INTRODUCTION

The transforming growth factor β (TGFβ) superfamily comprises a large group of structurally related proteins thought to be major regulators of normal growth and development (Roberts and Sporn, 1990). Nucleotide sequence analysis of the various gene members reveal two major subfamilies. The TGFβ subfamily includes three mammalian TGFβs (1-3) and the activins/inhibins (Roberts and Sporn, 1990). The DVR (Decapentaplegic-Vg-Related) subfamily (Lyons et al., 1991) consists of the Xenopus Vg-1 gene, Drosophila dpp and 60A (Doctor et al., 1992) genes, GDF-1 and six mammalian bone morphogenetic protein genes (BMPs 2, 3, 4, 5, 6 and 7).

In mammals, the temporal and spatial expression patterns of the three TGFβ genes have been studied extensively during embryogenesis, using in situ hybridisation and immunohistochemistry on serial sections, and whole-mount in situ hybridisation to complete embryos. TGFβ3 RNA was not seen in any of the tissue sections, but very low levels of the RNA were seen by whole-mount in situ hybridisation around the outflow tract of the heart at 8.5 days post coitum. TGFβ2 RNA is expressed at high levels in all cells with the potential to differentiate into cardiomyocytes. Additionally, the foregut endoderm, juxtaposed to the heart, and the neuroepithelium at the rostral extremity of the foregut, express very high levels of TGFβ2 RNA, between 8.5 and 9.5 days post coitum. As cardiomyogenesis proceeds, TGFβ2 RNA levels diminishes within the myocytes, with a concomitant increase in staining for TGFβ2 protein. TGFβ2 protein staining of cardiomyocytes persists throughout development and in the adult, in the absence of detectable levels of the corresponding RNA. Superimposed upon this myocardial pattern of expression, there is an upregulation of TGFβ2 RNA in the myocardium of the outflow tract and atrioventricular canal between 8.5 and 9.5 days post coitum, which returns to low levels by 11.5 days post coitum. The results are discussed in terms of a potential role of TGFβ2 in controlling cardiomyogenesis and in inductive interactions leading to cardiac cushion tissue formation.

Key words: TGFβ, embryogenesis, cardiogenesis, myogenesis, inductive interactions, in situ hybridisation
was added to a co-culture of chick AV endothelial cells with ventricular myocardium, in an in vitro collagen gel system, the AV endothelial cells were induced to undergo endothelial-mesenchymal cell transformation. If TGFβ1 was omitted the transformation did not occur. More recently, they have investigated this phenomenon further using modified anti-sense oligonucleotides designed to block translation of each of the four TGFβ chick isoforms specifically in this in vitro collagen gel system (Potts et al., 1991). Transformation of the AV endothelial cells was only blocked by addition of a TGFβ3 anti-sense oligonucleotide, whereas the other oligonucleotides did not interfere with this process. In the chick, TGFβ3 has thus been postulated as contributing to the myocardial induction signal.

In the mouse, we have shown that TGFβ1 RNA is expressed ubiquitously in immature endothelial cells and that, in the heart, this endothelial expression becomes limited, with time, to cells overlying cardiac cushion tissue (Akhurst et al., 1990). More interestingly, we have seen a highly restricted expression pattern of TGFβ2 RNA within the myocardium of AV and outflow tract regions of the heart, implicating this isoform as a candidate for part of the myocardial induction signal (Millan et al., 1991). It therefore appears, from the available data, that there are differences in TGFβ isoform utilisation in cardiogenesis between the chick and mouse.

To further our understanding of the role of TGFβs in mouse cardiogenesis, we have performed a detailed analysis of the expression of each of the three TGFβ isoforms in mouse embryos at earlier developmental stages than previously examined. Manova et al. (1992) have already presented preliminary data on the distribution of TGFβ2 RNA in 5.5 to 9.5 day p.c. embryos, though they made little comment on TGFβ2 expression with respect to cardiogenesis. In the present study, the localisation of TGFβ2 RNA is also compared with the translated polypeptide. By following the development of one system in detail, namely the heart, we are able to make tentative conclusions as to the cellular and molecular mechanisms establishing differential expression patterns for the RNA and protein, and the possible functions of TGFβ2 in this developing organ.

Preliminary observations relating to the current study have recently been discussed in a review (Akhurst et al., 1992).

**MATERIALS AND METHODS**

**Mouse stocks**

Mouse embryos were obtained from Parkes × NIH F1 females mated with NIH males. Noon on the day on which the copulation plug was found was considered as 0.5 days p.c. All embryos were fixed overnight in ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS), and then dehydrated and embedded in paraffin for use in immunohistochemistry and radioactive in situ hybridisation. Embryos to be used for whole-mount in situ hybridisation were dissected from their membranes, fixed as above, then dehydrated and rehydrated through a series of methanol and PBS containing 0.1% Tween-20, before following the protocol of Wilkinson (1992).

Staging of embryos for descriptive purposes was done according to Kaufman (1992), using plate numbers taken from his ‘Atlas of Mouse Development’. This allowed a very precise system of staging, which we considered essential for the description of the very rapidly developing heart.

