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Regulation of urokinase expression in the developing avian heart: a role for the Ets-2 transcription factor

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Abstract

During heart development, cells of the endocardial cushions undergo an epithelial-mesenchymal transformation and migrate into the surrounding extracellular matrix. This event is required for the normal formation of valves and chamber septation. Coincident with this phenotypic change is the expression of the serine protease urokinase by the mesenchymal cells. This protease plays an important role in remodeling of the matrix, promotion of cell migration by regulating cell-matrix interactions, and the activation of growth factors. To understand the mechanisms underlying the expression of urokinase during heart development, studies were designed to analyze the role of the Ets transcription factors in the regulation of the avian urokinase gene promoter. Deletion or mutagenesis of the Ets consensus sites significantly decreased the activity of the promoter in isolated cushion tissue cells. Proteins were identified by electrophoretic mobility shift analysis and UV-crosslinking which bound to a specific region of the promoter shown to be required for full transcriptional activity. Analyses based upon protein molecular weight and interaction with specific antibodies suggest a role for the Ets-2 protein in promoter binding and activity. The expression of Ets-2 in the cushion tissue cells was confirmed by RT-PCR analysis and in situ hybridization. The mRNA levels and the DNA binding activity of the Ets-2 protein were found to change during development paralleling the increase in urokinase activity. Overexpression of the full-length Ets-2 protein or a dominant-negative form of the protein altered the activity of the promoter and significantly affected the production of urokinase in these cells. The results from these studies suggest an important role for the Ets-2 protein in heart development and may contribute to a better understanding of the inductive factors present in the heart which facilitate the normal morphogenesis of this organ. © 1997 Elsevier Science Ireland Ltd.

Keywords: Urokinase; Ets-2; Transcription Factors

1. Introduction

Extracellular matrix (ECM) remodeling and cell migration are important events which occur during the normal development of tissues such as the heart. In the endocardial cushions of the atrioventricular canal (AVC) and the outflow tract (OFT), the endocardial cells respond to signals from the nearby myocardium and initiate a specific sequence of morphogenetic events (Krug et al., 1987; Potts and Runyan, 1989; Huang et al., 1995). A key feature of this response is an epithelial-mesenchymal transformation of the endocardium yielding a population of actively migrating mesenchymal cells which invade the surrounding cardiac jelly matrix (Markwald et al., 1977; Nakajima et al., 1994). This migration of cells is important in the normal formation of valves and septation of the heart into individual chambers, and may be partially regulated by the production of specific extracellular proteases. One extracellular protease which may have important functions during development is the serine protease, urokinase. This enzyme has been shown to play a major role in tissue remodeling, in the regulation of cell substrate interactions during cell migration, and in the release and activation of specific growth factors (Lyons et al., 1990; Naldini et al., 1992; Blasi, 1993). The activity and mRNA levels of this enzyme increase during heart development coincident with the transformation of endocardium to mesenchyme in the AVC and OFT regions (McGuire and Orkin, 1992; McGuire and Alexander, 1993). Urokinase is specifically expressed by the endocardium and migratory mesenchyme and is loca-

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lized to the cell surface where it may play a role in the regulation of cell-matrix interactions. Decreasing the synthesis of urokinase using antisense oligonucleotides inhibits the migratory abilities of these cells in vitro (McGuire and Alexander, 1993).

The avian urokinase promoter contains a number of Ets consensus elements (Leslie et al., 1990). These regulatory elements are also present in the gene promoters of other extracellular proteases and their inhibitors including collagenase, stromelysin and tissue inhibitor of metalloproteinase-1 (TIMP-1) (Gutman and Wasylyk, 1990; Edwards et al., 1992). Members of the Ets proto-oncogene family recognize a DNA-binding domain containing a common purine-rich trinucleotide core GGA (Wang et al., 1992; Wasylyk et al., 1993). These proteins are differentially expressed during murine embryogenesis, *Drosophila* development, *Xenopus* oocyte maturation, lymphocyte differentiation, and angiogenesis in humans. In order to facilitate maximal levels of transcription of some promoters, the Ets proteins may require interaction with other transcription factors (Gutman and Wasylyk, 1990; Wasylyk et al., 1990, 1993; Wernert et al, 1992; Kola et al., 1993; Maroulakou et al., 1994). Different proteins of the Ets family display distinct DNA binding specificities based upon the 3′ region flanking the consensus DNA binding site (Wang et al., 1992). Cells may express various Ets proteins which through their preferential DNA binding specificities are capable of differential gene regulation. The independent or concerted transcriptional activation of various genes through Ets binding and activity may be a key regulatory factor in the overall sequence of events involved in cell migration and ECM turnover during development.

We have begun to examine factors which bind the avian urokinase gene promoter and regulate the expression of this enzyme in the developing heart. These studies have focused on specific regions of this promoter which contain the consensus sequence 5′C[A/C]GGA[A/T]3′, to which members of the Ets family of transcription factors bind. The Ets-2 protein is produced by the endocardial derived mesenchymal cells. Its mRNA levels and DNA binding activity were found to increase coincident with the expression of urokinase in the heart, suggesting an important role for this protein in the morphogenetic events in this tissue.

2. Results

2.1. Regulatory regions of the urokinase promoter

These studies were designed to investigate the role of the Ets proteins in the regulation of urokinase expression in the developing avian heart. Sequence analysis of the 5′ region of the avian urokinase gene revealed three potential Ets consensus binding sites with the sequence C[A/C]GGA[A/T], at base pairs (bp) −1279 to −1273, −861 to −856 and −682 to −677. The chicken urokinase promoter pGC20 was ligated into the pGL-2 basic luciferase reporter plasmid resulting in the fusion construct, pGL-2-UK1.5. This construct showed high luciferase activity when transfected into cushion tissue cells isolated from the AVC of stage 15 embryos (Fig. 1). These cells have previously been shown to be the primary site of urokinase expression in the developing avian heart and produce urokinase in vitro (McGuire and Alexander, 1993).

The Ets sites were specifically mutagenized to determine the role of these individual elements in promoter activity while maintaining the overall structural integrity of the promoter (Fig. 1). Mutation of the 5′ Ets consensus sequence at bp −1279 to −1273 (pGL-2-UKΔA) resulted in a 72% decrease in promoter activity as measured by luciferase production. An even more dramatic effect was seen when the Ets site at bp −861 to −856 was altered (pGL-2-UKΔB) resulting in an 87% decrease in promoter activity compared to the control construct.

