Developmental changes of connexin40 and connexin43 mRNA distribution patterns in the rat heart

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Abstract

Objectives: Gap junctions have been demonstrated ultrastructurally in cardiac regions where connexin40 (Cx40) and connexin43 (Cx43) protein could not be detected immunohistochemically. We investigated therefore the distribution of their mRNAs with more sensitive techniques. Methods: In situ hybridizations with Cx40 and Cx43 cRNA probes were performed on sections of rat hearts from 9 embryonic days (ED 9) to adults. Results: From ED 13, Cx40 and Cx43 mRNA are detectable in atria and ventricles, but not in their flanking myocardium (inflow tract, atrioventricular canal and outflow tract). Even though Cx40 and Cx43 mRNA eventually become expressed in the inflow tract, they remain undetectable in the sinoatrial node, the atrioventricular canal (including atrioventricular node) and outflow tract. Expression of Cx40 is maximal in the fetal period and declines towards birth. Cx40 expression in the left and right ventricles evolves independently, its mRNA disappearing 4 days earlier from the right than from the left ventricle, and earlier from the free wall than from the trabeculations. Expression of Cx43 mRNA increases during development and changes postnatally from uniform to punctate. Prenatally, Cx43 mRNA was strongest in the subepicardial layer of the ventricular free wall. Nevertheless, we did not detect protein in this layer. Conclusions: Cardiac regions without detectable Cx40 or Cx43 mRNA either have extremely low levels of expression or express a different connexin. The temporally separate disappearance of Cx40 mRNA from the fetal ventricles implies that left and right ventricles mature independently with respect to gap-junctional communication. The division of the developing heart in compartments where Cx40 and Cx43 mRNA can and cannot be detected, implies pretranslationally regulated gene expression. The postnatally observed subcellular redistribution of Cx43 mRNA coincides with a reported increase in protein expression.

Keywords: Connexins; Rat, heart; Conduction system; Left/right patterning; Development

1. Introduction

Gap junctions consist of aggregates of hydrophobic cell-cell channels that allow the intercellular exchange of ions, metabolites and second messengers of up to 1 kDa in size. The aqueous pores are formed by serially linked hemi-channels (connexons) contributed by the apposing cell membranes. The connexon hemi-channel is composed of six transmembrane proteins called connexins (Cx). To date 13 different mammalian connexins are characterized. This gene family shares domains with sequence similarities that probably contribute to gap-junctional communication in general. The intracellularly localized carboxyl terminus is specific for each connexin and may be responsi-
during heart development may be twofold; they will establish impulse propagation, but they may also be involved in the regulation of cardiac morphogenesis. This last aspect deserves greater attention since it has become known that homozygotic Cx43-knockout mice survive until after birth when they die of a pulmonary outflow tract stenosis due to cardiac malformation [25]. In parallel, in humans an association has been found between a cardiac malformation syndrome featured by a.o. visceral heterotaxia and a mutation in the Cx43 gene [26].

In previous studies in the rat [12,13] it has been shown that the Cx43 protein in the heart is distributed heterogeneously during development up to the adult stage. During development the expression pattern of the protein correlates well with regional differences in conduction velocity [27]. However, our data were in apparent contrast with ultrastructural findings that showed the appearance of gap junctions in the developing mouse ventricle at embryonic day (ED) 10 [28], that is, before we could detect them immunohistochemically at ED 13 in the rat (which is comparable to ED 11 in the mouse). These early gap junctions were reported to be scarce, small and arranged in an atypical, immature configuration compared to the gap junctions found in the adult ventricle [29–31]. Recently, the distribution of Cx40 protein was studied in the developing mouse heart [32]. It shows that Cx40 is widely expressed in the ED 11 embryonic mouse heart (which is comparable to the ED 13 embryonic rat heart [33]). However, this developmental stage follows the stage where first gap junctions were found ultrastructurally. Therefore, the question remains of which connexin(s) the early gap junctions in the developing myocardium are composed.

Immunostaining of connexin isotypes is limited by the actual plaque size of gap junctions which makes the detection of small, immature gap junctions uncertain. Localization of their mRNAs with in situ hybridization is not limited by this constraint and, as a cytoplasmic labelling, provides a far better overview of connexin distribution than the discontinuous membrane staining of gap junctions. Nevertheless, it should be kept in mind that the presence of mRNA is only indicative and not proof for the presence of gap-junctional structures. Furthermore, to ensure monospecificity, all available connexin antibodies for immunostaining are raised against synthetic oligopeptides, some of them representing parts of the protein that can be modified posttranslationally, e.g. by phosphorylation [34]. The affinity of the antibodies for these epitopes may be affected by such modifications.

