Int. J. Dev. Biol. 39: 1033-1039 (1995)

Short Contribution

Differentiation of myoendocrine cardiac cells from presumptive heart mesoderm explants of *Bufo arenarum*

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ABSTRACT To investigate which factors are involved in the differentiation of *Bufo arenarum* heart myoendocrine cells, we studied the distribution of Atrial Natriuretic Peptide (ANP) immunoreactivity in hearts formed from presumptive cardiac mesoderm explanted at early embryo stages. Explants isolated from different embryonic stages throughout neurulation were cultured *in vitro* with or without the pharyngeal endoderm, and in other cases transplanted to the caudal region of embryos at similar stages. We utilized immunohistochemical and morphological techniques to assess myoendocrine cardiac cell differentiation. Development of heart beat and positive tropomyosin immunolabeling were considered signs of cardiac tissue differentiation. Our results confirm that explants of cardiac mesoderm cultured with endoderm showed a greater and more complete level of cardiac differentiation than those of mesoderm alone, and this coincides with the staining pattern of tropomyosin. ANP immunostaining revealed that cardiac cells containing ANP were scarce in those cultures without endoderm. On the contrary, in both cultured and grafted explants containing endoderm, ANP immunostaining was intensive and well-distributed in the cardiac tube, and in some cases restricted to one side of the formed heart. We conclude that the endoderm regulates cardiac cell differentiation, and in this way, is involved in the development of the heart myoendocrine system.

KEY WORDS: *ANP, heart development, cardiac cells, tropomyosin, induction*

The heart of mammals and submammals contains a family of regulatory peptides with potent natriuretic, diuretic and vasorelaxant properties. Atrial natriuretic peptides (ANPs) are present in secretory granules of cardiocytes located mainly in the atrium of the rat heart (De Bold *et al.*, 1981). The atrial natriuretic peptide (ANP) regulates blood pressure and extravascular fluid volume, inhibits the release of aldosterone and vasopressin, and has an antagonist action on functions induced by Angiostensin II (for a review see Brenner *et al.*, 1990). In submammalian vertebrates, immunoreactive ANPs were demonstrated in the atrial and ventricular cardiocytes (Chapeau *et al.*, 1985; Reinecke *et al.*, 1987a,b; Aoki *et al.*, 1988).

During development, ANPs appear to be some of the first hormones produced from mesodermal derivatives (Scott and Jennes, 1988). In mammals, ANPs immunoreactive cells are evident from the eleventh day of gestation (Thompson *et al.*, 1986; Scott and Jennes, 1988). In amphibia, immunocytochemical analysis showed the presence of myoendocrine cells at a very early stage of development, at embryonic stage 21 (gill circulation) (Casco *et al.*, 1992). In *Bufo arenarum* adult heart the ANP immunoreactivity was found strong in atria but weak in the ventricle (V.H. Casco, personal communication.).

In amphibia the heart forms from the lateral mesodermal plate, on either side of the prechordal plate, by the inductive interaction of the pharyngeal endoderm (Jacobson and Sater, 1988; Smith and Armstrong, 1990). The timing of cardiac induction varies among amphibian species: it is completed by late gastrula stage in the anuran *Xenopus laevis* (Sater and Jacobson, 1989; Naccone and Mercola, 1995), whereas in urodeles *(Taricha torosa)* it is not completed until tailbud stage (Jacobson and Duncan, 1968). In the Mexican axolotl, the mesoderm continues to be exposed to inductive pharyngeal endoderm during neurula and tail bud stage, when it is moving towards the ventral midline (Easton *et al.*, 1994). In *Bufo arenarum* the inductive interaction is completed by tailbud stage (Paz and Pisanó, 1990).

The mechanism of myoendocrine differentiation is not well known. In this respect it has been suggested the ANPs secretory cells are derived from mesoderm. However, endodermal pharyngeal cells could incorporate into the heart primordia and become forerunners of the endocrine cells (Scott and Jennes, 1988). It is well known that in vertebrates the endoderm has been shown to be a likely source of heart-inducing signals.

The aim of this study was to investigate what factors determine myoendocrine cell differentiation. One of the goals was to elucidate whether during embryogenesis inductive or noninduc-

Abbreviations used in this paper: ANP, atrial natriuretic peptide; PBS, phosphate buffer saline.

