ORIGINAL ARTICLE

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Immunolocalization of the transcription factor Slug in the developing avian heart

Abstract Slug is a transcription factor involved in processes such as the formation of mesoderm and neural crest, two developmental events that imply a transition from an epithelial to a mesenchymal phenotype. During late cardiac morphogenesis, mesenchymal cells originate from two epithelia – epicardial mesothelium and cushion endocardium. We aimed to check if Slug is expressed in these systems of epithelial-mesenchymal transition. We have immunolocated the Slug protein in the heart of quail embryos between Hamburger and Hamilton stages HH16 and HH30. In the proepicardium (the epicardial primordium), Slug was detected in most cells, mesothelial as well as mesenchymal. Slug immunoreactivity was strong in the mesenchyme of the endocardial cushions and subepicardium from its inception until HH24, but the immunoreactivity disappeared in later embryos. Only a small portion of the endocardial cells located in the areas of epithelial-mesenchymal transition (atrioventricular groove and outflow tract) were immunolabelled, mainly between HH16 and HH20. Endocardial cells from other cardiac segments were always negative, except for a transient, weak immunoreactivity that coincided with the development of the intertrabecular sinusoids of the ventricle. In contrast, virtually all cells of the epicardial mesothelium were immunoreactive until stage HH24. The mesenchymal cells that migrate to the heart through the spina vestibuli were also conspicuously immunoreactive. The myocardium was not labelled in the stages studied. Our results stress the involvement of Slug in the epithelial to mesenchymal transition. We suggest that Slug can constitute a reliable marker of the cardiac epithelial cells that are competent to transform into mesenchyme as well as a transient marker of the epithelial-derived mesenchymal cells in the developing heart.

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Introduction

The gene *slug* encodes a zinc finger protein of the snail family of transcription factors (Nieto et al. 1994). Snail was originally described in *Drosophila*, where it is required for mesoderm formation (Grau et al. 1984). In the chick embryo, Slug is expressed in the premigratory and migrating neural crest and in the mesodermal cells migrating from the primitive streak (Nieto et al. 1994). In mouse and rat embryos, the expression of Slug is not detected in premigratory primitive streak or neural crest cells, being prominent in migratory neural crest cells, sclerotome precursors cells, and mesenchymal components of several organs (Jiang et al. 1998; Savagner et al. 1998). The function of Slug has been related to the epithelial-mesenchymal transition (EMT; Duband et al. 1995; Savagner et al. 1997). In fact, Slug inactivation impairs the EMT and subsequent cell migration in avian embryos (Nieto et al. 1994).

Two well-known events of EMT occur during vertebrate cardiac morphogenesis (Markwald et al. 1996). The valvuloseptal tissue of the atrioventricular canal and outflow tract forms through localized transdifferentiation of the endocardium into mesenchyme (Eisenberg and Markwald 1995; Markwald 1995; Markwald et al. 1996). On the other hand, epicardial cells migrate to the subepicardium and contribute to a subepicardial mesenchyme that differentiates in the connective and vascular tissue of the heart (Muñoz-Chápuli et al. 1996; Markwald et al. 1996; Pérez-Pomares et al. 1997; Dettman et al. 1998; Gittenberger-de Groot et al. 1998; Vrancken Peeters et al. 1999). Both processes of formation of mesenchyme are essential for a proper development of the heart, and seem to be induced by a localized myocardial signal (reviewed in Mjaatvedt et al. 1999).

Although several growth factors have been related to the cardiac EMT (Lyons et al. 1990; Jones et al. 1991;

Nakajima et al. 1994, 1998; Song et al. 1999), very little is known about the transcription factors that might be involved in this process (reviewed in Mjaatvedt et al. 1999). We have shown elsewhere that the immunoreactivity of the transcription factor ets-1 increases in areas of EMT of the developing avian heart (Macías et al. 1998), but this factor is not specific to these areas.

The still undefined role played by Slug in the specification of epithelial cells into a mesenchymal phenotypic shift prompted us to check if the Slug protein is present in premigratory and migratory cells undergoing an EMT in the developing avian heart. We reasoned that this approach would allow for a better knowledge of the functions of this transcription factor and also that it might provide a specific marker of the cells that are committed in the cardiac EMT.

Materials and methods

The animals used in our research program were handled in compliance with the international guidelines for animal care and welfare. Quail eggs were kept in a rocking incubator at 38°C. The embryos were staged according to the Hamburger and Hamilton (1951) stages of chick development.