In addition to studies on the developmental distribution profiles of Cx43 and Cx40 protein [12,13,22,32,35,36] we wanted to establish connexin distribution in early heart development with in situ hybridization, using radioactively labelled cRNA probes specific for Cx43 and Cx40. The results presented in this study show for the first time both the temporal and the spatial distribution of Cx40 and Cx43 mRNA in the developing rat heart.

2. Materials and methods

2.1. Animals

Adult Wistar rats were purchased from the CPC Animal Farm, Austerlitz, The Netherlands. Embryos, fetuses and suckling rats were obtained from timed-pregnant rats, as described before [37]. Hearts from rats at 9, 10, 11, 13, 16, 18 and 20 days of embryonic development (ED) and hearts from 9-day-old suckling and 3-month-old adult rats were studied. From each developmental stage described three to five specimens were studied.

2.2. Histological procedures

Hearts in situ (ED 9, 10, 11, 13, 16, 18, and 20) and isolated hearts (suckling and adult) were fixed from 4 h to overnight in freshly made 4% formaldehyde. After dehydration in a graded series of ethanol followed by isobutanol, tissue was embedded in Paraplast Plus (Monoject, Kildare, Ireland). Serial sections of 7 μm were cut and affixed on 3-aminopropyltriethoxysilane-coated slides (AAS, Sigma A3648), which had been made ribonuclease-free with 0.1% diethylpyrocarbonate (DEP) prior to coating. Sections were stored at 4°C until use.

2.3. Synthesis of radioactively labelled RNA probes

Single-stranded antisense cRNA probes were made by in vitro RNA transcription. The rat Cx40 clone [8,38] in pBluescript consists of a cDNA of 970 bp, which represents almost the complete coding region, from the start codon to 100 bp short of the stop codon. The rat Cx43 clone [11] in pBluescript consists of a cDNA of 1.5 kb, which represents the complete coding region. To delineate the myocardium, probes were used that encode the α- and the β-myosin heavy chain (MHC) [47]. These cDNAs were kindly provided by Dr. K. Boheler, NHLI, London, UK. The α-myosin heavy chain clone in pBS consists of 120 bp of untranslated region in the 3' end of the mRNA. The β-myosin heavy-chain clone in pBS consists of 68 bp of untranslated region in the 3' end of the mRNA. All cRNA probes were made with T7 RNA polymerase. Sense RNA probes were used as negative controls.

The linearized cDNA was purified by extraction with phenol-chloroform. RNA probes with a specific activity of 2.5 × 10⁹ cpn/μg g were made as follows: in a volume of 10 μl, 0.5–1 μg DNA was incubated with 5 mM dithiothreitol, 30 U RNAse, 0.75 mM ATP, 0.75 mM CTP and 0.75 mM GTP, 2.5 μM [α-35S]UTP (specific activity of 1400 Ci/mmol (Amersham)), 40 mM Tris-HCl (pH 8.0), 25 mM NaCl, 8 mM MgCl₂, 2 mM spermidine and 100 U RNA polymerase (Gibeco, BRL) at 37°C for 1.5 h. The DNA template was hydrolysed with 1 μl of 1 U/μl of ribonuclease free deoxyribonuclease 1 (Promega) at 37°C for 15 min. The RNA strands were hydrolysed to frag-
ments of approx. 100 bp by adding 11 µl of 40 mM NaHCO₃, 60 mM Na₂CO₃ at 60°C for 10-20 min to the RNA preparation. The RNA was purified by phenol extraction after addition of 2 µg tRNA. The phenol phase was reextracted once. After ethanol precipitation, the RNA was redissolved in 10 mM Tris (pH 7.5), 0.1 mM EDTA, 10 mM DTT to a concentration of 1.0-1.5 × 10⁶ cpm/µl and stored at -20°C. Before the probe was used, the length of the probe was examined on a polyacrylamide gel.

2.4. Prehybridization of the sections

After deparaffination, the sections were air-dried. For a review on the protocol used, see Moorman et al. [39]. Briefly, prehybridization was as follows: (1) incubation in 0.2 N HCl at room temperature for 20 min, in order to extract proteins and thus improve probe penetration; (2) wash in bidistilled water for 5 min; (3) incubation in 2 × SSC (1 × SSC: 150 mM NaCl, 15 mM Na-citrate, pH 7.2) at 70°C for 10 min, which may improve the protease digestion in the next step, the unfolding of the RNA and the RNA accessibility in the tissue; (4) wash in bidistilled water for 5 min; (5) incubation in 1.35 U/ml Pronase E (Sigma type XIV No. P1547) in 50 mM Tris-Cl pH 7.5, 5 mM EDTA or 0.1% Pepsin (Sigma P7000) dissolved in 0.01 N HCl for 5 min (ED 10 and 13 embryos), for 15 min (ED 16, 18 and 20 embryos) or for 20 min (sucklings and