**Riboprobe synthesis**

The TGFβ1-specific riboprobe has previously been described by Akhurst et al. (1990). The TGFβ2-specific and TGFβ3-specific probes were described by Millan et al. (1991). The mouse cardiac α-actin riboprobe, a BamHI fragment containing the first non-coding exon of the gene from nucleotides −46 to +127, was kindly provided by M. Buckingham and is described by Sassoon et al. (1988). Various negative control probes were used, which were all sense riboprobes to mammalian or viral mRNAs.

35S-labelled antisense riboprobes were generated to a specific activity of 109 disintegrations/minute/µg using T3 or T7 polymerase, digested to an average length of 100 nucleotides by controlled alkaline hydrolysis and used at a final concentration of 30 pg/µl in the hybridisation mix.

Non-radioactive probes were synthesised using digoxigenin-labelled UTP, according to the protocol of Wilkinson (1992). These were not hydrolysed by alkali.

**In situ hybridisation to serial sections**

In situ hybridisation to 7 µm sections were performed at very high stringency as previously described (Akhurst et al., 1990). Autoradiographic exposure times were between 3 and 12 days. After development, slides were stained in haematoxylin and mounted. Photomicrography was performed using an Olympus BH-2 microscope and Ilford Pan F film.

**Whole-mount in situ hybridisations**

At least 10 embryos of each stage, of 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5 days p.c., were probed with either control probe, TGFβ1-, TGFβ2- or TGFβ3-specific digoxigenin-labelled antisense riboprobes, synthesised using T3 or T7 polymerases. Briefly, endogenous peroxidase in the embryos was blocked by washing in 6% hydrogen peroxide, followed by digestion with 10 µg/ml proteinase K. Embryos were then fixed in fresh 0.2% glutaraldehyde/4% paraformaldehyde before prehybridisation in 50% formamide, 5× SSC pH 4.5, 50 µg/ml yeast tRNA, 1% SDS, 50 µg/ml heparin at 70°C. For hybridisation, the digoxigenin-labelled RNA probes were added at 1 µg/ml.

Washes were performed as follows: twice in solution 1 (50% formamide, 5× SSC pH 4.5, 1% SDS) for 30 minutes at 70°C; once in a 1:1 mixture of solution 1 : solution 2 (0.5 M NaCl, 10 mM TrisHCl, pH 7.5, 0.1% Tween-20) for 10 minutes at 70°C; three times with solution 2 for five minutes at room temperature. The embryos were then treated twice with 100 µg/ml RNase A in solution 2 for 30 minutes at 37°C; washed twice with solution 2 for 5 minutes at room temperature and twice in 50% formamide, 2× SSC, pH 4.5 for 30 minutes at 65°C. Non-specific binding of the antibody to the embryos was blocked with 10% sheep serum before an overnight incubation at 4°C in preabsorbed alkaline phosphatase-conjugated sheep anti-digoxigenin antibody. The embryos were washed extensively before the colour reaction for alkaline phosphatase was initiated by the addition of 5-bromo-4-
chloro-3-indolyl-phosphate and 4-nitroblue-tetrazolium chloride. The embryos were left overnight and photographed using an Olympus stereomicroscope and Kodak Ektacolour Gold 160 film.

It was noted that this technique was slightly more sensitive than radioactive in situ hybridisation to sectioned material, presumably because of the larger mass of cellular material available for hybridisation.

**Immunohistochemistry**

A polyclonal antibody against TGFβ2 was raised in rabbits against the first 29 amino acid N-terminal portion of human TGFβ2. The specificity of the antibody has been previously determined by Western blot and ELISA assay, as described by Van den Eijnden-Van Raaij et al. (1990).

Sections adjacent to those hybridised with the TGFβ2 cRNA probe, were subjected to immunohistochemistry with the above anti-TGFβ2 polyclonal antibody, or with control preimmune serum or purified IgG, all at an equivalent IgG concentration of 20 μg/ml. Antibodies were localised to the above sections using an avidin-biotin peroxidase detection system (ABC, Dakopatts). After blocking endogenous peroxidase with 3% hydrogen peroxide, non-specific antibody binding was blocked with donkey serum (0.3%), mouse serum (0.3%) gelatin (0.1%) and BSA (0.1%) in PBS. The TGFβ2 antibody was applied in the blocking cocktail and left overnight at 4°C. The secondary antibody was biotinylated donkey anti-rabbit (Amersham) and the ABC system protocol was followed using diaminobenzidine as the chromogen. Sections were counterstained in haematoxylin, examined using an Olympus BH-2 microscope and photographed using Kodak Ektacolour Gold 160 film.