The spatial and temporal immunoreactive patterns of Slug was studied in a sample consisting of 27 embryos of quail (*Coturnix coturnix japonica*), which were collected at stages HH16(3), HHH17(1), HH18(2), HH19(4), HH20(4), HH21(2), HH22(2), HH23(1), HH24(3), HH25(1), HH27(2), HH30(2). A chick embryo, collected at stage HH10 was used as a positive control.

The embryos were excised and fixed in 4% paraformaldehyde in TRIS-phosphate-buffered saline (TPBS) for 30 min. After fixation, the embryos were washed, cryoprotected in 15% and 30% sucrose in TPBS, embedded in melted gelatin, and snap frozen in liquid nitrogen-cooled isopentane. Ten-μm sections were obtained with a Leica Frigocut cryostate and collected on poly-l-lysine coated slides. The sections were then post-fixed in 4% paraformaldehyde in TPBS for 15 min, and washed three times in TPBS for 15 min.

Endogenous peroxidase activity was quenched by incubation for 30 min with 3% hydrogen peroxide in TPBS. After washing, non-specific binding sites were saturated for 30 min with 16% sheep serum, 1% bovine serum albumin and 0.5% Triton X-100 in TPBS (SBT). Endogenous biotin was blocked with the avidin-biotin blocking kit (Vector, Burlingame, Calif.). The slides were then incubated overnight at 4°C in the anti-Slug supernatant diluted 1:150 in SBT (0.3 μg IgG/ml). Control slides were incubated in SBT only. Then, the slides were washed in TPBS (3×5 min), incubated for 1 h at room temperature in biotin-conjugated anti-mouse goat IgG (Sigma) diluted 1:100 in SBT, washed again and incubated for 1 h in avidin-peroxidase complex (Sigma) diluted 1:150 in TPBS. After washing, peroxidase activity was developed with Sigma Fast 3,3′-diaminobenzidine (DAB) tablets according to the indications of the supplier.

The anti-chicken Slug monoclonal antibody (Clone 62.1E6) was obtained from the Developmental Studies Hybridoma Bank. It has been used for the immunodetection of Slug protein in premigratory neural crest cells (Liem et al. 1995, 1997).

Because of the exclusively nuclear staining, Nomarski optics became necessary to observe and to photograph the sections at high magnifications.

Results

The immunostaining was always nuclear, probably indicating the accumulation of the transcription factor in the cell compartment, where it is active. Control sections incubated in SBT instead of the primary antibody were not stained.

Positive control, stage HH10

A chick embryo fixed at this stage showed a strong immunoreactivity in the neural crest cells and also in the margins of the neural plate, where premigratory neural crest cells were differentiating (Fig. 1A). A weaker staining was also recorded in the head mesenchyyme. The cardiac tube, already constituted of myocardium and endocardium, was unlabelled in this embryo.

Stages HH16–18

By HH16 only a few endocardial cells as well as the earliest cushion mesenchymal cells were immunoreactive. They were located in the atrioventricular canal and prospective outflow tract (Fig. 1B). By HH17 and HH18 most cushion mesenchymal cells were immunoreactive (Fig. 1C). A few immunoreactive endocardial cells were also observed but only at the level of the developing cushions. These Slug immunoreactive endocardial cells were hypertrophied or showed signs of ingression into the underlying matrix. Virtually all the proepicardial cells, either mesothelial or mesenchymal, and the early epicardial cells, were distinctly immunoreactive in these stages (Fig. 1C).