Fig. 1. Photomicrographs showing the mRNA expression of connexin43 (A, C, D and G), connexin40 (F) and β-MHC (B and E) in the ED 9 rat (A and B), in the ED 10 rat (C and D) and in the ED 11 rat (E, F and G). ec = ectoderm; me = mesoderm; he = myocardium of the heart; bp = branchial pouches; ao = aorta; ift = inflow tract; a = atrium; v = ventricle; oft = outflow tract. Connexin43 is abundantly expressed in the ectoderm, in the newly formed mesoderm and in the endoderm of the branchial pouches (A, C and D). At these stages neither connexin43 mRNA (A, D and G), nor connexin40 mRNA (F) can be detected in the myocardium of the heart, which is already characterized by an abundant expression of β-MHC as shown in serial sections (B and E). Bar, 50 µm.
Fig. 2. Photomicrographs showing mRNA expression of β-MHC (A, D and G), α-MHC (B, E and H), connexin40 (C), and connexin43 (F and I) in ED 13 rat hearts. ift = inflow tract; a = atrium; avc = atrioventricular canal; v = ventricle; oft = outflow tract; trab = trabeculations; ao = aorta; er = endocardial ridges. Panels A, B and C represent consecutive sagittal sections, showing the five compartments of the developing heart, i.e., the inflow tract, the atrium, the atrioventricular canal, the ventricle and the outflow tract. Panels D, E and F represent consecutive transverse sections of the heart at the level of the atrium, the ventricle and the outflow tract, while panels G, H and I represent consecutive transverse sections of the same heart at the level of the atrioventricular canal connecting the atrium and the ventricle. Both connexin40 (C) and connexin43 (F and I) are expressed in the atrium, the ventricle and the ventricular trabeculations, areas that are characterized as myocardium by the expression of β-MHC (A, D and G) and α-MHC (B, E and H). The atrioventricular canal and the outflow tract do not express either connexin above background level. Bar, 200 µm.
Fig. 3. Photomicrographs showing the mRNA expression of β-MHC (A), α-MHC (B), connexin40 (C) and connexin43 (D) in the ED 16 rat heart. moj = myo-osseous junction; ri = rib; ao = aorta; pa = pulmonary artery; sh = sinus horns; a = atrium; lv = left ventricle; rv = right ventricle; oft = outflow tract; trab = trabeculations. The panels represent consecutive sections showing the right and left atria, the right and left ventricles and the outflow tract of the heart. In the heart, the sinus horns and the atria are marked by the expression of α-MHC (B), while the ventricles and the outflow tract express β-MHC (A). Connexin40 is expressed in the atria and ventricles, heterogeneously in the sinus horns but not in the rim of myocardium that forms the outflow tract (C). In the ventricles a remarkable heterogeneity is observed, the free wall of the left ventricle, but not that of the right ventricle expressing connexin40. The trabeculations of both the right and the left ventricle express connexin40 (C). In contrast, connexin43 is homogeneously expressed in both the left and right ventricular free wall, but the level of expression is less abundant in the trabeculations and in the atria than in the ventricular free wall (D). In the sinus horns and the outflow tract little connexin43 can be detected (D). In the embryonic tissue that surrounds the heart, connexin40 is abundantly expressed in the myo-osseous junction, the vessel wall of the aorta and the pulmonary arteries (C). Connexin43 is expressed in the mesenchymal cells lining the developing ribs and vertebrae (D). Bar, 100 μm.
adults), to increase tissue accessibility; (6) inhibition of protease activity in 0.2% glycine in PBS (phosphate-buffered saline: 10 mM Na₂HPO₄/10 mM NaH₂PO₄, 150 mM NaCl pH 7.4) for 30 s; (7) two short wash steps of 30 s in PBS; (8) postfixation in 4% formaldehyde in PBS at room temperature for 20 min; (9) wash in PBS for 5 min; (10) incubation in 10 mM dithiothreitol in bidistilled water for 5 min, to prevent tissue oxidation and subsequent formation of S-S bridges between the ³⁵S-labelled probe and tissue proteins; (11) air-drying for at least 1 h.