**RESULTS**

We investigated the expression of TGFβ2 and TGFβ3 during early postimplantation mouse development from 6.5 to 12.5 days p.c. Radioactive anti-sense cRNA probes, specific for TGFβ2 and TGFβ3 (Millan et al., 1991), were hybridised to serial 7 μm transverse sections of mouse embryos to examine RNA localisations. A TGFβ1-specific gene probe (Akhurst et al., 1990) was used as a positive control, and a sense probe as a negative control. Intense expression of TGFβ2 RNA is seen in the regions of the embryo involved in heart formation, as detailed below. Expression of TGFβ3 RNA is not observed in any embryonic structure over this period of 72 hours using this technique, though a narrow band of decidual tissue adjacent to the muscular uterine wall expresses this RNA.

**Expression of TGFβ2 RNA in the cardiogenic plate**

We previously demonstrated that TGFβ1 RNA is expressed in the cardiac mesoderm cells within the heart at 7.0 days p.c. (Akhurst et al., 1990). In this study, TGFβ2 RNA expression, like that of TGFβ1, is seen in the allantois of late primitive streak stage embryos. However, no other expression of TGFβ2 RNA was seen in eight embryos that had been completely serial-sectioned, ranging from the advanced egg cylinder stage, 6.5 days p.c., to the late primitive streak stage, 7.2 days p.c. (Kaufman, 1992, plates 3-7). This is despite detection of very high levels of TGFβ2 RNA within the ciliated uterine epithelium around the decidual mass (Fig. 3A,B), indicating that the technique had worked satisfactorily. In two advanced primitive streak-stage embryos, examined by whole-mount in situ hybridisation, two regions of low TGFβ2 RNA expression were discernible, subjacent and lateral to the anterior neural groove (data not shown). It was not possible to discern, from the whole mounts, whether this is endodermal and/or mesodermal expression. Additionally, the allantois of these embryos was seen to express TGFβ2 RNA.

At around 7.25-7.5 days p.c., in the early head fold pre-somite stage embryo, the intraembryonic mesoderm, both rostrally and laterally, splits to form the intraembryonic coeloma (Kaufman and Navaratnam, 1981; Viragh and Challice, 1973; DeRuiter et al., 1992). The dorsal lining of somatic mesoderm forms a squamous mesothelial epithelium. The ventral splanchnic mesoderm differentiates into a cuboidal epithelium, the cardiogenic plate or promyocardium. Prior to foregut invagination, the cardiogenic plate extends in a crescent rostrally and ventrolaterally to the neural fold. Endothelial cells, which are known to express TGFβ1 (Akhurst et al., 1990), invade ventral to the cardiogenic plate (Kaufman and Navaratnam, 1981; Viragh and Challice, 1973; DeRuiter et al., 1992). Five serially sectioned embryos were examined over this period (Kaufman, 1992, plates 7-9). A low level of TGFβ2 RNA expression is seen in the promyocardium of two embryos at the 1- to 2-somite stage (Fig. 1B), and in a further two at the 4- to 5-somite stage. This TGFβ2 RNA expression within the cardiogenic region of presomite embryos is more clearly seen by whole-mount in situ hybridisation (Fig. 2A-D). As expected (Akhurst et al., 1990), TGFβ1 RNA is seen in the forming endocardial tube at this stage by whole-mount in situ hybridisation (Fig. 2E,F). Immunohistochemistry on embryo sections reveals no TGFβ2 protein staining within the embryo, despite staining of maternal decidual tissue and endometrial gland epithelium (data not shown).

**Formation of the primitive cardiac tube**

Between 7.5 and 8.0 days p.c., invagination of the foregut endoderm commences. Consequently, the cardiogenic region rotates through 180°, as it is pulled in a dorsocaudal direction with the foregut. Rostrally, the relative position of myocardium and endocardium are inverted, and the myocardium comes to engulf the endocardial cells to form the relatively symmetrical, but dorsally open, primitive heart tube (DeRuiter et al., 1992). The myocardium is still continuous along its length with the splanchnic epithelium, which is now on its dorsal side (dorsal mesocardium). The splanchnic epithelium continues to differentiate into early cardiomyocytes (Viragh and Challice, 1973).