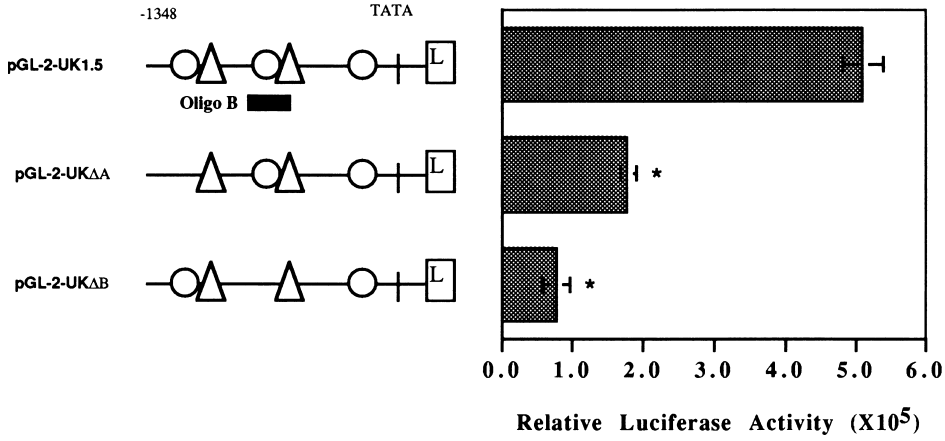

Fig. 1. Isolated cardiac mesenchymal cells were transfected with 3 μg of the indicated luciferase construct and 1.5 μg of pSV-β-galactosidase for each experiment. Cells from 3 coverslips transfected with individual luciferase constructs were harvested 24 h following transfection and assayed for luciferase and β-gal activity. Luciferase activity has been normalized to β-gal activity to correct for transfection efficiency. Diagrams of the full length and truncated urokinase promoter constructs are indicated at left. The data represent the mean ± SEM of $n = 3$ experiments. *O*, Ets consensus site; Δ, potential AP-1 site; L, luciferase. *Significantly different from control (pGL-2-UK1.5) at the $P \leq 0.05$ level.

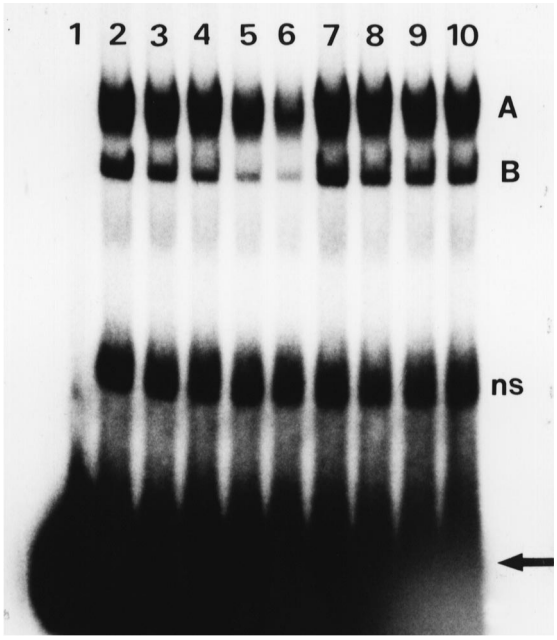

Fig. 2. Gel shift analysis of stage 18 heart extract using oligonucleotide B containing an Ets consensus site. Two shifted bands (A and B) are formed when 10 μg of whole heart extract is incubated with oligonucleotide B in a standard gel shift reaction (lane 2). This binding activity can be competed for by the presence of an increasing amount of unlabeled oligonucleotide B (lanes 3–6; 25, 50, 100 and 200 fold molar excess). A similar excess of unlabeled oligonucleotide containing a mutation of the Ets consensus site does not compete for binding (lanes 7–10). Lane 1, oligonucleotide B probe only. Arrow, free probe; ns, non-specific band.

2.2. Characterization of proteins interacting with the urokinase promoter and temporal regulation of binding

Since deletion of the consensus Ets site at −861 to −856 had such a profound effect on promoter activity, an oligonucleotide containing this site was used in an electrophoretic mobility shift assay to begin analyzing the factors which interact with the DNA to regulate transcription. Incubation of nuclear-containing extract from stage 18 hearts with oligonucleotide B (see Fig. 1) resulted in the appearance of two shifted bands following electrophoresis (Fig. 2). The specificity of protein binding to this oligonucleotide was confirmed by the addition of increasing amounts of unlabeled oligonucleotide B or an oligonucleotide containing a mutation of the Ets binding site. Addition of unlabeled oligonucleotide B resulted in the progressive disappearance of the shifted bands, while the mutant oligonucleotide did not affect protein binding or the formation of shifted bands. In addition, binding reactions with a random oligonucleotide gave no shifted bands (not shown) further confirming the specificity of binding to oligonucleotide B.

In order to characterize the proteins present in the developing heart which interacted with oligonucleotide B in this assay, extract from stage 18 hearts was incubated with oligonucleotide B in a standard mobility shift assay and crosslinked by UV irradiation. Protein-oligonucleotide complexes corresponding to molecular weights of 73–76 kDa were resolved following electrophoresis and autoradiography (Fig. 3). The approximate molecular weights of the proteins in the major complex were determined by subtracting the molecular weight of the radiolabeled oligonucleotide (14.5 kDa) resulting in proteins with approximate molecular weights of 58 and 62 kDa which have been reported for the Ets-2 protein (Boulukos et al., 1988). The presence of Ets-2 in the gel shift complex was further confirmed by the addition of an Ets antibody to the gel shift reaction. This antibody is directed against the DNA binding domain of Ets-1 and Ets-2 and would therefore be expected to inhibit the interaction of the protein with the DNA as opposed to producing a supershifted complex. A significant decrease was seen in the formation of the DNA/protein complex upon the addition of 100 ng of Ets antibody during the gel shift reaction compared to the addition of an equivalent amount of a non-specific rabbit IgG (Fig. 4).

The gel shift reaction was used semi-quantitatively to determine if the binding of protein to oligonucleotide B changed during progressive stages of heart development. In this assay band intensity following autoradiography was related to the amount of specific protein present in the extract which can effectively bind to the DNA probe. A significant increase in the amount of protein binding to oligonucleotideB was present in extracts of stage 18 hearts compared to that from the earlier stage 12 hearts (Fig. 5). This increase in protein binding correlated with the period of development showing elevated urokinase expression and the induction of cell migration in the cushion tissues. The DNA binding activity of the protein decreased slightly by stage 23 but remained elevated when compared to stage 12.