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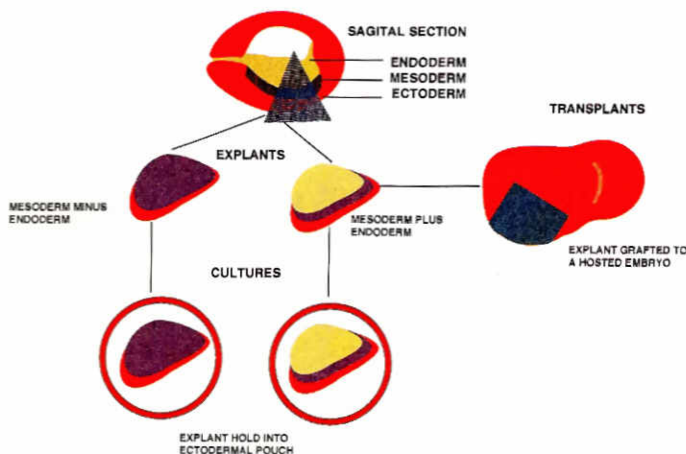


Fig. 1. Schematic representation of the experimental procedure. *The presumptive cardiac area was isolated from the region indicated by the dotted line at three different embryo stages (neural groove, neural tube and tail bud). The explanted area was presumptive cardiac mesoderm and included the foregut endoderm. In some cases the endoderm was removed after explantation as described in the text. Explants were closed into ectodermal pouch, cultured for 7 days and scored for the formation of a beating heart. In other cases the explant (mesoderm plus endoderm) was transplanted to the caudal side of a other embryo and maintained till reach larval stage.*

tive territories adjacent to the prospective heart mesoderm are involved in the cardiac endocrine differentiation. In this work we compared combinations of tissues containing endoderm cells with others in which endoderm was not included, testing them for the formation of a beating heart and for tropomyosin immunolabeling to ensure they reached a terminal myocardial differentiation. Explants of prospective heart mesoderm were isolated from different embryonic stages throughout neurulation. In some cases cardiogenic mesoderm and inductive endoderm were transplanted to the ventrocaudal side of a host embryo.

The inducing capacity of the anterior endoderm is necessary to form a complete, well developed heart

The critical events of heart induction, like the inductive role of the endoderm on the heart primordia, occur at different stages depending on the species (Muslin and Williams, 1991).

To evaluate the inductive process in *Bufo arenarum* embryos we performed two kind of cultures: a) those in which only heart-forming mesoderm was explanted, and b) those in which adjacent pharyngeal endoderm was included in the sandwich (Fig. 1). All explants were cultured and monitored daily for the formation of beating hearts over a period of 7 days. The results are summarized in Figure 3. Explants of precardiac mesoderm removed from embryos at neural groove stage (NG, stage 14) and cultured alone, developed beating hearts only in 8% of total explants. When cardiac mesoderm was explanted at neural tube (NT, stage 16), 40% showed beating tissue and from tailbud (TB) 68% formed beating hearts. By contrast, those cultures which included pharyngeal endoderm developed a higher frequency of differentiated beating cardiac tissue, NG: 75%; NT: 82% and TB: 83% (see Fig. 2).

The histological analysis of beating sandwiches of cardiac mesoderm cultured alone from NT stage showed, after 7 days of culture, scarce morphological differentiation (Fig. 3). Hearts formed from mesoderm and endoderm cultures were structurally more complex, with the differentiation in chambers or regions of the heart (Fig. 4).

Although recent experiments have revealed that heart induction in *Xenopus laevis* may occur via the stepwise actions of the Spemann Organizer and endoderm signals (Naccone and Mercola, 1995), our experiments demonstrated the inductive action of endoderm in mesoderm explants from early neural stage increasing the frequency of beating hearts and the complexity of their structures. This suggests that to complete its development, the heart primordia still depends on the inductive endoderm at later stages than neurula. Similar results were obtained in *Xenopus laevis* by Sater and Jacobson (1989).

Foregut endoderm is crucial for the myofibrillogenesis

In *Ambystoma mexicanum* embryos with heart that fail to initiate beating (gene c homozygous) contain contractile proteins but the myofibrillogenesis fails (Lemanski, 1979). Immunofluorescence and electron microscopic studies have shown that in these lethal mutant hearts the normal myofibrillogenesis may be altered. Mutant hearts had normal amounts of actin, but significantly lower than normal amounts of tropomyosin (Starr *et al.*, 1989). Smith and Armstrong's studies (1990) of the cardiac failure in *Ambystoma* concluded that in axolotl cardiac induction of heart-forming mesoderm is a two-step process: the first signal directs the cells to begin differentiating in cardiomyocytes; the second signal promotes the process of myofibrillogenesis. The beating failure in mutant embryos is apparently due to the lack of the second signal.

