Embryonic stem cell-derived cardiomyocytes
Fijnvandraat, A.C.

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Non-Radioactive In Situ Detection of mRNA in ES Cell-Derived Cardiomyocytes and in the Developing Heart

Arnoud C. Fijnvandraat, Piet A.J. de Boer, Ronald H. Lekanne Deprez and Antoon F.M. Moorman
Experimental and Molecular Cardiology Group, Academic Medical Center, Amsterdam

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ABSTRACT

Non-radioactive in situ hybridisation is an excellent method to visualise mRNA molecules within their topographical context. Recently we have reported a new non-radioactive in situ hybridisation procedure on tissue sections that is essentially based on the whole mount in situ hybridisation procedure. This method is superior in spatial resolution and sensitivity compared to the radioactive in situ hybridisation procedure. Generally, low levels of gene expression, such as found with the developmental onset of gene expression and in differentiating embryonic stem cells, are difficult to detect by in situ hybridisation. Here an application of the protocol is presented which is based on tyramide signal amplification, which enables the detection of very low abundant mRNAs. The significance of this method is two-fold: (1) the molecular phenotype of embryonic stem cell-derived cardiomyocytes can be examined at the cellular level with high sensitivity, and (2) the number of cells that express the gene of interest can be assessed.
INTRODUCTION

Development is controlled by complex molecular events in which cell-cell interactions play an important role. Knowing the spatio-temporal relationships of the genes involved provides insight in the precisely orchestrated process of tissue development and organogenesis. Detection methods of gene expression should be sensitive and the cellular resolution high, as the morphological context provides indications of functional interactions between cells. This is particularly important in an organ like the heart that comprises distinct compartments and has an intricate three-dimensional architecture. The developmental interactions between endocard, myocard and epicard in relation to the formation of the working myocardium of the chambers and the formation of the nodes and the ventricular conduction system, are far from understood and are still subject to much controversy.

Embryonic stem (ES) cells can be differentiated in vitro and potentially can give rise to all cell types of an organism. Several groups showed the presence of cells representing the cardiac lineage (Müller et al. 2001; Doevendans et al. 2000; Hescheler et al. 1997; Doevendans et al. 2000; Hescheler et al. 1997; Nascone and Mercola, 1996). In terms of its use as a model for cardiogenesis the question can be justifiably asked whether and to what extent in vitro differentiation of ES cells represents in vivo cardiogenesis. Moreover, when ES cell-derived cardiomyocytes are applied for clinical transplantation, validation of the model system by characterisation of the cardiomyocytes is required. Therefore, it is essential to assess changes in the molecular phenotype of the in vitro differentiated cells. Comparison of the cardiac phenotypes observed after ES cell differentiation with the in vivo developmental cardiac phenotypes might elucidate the way in which the in vitro system serves as a model for developmental processes in vivo.

Real-time reverse transcription polymerase chain reaction (RT-PCR) is one of the most sensitive methods as yet to quantify the developmental changes in mRNA levels. Because this method is applied on mRNA extracted from tissue homogenates, differences in mRNA levels owing to changes in cellular diversity are masked. Thus, high expression in a few cells cannot be distinguished from low expression in a large number of cells. Moreover, it cannot be assessed whether increase in expression is caused by higher expression in the same amount of cells or due to recruitment of expressing cells. In situ hybridisation (ISH) potentially could overcome this problem.

Classic ISH techniques use radioactively labelled probes and a photographic emulsion to visualise the location of hybridisation in the tissue section. The method is very sensitive and quantitative (Moorman et al. 2000a; Jonker et al. 1997; Jonker et al. 1997). However, the field of sharpness of the section and of the developed silver grains in the photographic emulsion are different, thereby decreasing cellular resolution.
In this paper we describe a sensitive detection system to detect low copy mRNA on sections and cells using non-radioactive (non-RA) ISH. We increased the sensitivity even more by using tyramide signal amplification, also called catalysed reporter deposition (Yang et al. 1999). This amplification step proved essential to detect in situ mRNA sequences in embryoid bodies or cells but gained no improvement on sections. In this way we optimised the protocol for ES cell-derived cardiomyocytes that show low levels of gene expression.