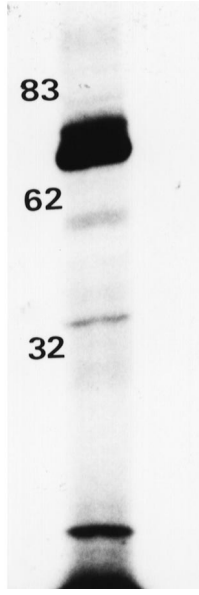

Fig. 3. The relative molecular weight of proteins binding to oligonucleotide B was determined by UV-crosslinking and SDS-PAGE. Heart extract (20 μg) from stage 18 heart was bound to oligonucleotide B, UV-crosslinked, and electrophoresed under reducing conditions as described. Molecular weight standards are indicated.

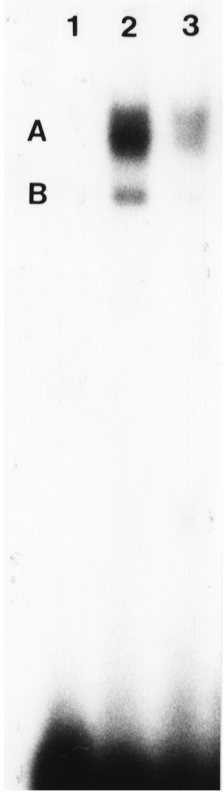

Fig. 4. Identification of Ets proteins interacting with oligonucleotide B. Stage 18 heart extract (10 μg) in binding buffer was incubated with the [^{32}P]-end labeled oligonucleotide in the presence of 100 ng of Ets1/2 antibody (lane 3), or an equivalent amount of non-immune rabbit IgG (lane 2). Lane 1, probe only.

The presence of a similar binding activity was also found in the isolated cushion tissue cells. When cell extract was crosslinked to the radiolabeled oligonucleotide a doublet of bands was observed, similar to that seen when whole heart extract was used (Fig. 6A, lane 1). When the Ets antibody was included in the binding reaction prior to crosslinking, the uppermost band of the doublet disappeared, again confirming that Ets-2 was a component of this complex (Fig. 6A, lane 2). This antibody was also used in a western blot analysis of the cell extract and specifically recognized the 62 kDa Ets-2 protein (Fig. 6B).

A full-length cDNA of avian Ets-2, as well as a cDNA encoding a truncated form of the protein containing only the DNA binding domain and nuclear localization signals, was cloned by RT-PCR (Fig. 7A). The cDNA constructs were subcloned into the pSI expression plasmid and transfected into Cos-1 cells to express the recombinant proteins. Both the full-length and truncated proteins were expressed and found to bind to an Ets consensus site in a mobility shift assay, and this interaction was inhibited with the Ets DNA binding domain antibody (data not shown). These constructs were subsequently co-transfected along with the urokinase promoter luciferase construct pGL-2-UK1.5 into isolated cardiac cushion tissue cells. The overexpression of the Ets-2 DNA binding domain greatly attenuated the activity of the urokinase promoter. This result suggests that the truncated protein can successfully act in a dominant-negative manner to inhibit the function of the wild-type Ets-2 protein in these cells (Fig. 7B). In contrast, overexpression

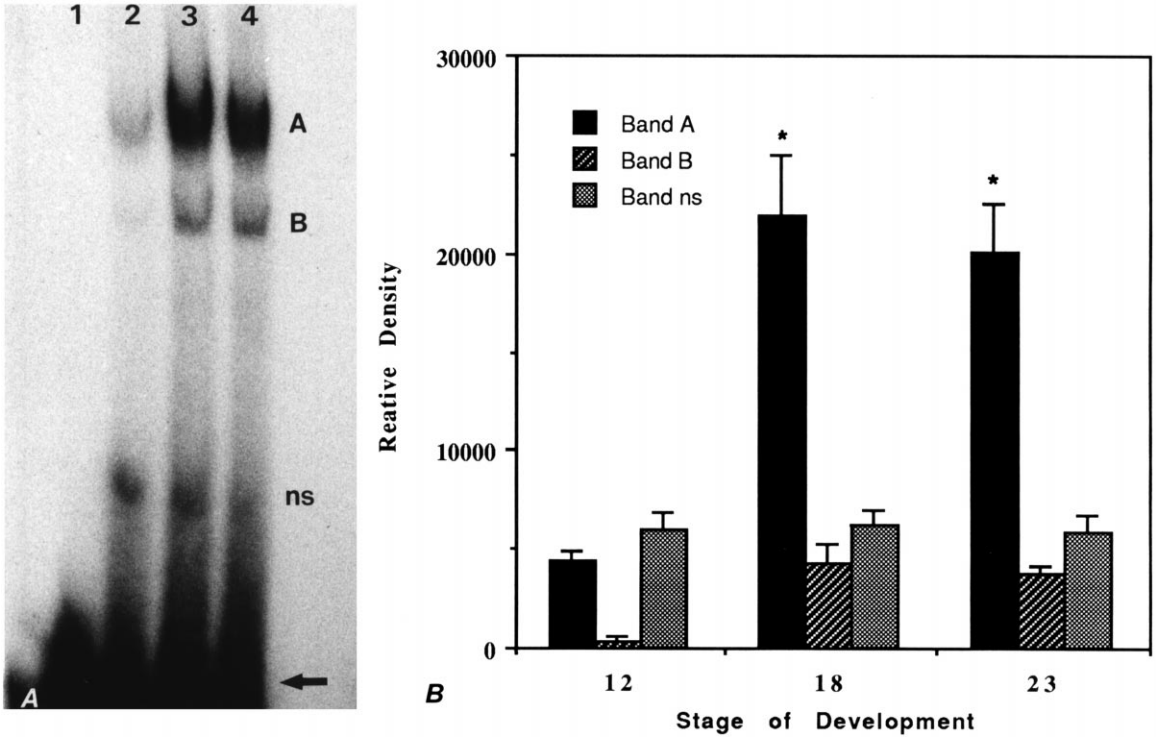

Fig. 5. Temporal binding of regulatory proteins to oligonucleotide B. (A) Gel shift analysis of heart extract from stages 12 (lane 2), 18 (lane 3) and 23 (lane 4). Lane 1, probe only. Arrow, free probe; ns, non-specific band. (B) Quantitation of band density using the NIH Image analysis software. Values are the mean ± SEM for $n = 3$ experiments. *Significantly different from stage 12 at the $P \leq 0.05$ level.