Eight 5- to 7-somite embryos (Kaufman, 1992 plate 10) were examined by serial sectioning. Intense expression of TGFβ2 RNA is seen in the caudal splanchnic epithelium, lining the left and right pericardio-peritoneal coeloma (Fig. 1E,F). In continuity with this, the promyocardium of the vitelline veins and sinus venosus also express TGFβ2 RNA, but at lower levels (Fig. 1G-J). There is a rostrocaudal gradient of TGFβ2 RNA expression within the primitive heart tube, such that rostrally, in the outflow tract and primitive ventricle, TGFβ2 RNA levels are barely detectable (Fig. 1K,L). Intense TGFβ2 RNA expression is seen in tissues all around the pericardial cavity, including splanchnic and somatic epithelia, and the ventral foregut endoderm and
The expression of TGFβ1 (C,D) and TGFβ2 (A,B,E-N). (A) Sagittal section through a precardiac mouse embryo to show morphology of the heart at low magnification (Kaufman, 1992, plate 64-d-f); (B) Transverse section of similar embryo at higher magnification to show TGFβ2 RNA expression restricted to the promyocardium (Kaufman, 1992, plates 7, 8). (C-L) Serial transverse sections through a 8.0 day p.c. embryo (Kaufman, 1992, plate 10), from a caudal to rostral direction. Expression of TGFβ1 is shown in the endothelial components of the vitelline veins and heart (C,D). TGFβ2 RNA is seen in the splanchnic epithelium of the pericardio-peritoneal coeloma (E,F), the ventral foregut endoderm, vitelline veins, somatic and splanchnic epithelia (G,H), and the promyocardium of the sinus venosa (I,J). Note also the expression of TGFβ2 in the budding thyroid diverticulum (IJ) and, at much lower levels in the promyocardium of the primitive ventricle (K,L). (M,N) A slightly later stage embryo (Kaufman, 1992, plate 11) sectioned transversely to show TGFβ2 expression in the myocardial cells of the primitive heart tube and the dorsal mesocardium. ce, coelomic epithelium; cj, cardiac jelly; d, decidua; dm, dorsal mesocardium; e, endocardium; fe, foregut endoderm; hm, head mesenchyme; m, myocardium; nc, non-cellular material which binds non-specifically to radioactive probe; nf, neural fold; pm, promyocardium; psm, primitive streak mesoderm; pv, primitive ventricle; se, splanchnic epithelium; sm, somatic mesoderm; sv, sinus venosa; td, thyroid diverticulum; vv, vitelline vein. Scale bar in A, 100 µm. Scale bar in B, 50 µm. Scale bar in C-M (shown in E), 200 µm.

Fig. 1. TGFβ2 expression in the cardiogenic plate. Bright-field and dark-field images of sections hybridised with gene probes specific for TGFβ1 (C,D) and TGFβ2 (A,B,E-N). (A) Sagittal section through a precardiac mouse embryo to show morphology of the heart at low magnification (Kaufman, 1992, plate 64-d-f); (B) Transverse section of similar embryo at higher magnification to show TGFβ2 RNA expression restricted to the promyocardium (Kaufman, 1992, plates 7, 8). (C-L) Serial transverse sections through a 8.0 day p.c. embryo (Kaufman, 1992, plate 10), from a caudal to rostral direction. Expression of TGFβ1 is shown in the endothelial components of the vitelline veins and heart (C,D). TGFβ2 RNA is seen in the splanchnic epithelium of the pericardio-peritoneal coeloma (E,F), the ventral foregut endoderm, vitelline veins, somatic and splanchnic epithelia (G,H), and the promyocardium of the sinus venosa (I,J). Note also the expression of TGFβ2 in the budding thyroid diverticulum (IJ) and, at much lower levels in the promyocardium of the primitive ventricle (K,L). (M,N) A slightly later stage embryo (Kaufman, 1992, plate 11) sectioned transversely to show TGFβ2 expression in the myocardial cells of the primitive heart tube and the dorsal mesocardium. ce, coelomic epithelium; cj, cardiac jelly; d, decidua; dm, dorsal mesocardium; e, endocardium; fe, foregut endoderm; hm, head mesenchyme; m, myocardium; nc, non-cellular material which binds non-specifically to radioactive probe; nf, neural fold; pm, promyocardium; psm, primitive streak mesoderm; pv, primitive ventricle; se, splanchnic epithelium; sm, somatic mesoderm; sv, sinus venosa; td, thyroid diverticulum; vv, vitelline vein. Scale bar in A, 100 µm. Scale bar in B, 50 µm. Scale bar in C-M (shown in E), 200 µm.

TGFβ2 in heart development

Regionalisation of the cardiac tube

Between 8.0 days p.c. (5-7 somites) and 8.5 days (11-13 somites) p.c., as the embryo undergoes ‘turning’, the heart expands rapidly, bends and dilates, due to differential growth rates along the tube, and constraints imposed by the pericardial cavity. Over this period, the dorsal mesocardium degenerates along the length of the myocardium, remaining in continuity with the heart only at the caudal and rostral ends.

Eight serially sectioned embryos, at various stages of turning (Kaufman, 1992, plates 11-14), were examined by in situ hybridisation for TGFβ1, TGFβ2 and TGFβ3 RNA expression and by immunohistochemistry for TGFβ2 polypeptide (data not shown). TGFβ2 RNA persists in the neural epithelium and ventral foregut endoderm apposed to the heart. It is present along the length of the coelomic epithelium, including that which feeds into the inflow and outflow regions of the myocardium. Thus, mesodermal expression is seen around the first branchial arch arteries, and in the myocardium of the aortic sac and outflow tract. Ventricular and atrial myocardia do not show strong hybridisation with the TGFβ2 probe, though this RNA is present at high levels in the sinus venosus. At about this time in development, the septum transversum appears as a proliferation of splanchnopleuric mesoderm ventrocaudally to the heart, and continuous with the myocardium. This structure ultimately contributes to both the liver and diaphragm, as it is invaded successively by the hepatic diverticulum and by skeletal myoblasts (Kaufman, 1992). The septum transversum is the richest source of TGFβ2 RNA at this stage. These results were confirmed by whole-mount in situ hybridisation (Fig. 5A-C).