MATERIALS, METHODS, AND ANIMALS

Cell Culturing
The ES cell line HM1 (Magin et al. 1992) was kept in an undifferentiated state by culturing on a feeder layer of irradiated primary cultures of mouse embryonic fibroblasts (MEF). Cells were cultivated on 0.1% gelatin coated tissue culture plastics (COSTAR) on GMEM (GibcoBRL), 10% FCS (v/v) (GibcoBRL), L-Glutamine (2 mM, GibcoBRL), sodium pyruvate (1 mM, GibcoBRL), non-essential amino acids (1x, GibcoBRL), 5x10^{-5} M β-mercaptoethanol (GibcoBRL), Leukemia Inhibitory Factor (LIF) (10^3 U/ml, GibcoBRL).

Cardiac differentiation was evoked by culturing the cells as embryoid bodies, using the hanging drop assay, essentially as described by Maltsev et al. (1994) with some small modifications. These drops, consisting of 600 cells/20 µl ISCOVE'S MDM culture medium (GibcoBRL), enriched with 20% FCS (Bodinco, catalogue number 39454), 1x amino acids and 1x penicillin/streptavidin (both from GibcoBRL), were pipetted on the lid of bacterial petri dishes. The lids were placed back on the dish that contained PBS (GibcoBRL) to prevent evaporation of the drops and were cultured at 37°C and 5% CO2. After 3 (indicated as day 3+0) days of incubation in hanging drops, aggregates of differentiating ES cells, called embryoid body (EB), were transferred to a floating culture, one EB per culture well (24 wells plate, COSTAR, O approximately 1.5 cm). After 2 days in suspension culture (day 3+2), approximately 90% of the EBs showed beating activity, encompassing over 50% of the total area of the EB. After several days, beating embryoid bodies were harvested from suspension culture, rinsed twice in PBS and fixed in 4% formaldehyde in PBS for 30 minutes at RT. Groups of EBs were embedded in 1% sterile agarose at 50°C and stored in 70% ethanol at 4°C until further treatment. Agarose blocks were dehydrated in a graded ethanol series and butanol and embedded in paraplast. Serial sections of 15 µm were mounted onto microscope glasses coated with 3-aminopropyltriethoxysilane.
TABLE 1. Specificities of Dig-UTP Labelled Probes Used for Non-RA ISH

<table>
<thead>
<tr>
<th>Probe</th>
<th>A-residues in template</th>
<th>Nucleotides</th>
<th>Species</th>
<th>Rat/mouse nucleic acid homology (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anf</td>
<td>156</td>
<td>1-580</td>
<td>Rat</td>
<td>92%</td>
<td>Seidman et al. (1984)</td>
</tr>
<tr>
<td>Mlc2v</td>
<td>181</td>
<td>1-640</td>
<td>Rat</td>
<td>94%</td>
<td>O’Brien et al. (1993)</td>
</tr>
<tr>
<td>cTnl</td>
<td>243</td>
<td>1-830</td>
<td>Rat</td>
<td>95%</td>
<td>Ausoni et al. (1991)</td>
</tr>
</tbody>
</table>

Animals

FVB mice were obtained from timed-pregnant animals and used for RNA isolation and ISH. Noon of detection of the vaginal plug was considered embryonic day 0.5. Embryos were fixed for 4 hours to overnight in 4% formaldehyde in PBS by rocking at 4°C. Embryos were dehydrated in a graded ethanol series and in butanol and embedded in paraplast. Serial sections of 15 μm were mounted on microscope glasses coated with 3-aminopropyltriethoxysilane.