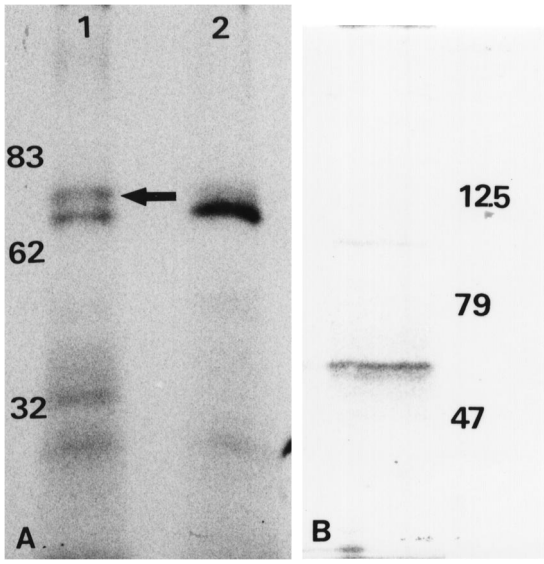

Fig. 6. Identification of mesenchymal cell proteins binding to oligonucleotide B. (A) 20 μg of cell extract was incubated in a gel shift reaction with radiolabeled oligonucleotide B, UV-crosslinked, and analyzed by SDS-PAGE (lane 1). The addition of 100 ng of Ets1/2 antibody to the reaction (lane 2) disrupted the binding of the band corresponding to the 62 kDa protein (arrow in lane 1). (B) Western analysis of Ets-2 protein in isolated cardiac mesenchymal cell extract. A specific band of 62 kDa is present.

of the full-length Ets-2 wild-type protein in these cells increased promoter activity by 54% (Fig. 7B).

The effect of overexpression of each of the Ets-2 constructs on urokinase enzyme production in these cells was then determined using a retroviral system. An RCAS retrovirus encoding either the full-length or truncated construct was produced and used to infect the isolated cushion tissue cells. Use of the RCAS system, unlike the pSI expression vectors, allowed for overexpression of the appropriate Ets protein in virtually every cell in the culture 24 h after the initial infection. As expected, overexpression of the dominant-negative form of the protein, Ets-2 (dbd), caused a significant reduction in the level of urokinase activity in the cultures, whereas expression of the full-length Ets-2 protein nearly doubled the urokinase activity associated with the cells (Fig. 8).

2.3. Ets protein expression in the developing heart and isolated mesenchymal cells

The previous data suggests that Ets-2 regulates expression of the urokinase gene in cells of the developing heart. However, other studies have shown that Ets-1 may also act on this promoter in other cell types and systems (Janknecht and Nordheim, 1993; Hromas and Klemsz, 1994; Wernert et al., 1992). RT-PCR analysis using specific primers for Ets-1 and Ets-2, was performed in order to confirm which of these proteins are expressed in the heart and in isolated cardiac mesenchymal cells. Both Ets-1 and Ets-2 messages were detected in the whole heart, however, only the Ets-2 message was present in the isolated cardiac mesenchymal cells (Fig. 9). These cells have previously been shown by in situ hybridization to be the major source of urokinase in the developing heart (McGuire and Alexander, 1993). The localization of Ets-2 in the heart was further investigated by in situ hybridization, and in the stage 18 AVC the message for Ets-2 was found localized to the endocardial layer and the associated mesenchyme (Fig. 10A). The myocardial layer of cells in this region were not expressing Ets-2 as shown by a lack of specific labeling.

RT-PCR was also used in a semi-quantitative manner to determine the temporal and spatial expression of Ets-2 mRNA in the developing heart. This was done by including primers for the avian GAPDH in the same PCR reaction (Fig. 11). Significantly higher levels of Ets-2 mRNA were detected in the stage 18 heart compared to stage 12, which correlated with the increase in protein binding seen in the mobility shift assays (compare with Fig. 5). Spatial differences were also noted in the levels of Ets-2 mRNA at stage 18. Higher levels of Ets-2 mRNA were found in the AVC and OFT regions which undergo the epithelial mesenchymal transformation and express high levels of urokinase, compared to the ventricle which does not form extensive

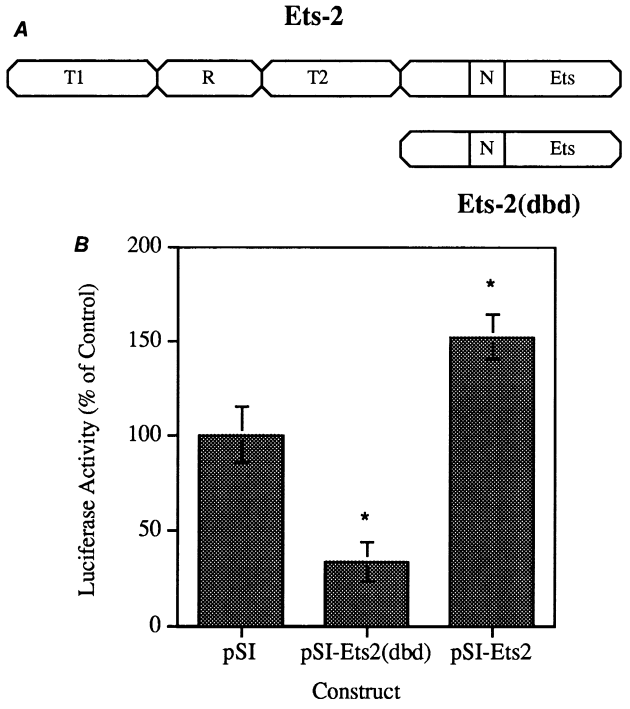

Fig. 7. Cloning and expression of recombinant Ets-2 proteins. (A) Domain organization of the Ets-2 protein adapted from Crepieux et al. (1994). T1 and T2, transactivation domains; R, regulatory domains; Ets, DNA-binding domain; N, nuclear localization signal domain. The full-length Ets-2 protein and the truncated protein Ets-2(dbd) are shown. (B) Isolated cardiac cushion tissue cells were transfected with the pSV-β-galactosidase, pGL-2-UK1.5 luciferase reporter plasmid, and either the pSI vector alone, or pSI containing either the truncated Ets-2 construct Ets2(dbd) or the full length Ets2 construct. Cells were collected 24 h following transfection and analyzed for luciferase activity. Data are the mean ± SEM normalized to β-gal for three independent experiments. *Significantly different from control (pSI) at the $P \leq 0.05$ level.