2.5. In situ hybridization

Each slide contains six serial sections; on each section a different probe was applied, using the droplet method [39]. The ³⁵S-radiolabelled probe was diluted in a hybridization mixture (50% formamide, 10% dextran sulphate, 2 X SSC, 0.1% Triton X-100, 10 mM dithiothreitol, 200 ng/μl herring sperm DNA) to 5-7 X 10⁴ cpm/μl. Hybridization took place at 51°C for approx. 17 h. The washing protocol consisted of the following steps: (1) rinse the hybridization mixture from the slides with a Pasteur pipet; (2) wash two times in 0.50% formamide/1 X SSC for 15 min at 51°C; (3) incubate in ribonuclease A (10 μg/ml) at 37°C for 30 min; (4) wash two times in 1 X SSC for 10 min at 51°C; (5) wash in 0.1 X SSC for 10 min at 51°C; (6) dehydrate in a graded series of 0.3 M NH₄Ac in ethanol; (7) air-dry the sections.

For the detection of the radioactive signal the slides were dipped in a photosensitive emulsion (Ilford Nuclear Research Emulsion G5), and exposed for 5-7 days. After development, the sections were counterstained with 0.01% toluidine blue or nuclear fast red. After dehydration in a graded series of ethanol and xylol the sections were mounted in malinol and photographed using bright field illumination.

3. Results

3.1. Embryonic period: 9-16 days of gestation

From the earliest stages of development the myocardium can be identified by the expression of α- and/or

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Fig. 4. Photomicrographs showing the expression of B-MHC (A), α-MHC (B), connexin40 mRNA (C) and connexin43 (D) in the ED 18 rat heart. san = sinoatrial node; a = atrium; rv = right ventricle; lv = left ventricle; lscv = left superior caval vein. Panels A, B, C and D represent consecutive sections, showing the sinoatrial node at the border of the right superior caval vein and the right atrium, the pulmonary vein draining into the left atrium, the right and left atrioventricular connections and the basal parts of both ventricles. The myocardium of the atria, the pulmonary veins and the sinus horns, and the myocardium of the ventricles are characterized by the expression of α- or β-MHC (A and B). Connexin40 (C) and connexin43 (D) are expressed in the right and left atrium, the proximal parts of the pulmonary veins and the sinus horns. No expression can be detected in the sinoatrial node, the atrioventricular canal and the atrioventricular bundle. In the ventricles, connexin40 is expressed in the ventricular trabeculations and, to a lesser extent, in the free wall of the left ventricle. No expression is found anymore in the free wall of the right ventricle and only a low level of expression in its trabeculations (C). Connexin43 is expressed in the free walls of both ventricles and to a lesser extent in the trabeculations (D). Bar, 200 μm.
0-MHC. In the ED 9 embryo the cardiogenic plate in the visceral wall of the pericardial coelom can be distinguished by the expression of β-MHC mRNA (Fig. 1B). In a serial section no Cx40 or Cx43 mRNA can be detected in this particular part of the embryo, while the ectoderm is marked by a high labelling of Cx43 mRNA (Fig. 1A).
At ED 10, the tubular heart is recognizable in the pericardial cavity at the ventral side of the developing embryo (Fig. 1D). The expression of both Cx40 and Cx43 mRNA (Fig. 1D) does not exceed background levels, while Cx43 mRNA is highly expressed in the neural ectoderm, the endoderm of the branchial pouches and in
Fig. 7. Photomicrographs showing the expression of desmin protein (A), connexin43 mRNA (B, C and D) and a haematoxylin-azophloxin staining (E) in the adult rat heart. Panels D' and E' show a larger magnification of part of panels D and E (bar = 200 μm). vcs = superior caval vein; san = sinoatrial node; a = atrium; ias = interatrial septum; v = ventricle; avc = atrioventricular canal; avn = atrioventricular node; avb = atrioventricular bundle. The figure in panel A represents a section at the level of the junction between the right atrium and the right ventricle. Desmin, a myocardial marker (A), is abundantly expressed in the atrioventricular node (arrow) at the basal part of the interatrial septum. The figure also shows the sinoatrial node at the border of the superior caval vein and the right atrium (arrow). The enlargements of areas of the sinoatrial node (B) and the atrioventricular node (C) show that connexin43 is expressed in the surrounding myocardium, but is not detectable in the nodes themselves. Furthermore, no expression can be detected in the atrioventricular bundle and the bundle branches (D), whereas the surrounding myocardium of the atrium and the ventricle do express connexin43. Note that connexin43 is expressed in a distinct spotty pattern (D, D' shows larger magnification). In a consecutive section (E), the classical haematoxylin staining shows the cell nuclei (E' shows larger magnification). Bar, 200 μm.

At ED 11 the tubular heart has looped, the myocardium is marked by the expression of β-MHC and the different segments of the heart can be clearly distinguished (Fig. 1F). No clear labelling of either Cx40 (Fig. 1F) or Cx43 (Fig. 1G) mRNA can be recognized in these parts. Cx40 mRNA labelling can be observed in the endothelial lining of the aorta (Fig. 1F). The labelling of Cx43 mRNA in the neural ectoderm has become less intense (Fig. 1G).