Fig. 1A–F Slug immunoreactivity in avian embryos from stages HH10 to HH20. **A** Chick embryo, HH10, transverse section. Slug immunoreactivity is strong in premigratory and migrating neural crest cells (*NC*), and weak in the head mesenchyme (*HM*). The lateral and ventral walls of the neural tube (*NT*) are unlabelled, as well as the heart tube (*H*; *F* foregut). ×180. **B** Quail embryo, HH16, sagittal section. In the developing heart, Slug immunoreactivity is only detected in a few endocardial cells (*arrowhead*) of the atrioventricular (*AV*) canal and in the earliest cushion mesenchyme cells (*CM*). A strong immunoreactivity is present in the splanchnic mesenchyme (*SP*; *SA* sinoatrial region, *OFT* outflow tract). ×170. **C** Quail embryo, HH18, sagittal section. The cushion mesenchyme (*CM*) of the outflow tract (*OFT*) and the atrioventricular canal (*AV*) is intensely labelled with the antibody. The immunoreactivity is also distinct in the developing epicardium (*EP*) and its primordium, the proepicardial villi (*PE*). The labelling involves virtually all the proepicardial and epicardial cells, epithelial as well as mesenchymal. Note the strong immunoreactivity of the mesenchyme that surrounds the branchial pouches (*BP*) and the liver diverticuli (*L*; *A* atrium, *AA* aortic arch, *DV* ductus venosus). ×125. **D–F** Quail embryo, HH20, transverse sections. Slug immunoreactivity is strong in the mesenchyme of the outflow tract (*OFT*) cushions, inferior and superior atrioventricular cushions (*IAV* and *SAV*, respectively), epicardium (*EP*) and