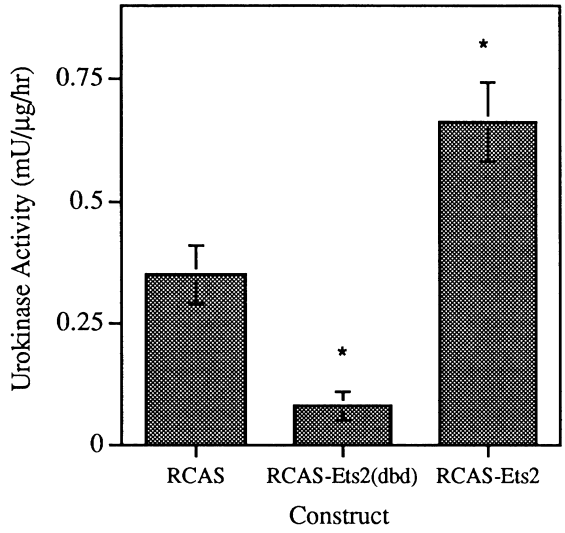

Fig. 8. Urokinase expression in retrovirus infected cells. Isolated cushion tissue cells were infected with either RCAS virus alone, or RCAS containing either the truncated Ets-2 construct Ets-2(dbd) or the full length Ets-2 protein. The cells were collected 24 h later, extracted and analyzed for urokinase activity by the chromogenic microtiter plate assay. Data are the mean ± SEM from three independent experiments.

amounts of mesenchyme and expresses only low levels of the enzyme (McGuire and Orkin, 1992).

3. Discussion

A key feature of tissue remodeling during embryonic development is the transformation of primary epithelium to mesenchyme which occurs in specific regions of the embryo at defined stages of development. Examples include the somite-derived mesenchyme which gives rise to bone,

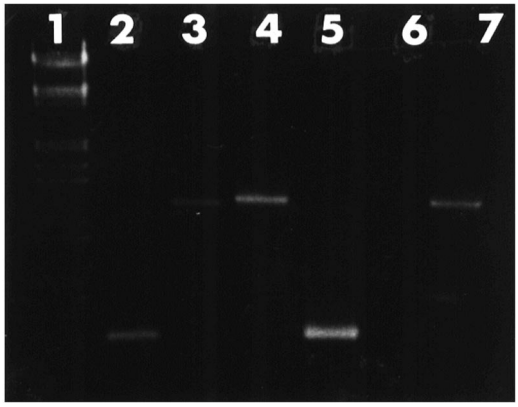

Fig. 9. RT-PCR analysis of Ets-1 and Ets-2 expression in the heart and isolated cells. First strand cDNA was synthesized from total RNA from either whole heart or isolated cardiac mesenchymal cells and used as the template in PCR reactions with specific primers for Ets-1 (1141 nt product), Ets-2 (1199 nt product) and GAPDH (300 nt product). Lane 1, DNA ladder; lanes 2–4, GAPDH, Ets-1 and Ets-2 from whole stage 18 heart; lanes 5–7, GAPDH, Ets-1 and Ets-2 from isolated cardiac mesenchymal cells.

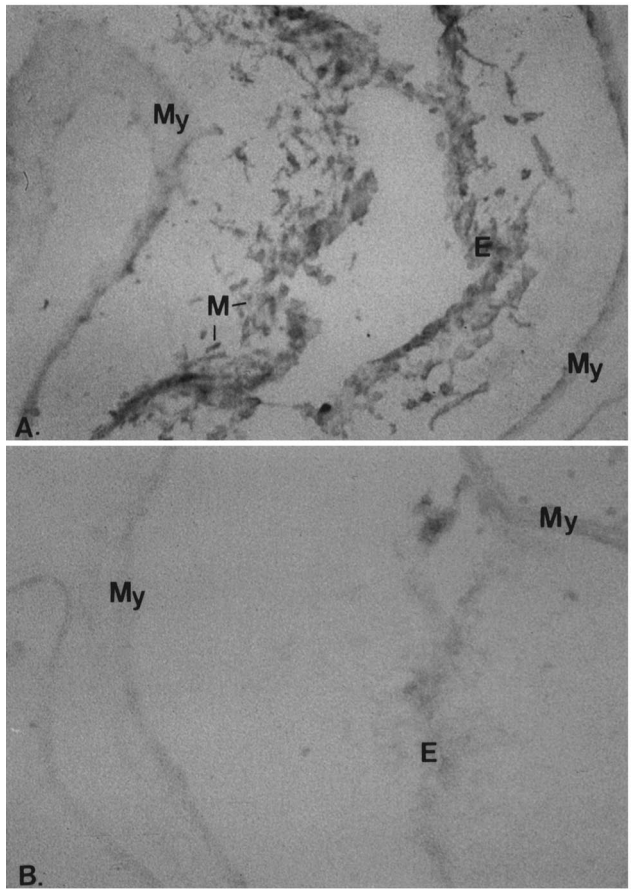

Fig. 10. In situ hybridization analysis of Ets-2 expression. (A) Dig-labeled antisense riboprobe hybridizes to the endocardial lining (E) and migratory mesenchymal cells (M) of the stage 18 atrioventricular canal. The outer layer of myocardium (My) shows no labeling. (B) Sections incubated with a sense riboprobe show only background labeling. Scale bar, 55 μm.

cartilage, muscle and connective tissue of the dermis, the neural crest, and the midline epithelial seam of the remodeling palatal shelves (Hay, 1990). This transformation also plays a key role in the morphogenesis of the embryonic heart. The endocardial cells lining the atrioventricular canal and outflow tract undergo an epithelial-mesenchymal transformation in response to factors derived from the nearby myocardium and migrate into the surrounding extracellular matrix. This formation and migration of cushion mesenchyme is postulated to be a critical step leading to normal valve morphogenesis and chamber septation (Markwald et al., 1977; Carmi et al., 1992; Huang et al., 1995). A characteristic of the endocardial-derived mesenchymal cells is the elevated expression of the serine protease urokinase (McGuire and Orkin, 1992). The inhibition of this enzyme has been shown to effect mesenchymal cell attachment and motility in vitro (McGuire and Alexander, 1993). The increase in urokinase activity associated with actively migrating cells is not unique to the heart. Previous studies from this laboratory have also demonstrated the elevated expression of this enzyme in the sclerotome cells derived