Probes

Digoxigenin-labeled probes were made according to the manufacturer’s specifications, using Dig-UTP (Roche; Mannheim, Germany). Full-length probes were used complementary to the mRNA coding for cardiac troponin inhibitor (cTnl) (Ausoni et al. 1991), myosin light chain (Mlc)2v (O’Brien et al. 1993) and atrial natriuretic factor (Anf) (Seidman et al. 1984). In contrast to probes used for classical radioactive ISH, which are alkaline hydrolysed to a length of approximately 150 nucleotides for optimal hybridisation and penetration of the tissue, no difference was observed between full-length and alkaline hydrolysed digoxigenin labelled probes. The amount of adenosine residues in the template is indicative for the specific UTP-Dig labelling of the probe. Probe characteristics are cited in Table 1.

Non-Radioactive In Situ Hybridisation on Tissue Sections

Sections were deparaffinized in xylene and a graded series of ethanol. After two washes in PBS for 5 minutes, the sections were treated with proteinase K (20μg/ml in PBS) for 8 minutes to open the cells by proteolytic digestion. Proteinase K enzyme activity was competitively inhibited during incubation in 0.2% glycine/PBS for 5 minutes. Cells were rinsed twice in PBS for 5 minutes and post fixed for 20 minutes in 4% formaldehyde/0.2% glutaraldehyde in PBS to increase attachment of the sections to the microscope slide. After 2 x 5 minutes washing with PBS, cells were prehybridised with hybridisation mix without probe for 1 hour at 70°C in a moist chamber: Plastic incubation boxes (13 x 8.5 x 1.5 cm) (Heraeus, Quadriperm) containing maximally 4 horizontally placed microscope glasses with the sections on top were put in a closed plastic box (15 x 11 x 7 cm) containing 50% formamide soaked paper tissues. The hybridisation mix is composed of 50% formamide, 5 x SSC, 1% block solution (Roche), 5
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mM EDTA, 0.1% Tween-20, 0.1% CHAPS (Sigma, St. Louis, MO), 0.1 mg/ml heparin (Becton-Dickinson; Mountain View, CA), and 1 mg/ml yeast total RNA (Roche). Hybridisation mix (3-5 µl) was applied to each section. Pre-hybridisation mix was replaced by fresh hybridisation mix containing digoxigenin labelled probe (1 ng/µl) and hybridisation was performed overnight at 70°C. Again, hybridisation was performed in a moist chamber to prevent evaporation, in this way overcoming the need for cover slips, which has the risk of damaging the sections. It is crucial to demarcate each section with a hydrophobic contour that can resist high temperatures, to prevent mixture or leaking away of hybridisation mix during incubation. In our hands, the ImmEdge pen (Vector, Vector Laboratories, Inc., Burlingame, CA; catalogue number H-4000) worked appropriately.

After hybridisation, sections were rinsed in 2 x SSC, pH 4.5, washed (2 x 15 minutes) with 50% formamide/2 x SSC, pH 4.5 at 65°C and washed three times 5 minutes with PBS-T (PBS + 0.1% Tween-20 v/v). Immunological detection of the hybridised digoxigenin labelled probe was done as follows: Sections were blocked for 30 minutes with B-Block (2% Blocking Reagent (Roche 1096 176) + 10% goat serum in PBS-T) to reduce non-specific binding of antibodies and incubated with sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Roche), 500 mU/ml, in B-Block for 2 hours at RT (7-10 µl per section), and finally washed 3 x 5 minutes with PBS-T followed by two washes in NTM-T (100 mM NaCl, 100 mM TRIS pH 9.5, 50 mM MgCl₂ + 0.05% Tween-20) for 5 minutes. After removal of NTM-T, NBT/BCIP was used as a chromogenic substrate (Roche), diluted 1:50 in NTM-T, according to the manufacturers protocol. The reaction time was 4-18 hours. Sections were dehydrated in a graded series of ethanol and xylene and embedded in Entellan.