In the ED 13 embryo both Cx40 and Cx43 mRNA
exceed the background level of staining in the atrial and ventricular myocardium that is again delineated by the labelling of α- (Fig. 2B,E,H) and β-MHC (Fig. 2A,D,G). Most of the Cx40 mRNA labelling can be detected in the roof of the embryonic atrium and in the venous valves (Fig. 2C). Towards the atrioventricular canal the expression of Cx40 mRNA gradually decreases. In both ventricles, the free wall and the trabeculations express Cx40 mRNA to a comparable extent.

Cx43 mRNA labelling in the atrial myocardium is detectable, but faint (Fig. 2F,I). In the ventricle the labelling of Cx43 mRNA is more pronounced in the free wall as compared to the trabeculations. In the other segments of the developing heart, i.e., the inflow tract (including the myocardial part of the sinus venosus and, later in development, the caval and pulmonary veins), the atrioventricular canal and the outflow tract (comprising the myocardium interposed between the ventricles and the great arteries), the expression of neither Cx40 (Fig. 2C) nor Cx43 (Fig. 2F,I) mRNA exceeds background levels.

In the late embryonic stage (ED 16) the heart has almost completed its remodelling towards a four-chambered blood pump; the right and left sides have their own inflow and outflow components. The α-MHC marks the myocardium of the inflow tract and the atria (Fig. 3B), while β-MHC marks the ventricles and the outflow tract (Fig. 3A). Compared to younger embryos, both Cx40 and Cx43 mRNA are easier to detect in various compartments of the heart. Cx40 mRNA is randomly expressed in the myocytes of the sinus horns, i.e., some cells do and some cells do not show hybridization signal. In both the left and the right atrium, abundant homogeneous labelling of Cx40 mRNA can be detected (Fig. 3C). However, between the two ventricles a remarkable heterogeneity is evident so that a distinction has to be made between left and right. In the right ventricle, Cx40 mRNA can be observed in the trabeculations but no longer in the ventricular free wall. In the left ventricle, Cx40 mRNA is still detectable in both the trabeculations and the free wall. In the interventricular septum the intensity of Cx40 mRNA hybridization does not exceed background staining.

Cx43 mRNA labelling in the inflow tract and the atria exceeds background labelling but is still difficult to detect (Fig. 3D). In both ventricles labelling can be detected mainly in the free wall, while Cx43 mRNA in the trabeculations is less obvious. The rim of myocardium that forms the outflow tract and which is clearly distinguishable by the labelling with β-MHC mRNA (Fig. 3A), does not show labelling for either Cx40 (Fig. 3C) or Cx43 (Fig. 3D). The same holds true for the myocardium of the atrioventricular canal (not shown).

Besides the labelling in the heart, Cx40 mRNA can also be detected in the endothelial lining of the vessel walls and in the developing skeletal muscle at the transition between developing bone and the side where myoblasts fuse to form myotubes (the myo-osseous junction; Fig. 3C). This observation is in line with earlier observations of Dahl et al. [22]. Cx43 mRNA can also be detected in chondroblasts and osteoblasts in the developing bones (Fig. 3D).

3.2. Fetal period: 18–22 days of gestation

In this stage of development morphogenesis of the heart is completed, although the process of maturation continues. The myocardium is clearly delineated by the expression of α-MHC in the atria and β-MHC in the ventricles (Fig. 4A). To a large extent the distribution patterns of the connexin mRNAs in the fetal period do not change when compared to the late embryonic period. Cx40 mRNA is still expressed in the inflow tract and the atria (Fig. 4C). An exception is formed by the ventricles which display a very dynamic distribution pattern of Cx40 mRNA. In the ED 16 heart, Cx40 mRNA could be detected in the trabeculations of the right ventricle but not in the ventricular free wall. In the left ventricle, however, both the trabeculations plus the free wall of the ventricle showed hybridization of the Cx40 cRNA probe (Fig. 3C). Two days later in development, at ED 18, the labelling of Cx40 mRNA has also diminished in the free wall of the left ventricle (Fig. 4C). At ED 20, Cx40 labelling in the left ventricle has become confined to the trabeculations, just like in the right ventricle (Fig. 5C).

Cx43 mRNA remains clearly present in the ventricular free wall (Fig. 4D). The hybridization signal of Cx43 mRNA stays relatively low in the inflow tract, the atria and the ventricular trabeculations (Fig. 4D). Although the atrioventricular canal (Fig. 4C,D) and outflow tract (not shown) are diminished in length relative to the atria and ventricles, significant levels of Cx40 and Cx43 mRNA remain undetectable.