In our experiments the immunolabeled pattern of tropomyosin varied according to the degree of heart development. The non-

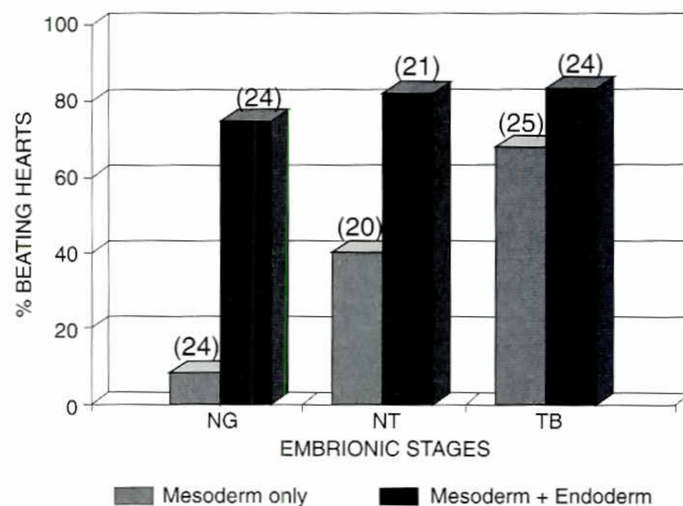


Fig. 2. Cultures from each embryonic stage: neural groove (NG), neural tube (NT) and tail bud (TB), *with and without endoderm were classified as having beating hearts and failing to undergo heart formation after 7 days in culture. The effects of foregut endoderm on heart formation is presented as a percentage of total cases undergoing heart formation. The number in parentheses (n) represents the total number of explants.*