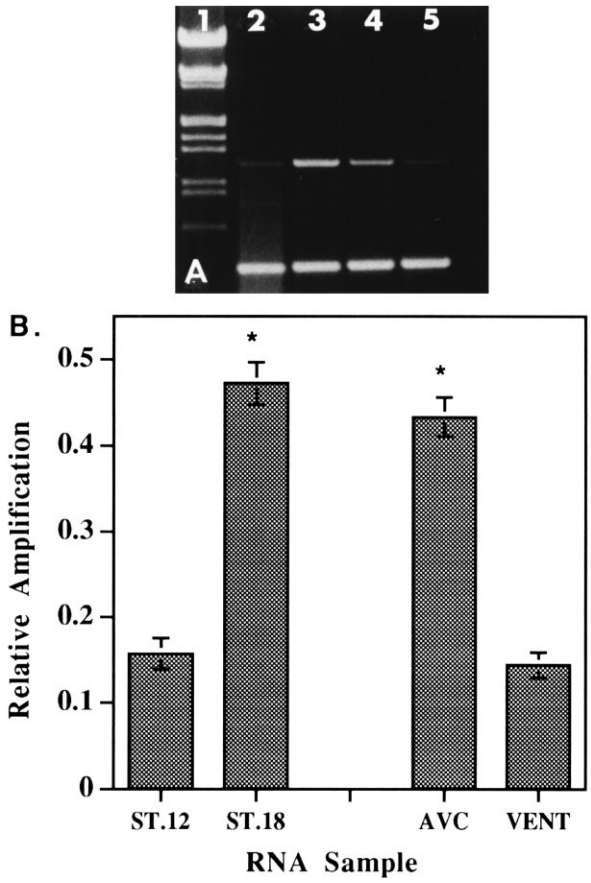

Fig. 11. Semi-quantitative analysis of Ets-2 in developing heart. (A) Levels of Ets-2 mRNA were normalized to GAPDH for each sample by including both Ets-2 and GAPDH specific primers in the same PCR reaction. Ets-2 and GAPDH products in stage 12 (lane 2) and stage 18 (lane 3) whole hearts. Ets-2 and GAPDH messages in the AVC (lane 4) and the ventricle (lane 5). (B) Quantitation of band density. Values are the mean ± SEM for $n = 4$ experiments. *Significantly different at the $P \leq 0.05$ level.

from the epithelial-mesenchymal transformation of the somites (McGuire and Alexander, 1992). In the present study we have begun to examine the factors which regulate the expression of urokinase in the developing heart and have demonstrated the importance of the Ets-2 protein in the transcriptional regulation of the urokinase gene.

The Ets proteins have been shown to participate in the induction of urokinase gene transcription in various human tumor cell lines as well as in cells stimulated with a variety of agents including epidermal growth factor, granulocyte monocyte colony stimulating factor and phorbol esters (Nerlov et al., 1992; Rorth et al., 1990; Stacey et al., 1995). In addition, Ets proteins have been shown to be transcriptional activators of other proteases (stromelysin, collagenase) and their inhibitors (TIMP-1) (Edwards et al., 1992; Wasylyk et al., 1990; Nerlov et al., 1992; Crepieux et al., 1994). Analysis of the avian urokinase promoter revealed three Ets consensus sites with the sequence (5′C-[A/C]GGA[A/T]3′) suggesting a potential role for Ets proteins in the transcriptional regulation of this gene during development. Using a luciferase reporter system transfected into isolated cardiac mesenchymal cells, we determined that the presence of the Ets consensus sequences accounts for a significant degree of total promoter activity. A functional Ets-binding site at bp 861–856 appears to be critical for high promoter activity in this cell type. Mutation of the Ets sites alone and the resultant loss of promoter activity initially suggests a significant role for the Ets proteins in the regulation of the urokinase gene promoter. This does not however, rule out the possibility that the loss of Ets binding also effects the interaction of other transcription factors with their specific sites in the promoter (Crepieux et. al., 1994).

Electrophoretic mobility shift analyses were performed using an oligonucleotide containing the Ets consensus site (bp −861 to −856). Proteins from embryonic heart extract were found to interact with this oligonucleotide probe producing two distinct shifted bands in this assay. Based upon an analysis of molecular weights and altered oligonucleotide binding in the presence of specific antibodies, these protein/DNA complexes were shown to contain Ets proteins.

The temporal DNA binding activity of these proteins was examined in hearts at stages prior to, during, and following the epithelial-mesenchymal transformation of the endocardium. Protein binding increased coincident with the epithelial-mesenchymal transformation of the embryonic endocardium and peak urokinase expression (McGuire and Orkin, 1992). The amount of protein bound to the oligonucleotide remained elevated at later stages of development. This may be attributed to a continued requirement for urokinase for the completion of endocardial cushion remodeling (Chin et al., 1992; Huang et al., 1995). This increase in Ets protein binding, as observed by mobility shift assay, may be due to either increased expression of the protein in the developing heart, or to a change in the activity of preexisting protein (i.e. phosphorylation/dephosphorylation of the protein or masking/unmasking of the DNA binding domain) (Fisher et al., 1991; Crepieux et. al., 1994).

PCR studies were conducted to determine which Ets proteins were present in the heart during specific stages of development. Ets-1 and Ets-2 messenger RNA were both found to be present in the embryonic heart at stage 18 of development. Since the DNA binding domains of both of these proteins are similar, either one could potentially recognize and bind to the Ets motif in the urokinase promoter. One possible mechanism of dealing with the specificity of transcription factor binding and urokinase regulation in the developing heart may be through cell-type-specific expression of the Ets proteins as seen in other systems (Nerlov et al., 1991). Upon further analysis it was determined that only Ets-2 mRNA was expressed in the cardiac mesenchymal cells. These cells have previously been shown to be the primary source of urokinase in the developing heart (McGuire and Alexander, 1993).

Subsequent studies of Ets-2 expression in the heart between stages 13 and 18 revealed increasing levels of

Ets-2 mRNA which may account for the increase in protein binding seen by mobility shift assay. Ets-2 was also found to be regionally expressed in the developing heart. In the regions in which the endocardium is actively forming mesenchyme (the AVC and OFT) the level of Ets-2 mRNA was found to be nearly 2 fold higher when compared to the ventricular region. The endocardium of the ventricle, which contains lower levels of urokinase activity compared to the AVC and OFT (McGuire and Orkin, 1992), does not form a significant amount of mesenchyme. Rather, it evaginates as a continuous sheet of cells toward the myocardial layer in the process of trabeculation (Icardo and Fernandez-Teran, 1987). These findings suggest that Ets-2 has a primary role in the regulation of urokinase production, and that changes in Ets-2 gene expression may be responsible for the differing levels of enzyme activity found in the heart at specific times and locations during development.

Studies have shown that the Ets proteins are also expressed in a spatially and temporally restricted pattern in the developing mouse embryo (Bhat et al., 1987; Kola et al., 1993; Maroulakou et al., 1994). The importance of these proteins to normal development is illustrated by the recent studies of Sumarsono et al. (1996) in which the Ets-2 protein was overexpressed in transgenic mice. The mRNA for Ets-2 in the transgenic animals was found to be 1.1–1.8 fold higher in some organs compared to controls. Mice expressing higher levels of Ets 2 were found to consistently display abnormalities of the musculoskeletal system similar to those found in humans with Down's syndrome. Previous studies of the normal developing embryo have demonstrated the transient expression of Ets-2 in bone and cartilage. Following the differentiation of these tissues, levels of Ets-2 are markedly reduced suggesting that sustained elevated levels of this protein may be deleterious to normal skeletal morphogenesis (Maroulakou et al., 1994).