In the fetal period, parts of the conduction system, as they are defined in the adult heart, become recognizable. In the sinuatrial node, which is topographically located at the transition of the vena cava superior and the right atrium, neither Cx40 (Fig. 4C and Fig. 5C) nor Cx43 (Fig. 4D) mRNA can be detected. The node is characterized by the expression of some β-MHC mRNA (Fig. 4A and Fig. 5A) and abundant α-MHC (Fig. 4B and Fig. 5B) mRNA. In the atrioventricular bundle and the top of the bundle branches, which are located at the top of the interventricular septum, some Cx40 mRNA is labelled (Fig. 4C and Fig. 5C). However, no Cx43 mRNA labelling was observed (Fig. 4D). More distal from the atrioventricular bundle, where the bundle branches are located, both Cx40 and Cx43 mRNA labelling can be observed.

At the end of fetal development, Cx40 and Cx43 mRNA can be readily detected in the walls of the pulmonary artery and the aorta, which are recognizable as elastic vessels in which neither α-MHC nor β-MHC can be detected (Fig. 5D,F). Cx40 mRNA is confined to the endothelial lining of the large arteries and coronary arteries (Fig. 5E). Interestingly, Cx40 mRNA is not expressed in
Table 1
Expression patterns of connexin40 and connexin43 mRNA and protein during development in various parts of the heart

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MRNA mRNA only mRNA and PROTEIN
the endothelium that covers the developing semilunar valves, revealing that this endothelium behaves as true endocardium. Similar observations were made by Bastide et al. [14]. The hybridization signal of Cx43 mRNA can be detected more broadly over the entire profile of the vessel wall, probably representing layers of smooth muscle cells (Fig. 5G).

3.3. Suckling period and adult

Compared to the fetal period, only a faint expression of Cx40 mRNA remains in the atria of the suckling rat (Fig. 6B). This expression gradually extinguishes and is no longer detectable in the adult. In both the right and the left ventricle, the expression of Cx40 mRNA (Fig. 6H) has become confined to the subendocardial myocyte fibres. These fibres can be identified as myocardial by the expression of α-MHC mRNA or desmin protein are the conducting fibres of the ventricular conduction system. No Cx40 mRNA can be detected in the working myocardium of adult ventricles.

Prenatally, the expression of Cx43 mRNA is more pronounced in the myocardium of the ventricular free wall than in either that of the ventricular trabeculations or the atria. Compared to these younger developmental stages, Cx43 mRNA becomes expressed more abundantly within the myocardium of the atria and of the ventricular trabeculations in the neonatal and suckling period, so that the difference in staining intensity between the trabeculations and the myocardium of the free wall has become less pronounced. More interestingly, the pattern of expression of Cx43 mRNA changes during the suckling period from a homogeneous distribution of grains towards a more spotty configuration. In the adult heart, the clustering of grains has become so pronounced that the expression of the Cx43 mRNA is confined to discrete spots (Fig. 7B,C,D,D'). Going from the subendocardium towards the subepicardial side of the ventricles, the concentration of spots increases. Comparison of the in situ hybridization and haematoxylin-eosin images (Fig. 7D,E) shows that the number of spots does not correspond with the number of nuclei. For more detail, compare larger magnification in Fig. 7D',E'. We assume that the Cx43-positive spots are confined to cardiomyocytes, while the HA-stained nuclei represent in addition endothelial cells and fibroblasts. We have not observed this punctuated expression pattern for several other mRNAs, including that of Cx40, in parallel hybridizations of consecutive sections. Showing it to be specific for Cx43 and not due to a histological artifact. Due to the proteolytic enzyme treatment of the sections prior to hybridization, we have not yet been able to localize the subcellular localization of Cx43 mRNA within the myocytes more precisely.

5.4. The conduction system

After birth, during the suckling period as well as in the adult heart, the myocardium of the superior caval vein, as delineated by the expression of α-MHC mRNA (Fig. 6A) or desmin protein (Fig. 7A), were found to express Cx43 mRNA (Fig. 6C and Fig. 7B), but slight Cx40 mRNA (Fig. 6B). In the sinoatrial node at the junction of the superior caval vein and the right atrium and in the atrioventricular node at the bottom of the interatrial septum, no detectable levels of either Cx40 (Fig. 6B,E) or Cx43 (Fig. 6C,F and Fig. 7B,C) mRNA were noticed.