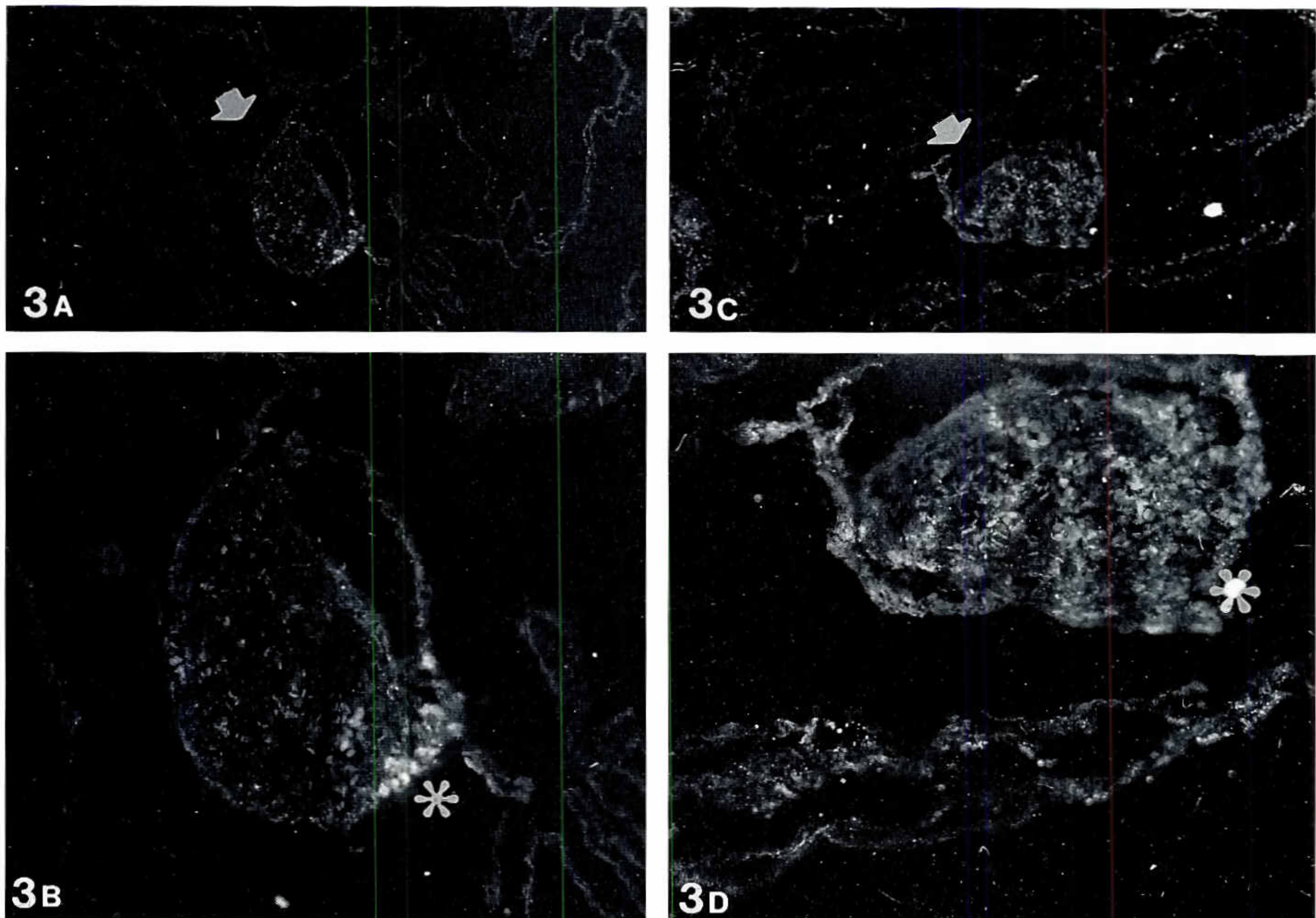


Fig. 3. Sections of precardiac mesoderm cultured in ectodermal sandwiches. *Precardiac mesoderm fragments were dissected from 17 stage embryo and the sandwich constructed as in Fig. 1, cultured for 7 days at 20°C, fixed and processed as described in Experimental Procedures. Sections were immunostained with anti-ANP or anti-tropomyosin antibody.* **(A)** *and* **(C)** *are adjacent sections.* **(A)** *The precardiac mesoderm cultured alone formed a compact cell mass (arrow), the ectoderm formed numerous folded epidermal membranes.* **(B)** *Shows a higher magnification of* **(A)**, *at cardiac tissue level the ANP expression was scarce and distributed in patches (asterisk).* **(C)** *Tropomyosin expression into the cardiac tissue (arrow).* **(D)** *At high magnification the cardiac cell mass displayed tropomyosin expression without an organized array (asterisk). Original magnification: A,C, x100; B,D, x200.*

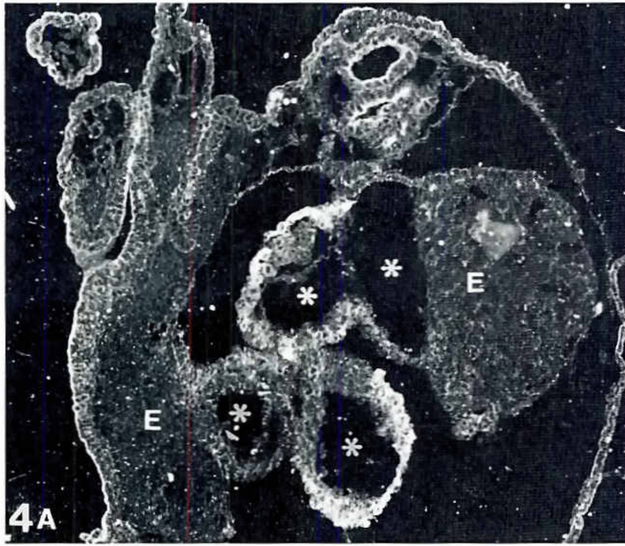
beating hearts developed from mesoderm alone explanted at NT stage showed very little staining for tropomyosin. Cardiac mesoderm from NG stage maintained in culture without endoderm, formed a compact cellular mass which expressed tropomyosin (Fig. 3), although it did not exhibit the normal pattern described during amphibian embryonic heart development (La France and Lemanski, 1994). At NT and TB stages, results were similar to those described for NG stage, showing a weak tropomyosin immunostaining (not shown).

Cultures of endoderm and cardiac mesoderm coexplanted from NT and TB embryos formed a contractile multilayered tube which expressed tropomyosin in a well-defined manner. Grafts containing cardiac mesoderm and endoderm from tail bud embryo stage showed tropomyosin in a sarcomeric staining pattern similar to the normal morphological development (Fig. 5).