TGFβ2 immunohistochemistry, performed on adjacent sections, reveals disparity between the localisation of RNA and polypeptide, the two patterns being almost mutually exclusive. TGFβ2 polypeptide staining is only observed in cardiomyocytes of the bulbus cordis, ventricle and atrium, whereas the neural epithelium, foregut endoderm, coelomic epithelium and sinus venosus showed no staining. The septum transversum contained very low levels of immunohistochemically detectable material (Fig. 4F).

Nine serially sectioned embryos were examined spanning 8.5 days to 9.5 days p.c. (Kaufman, 1992, plates 15-19), by which time regionalisation of the heart is even more pronounced, with very clear divisions between bulbus cordis, primitive ventricle, atrium and sinus venosus. At around 8.5 days p.c., the acellular cardiac jelly, which forms between endocardium and myocardium, remains pronounced only within the regions of the outflow tract and AV junction. By 9.0-9.5 days p.c., mesenchymal cells have begun to delaminate from the endocardium within the AV canal and proximal outflow tract, before invading the underlying cardiac jelly to generate cardiac cushion tissue (Fig. 4). In these two regions, expression of TGFβ2 RNA is upregulated in the myocardium underlying cardiac jelly/cushion tissue, whereas in the atrium and ventricle, myocardial TGFβ2 RNA expression remains at low levels (Fig. 4).

At these stages, TGFβ2 RNA is still expressed at high levels in the ventral endoderm of the gut, coelomic epithelium, enlarged septum transversum, distal sinus venosus and at a lower level in the ventral floor plate of the neural tube. At 9.5 to 10.5 days p.c., the caudal portion of pharyngeal endoderm within the first branchial pouch expresses high levels of TGFβ2 RNA (data not shown), as does the...
lateral plate mesoderm of the body walls which extend caudally from this pharyngeal region. This is seen as two 'rods' of expression, on either side of the heart, in whole-mount in situ hybridisation (Fig. 5E-H). At this stage, TGFβ2 hybridisation to the mesoderm of the forelimb buds is also seen (Fig. 5G,H). Hepatic cells invading the septum transversum, do not express TGFβ2 RNA (data not shown).

Adjacent sections, subjected to immunohistochemistry with the TGFβ2 antibody (Fig. 4), demonstrate a similar qualitative pattern of TGFβ2 protein distribution to that

Fig. 2. Early cardiac expression of TGFβ1 and TGFβ2 expression. Whole-mount in situ hybridisation of early head fold stage embryo (Kaufman, 1992, plate 9) showing expression of TGFβ2 RNA in the epithelia of the coelomic cavity (A-D). TGFβ1 RNA is seen in the endocardial cells of the early heart (E,F). Bright-field and dark-field images of an 8.0 day p.c. (Kaufman, 1992, plate 10) hybridised with TGFβ2, showing expression in the epithelia of the coelomic cavity and in the neural groove. The allantois and some somites are also positive for TGFβ2 (G,H). (I) Three embryos of 8.0-8.25 days p.c. (Kaufman, 1992, plate 11) showing low myocardial expression in the early heart tube. a, allantois; ce, coelomic epithelium; e, endocardial cells; efl, early foregut invagination; ht, heart tube; ne, neural epithelia; s, TGFβ2 expressing somites. Scale bar, 160 µm.
seen at 8.5 days p.c., namely in cardiomyocytes only. At this stage, there is clearly a gradient of protein concentration, with highest levels in the most differentiated ventricular myocardium, and diminishing quantities towards the outflow tract and sinus venosus regions, respectively (e.g. Fig. 4C,F). At the distal end of the outflow tract, some myocardial cells express both the protein and RNA, as do the myocardial cells of the AV region. TGFβ2 protein was also detected at low levels in the mid-gut endothelium (Fig. 4L).

TGFβ3 RNA expression is not observed by in situ hybridisations to tissue sections; however, a very low level of expression is detected in cells around the outflow tract of 8.5-9.0 day p.c. embryos by whole-mount in situ hybridisation (Fig. 5C). Though it is difficult to distinguish cell type, in whole mounts, this hybridisation signal is probably in early pericardial cells (Millan et al., 1991).

Whole-mount in situ hybridisation with the TGFβ1 probe at 8.5 days p.c. shows hybridisation to the endocardium, and to the capillary networks forming around the somites and in the head mesenchyme (Fig. 5C), as would be expected from our previous work (Akhurst et al., 1990). At 9.0-9.5 days p.c., and on sectioned material at 10.5 days p.c., TGFβ1 hybridisation was seen in the endocardium of the AV region and outflow tract and, at a lower level, in the endothelial lining of the ventricular trabeculae (data not shown), which was not noted in our earlier study (Akhurst et al., 1990).