In the neonatal period, the expression of Cx40 mRNA and protein becomes confined to the subendocardial myocyte fibres in both ventricles. These fibres, which can be identified as myocardial by the expression of α-MHC mRNA (Fig. 6G), are probably the conducting fibres of the ventricular conduction system. Some Cx40 is detectable in the atrioventricular bundle at the mRNA level (Fig. 6H) as well as at the protein level (not shown). However, as described previously for the Cx43 protein, no Cx43 mRNA could be detected in the atrioventricular bundle and the proximal part of the bundle branches on top of the interventricular septum (Fig. 6I and Fig. 7D). In agreement with previous studies [15,21], more distally in the conduction system, in the subendocardial fibres of the bundle branches, the Cx40 protein is more abundantly expressed when compared to the more proximal atrioventricular bundle (not shown), but only a faint expression of Cx40 mRNA is seen (Fig. 6H). In these distal subendocardial Purkinje fibres, the Cx40 is co-expressed with the Cx43.

4. Discussion

The aim of this study was to map the spatiotemporal expression pattern of Cx40 and Cx43 mRNA, to substantiate the developmental appearance and distribution of the respective proteins. Commonly, the distribution pattern of
connexins is studied using immunohistochemistry as a labelling technique; however, conclusive remarks about the presence or absence of connexin isotypes are hard to make because immunostaining greatly depends on the plaque size of gap junctions. It has been calculated that the gap junctions containing 40–100 channels form the upper limit of detection by immunohistochemistry [12, 23]. In contrast, in situ hybridization with a radioactive probe provides a sensitive tool that is able to detect the presence of only 150–300 mRNA molecules per 100 μm² [40]. Furthermore, since it produces cytosolic labelling, it provides a clear overview of the expression pattern of genes, which is particularly helpful for connexins as these proteins are present as small aggregates in membranes. Table 1 summarizes Cx40 and Cx43 mRNA expression patterns during development as they are described in this study. They are combined with previously published studies on the expression patterns of Cx40 [32] and Cx43 [12] protein.

4.1. Comparison of Cx43 mRNA and protein distribution

As could be expected, myocardium that expresses Cx43 protein, i.e., the working myocardium of the atria and ventricles, and later in development the myocardium of the inflow tract (except the sinus node), is also labelled with the Cx43 cRNA probe. However, we were not able to detect Cx43 mRNA exceeding background levels in areas where we could not detect Cx43 protein in development, i.e., the early inflow tract, the atrioventricular canal and the outflow tract, and later in prenatal life and in the postnatal heart the sinus node, the atrioventricular node and bundle and proximal part of the bundle branches. This observation strengthens the idea that early gap-junctional structures and the gap junctions that are found in the sinus node, atrioventricular node and bundle and the top of the bundle branches are formed by a connexin isotype other than Cx43.

Quantitatively, the hybridization signal becomes more intense during development which suggests an increase in Cx43 mRNA transcription. Using Northern and Western blots, Fishman et al. [41] and Fromaget et al. [42] measured the developmental changes in the relative abundance of Cx43 mRNA and protein concentrations in the rat and mouse heart between the early fetal period and 6 weeks after birth. Cx43 mRNA and protein show an eight- to 15 fold increase. The cardiac Cx43 mRNA content in the rat rapidly increases from the earliest measurement at ED 17 to its maximum level at about 1 week after birth. The Cx43 protein content rises directly after birth and peaks at 3 weeks after birth, i.e., 2 weeks later than the mRNA. This temporal difference in the accumulation of mRNA and protein suggests that at early stages of heart development the levels of expression of Cx43 protein are regulated by the abundance of Cx43 mRNA, i.e., by pretranslational regulatory mechanisms, but also that translational efficiency is still low. After birth, the relative levels of protein increase faster than those of mRNA, suggesting that the Cx43 protein abundance depends to a large extent on increased protein stability and/or increased translational efficiency, i.e., posttranscriptional regulatory mechanisms [42].

The postnatal increase in translational efficiency or protein stability is accompanied by a marked change in the subcellular staining pattern of Cx43 mRNA, viz. a progressive clustering of silver grains, so that beyond the weaning period the Cx43 mRNA staining pattern has become confined to discrete spots. Puranam et al. [43] described the trapping of Cx43 protein in the Golgi apparatus on its way to the plasma membrane. The clustering of Cx43 mRNA in discrete dots may reflect the area of translation, possibly near the Golgi apparatus. This developmental change in the subcellular distribution of the Cx43 mRNA appears to provide an attractive explanation for the postnatal increase of the Cx43 protein/mRNA ratio [41, 42] by suggesting that the change in this ratio is due to an increased efficiency of mRNA translation rather than an increased stability of the protein.