Non-Radioactive In Situ Hybridisation on Sections of EBs
Signal amplification for low abundant messenger detection in EBs was performed by the NEN (Boston, MA) Renaissance Tyramide Signal Amplification kit. The pre-treatment and hybridisation procedure was generally the same as described above. After the graded series of ethanol, a 30-minute incubation in 1% hydrogen peroxide in PBS was introduced to block endogenous peroxidase. Sections of EBs were treated with proteinase K for 4 minutes. After the wash with 50% formamide/2 x SSC, sections were washed 3 x 5 minutes in TNT (100 mM TRIS pH 7.5, 150 mM NaCl, 0.05% Tween20) and were blocked for 30 minutes with TNB (100 mM TRIS pH 7.5, 150 mM NaCl, 0.5% Blocking reagent from NEN). A graded range of probe concentrations was tested. A probe concentration of 2 ng/µl proved optimal. Subsequently, sections were incubated with sheep antidigoxigenin Fab fragments conjugated to horseradish peroxidase (Roche), 1U/ml in TNB for at least 2 hours at RT and washed in TNT (3 x 5 minutes). Signals were amplified by incubating the sections with Biotinyl-Tyramide (NEN™ Life Sciences...
Products) (1:50 in amplification solution) for 15 minutes. After washing 3 x 5 minutes in TNT, biotin was detected by overnight incubation with streptavidin conjugated to an alkaline phosphatase (Roche) 500 mU/ml in TNB followed by 3 x 5 minutes washing in TNT. Sections were washed with NTM-T and incubated with NBT/BCIP as described for the non-signal amplification detection method. The time of the staining reaction was 30 minutes. The staining reaction was terminated by washing the sections in double-distilled water. Sections were dehydrated in a graded ethanol series and xylene, and embedded in Entellan.

**Non-Radioactive In Situ Hybridisation on Single Cells**

Beating EBs of day 3+10 were collected and washed in low-Ca\(^{2+}\) buffer. Low-Ca\(^{2+}\) buffer contains 120 mM NaCl, 5.4 mM KCl, 5 mM MgSO\(_4\), 5 mM Na pyruvate, 20 mM glucose, 20 mM taurine, 10 mM HEPES. The pH was adjusted to 6.9 by addition of NaOH (Maltsev et al. 1994). Aggregates were dissociated in low-Ca\(^{2+}\) buffer, supplemented with 1mg/ml collagenase B (Boehringer Mannheim) and 30 μmol/l CaCl\(_2\). Dissociation was performed for 30 minutes at 37°C and aggregates were mechanically dispersed by carefully pipetting up and down with a 200-μl Gilson pipette every 10 minutes. The suspension was diluted in differentiation medium and plated onto 3-aminopropyltriethoxysilane coated microscope slides (2.4 x 2.4 cm, Rofa-Mavi) in a culture well (Ø 3.5 cm Becton Dickinson). Cells were cultured o/n at 37°C and 5% CO\(_2\). The next day, medium was removed and the cells were washed twice with PBS, following fixation with 4% formaldehyde in PBS for 30 minutes at RT. Cells were rinsed with PBS and stored under 70% (v/v) ethanol in water at 4°C. After hydration by rinsing in 50% ethanol and washing with PBS, the cover slips could be used for ISH. The ISH procedure is the same as described for EBs except that deparaffination is not necessary and proteinase K treatment was omitted since this leads to loss of attached cells.

**Imaging**

Images were taken using a Digital Nikon Coolpix camera attached to a Zeiss Axiophot microscope, flat field correction was applied, and digitised files were recorded.