The Ets-2 gene has been localized within a cluster of genes on the distal region of human chromosome 21 and is also present in a similar region of chromosome 16 in the mouse (Watson et al., 1986). This group of conserved genes has been defined as the minimal Down's syndrome region and is sufficient to generate the recognizable features of the disorder (Sacchi et al., 1988; Korenberg, 1991). In addition to skeletal dysmorphogenesis, another consequence for humans with trisomy 21 and mice with trisomy 16, is the failure of the endocardial cushion tissues of the heart to fuse resulting in an atrioventricular septal defect (AVSD) (Marino et al., 1990; Miyabara, 1990). A study by Hiltgen et al. (1996) has demonstrated that the number and density of cardiac mesenchymal cells is significantly reduced in the endocardial cushions of the Trisomy 16 mouse, and postulate that the high incidence of AVSD in this model may be due to an alteration in the epithelial-mesenchymal transformation of the endocardium and the subsequent activities of the cardiac mesenchyme.

These studies all suggest that Ets-2 plays an important role in normal development and that alterations in the expression of Ets-2 may lead to alterations in the normal morphogenesis of specific organ systems. It remains unclear however, what developmentally important genes are regulated by this transcription factor. The results of the present study demonstrate a role for the Ets-2 protein in the regulation of urokinase production in the cardiac mesenchyme of the developing endocardial cushions. Urokinase plays important roles in tissue remodeling, regulation of cell migration, and the activation and release of specific growth factors. We would hypothesize therefore, that alterations in the levels of Ets-2, and subsequent changes in urokinase production, may affect some or all of these processes leading to aberrant morphogenesis of the tissues in which these proteins are expressed.

4. Experimental procedures

4.1. Embryos and cell cultures

Hearts were collected from quail embryos (Coturnix coturnix japonica) at stages 12, 18 and 23 of development (Zacchei, 1961). Extracts or total RNA were prepared from whole hearts, atrioventricular canals, outflow tracts, and ventricles. Endocardially derived mesenchymal cells were isolated from AVC explants of 50–56 h quail embryos as described (Bernanke and Markwald, 1982; McGuire and Orkin, 1992). Cells from the primary explant were subcultured onto coverslips and grown to confluence in M199 medium containing 10% fetal calf serum and antibiotics. The identity of the cells was confirmed using the QH1 monoclonal antibody (Pardanaud et al., 1987) which positively labeled >98% of the isolated cells.

4.2. Luciferase analysis

The fragments of the urokinase promoter corresponding to bp −1348 to +139 and bp −1186 to +139, including the TATA box, were excised from the pGC20 plasmid and directionally ligated into the luciferase reporter plasmid pGL-2 Basic (Promega) yielding the constructs pGL-2-UK1.5 and pGL-2-UK1.3. A third fragment of the urokinase promoter from bp −752 to +14, was synthesized via PCR using the pGC20 plasmid as a template and ligated into pGL-2 Basic yielding the construct pGL-2-UK0.76. The construct containing the PCR amplified fragment was sequenced using Sequenase 2.0 to confirm that no PCR-induced mutations had occurred.

Mutagenesis of Ets consensus sequences in the avian urokinase promoter was carried out using either the Quick Change or the Chameleon Double-Stranded Site Directed Mutagenesis Kits (Stratagene) and pGL-2-UK1.5. The purine rich core sequence 5′GGA3′ was mutated to 5′TTG3′ or the consensus sequence was deleted. Plasmids were sequenced to confirm the presence of a mutation in the Ets consensus region.

Endocardial derived mesenchymal cells were subcultured onto 15 mm plastic coverslips and grown to 80–90% confluence. Each coverslip of cells was transiently cotransfected using optimum concentrations of lipofectamine (20 μg; GibcoBRL), 3.0 μg of luciferase reporter plasmid and 1.5 μg of pSV-β-galactosidase plasmid (Promega) in serum free medium. After 6 h the cells were rinsed twice with serum free medium and 1 ml of the complete growth medium was added. The cells were then incubated at 37°C in a CO_2 incubator for 18–24 h, collected and assayed for luciferase and β-galactosidase activity. Cells were washed and harvested in PBS, pelleted, and resuspended in lysis buffer (0.1 M KPO_4, 1 mM DTT, pH 7.8) followed by freeze/thawing in liquid nitrogen and a 37°C water bath. The cell lysates were assayed for luciferase activity using a Lumat Luminometer (Berthold). Each luciferase assay sample contained 100 μl of the cell lysate, 100 μl of the luciferase assay reagent (5 mM γ ATP, 15 mM $MgSO_4$, 0.1 M KPO_4, pH 7.8) and 100 μl of 1 mM luciferin in 0.1 M KPO_4, 1 mM DTT at pH 7.8. Luciferase activity was measured over a period of 15 s. β-Galactosidase was assayed in cell lysates, with o-nitrophenyl-β-D-galactopyranoside in 0.1 M phosphate buffer, at 37°C for 30 min to 2 h. The activity was determined at 420 nm using commercially available β-galactosidase (Sigma) as the standard. Luciferase assay results were normalized to β-galactosidase activity for efficiency of transfection. The data from these and subsequent experiments was analyzed by Student's *t*-test.

4.3. Gel shift analysis

Oligonucleotide B was used for gel shift analyses. This oligonucleotide, corresponding to bp –868 to –847 of the urokinase promoter contains a consensus Ets binding site (5′CCAACCGCAGGAAACCTGCCTC3′) which when mutated caused a significant reduction in promoter activity. Double stranded probes were produced for the gel shift assays by heating complementary oligonucleotides at 80°C for 10 min in the presence of 200 mM NaCl and cooling slowly to 37°C. Fifty nanograms of each double stranded oligonucleotide was 5′ end labeled with [γ^{32}P]-ATP (ICN, California) using 500 U bacterial T4 polynucleotide kinase (Promega).

Extracts were prepared from mesenchymal cells and whole hearts by homogenization at 4°C in extraction buffer (20 mM HEPES pH 7.8, 450 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 25% Glycerol] containing the protease inhibitors leupeptin and PMSF (20 μg/ml). Protein concentrations of the extracts were determined using the micro-BCA protein assay (Pierce).