Our results indicate that, in addition to temporal differences, regional differences in the regulation of Cx43 expression exist: the presence of myocardial areas that do and do not express Cx43 mRNA indicates the presence of a pretranslational control mechanism in the heart. Keeping in mind the earlier mentioned drawbacks of immunohistochemistry, we observed that both Cx43 protein and mRNA labelling show differences in regional abundance. By comparing regional protein abundance with regional mRNA abundance we observed a relatively high expression of Cx43 mRNA, but no detectable protein [12] in the free wall of the ventricle in the embryonic period and in the subepicardial layer of the ventricular free wall in the fetal period. In contrast, we observed a relatively faint expression of Cx43 mRNA, but a relatively abundant expression of Cx43 protein in the myocardium of the atria and the ventricular trabeculations in the fetal period. Both observations suggest the presence of regional differences in regulation of expression at a posttranscriptional level. Comparison of the studies of Yancey et al. [35] and Dahl et al. [22] with that of Ruangvoravat et al. [44], who respectively report on the expression of Cx43 protein and mRNA in mouse hearts, shows similar regional regulation of Cx43 expression in the ventricle: Cx43 protein labelling is abundant in the trabeculae and weak in the epicardial myocardium, while Cx43 mRNA labelling is homogeneously abundant. This kind of observations nicely illustrates that a morphological analysis of gene expression is a required complement to biochemical analysis lest a too simple picture of the level of regulation of Cx43 expression emerges.

4.2. Comparison of the Cx40 mRNA and Cx43 mRNA distribution

This study aimed in part to establish whether gap junctions in early development (before ED 13) and gap
junctions in the inflow tract, the atrioventricular canal and the outflow tract of the developing heart are composed of Cx40, since no Cx43 protein could be detected in these parts. The answer is in the negative. Recently, Delorme et al. [32] studied the distribution pattern of Cx40 protein in the developing mouse heart. Comparison of our and their study showed that spatial and temporal distribution patterns of Cx40 mRNA and protein in the developing rat and mouse are highly comparable. Furthermore, other less detailed data on the expression of Cx43 [44] and Cx40 mRNA [22] in heart and other tissues during development are in line with our findings. In general, no major regional differences were found to exist between the pattern of distribution of Cx40 and Cx43 in prenatal life. Postnatally, in the rat low levels of expression of Cx40 protein in the atrioventricular node, and little Cx43 protein in the sinoatrial node, the atrioventricular node and the atrioventricular bundle have been reported in CLSM studies [13,15]. However, we have not been able to demonstrate Cx40 and Cx43 mRNA in these respective structures. These data can only be reconciled if translation of gap-junction mRNA and/or stability of the gap-junction protein is very high in the nodes.

Based on the expression of Cx40 and Cx43 mRNA in the developing rat myocardium two populations of myocytes can be distinguished, viz. cardiomycocytes that do not express Cx40 and Cx43, and cardiomycocytes that do express both. The first group includes the myocardium of the sinoatrial node, the atrioventricular canal (including the atrioventricular node) and the outflow tract. The second population includes the working myocardium of the atria and the ventricles, and, later in development, the myocardium of the inflow tract, excluding the sinoatrial node. Nevertheless, due to the overview that is accomplished by the in situ hybridization signal, it is clear that the temporal pattern of expression between Cx40 and Cx43 differs greatly. Both genes become expressed at detectable levels in the heart at ED 13, but expression of Cx40 peaks at ED 16 and subsequently declines. In contrast, the expression of Cx43 continues to increase, concomitant with increasing conduction velocity in the heart.

Because Cx40 expression begins to decline in the working myocardium before maximum conduction velocities have developed, it seems very well possible that Cx40 in the developing heart is responsible for other functions of gap junctions that have been identified, such as growth [45] and/or differentiation [46]. In this context, the locally different rate of decline of Cx40 mRNA that we found in the ventricles may be of importance. Up to ED 14, Cx40 mRNA is expressed with comparable abundance in both ventricles. Between ED 14 and ED 20, Cx40 mRNA gradually disappears from the ventricular myocardium, first from the right ventricular free wall towards the trabeculations, and later also from the left ventricular free wall. Cx40 mRNA remains expressed in the apical part of the trabeculations, presumably the future subendocardial conduction fibres. This asymmetric distribution pattern of Cx40 mRNA in the ventricles, which was also observed for Cx40 protein in mouse ventricular development [32], suggests a separate, individual development of the left and right ventricles with respect to gap-junctinal structures, even though a coordinated depolarization is required for a synchronous contraction. The idea that gap junctions may be involved in the left/right patterning of the heart is also endorsed by the findings on the hearts of the Cx43-knockout mice [25]. They die, just after birth, because overgrowth of tissue in the right ventricle results in anatomical blockage of normal blood flow to the lungs. The fact that Cx43-knockout mice follow an apparently normal developmental programme may be ascribed to the rescue of Cx43 by other connexins, possibly Cx40. In this context, besides the necessity to establish which connexin is expressed in areas where we were not able to detect either Cx43 or Cx40 and to study the functional implications of connexin diversity, it seems of interest to know whether expression of different connexins may be mutually regulated.

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