RNA isolated from liver of ROSA5 heterozygotes, using a primer specific for the *lacZ* sequences (5'-ATGCGCTCAGGTCAAATTCAGACGG-3') and standard procedures (Pharmacia U-prime cDNA synthesis kit). The resulting double-stranded cDNA was ligated into the *Eco*RI site of λ ZapII (Stratagene). Plaques (150,000) were screened with a labeled oligonucleotide (5'-GAAAGACCGCGAAGAGTTTGTCCTCAA-CCG-3') complementary to sequences immediately 3' to the splice acceptor of ROSA β -geo. Four positive plaques were purified, and the one with longest insert (cRS-1) was used to recover the cloned insert by phagemid rescue.

The full-length TEF-1 cDNA was identified by screening a random-primed ES cell cDNA library, constructed as follows. Total cellular RNA was isolated from AB1 ES cells (McMahon and Bradley 1990) grown in the absence of feeder cells in leukemia inhibitory factor (LIF)-supplemented ES medium. Poly(A)⁺ RNA was isolated by purification using the PolyA-Track mRNA isolation system (Promega). Five micrograms of poly(A)⁺ RNA was mixed with random hexamers (Pharmacia) at a final concentration of 6 μ g/ml, and cDNA was synthesized according to standard procedures. After ligation to two different adapters (Elledge et al. 1991), cDNA was run through a 1% low-melt agarose gel to select cDNA in the size range of 0.6–4 kb. After purification by phenol/chloroform extraction and ethanol precipitation, 0.1 μ g of cDNA was ligated to 2 μ g of T-filled λ YES-R vector (Elledge et al. 1991) and packaged using a Giga-

pack Gold packaging extract (Stratagene). The original cDNA library represents 12×10^6 individual plaques.

A 260-bp *EcoRI*–*ClaI* DNA fragment from cR5-1 was used to screen the ES cDNA library. Among 0.5×10^6 colonies screened, 13 positive clones were identified. The inserts from these positive clones were ligated into pKS Bluescript II (Stratagene). Two clones with 2.4- and 1.4-kb inserts were sequenced after Exonuclease III/S1 nuclease nested deletions.

Genomic cloning and genotyping

ROSA5 genomic DNA was partially digested by *MboI* and 15- to 23-kb fragments were cloned at the *Bam*HI site of λ DashII (Stratagene). Following in vitro packaging, the phage library was screened with a neo probe. None of the five clones isolated, spanning ~20 kb of the insertion locus, hybridized to the TEF-1 cDNA, suggesting that the provirus has inserted into a large intron. For genotyping, a 250-bp *PstI* genomic DNA fragment 0.7 kb downstream of the proviral insertion site was used. After digestion of genomic DNA with *SacI*, the probe detects a 3.6-kb band from wild-type allele and a 4.0-kb band from the mutant allele.

RT-PCR

To amplify fragments of the TEF-1 cDNA and the TEF-1/ β -geo fusion cDNA, total embryo RNA isolated from E9.5 embryos was mixed with 10 pmoles of either a *lacZ* reverse primer 5'-ATGCGCTCAGGTCAAATTCAGACGG-3' or a TEF-1 5' reverse primer 5'-GGAAGGTGGGCGGTGGAA-3' and denatured by heating at 70°C for 3 min. RT reactions were performed at 37°C for 60 min with 200 units of Moloney murine leukemia virus reverse transcriptase in 20 μ l of 1 $\times$ PCR buffer plus 0.5 mM of each dNTP, 10 mM DTT, and 10 units of RNasin (Promega). After the reverse transcriptase reaction, the volume of each reaction was increased to 100 μ l by adding 80 μ l of 1 $\times$ PCR buffer containing 10 pmoles of a TEF-1 5' forward primer 5'-CTGGAGTAACGCGGGGGC-3' and 5 units of AmpliTaq DNA polymerase (Perkin-Elmer Cetus). Forty cycles of PCR was performed with the following temperature profile: denaturation at 94°C for 1 min, annealing at 64°C for 1 min and extending at 72°C for 1.5 min.

To detect 3' exons of the TEF-1 cDNA, RT-PCR was performed essentially as above except that (1) 100 pmoles of random hexamers were used in the reverse transcriptase reaction; and (2) forward (5'-AACACGGAGCACAGCACCA-3') and reverse (5'-TCTACCACAACAGGAAACAG-3') TEF-1 3' oligonucleotides were employed.

For cTnC RT-PCR, forward (5'-AATGGATGACATCTACAAAG-3') and reverse (5'-TGAGCTCTTCAATGTCATCT-3') oligonucleotides were used in a PCR reaction consisting of 40 cycles of denaturation at 93°C for 30 sec, annealing at 54°C for 40 sec, and extension at 65°C for 40 sec. Amplification with this set of primers generates a 410-bp cTnC cDNA fragment.

Protein isolation and Western blot analysis

Protein was isolated from E10.5 embryos by homogenization in Laemmli buffer followed by boiling for 5 min. Forty micrograms of protein was loaded per lane and separated by SDS-PAGE. After blotting to nitrocellulose, Western blot analysis was performed with the following antibodies: MF-20 recognizing all myosin isoforms, mAb 13-11 specific for cTnT, and T1-4 recognizing both cTnI and skeletal troponin I (Saggin et al. 1989).

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