proepicardial villi (*PE*). Only a few endocardial cells are stained in the atrioventricular cushions (*arrowheads* in **E**). However, immunoreactive endocardial cells were more abundant in the outflow tract, as shown in **F** (*arrowheads*; *CM* cushion mesenchyme). **D** ×125, **E** ×250, **F** ×550
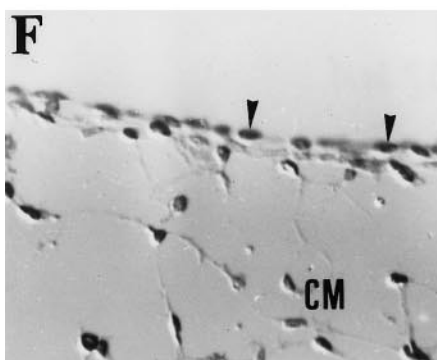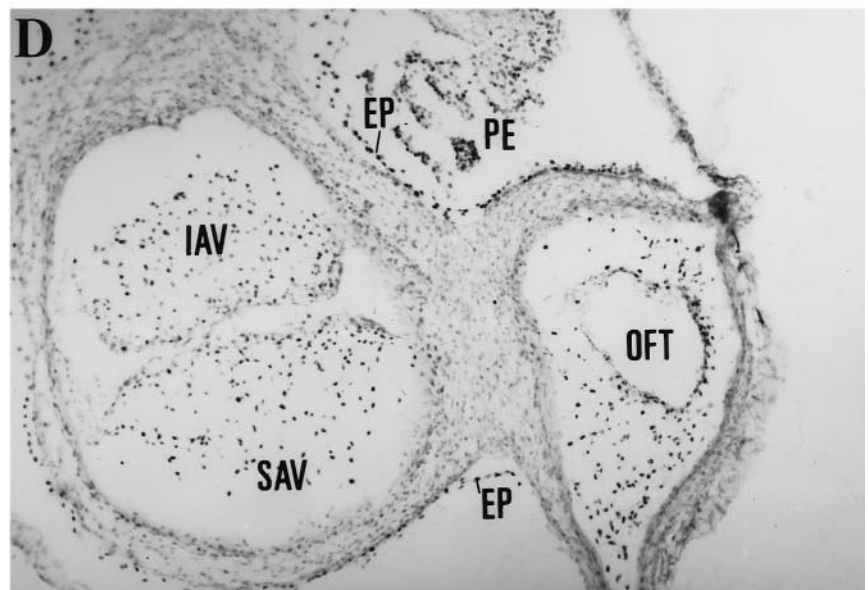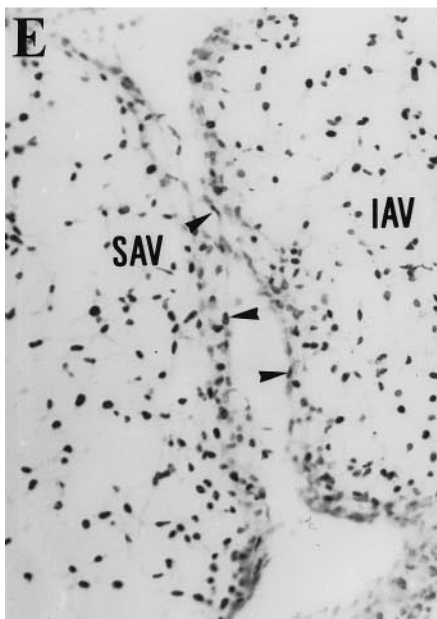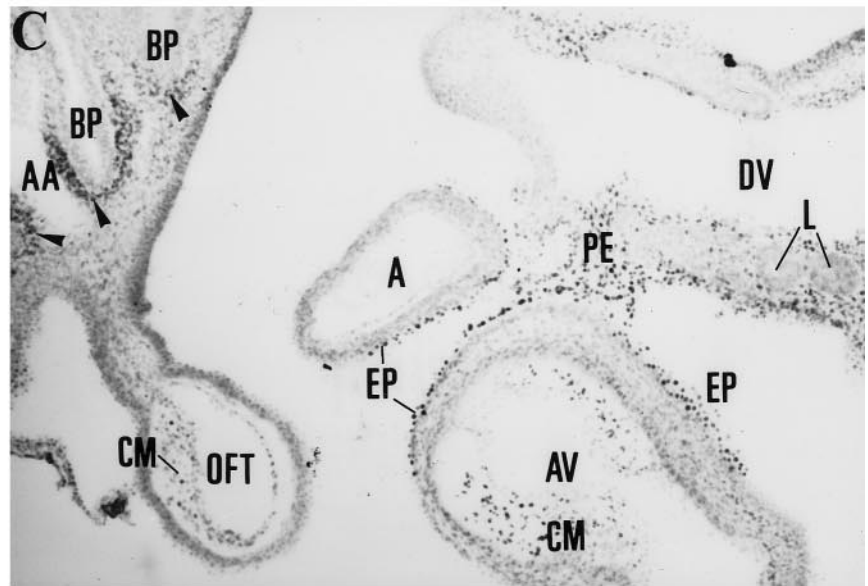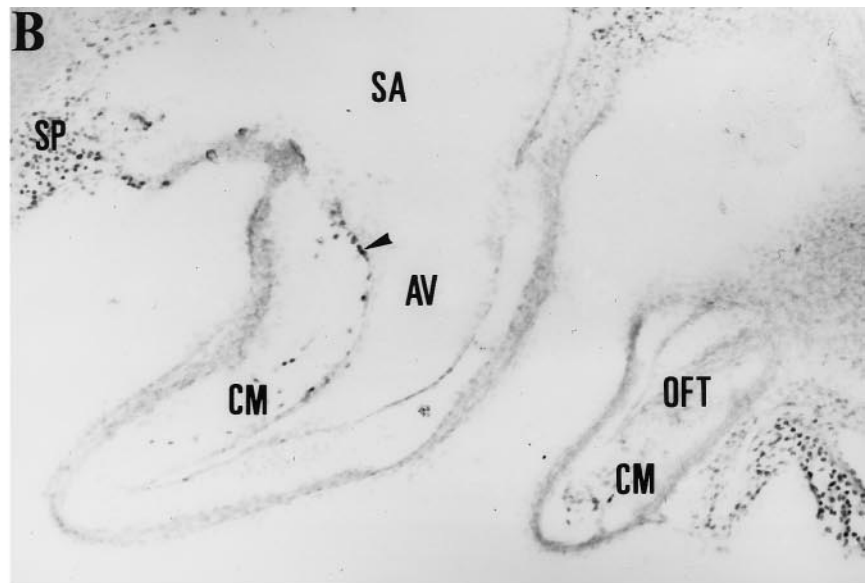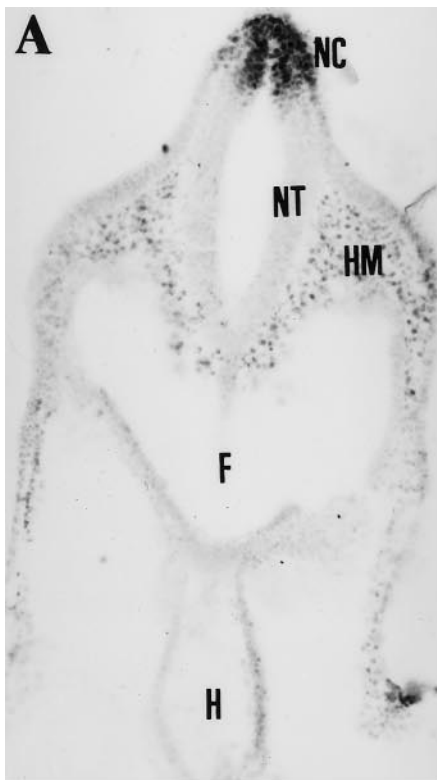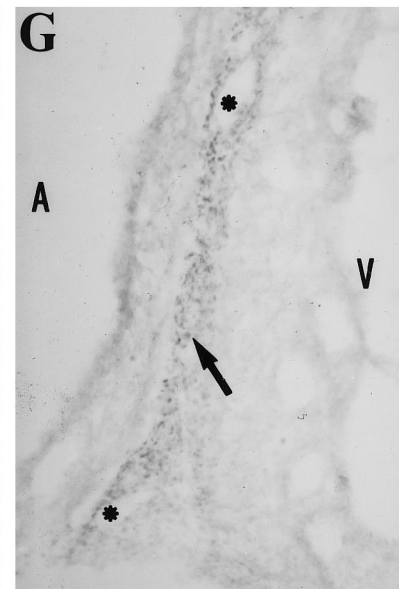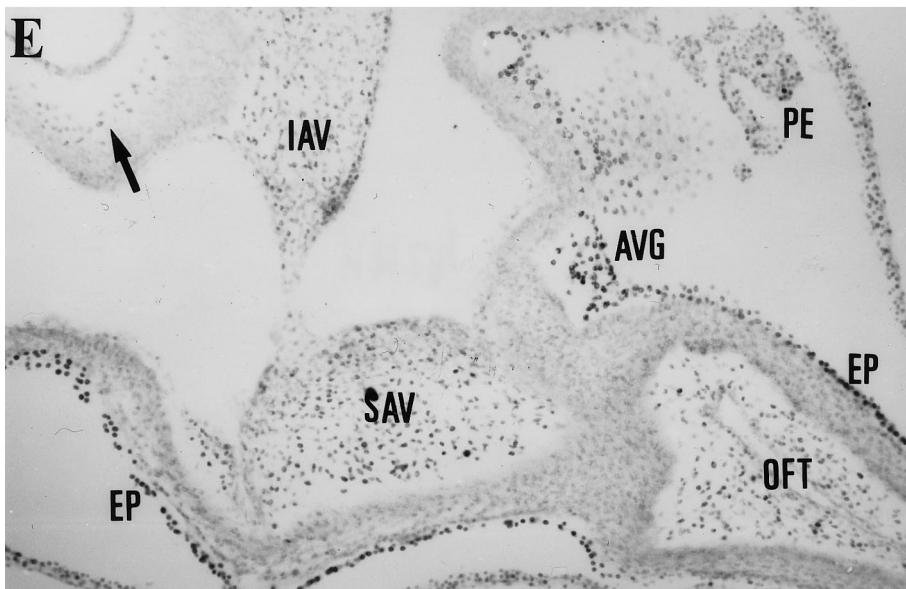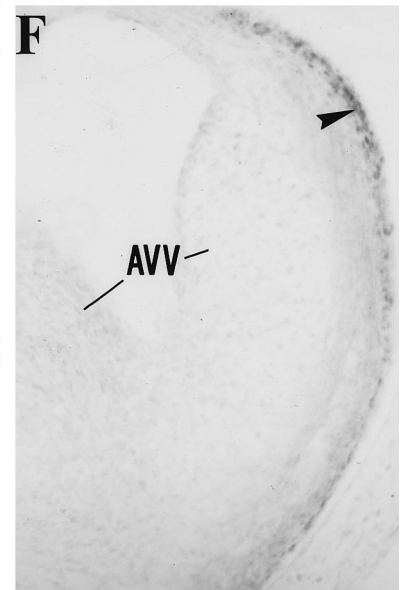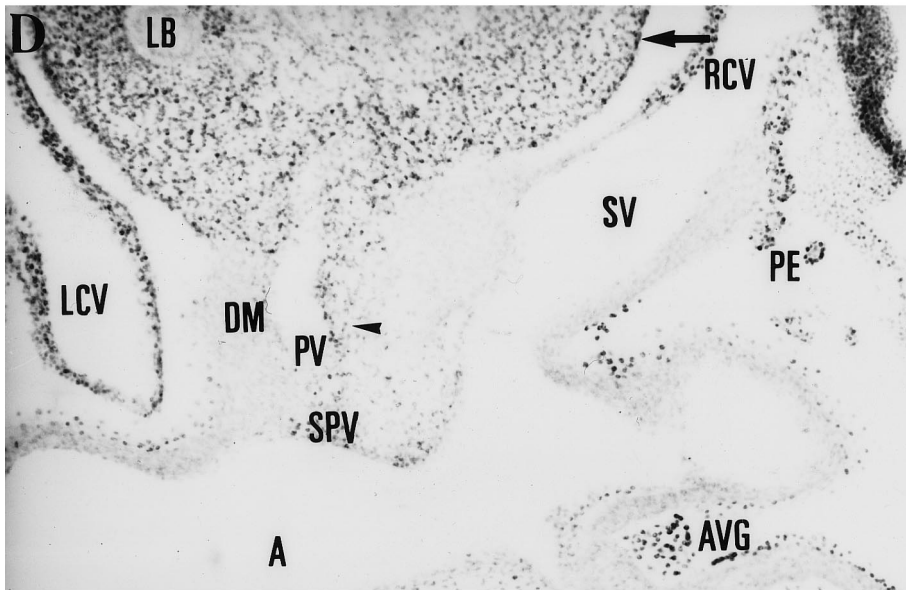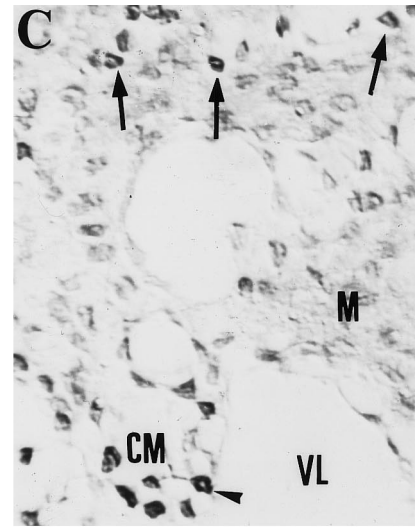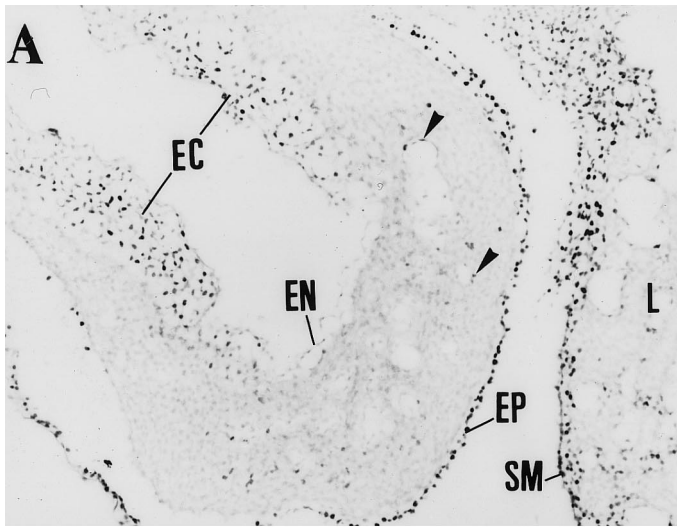
Stages HH19, 20