**Completion of cardiac septation**

By 10.5 days p.c., the AV cushions have expanded by mesenchymal cell proliferation and are beginning to fuse, contributing to the septation of both the atria and the ventricles. Three embryos were examined at this stage. As reported earlier (Millan et al., 1991), elevated myocardial TGFβ2 RNA expression persists in the region of the AV canal and outflow tract, although the intensity of the signal is reduced relative to that 24 hours earlier (Fig. 6A-C). By
Fig. 4
11.5 days p.c., TGFβ2 RNA expression was greatly diminished and restricted to a narrow band of specialised cardiomyocytes adjacent to the cushion tissue (Fig. 6D-F), some of which contribute to the AV conduction system (Viragh and Challice, 1977a,b). The septum transversum still expresses low levels of TGFβ2 RNA. By 12.5 days p.c., there is no longer any expression of TGFβ2 RNA within the myocardium, although the mesenchymal cells of the cushion tissue now express low levels of this RNA (Fig. 6G,H and Millan et al., 1991). Immunohistochemistry performed on 10.5 to 12.5 days p.c. hearts, demonstrates the presence of TGFβ2 protein in the entire myocardium, with absence of staining of the mesenchyme (Fig. 6LJ). Cardiomyocyte staining with the TGFβ2 antibody persists into the adult (data not shown).

**TGFβ2 expression during early skeletal myogenesis**

During this study, we noticed that a number of somites, from as early as 8.25 days p.c., express TGFβ2 RNA (see Figs 2G, 5A,C). Furthermore, it was noticeable that the TGFβ2 antibody stained muscle in the uterine wall (Fig. 3C), and in the body wall of later embryos (data not shown). To investigate the possibility that TGFβ2 might be involved in skeletal myogenesis, we performed in situ hybridisation on serial transverse sections of 10.5, 11.5 and 12.5 days p.c. embryos, in the region of the cervical somites, using the TGFβ2- and TGFβ3-specific probes, and a probe for α cardiac actin, which is expressed at early stages of both cardiomyogenesis and skeletal myogenesis (Sasoon et al., 1988). Adjacent sections were subjected to immunohistochromy with the TGFβ2 antibody.

TGFβ2 RNA expression is detected at low levels in the dermamyotome component of the cervical somites at 9.5 days p.c., though no protein staining is observed at this time. By 10.5 days p.c., the dermamyotome has differentiated into two distinct components, the myotome and dermatome, which can be distinguished molecularly by differential expression of the α cardiac actin gene (Fig. 7C,D). Low levels of TGFβ2 RNA are present in both of these components (Fig. 7A,B), and very light immunostaining of only the myotomal component is seen with the TGFβ2 antibody (data not shown). By 12.5 days, expression of TGFβ2 RNA is predominantly in the dermatome, though very weak hybridisation is still seen in the myotomes (Fig. 7E,F). At this stage, the antibody still stains only the myotome, albeit very weakly.

**DISCUSSION**

This study presents a detailed analysis of the localisation of TGFβ2 and TGFβ3 RNA during early postimplantation mouse development (6.5–9.5 days p.c.). Virtually no TGFβ3 RNA expression was seen in the embryo over this period of time. Mahmood et al. (1992) also noted that TGFβ3 protein was not expressed strongly at any of these early stages.

We have also examined immunolocalisation of TGFβ2 polypeptide during cardiogenesis. We have localised the polypeptide to the cardiomyocytes of the embryo, and demonstrated that this immunostaining increases from 8.5 to 12.5 days p.c. and persists in the adult heart. Recently other reports of the immunolocalisation of TGFβ2 and TGFβ3 polypeptides during murine embryogenesis have been made, using independent antibody preparations (Pelton et al., 1991; Flanders et al., 1991; Mahmood et al., 1992). In some cases, the data are very similar to those of this study, but differences are also notable. Mahmood et al. (1992) and Pelton et al. (1991) both observed TGFβ2 protein in cardiomyocytes at some stage though, unlike us, they noticed down regulation of myocardial TGFβ2 protein staining at 10.5 and 17.5 days p.c. respectively. Furthermore, Heine et al. (1991) only detected very weak staining of adult rat myocardium with a TGFβ2 antibody. The TGFβ2 polypeptide distribution described by Mahmood et al. (1992) is more widespread than that seen by us, but similar to the combined localisations of TGFβ2 RNA and protein described in the present study. These differences in TGFβ2 protein distributions seen between different groups, could be explained by the use of different immunostaining procedures, or different antibody preparations recognising different conformational forms of the same protein, as has been seen for TGFβ1 (see Flanders et al., 1989 and Fowlis et al., 1992 for discussion). Indeed, based on the three-dimensional model of TGFβ2 protein recently published by Schunegger and Grutter (1992), the anti-TGFβ2 antibody used in this study (Van den Eijnden-Van Raaij et al., 1990) would have access to more exposed amino acid residues than that used by other workers (Flanders et al., 1991; Mahmood et al., 1992). Furthermore, since all the antibodies used to date have been polyclonal, one cannot rule out the possibility of minor non-specific IgG components.