As suggested by Davis and Lemanski (1987) and Smith and Armstrong (1991) those hearts formed without endoderm at neural plate, neural tube and tail bud embryo stages, had the same inability as the mutant hearts to form organized myofibrils, suggesting that the second signal of the induction process proposed by Smith and Armstrong (1990, see above) was missing. This was not observed in the explants including endoderm (both cultured and transplanted), indicating that the endoderm could be an important effector of terminal myofibrillogenesis in the heart of *Bufo arenarum*. In chick embryos, recent studies have indicated that endoderm is necessary for the generation of stable sarcomeric protein expression, organized myofibrils and beating tissue from early embryonic stages (Gannon and Bader, 1995).

Endoderm and ANP synthesis

The cardiogenic effects of the anterior endoderm on the heart primordia aiding in the development of a functional heart is quite clear. We now raise the question whether adjacent tissues like pharyngeal endoderm – the best heart inducer – are involved in the promotion of the endocrine heart functions.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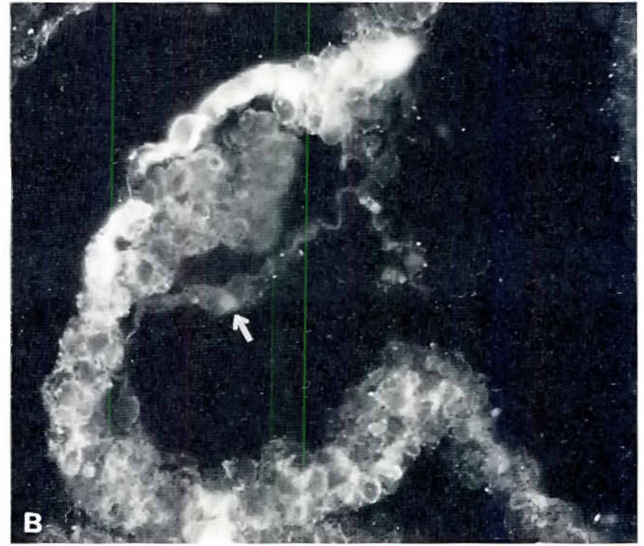
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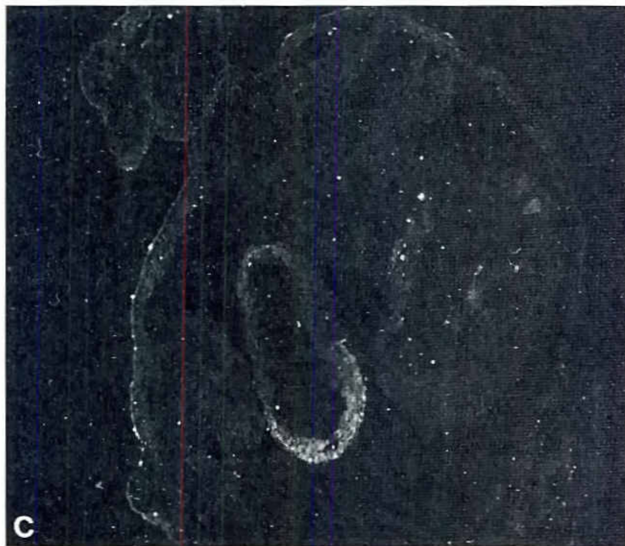
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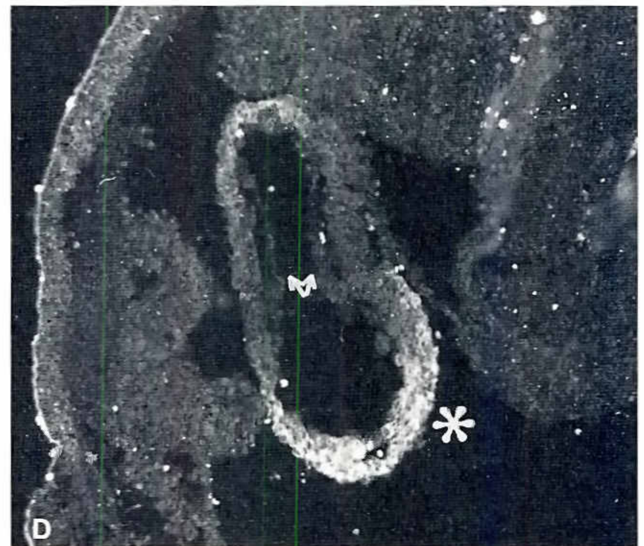
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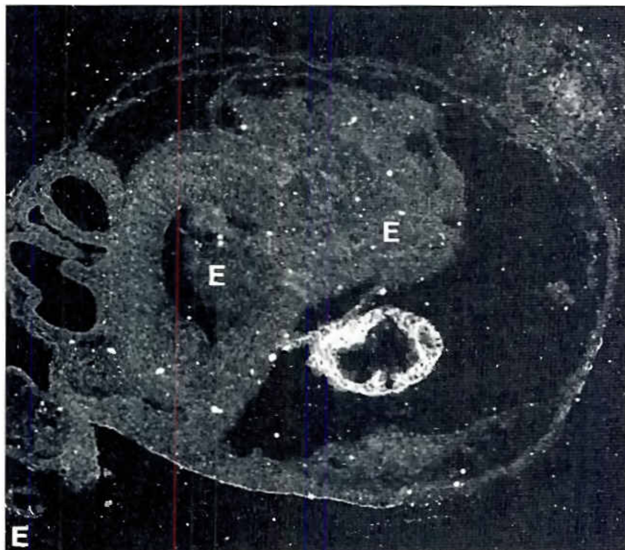
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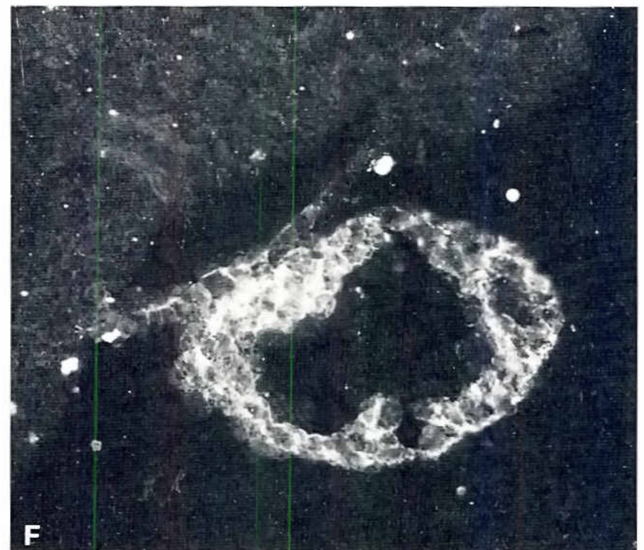
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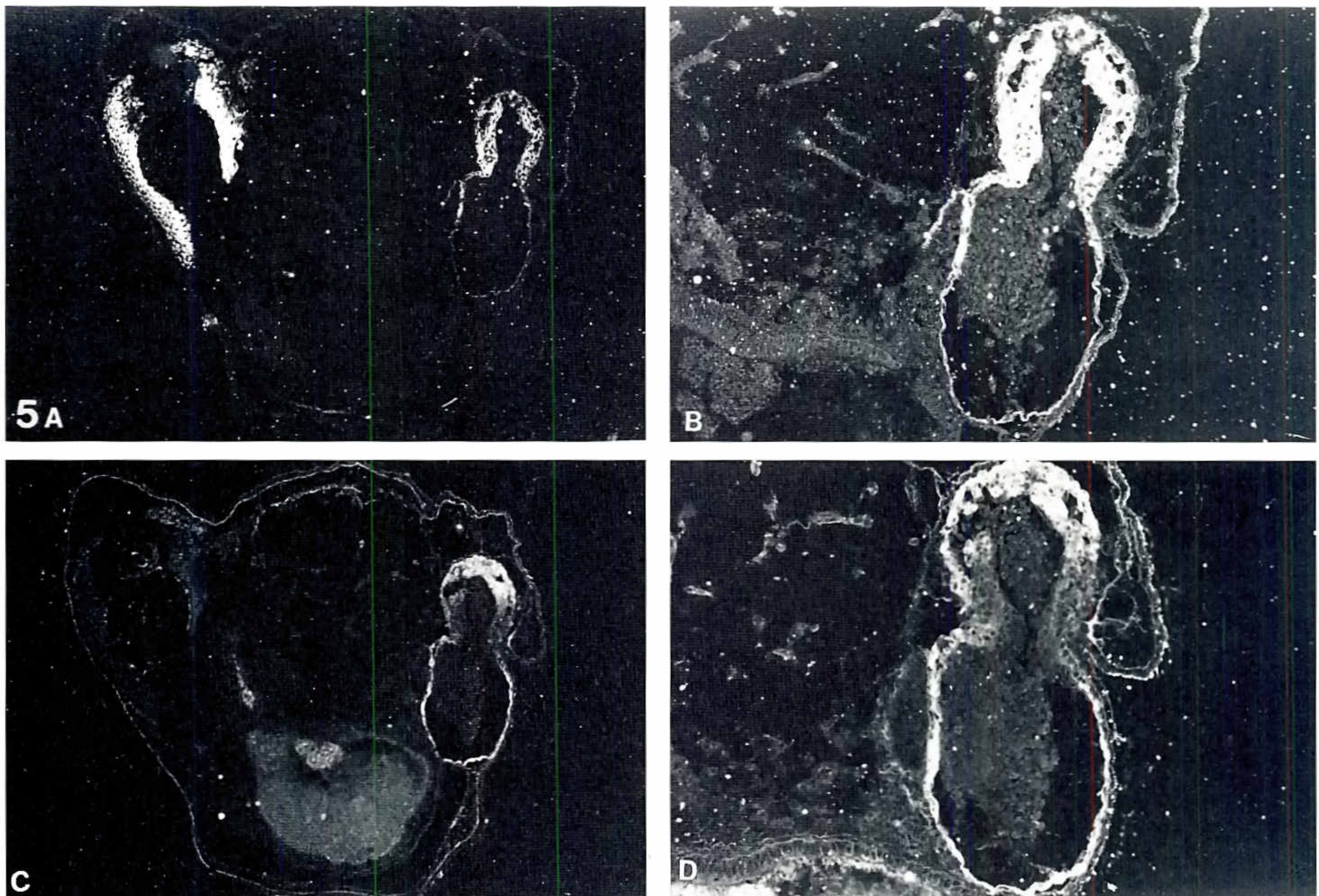