In the embryos of stages HH19 and HH20, Slug immunoreactivity was strong in some endocardial cells of the atrioventricular and outflow tract cushions and in most cushion mesenchymal cells (Fig. 1D–F). Immunoreactive endocardial cells were precisely located in those cushion areas overlying a larger number of mesenchymal cells. Endocardial cells were not labelled in the main atrial and ventricular cavities. The proepicardial villi (mesothelium and mesenchyme), the epicardial and the subepicardial mesenchymal cells were intensely labelled with the antibody (Fig. 1D).

Stages HH21–24

By HH21–24, Slug immunoreactivity was strong in the cushion mesenchyme, epicardial and subepicardial cells (Fig. 2A–E). Slug immunoreactive mesenchymal cells were found in a continuous band that extended from the pulmonary mesenchyme, where the immunoreactivity was strong, up to the inferior atrioventricular endocardial cushion, passing through the dorsal mesocardium and the spina vestibuli (Fig. 2D). In these embryos the immunoreactivity of the epicardial mesothelium has decreased in the dorsal part of the atrium, but it is still intense in the atrioventricular groove, ventricle and outflow tract (Fig. 2B, D, E). Some endocardial cells showed a weak immunoreactivity in the developing intertrabecular sinusoids (Fig. 2A, C). No other endocardial cells were labelled throughout the heart, except for some cells located in the outflow tract cushions. The coelomic mesothelium lining the pulmonary mesenchyme showed an intense immunoreactivity (Fig. 2D).

Stages HH27 and HH30

In embryos of these stages, Slug immunoreactivity has markedly decreased from most of the heart. Only a faint labelling can still be observed in the mesenchymal cells of the subepicardium of the atrioventricular canal, especially in the HH27 embryos (Fig. 2F, G).