**RNA Isolation and Primer Design**

Total RNA was isolated using an RNA isolation kit (RNeasy, Qiagen) according to the instructions of the manufacturer. To remove contaminating genomic DNA, the RNA preparation (10-100 μg) was subsequently incubated with 10 U RQ1 RNase-free DNase (Promega) for 30 minutes at 37°C, extracted with phenol and chloroform and finally precipitated and dissolved in 3mM Tris-HCl pH 7.5/0.2mM EDTA. Total RNA concentration was determined spectrophotometrically at 260 nm. RNA integrity was
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TABLE 2. Characteristics of Primers Used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genbank access, no.</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anf</td>
<td>K02781</td>
<td>593-612</td>
<td>831-809</td>
<td>136</td>
</tr>
<tr>
<td>M62e</td>
<td>M91602</td>
<td>398-418</td>
<td>548-527</td>
<td>151</td>
</tr>
<tr>
<td>cTnl</td>
<td>NM_009406.1</td>
<td>511-527</td>
<td>613-596</td>
<td>103</td>
</tr>
<tr>
<td>I83'</td>
<td>X00686</td>
<td>899-918</td>
<td>1049-1029</td>
<td>151</td>
</tr>
</tbody>
</table>

1Coordinates according to Genbank.

verified electrophoretically. RNA was isolated from 10 beating EBs cultured for 3+14 days, hearts from embryonic day 16 (E16) and adult (3 month) FVB mice.

Complementary DNA PCR primers for mouse were designed using Oligo primer analysis (version 4.1, National Biosciences) and Primer Express (version 1.0, PE Applied Biosystems) software from DNA and RNA sequences obtained from GenBank (Table 2). All primer sets had a calculated annealing temperature of 58°C (nearest neighbour method). Primers were ordered from Biolegio (The Netherlands).

Reverse Transcription

First-strand complementary DNA was synthesized from 1 μg total RNA by priming with a mixture of 2 pmol/μl Anf reverse and 125 pmol/μl oligo-dT14VN (Biolegio, The Netherlands) primer. Oligo-dT14VN, containing a lock docking site (VN) at the 3'end, was obtained by making each of the 12 hexadecamer combinations separately, which were added together in equimolar amounts. This primer has the advantage that cDNA synthesis starts at the boundary polyA-tail and mRNA. In other experimental systems the introduction of this site improved the detection sensitivity significantly (Kahn et al. 1990; Liang et al. 1993; Liang et al. 1993). The reverse transcription was performed in two steps. First the priming mix was annealed to 1 μg of total RNA in a total volume of 10μl by incubation at 70°C for 10 minutes and cooling down to 4°C (PTC 200, MJ research). In the second step first strand cDNA was made in a total volume of 25μl using the following reaction conditions: The annealed RNA, 5 mM CaCl2, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 0.5 mM dNTP, and 100 U superscript II (GibcoBRL) were added together and incubated at 42°C for 60 minutes, heated to 70°C for 15 minutes and cooled to 4°C (PTC 200, MJ research). Finally, the cDNA reaction was diluted by adding 30 μl of 3 mM Tris-HCl pH 7.5/0.2mM EDTA. All samples to be analysed were prepared using the same master-mixes. For each sample two separate cDNA synthesis reactions were performed. To determine the presence of contaminating genomic DNA, reverse transcriptase was omitted in the cDNA synthesis reaction. In none of these reactions was a specific product observed.
Real-time PCR

PCR amplification and analysis were achieved using a LightCycler Instrument (Roche) and software version 3.0, respectively (Roche). The reaction mixture consisted of cDNA (1 µl), 0.5 mM of each primer, 1 µl LightCycler FastStart DNA Master SYBR Green 1 mix (Roche, 2239264) and 4 mM MgCl₂ in a total volume of 10 µl. cDNA conditions to be compared were made with the same PCR master-mixes. All templates were amplified by using the following LightCycler protocol. The fluorimeter gain for channel 1 was set to 5. The FastStart polymerase was activated and cDNA denatured by a preincubation for 10 minutes at 95°C, the template was amplified for 40 cycles of denaturation programmed for 15 seconds at 95°C, annealing of primers at 58°C programmed for 5 seconds, and extension at 72°C programmed for 10 seconds. Fluorescent data were acquired during each extension phase. After 40 cycles a melting curve was generated by heating the sample to 95°C programmed for 0 seconds followed by cooling down to 65°C for 15 seconds and slowly heating the samples at 0.1°C/s to 95°C while measuring fluorescence continuously. Fast loss of fluorescence is observed at the denaturing/melting temperature of a DNA fragment, which is a unique feature of that fragment. The melting peak can be obtained by plotting the negative first derivative of fluorescence against temperature. Finally, the samples were cooled down to 40°C for 30 seconds. Product identity was confirmed by electrophoresis on a 10% non-denaturing polyacrylamide gel stained with ethidium bromide afterwards. All analysed samples showed a specific melting peak and the expected fragment size in gel. Standard curves were generated from concentration series (10⁰, 10⁴, 10³, 10² and 10¹ molecules) of PAGE gel purified PCR fragment. The concentration of the fragments was measured in a fluorometer (TD-360 Mini Fluorometer-Turner Design) using PicoGreen dsDNA (Molecular Probes) as quantitation reagent. Absolute concentrations were determined in 2 independent RNA samples per condition corrected for 18S and expressed relative to the Anf level detected in EB 3+14 (fold difference in mRNA concentration).