For EMSA analysis 7.5–20 μg of extract was added to 30 000 cpm of [^{32}P]-end labeled oligonucleotide in binding buffer (20% Glycerol, 40 mM Tris–Cl pH 7.9, 100 mM KCl, 2 mM EDTA, 2 mM DTT, 0.1% NP-40, 0.01% poly dI–dC) and incubated for 30 min on ice. For some experiments a 25–200 fold molar excess of cold, unlabeled oligonucleotide was added to the binding reactions with the labeled oligonucleotide. In other experiments, 100 ng of Ets antibody (Santa Cruz), or non-immune rabbit IgG were added to reactions 30 min prior to the addition of the labeled oligonucleotide. Samples were electrophoresed on an 8% native polyacrylamide gel in 1 × TBE at 35 mA for 1 h, dried, and exposed to x-ray film at –70°C. Quantitation of autoradiograms was done using the NIH Image Analysis software.

4.4. UV-crosslinking

In order to partially characterize the proteins binding in the EMSA reactions, 20 μg of whole cell or tissue extract was incubated with [^{32}P]-end labeled oligonucleotide B in a mobility shift binding reaction as previously described. After 30 min, the samples were crosslinked by UV irradiation for 1 h at a distance of 15 cm from the light source in a Stratalinker (Stratagene). The samples were electrophoresed on a 7.5% SDS-polyacrylamide gel under reducing conditions, and exposed to x-ray film at –70°C for 24–48 h. In some experiments, 100 ng of the Ets antibody was added to the binding reaction prior to UV-crosslinking.

4.5. RT-PCR analysis

First strand cDNA was prepared from 0.5 μg total RNA using an oligo dT primer and Superscript reverse transcriptase (GibcoBRL). For semi-quantitative PCR, 1 μl of each first strand reaction was amplified using oligonucleotide primers specific for chicken Ets-1 (1141 bp product), chicken Ets-2 (1199 bp product), and chicken GAPDH (300 bp product). Standard PCR amplification was carried out at 94°C, 1 min, 60°C, 1 min, 72°C, 1 min for 27 cycles, which was determined to be within the linear range of product amplification. Upon completion, 10 μl of the reactions were analyzed by agarose gel electrophoresis and ethidium bromide staining to determine the presence or absence of transcripts specific for Ets-1 and/or Ets-2 in the various cells and tissues, as well as the levels of Ets-1 and Ets-2 relative to the control transcript GAPDH. All products were subcloned into the pCR2.1 cloning vector (InVitrogen) and sequenced. Quantitation of band density was accomplished using the NIH Image analysis software. Primers for these analyses are as follows:

Chicken GAPDH:	5′ACCATCAAGTCCACAACACG3′
	5′AAGGTCATCCCAGAGCTGAA3′
Chicken ETS-1:	5′ATTGGCAGCACGGGTCCTTATC3′
	5′TGAGCAGCTCCAGCAGGAATTG3′
Chicken ETS-2:	5′CGCTAAATGAAGATCAAACGCTCC3′
	5′CGAGGTCTTGTGGATGATGTTCTTG3′

4.6. In situ hybridization

Stage 18 quail embryos were fixed with 4% paraformaldehyde, frozen in OCT compound, and sectioned. Sections were

treated with proteinase K (20 μg/ml) in PBT (PBS with 0.1% Tween 20) for 5 min at room temperature and washed with 2 mg/ml glycine in PBT. Sections were subsequently treated with 0.1% sodium borohydrate in PBT, washed, and prehybridized in HYB solution (50% formamide, 0.75 M NaCl, 10 mM PIPES, pH 6.8, 1 mM EDTA, 100 μg/ml tRNA, 0.05% heparin, 0.1% BSA, and 1% SDS) for 1 h at 55°C. The sections were incubated overnight at 55°C in HYB solution containing 1 μg/ml of Dig-labeled sense or antisense riboprobe. Following hybridization, the sections were treated with 100 μg/ml of RNase A for 5 min at room temperature, and washed with solutions of SSC at increasingly higher stringency. Sections were blocked with 10% goat serum in PBT for 1 h and incubated overnight at 4°C with a 1:2000 dilution of goat anti-Dig antibody conjugated to alkaline phosphatase (Boehringer Mannheim). Sections were washed extensively with PBT and equilibrated in AP buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, and 0.1% Tween-20) containing 2 mM levamisol. Alkaline phosphatase was detected using NBT and BCIP following a 5 h incubation at room temperature. Dig-labeled sense and antisense riboprobes were synthesized from a linearized quail Ets-2 plasmid using a Dig-nucleotide mixture (Boerringer Mannheim) and the Riboprobe Gemini Kit (Promega).

4.7. PCR cloning and overexpression studies

PCR primers were designed to amplify either the full-length Ets-2 cDNA (5′TGTGCCACATCGAGGATATTTTGGC3′ and 5′ATCACAGTTGCACCCATCCGAACAG3′) or a truncated Ets-2 cDNA encoding only the DNA binding domain and nuclear localization signals (5′CCATCGATGGCTCAGTCCTCGGTGTCAGG3′ and 5′CCATCGATATGGGCAGTGGACCTATACAG3′). RNA was extracted from stage 18 hearts and first strand cDNA was prepared using an oligo dT primer and Superscript reverse transcriptase. PCR reactions were set up containing 1 μl of first strand cDNA, 200 μM of each dNTP, the appropriate primer pairs, and 2.5 U of Expand High Fidelity polymerase (Boehringer Mannheim). The PCR conditions consisted of 27 cycles of 60 s at 94°C, 60 s at 60°C, 60 s at 72°C, and a final extension at 72°C for 10 min. The PCR products were analyzed on a 1.2% agarose gel and the products ligated into the pCRII cloning vector (InVitrogen). Sequencing of the PCR products was performed using the dideoxy-chain termination method and Sequenase 2.0 (Amersham).

For expression studies, the Ets-2 cDNAs were subcloned into the pSI expression vector, and transfected into isolated cushion tissue cells along with the luciferase reporter plasmid and the pSV-βgal plasmid. Cells were collected 24 h later and analyzed for luciferase and β-gal activities as described above.

4.8. Retrovirus construction

The RCASBP(A) retroviral vector and adaptor plasmids were obtained from Dr. Stephen Hughes (NCI-Frederick Research and Development Center) and used for the production of replication-competent retrovirus. The full-length Ets-2 and truncated Ets-2 cDNAs were ligated into the pCla12 adaptor plasmid and subsequently subcloned into the Cla1 site of RCASBP(A). For the preparation of retrovirus, the recombinant RCAS plasmids were transfected into Line 0 chicken embryo fibroblasts (USDA-ARS) using lipofectamine and standard transfection procedures. The conditioned medium from transfected cells at passage 5 or 6 was collected and stored at −80°C. Virus-containing medium was added to isolated cushion tissue cells and 24 h later the cell layer and medium were collected and analyzed for urokinase activity as described above.

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