Fig. 5. Transverse sections of the caudal side of larvae (stage II) with a well developed beating heart formed from a graft of presumptive cardiac mesoderm and inductive endoderm explanted at neural groove embryo stage. (A) *Tropomyosin expression in two defined areas: somitic (S) area around the notochord and neural tube with an intensive label and at the new formed heart (H).* **(B)** *Higher magnification of the immunolabeled heart showing a normal histology with 2 well formed atria (A) and ventricle (V) chambers. Staining of Tropomyosin in both chambers showed a normal pattern, more intense at the ventricle trabeculae. Note the presence of blood cells in the heart.* **(C)** *Adjacent (A) section showing ANP expression confined in the grafted heart.* **(D)** *At higher magnification ANP staining is intensive in the atrial and ventricle cardiocytes. ANP expression is absent at the cardiac cushion level. Original magnification: A,C, x100; B,D, x250.*

Cultures of heart-forming mesoderm alone explanted from NG, NT or TB stages showed little spots of ANP immunostaining (Fig. 3A,B). Cultures of cardiac mesoderm with anterior endoderm showed ANP expression at myocardial level but not at the differentiated endocardium (Fig. 4). Those cultured from TB stage developed well-defined structures like vesicles, which showed ANP immunostaining (not shown). Some explants formed a constriction in the primitive tubular heart and the immunolabeling was restricted to one side of the tube (Fig. 4C,D).

In transplant experiments a well-defined heart developed from cardiac grafted tissue with two differentiated vesicles: atrium and ventricle which expressed ANP and tropomyosin in adjacent sections (Fig. 5). The presence of ANP in hearts obtained by culture and transplantation techniques confirms that the environment of normal heart differentiation does not condition ANP synthesis. However, in hearts developed without endoderm, the immunolabeled cells containing ANP were scarce and with a rudimentary pattern of distribution. This suggest that the endo-

Fig. 4. Sections of precardiac mesoderm co-cultured with foregut endoderm using the sandwich methodology. *The sections were immunostained for ANP* **(A-D)** *or tropomyosin* **(E-F). (A)** *The cardiac tissue differentiated in a heart tube folded and with cardiac chambers (asterisks). ANP immunoreactivity was distributed in the chambers. The endoderm* **(E)** *was not positive for ANP.* **(B)** *Higher magnification, while ANP distribution was intensely expressed in the myocardial layer, no immunostaining was observed in the endocardial layer (arrow).* **(C)** *Section of a culture where the cardiac mesoderm differentiated in a tubular heart with a constriction, the immunolabeling was restricted to one side of the tube.* **(D)** *Higher magnification, while ANP distribution was sparse in one side of the tube, it was intensely expressed in the other side (asterisk). The endocardium was not stained (arrows).* **(E)** *Tropomyosin distribution in a tubular heart formed after 7 days of culture. The endoderm* **(E)** *was not immunostained.* **(F)** *At higher magnification the tropomyosin was intensely expressed in the tube. Original magnification: A,C,E, x100; B, x300; D,F, x250.*

derm could be an important effector of terminal myoendocrine differentiation.