◀ **Fig. 2A–G** Slug immunoreactivity in quail embryos from stages HH22 to HH30. **A–C** Quail embryo, HH22, sagittal sections. Immunoreactive cells are abundant in the endocardial cushions (*EC*) and epicardium (*EP*). The epicardial labelling included primitive epicardial cells (*EP* in **B**) as well as the subepicardial mesenchyme (*SE* in **B**). Note also the strong immunoreactivity of the splanchnic mesothelium (*SM*) and the mesenchyme of the liver (*L*). A weak immunoreactivity was observed in endocardial cells from the developing sinusoids (*arrowheads* in **A**, *arrows* in **C**), but not in the endocardium (*EN*) lining the main ventricular lumen (*VL*). An immunoreactive endocardial cell, apparently migrating into the endocardial cushion, is shown by an *arrowhead* in **C** (*CM* cushion mesenchyme, *M* myocardium). **A** ×125, **B** ×550, **C** ×550. **D** Quail embryo, HH24, transverse section. Abundant Slug immunoreactive mesenchyme surrounds the lung buds (*LB*) and fills the dorsal mesocardium (*DM*) contacting (*arrowhead*) with the mesenchyme of the spina vestibuli (*SPV*) along the margins of the developing pulmonary veins (*PV*). Note the immunoreactive cells in the coelomic epithelium (*arrow*). Immunoreactive cells can also be seen in the walls of the left (*LCV*) and right (*RCV*) common cardinal veins, but not in the wall of the sinus venosus (*SV*). As in previous stages, the proepicardial villi (*PE*) and the subepicardium of the atrioventricular groove (*AVG*) are stained (*A* atrium). ×125. **E** Section of the same embryo, located at a more posterior level. The strongest immunoreactivity and the largest number of positive cells are located in the epicardium and subepicardium of the atrioventricular groove (*AVG*) and outflow tract (*OFT*). However, the epicardium of the dorsal area of the atrium is only weakly stained (*arrow*). The immunoreactivity is also weaker in the atrioventricular (*IAV*, *SAV*) and outflow tract (*OFT*) cushions. ×140. **F** Quail embryo, HH27, transverse section. Slug immunoreactivity has disappeared from the atrioventricular valves (*AVV*) and remains faintly in the subepicardial mesenchyme of the atrioventricular groove (*arrowhead*) ×140. **G** Quail embryo, HH30, transverse section. Only a few subepicardial cells of the atrioventricular groove are immunoreactive (*arrow*). These cells are surrounding the developing cardiac vessels (*asterisks*; *A* atrium, *V* ventricle). ×125

Discussion

The precise function of Slug has been related to the epithelial-mesenchymal transition, either by specifying EMT competence in epithelial cells (Duband et al. 1995) or by maintaining the mesenchymal phenotype and repressing differentiation processes (Ros et al. 1997). In the chick embryo (but not in mice embryos) Slug expression starts in the epithelial cells during the premigratory stage, and this event seems to be a prerequisite for the transformation. Significantly, the incubation of chick blastoderms with antisense Slug oligonucleotides inhibits the migration of the neural crest cells (Nieto et al. 1994). Furthermore, transfection of Slug cDNA in epithelial cells results in the disappearance of desmosomal markers and cell spreading, while antisense Slug cDNA inhibits the EMT induced by fibroblast growth factor-1 or hepatocyte growth factor (Savagner et al. 1997). All these data suggest that Slug plays a key role in the epithelial-mesenchymal transition of the chick embryos. However, Slug seems to be not essential for mesodermal or neural crest development in mice (Jiang et al. 1998), an observation that might be related to the different expression patterns of Slug and Snail in the early development of the chick and mouse embryo (Sefton et al. 1998).

According to our observations in the developing avian heart, the transcription factor Slug is expressed in very precise sites where events of epithelial-mesenchymal transitions have been reported. Ets-1, another transcription factor expressed during epithelial-mesenchymal transitions in the chick embryo (Fafeur et al. 1997), shows a more generalized pattern of expression in the embryonic heart, including the myocardium, endocardium and epicardium, although ets-1 protein seems to be more abundant in the cardiac areas of epithelial transdifferentiation (Macías et al. 1998).

The anti-Slug antibody did not label the differentiated myocardium in the stages studied. Slug immunoreactivity was mainly observed in the undifferentiated mesen-

chymal cells of the endocardial cushions, subepicardium and spina vestibuli. Interestingly, a number of epithelial cells, endocardial as well as epicardial, were also labelled. It is important to remark that endocardial cells were mainly labelled in those areas where an epithelial to mesenchymal transition is occurring, i.e. the atrioventricular canal and outflow tract, and predominantly in early stages, between HH16 and HH20. Some immunoreactivity was also transiently recorded in the endocardium lining the developing sinusoids, i.e. coinciding with an invasive behaviour of the endocardium and also with the expression of HGF/SF by the trabeculate myocardium (Song et al. 1999). However the endocardium of the main atrial and ventricular cavities was not labelled in the stages studied. About the labelling of the mesenchyme in the dorsal mesocardium and spina vestibuli, it is important to remark that at least a part of this mesenchyme has been proposed to arise from the epithelial-mesenchymal transition of the coelomic epithelium (Markwald et al. 1996), an epithelium in which we have also recorded an intense Slug immunoreactivity.