In a previous study from one of our laboratories (Slager et al., 1991), TGFβ2 protein was detected in visceral embryonic and extraembryonic endoderm of 6.4 to 7.5 days p.c. embryos, as determined by whole-mount immunofluorescence with the aid of a confocal laser microscope. In the present study, there was no obvious immunolabelling of endoderm cells in sectioned embryos of 6.5–7.5 days p.c.. This might be explained by the differential sensitivity of the techniques used. There is far more cellular material available for reactivity in a whole-mount procedure, compared to that in 7 μm tissue sections, and the use of the confocal laser microscope would also enhance sensitivity.
Fig. 5
RNA and protein distributions of TGFβ2 RNA. At 8.0 days p.c., there is obvious continuity between the splanchnic mesoderm and the myocardium, which has down-regulated this mRNA. There is also a caudorostral gradient of TGFβ2 RNA. At around 24 hours after the first appearance of the RNA, and is localised to cardiomyocytes. Only very low levels of TGFβ2 protein are seen in the lateral mesoderm and septum transversum. Most intense protein staining occurs in the ventricular cardiomyocytes, and reduced staining in the outflow tract and atrium. The intensity of staining with the TGFβ2 antibody is therefore directly proportional to the extent of differentiation of the cardiomyocyte. It is evident that high steady state levels of TGFβ2 RNA are characteristic of potential myocardial progenitor cells and that, as differentiation ensues, the RNA is down-regulated but protein accumulates, thus generating two opposing gradients of TGFβ2 protein and RNA within the heart tube.

The differential distribution of TGFβ2 RNA and protein could be explained as a consequence of autocrine production of the growth factor by rapidly moving cells. We have previously demonstrated that there is a temporal delay of at least 18 hours between the first appearance of TGFβ1 RNA and immunohistochemically detectable protein in two different biological systems (Akhurst et al., 1990; Fowlis et al., 1992). Notably, this time scale is similar to the period of early cardiogenesis under study here. The differential distributions of TGFβ2 RNA and protein in the heart would also be amplified if one postulated that there was a switch in translational control of TGFβ2 synthesis as the progenitor cell progresses to the cardiomyocyte. It is known that TGFβ2 is under translational control in other systems (Glick et al., 1989).

An alternative explanation is that TGFβ2 protein, synthesised in the splanchnic mesoderm and ventral foregut endoderm which together ensheath the heart, is taken up by cardiomyocytes in a paracrine fashion. Pelton et al. (1989) suggested paracrine regulation to explain the differential RNA and protein distributions of TGFβ2 in the developing inner ear, and this could also be an explanation for the disparate TGFβ2 RNA and protein localisations in the uterine epithelium and endometrial glands. We do not know whether the antibody used in this study recognises latent or active TGFβ2 (or some other conformation) and, in the absence of data on TGFβ receptor localisations, it is impossible to discriminate between the possibility of autocrine and/or paracrine mechanisms.
In considering a paracrine function for TGFβ2, it is striking that the ventral foregut endoderm, apposed to the forming heart, is a rich source of TGFβ2 RNA. This growth factor has been shown to have mesoderm-inducing capability in a *Xenopus* animal cap assay (Melton, 1991) and, in Urodeles, the foregut endoderm has been implicated in inducing heart formation (reviewed in Jacobson and Sater, 1988). Thus, in the mouse, endodermally derived TGFβ2 might be important in early induction of the heart and may function in supporting cardiomyogenesis up to 10 days p.c., when foregut expression ceases (see below).

A role for TGFβ2 in myogenesis?
Although expression of TGFβ2 is far less pronounced during skeletal myogenesis than during cardiomyogenesis (see Millan et al., 1991), there is a similarity in the temporal and spatial sequence of expression of the RNA and protein. Low levels of TGFβ2 RNA are first seen in the dermamyotome component of the somite. Later, as the myotome migrates out of the somite, low levels of RNA and protein colocalise in the myotome. Still later, although myoblasts retain weak protein staining, TGFβ2 RNA is down-regulated in these cells, and the major source of TGFβ2 RNA becomes adjacent tissue, namely dermatome and surrounding mesenchymal tissue, as previously noted by Gatherer et al. (1990) in the human. Whether or not this growth factor appears in cardiac and skeletal myocytes via autocrine or paracrine routes, its expression patterns are
suggestive of playing a role in cardiomyogenesis and possibly, to a lesser extent, in skeletal myogenesis.

Until recently, it was generally accepted that TGFβs are inhibitors of skeletal myogenesis, since TGFβ1 inhibits differentiation of primary myoblasts and skeletal myoblast cell lines when cultured under the differentiation-inducing conditions of low serum concentration (Massague et al., 1986; Olson et al., 1986; Florini et al., 1986). This differentiation-inhibitory effect has been demonstrated to be mediated directly via down-regulation of myogenic factors (Vaidya et al., 1989; Heino et al., 1989; Salminen et al., 1991) and, indirectly, via effects on the extracellular matrix (Heino et al., 1989). Lack of appropriate cell lines has made a similar study of cardiomyogenesis very difficult. Parker et al. (1991), have shown that TGFβ1, rather than inhibiting differentiation, alters the profile of contractile protein gene expression in cultured neonatal rat cardiomyocytes, shifting it towards a more fetal phenotype.