Some authors have suggested a connection between ANP immunoreactive cells and the conducting system in fetal hearts. These suggestions could indicate a close relationship between blood flow and the promotion of endocrine cells differentiation (Scott and Jennes, 1988). The point was analyzed using transplants of cardiac mesoderm as described in Figure 1. A great level of developmental commitment is represented by the ability of a tissue to undergo organ formation when transplanted to a new site in the embryo, since under this condition, the tissue is exposed to novel environmental influences (Sater and Jacobson, 1989). Our results indicate that the heterotopic transplant of the heart mesoderm (at neurula or later stages) from its original embryonic environment to develop in a new place with different conditions, did not avoid ANP synthesis.

In conclusion, the discovery of ANP expression in cultures without any participation of the vascular system and also in the transplanted hearts, indicates that a circulatory system development in close relation to the heart is not crucial for ANP synthesis.

Experimental Procedures

Embryos

Bufo arenarum embryos were obtained by injecting females with a suspension of homologous hypophysis and carrying out *in vitro* fertilization (Ruiz *et al.*, 1994). Embryos of different stages were maintained in 10% Holtfreter's solution. The embryonic stages were recognized applying the Del Conte and Sirlin (1952) table.

Microdissection

The experimental design is summarized in Figure 1. Dejelled embryos of early stages (neural groove, NG; neural tube, NT; tail bud, TB), were transferred to sterile Steimberg solution supplemented with 50 ug ml^{-1} gentamycin sulfate and 50 ug ml^{-1} Nycostatin (SIGMA Chemical Co, St. Louis, USA). Explants containing mesoderm, endoderm and ectodermal epithelium were removed with hair loops, watchmaker's forceps and eyebrow hair knives. All microdissection was performed in sterile Steimberg solution. The separation of mesoderm and endoderm was performed in Steimberg solution containing 0.01% Trypsin (Sigma type IX) and the explants were subsequently incubated in 0.02% soybean trypsin inhibitor (Sigma type II-S) in 100% Steimberg solution for several minutes before they were transferred to culture dishes containing sterile 50% Steimberg solution. Presumptive cardiac explants were enclosed in epithelia removed from the back zone of the same embryo which formed an epithelia vesicle *(Sandwich methodology)*. Cultures were kept in sterile 10% Steimberg solution supplemented with 50 mg ml^{-1} gentamycin at 17°C. Other explants were preserved in 50% sterile Steimberg solution before being grafted to the caudal side of host embryos at similar stages, and kept in 10% Holtfreter's solution at room temperature with glass handles till their cicatrization. Observations were made daily under a Wild Heerbrugg Stereomicroscope.

Immunocytochemistry

Cultures, control embryos, and embryos containing grafts, were held until they reached the first larval stage (7 days of culture). Immunostaining was carried out as described in detailed elsewhere (Casco *et al.*, 1992). For ANP immunostaining, a well characterized anti-rat ANP (99-126) polyclonal antibody was used (gift from Dr. W.G. Forssmann, Hannover, Germany, through Dr. A. Aoki, Córdoba, Argentina). For tropomyosin a monoclonal anti-sarcomeric tropomyosin (Sigma) was used. Control sections were obtained by replacing the primary antibody with the blocking agent or the preabsorption of the first antiserum with 7-10 ug r-ANP at the working dilution for 24 h at 4°C. Slides were mounted with 50% glycerol in PBS with 0.2% N-propyl galate and photographed using a Reicher Polyvar microscope with epifluorescence and appropriate filters.

Acknowledgments

Special thanks to Dr. Victor Casco for his critical reading, Drs. A. Aoki and W.G. Forssmann for providing the anti-ANP antibody, Mrs. P. Roig for photographic help and Mrs. M.E. Cincallegro for typing the manuscript. This work was supported by Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), (D.A.P and A.P. are career members of CONICET).

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Accepted for publication: November 1995