The low number of immunolabelled endocardial cells observed in the endocardial cushions might be related to the low ratio of endocardial cells (about 10%) that are JB3 immunoreactive within the developing tubular heart of the avian embryo (Wunsch et al. 1994; Mjaatvedt et al. 1999). JB3, a fibrillin-2 related antigen (Rongish et al. 1998), is only expressed in the subset of endothelial cells competent to transdifferentiate into cushion mesenchyme. Thus, it is tempting to suggest that the JB3 immunoreactive and the Slug immunoreactive endocardial cells constitute the same population.

Virtually all the epicardial cells were immunostained between stages HH17 (when they were still forming the extracardiac proepicardium or epicardial primordium) and HH24. Although this result is in agreement with our previous suggestion of an EMT that occurs in the proepicardium and epicardial mesothelium (Pérez-Pomares et al. 1997, 1998), it is remarkable that, in contrast to our findings in the endocardium, Slug immunoreactivity involves all the mesothelial cells of the embryonic epicardium, as discussed below.

Slug immunoreactivity abruptly decreased in the cardiac tissues between HH24 and HH27, coinciding with the differentiation of the cardiac mesenchyme in valvuloseptal, connective and vascular tissue. It might be argued that the epicardial contribution to the subepicardial mesenchyme continues after HH24, i.e. when Slug immunoreactivity sharply decreases. However, we think that the transactivating functions of Slug may only be necessary in the early phase of the process, rendering the cell responsive to the triggering signal for the EMT. An alternative explanation is that the presence of Slug is not indispensable for the epicardial EMT.

It is tempting to suggest that Slug plays a direct role in the shift from an epithelial to a mesenchymal phenotype during the cardiac EMT, a role that would be clearly substantiated by our results. Slug immunoreactivity was only found in epithelial cells from the areas and stages of the developing heart where an EMT occurs (proepicardium, cushion endocardium, and epicardium). However, the small proportion of Slug immunoreactive endocardial cells contrasts with the extensive immunoreactivity of the primitive epicardial cells, and this is an observation that deserves attention. It is possible that Slug expression may be a necessary but not sufficient condition for the cardiac EMT. In this case, we could hypothesize that Slug expression in the epicardial mesothelium indicates a generalized competence to transform into mesenchyme, although only a subset actually achieves such a transformation, probably in response to extrinsic, regional signals. It would agree with our observations on the chimeric epicardium obtained after grafting quail proepicardial tissue into chick embryos (Pérez-Pomares et al. 1998). The donor-derived epicardium only transformed to mesenchyme in definite areas (atrioventricular canal and outflow tract), but not in the dorsal part of the atrium, where the epicardial cells are probably responsive but lack the triggering signal. This situation would be different in the case of the endocardium, since only a small fraction of the endocardial cells would be competent for the transformation; but all these cells probably receive the triggering signal from the myocardium, complete the process of transition and migrate into the cushions as mesenchymal cells. In fact, only endothelial cells from the atrioventricular or proximal conus region of the heart give rise to cushion mesenchyme in culture (Mjaatvedt et al. 1987), and only a fraction of the endothelial cells of these regions leave the monolayer to invade the underlying cushion matrix.

These observations, combined with our report of a small proportion of Slug immunoreactive endocardial cells, suggest that Slug might to be a reliable and specific marker of the endocardial cells that have been fated to transform into mesenchyme, and the first directly related with the genetic control of the transformation process. Furthermore, the presence of Slug in the epicardial mesothelium might be indicating a latent competence that would be compatible with the suggested pluripotential character of the mesothelial-derived cells in the developing embryo (Muñoz-Chápuli et al. 1999). Functional knockout of the Slug transcripts might provide new and interesting information about the cardiac EMT and the developmental fate of the mesenchyme derived from the endocardium and the epicardium.

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Note added in proof

Romano and Runyan [Dev Biol (1999) 212:243–254] have recently shown that Slug is required for the transformation of the endocardium in the endocardial cushions of chicken embryos since treatment of atrioventricular canal explants with antisense Slug oligodeoxynucleotides prevents endothelial cell-cell separation