More recently, several reports have suggested that TGFβs can be inducers of both skeletal and cardiac myogenesis, under appropriate culture conditions. In the axolotl, TGFβ enhances cardiac differentiation of cultured cardiac mesoderm (Muslin and Williams, 1991). In mammalian cells, Zentrella and Massague (1992) have shown that the skele-

Fig. 7. TGFβ2 expression during early skeletal myogenesis. (A,B) Transverse section through a 10.5 day p.c. embryo showing TGFβ2 RNA expression in both the dermatome and myotome components of the cervical somites. (C,D) Adjacent sections showing myotome-specific expression of cardiac α-actin. (E,F) Transverse section of a 12.5 day p.c. embryo hybridised with TGFβ2 probe, showing expression in the dermatome and, at a low level, in the myotomes. (G,H) Section adjacent to E,F probed with cardiac α-actin probe to show expression in the myotomes. dm, dermatome; my, myotome. Scale bar, 200 μm.
tal myoblast cell line, L6E9, is induced to differentiate by TGFβ1 when actively growing in mitogen-rich medium. This is a consequence of down-regulation of c-myc expression, with consequent withdrawal from the cell cycle, and of down-regulation of the inhibitor of myogenic differentiation, Id (Zentrella and Massague, 1992). It has also been reported that TGFβ1 can convert cardiac fibroblasts, isolated from adult rat heart, into cells with a cardiac myocyte phenotype which are still capable of proliferation. This effect was seen in both mitogen-rich and mitogen-poor media (Eghbali et al., 1991). Finally, one of us (Slager, 1992) has recently demonstrated that TGFβ1 and TGFβ2 stimulate embryonic stem cells, in culture, to differentiate into cardiac and skeletal muscle cells. Moreover, TGFβ2 increases the rate at which beating (cardiac) muscle is formed.

On the basis of this evidence, it is likely that TGFβ2, expressed in the region of the developing heart, is involved in regulating myogenesis. At early stages of development, when cell proliferation is marked (Zentrella and Massague, 1992), this growth factor may be differentiation-inducing, involved in switching on the differentiation programme of the fetal cardiomyocyte (Parker et al., 1991). Additionally (or alternatively) there may be a specialised function for TGFβ2 in the mature cardiomyocyte (see for example Thompson et al., 1988; Heine et al., 1991; Giannini et al., 1992), since protein staining persists in the adult heart.

The role of TGFβs in induction of mesenchymal cushion tissue

Our previous work showed that TGFβ2 RNA has a myocardial expression pattern restricted to the AV canal and outflow tract at 9.5 days p.c. (Millan et al., 1991). As discussed in the Introduction, this suggests that TGFβ2 might be a component of the regional myocardial induction signal which is necessary for cardiac cushion tissue formation. In the present study, we have demonstrated that AV expression of TGFβ2 RNA appears at around 8.5 days p.c., is maximal at 9.0 days p.c. when endothelial cells begin to delamate from the endocardium, and rapidly disappears again, so that by completion of the transformation event very little RNA persists. This is entirely compatible with TGFβ2 contributing to the inductive signal. We did not observe TGFβ2 immunostaining within the cardiac jelly, mesenchymal cushion tissue or the endocardial cells at these stages, which might have been expected if the growth factor was acting in a paracrine manner on the overlying cells. However, this could be due to the choice of antibody used in the experiments and detection sensitivity. It should also be noted that we did not see TGFβ3 RNA expression within the AV canal at this time, which is not concordant with the results of Potts et al. (1991). However, it is reasonable to suppose that there may be differential isoform usage between chick and mouse.

In view of the possibility that TGFβ2 may be regulating cardiomyogenesis in the early heart, an alternative explanation for the persistent expression of TGFβ2 RNA within the AV canal is that this gene expression is related to the specific differentiative state of these myocardial cells. Indeed, cardiomyocytes of the AV canal of the 9 to 10 day p.c. heart are quite distinct from the rest of the myocardium, being specialised to contribute to the primitive AV conduction pathway (Viragh and Challicke, 1977a). At 11.0 days p.c., cardiomyocytes contributing to the AV node and bundle have been characterised as proliferative and immature, with poorly developed myofibrils (Viragh and Challicke, 1977b). It should be stressed, however, that the two alternative explanations for TGFβ2 RNA expression in the AV region are not mutually exclusive.

In terms of the role of this growth factor in inductive interactions in the AV canal, it is notable that members of the major classes of molecules that have been implicated in much earlier inductive interactions (Melton, 1991) are expressed in this region of the developing heart. These include other TGFβ-related molecules, such as BMP2 (Lyons et al., 1990) and BMP4 (Jones et al., 1991), other growth factors, such as bFGF (Parlow et al., 1991), HOX genes (Robert et al., 1989) and the retinoid binding protein genes, RARα, CRBP and CRABP (Dolle et al., 1990).

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