RESULTS

Information gained from the spatio-temporal expression of cardiac genes in the embryonic heart can be used to define its developmental stage and (molecular) phenotype (i.e. atrial- or ventricular-like or embryonic myocardium). This information is important to determine the phenotype of cardiomyocytes derived from differentiated ES cells. To this end non-RAISH was performed on sections from both embryonic mouse hearts and EBs. Although we used a specific and sensitive protocol based on hybridisation in whole mounts as recently described (Moorman et al. 2001), it turned out to be not sensitive enough to detect specific signal in cardiomyocytes present in EBs. Therefore, this
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The technique was modified to allow specific and sensitive probe detection in ES cell-derived cardiomyocytes. The probes against cTnl, Mlc2v and Anf were used to validate the method.

Non-Radioactive In Situ Hybridisation on Embryo Sections

Figure 1a shows the expression of cTnl at embryonic day 11.5 as a representative example. An almost equal expression of cTnl is observed in all cardiac compartments, atrium, atrio-ventricular canal, ventricle, and outflow tract, of the developing heart. In addition, cTnl is expressed in the heart only. Therefore it was used as a marker to demarcate cardiomyocytes in EBs. The expression of Anf is restricted to the atria and the trabeculated part of the ventricle during embryonic development. Anf is not observed in the atrio-ventricular canal and the out-flow tract (Fig. 1b). Therefore, Anf can be regarded as a marker for chamber myocardium in the developing heart. Mlc2v is initially expressed in the anterior part of the primary tube at embryonic day 8 (Christoffels et al. 2000; O'Brien et al. 1993) and becomes confined to the atrio-ventricular canal, the ventricle and proximal part of the out-flow tract during development as can be seen in Figure 1c.

Non-Radioactive In Situ Hybridisation on Sections of EBs

As a first approach to detect gene expression at the cellular level, we investigated whether the protocol as described for non-RA ISH on tissue sections worked comparably well on EBs. To visualise the relative contribution of cardiac cells to the EB serial sections of EBs were hybridised with a probe against cTnl. The staining reaction was performed overnight. The area in which cTnl expression could be detected was unexpectedly small and the intensity of precipitate accumulated by the action of alkaline phosphatase was low. Even when the incubation time for the staining reaction was extended to 48 hours, the area remained poorly stained. This suggests that the concentration of cTnl mRNA in EBs is low (Fig. 2a).

To increase the sensitivity of the method we compared sections treated with and without tyramide signal amplification. Using tyramide mediated amplification, a strong and much more extended signal was observed within 30 minutes staining time only (compare Figs. 2a and c). Signal was not detected on serial sections hybridised with hybridisation mix without probe while the amplification procedure was performed (Fig. 2b). This indicates the specificity of the procedure. More examples of tyramide mediated amplification on serial EB sections are shown in Figure 3. Again, almost the whole tissue area of the EB is cTnl positive (Fig. 3b), which is in agreement with the observation that beating EBs show contracting activity in 50-100% of their area. In contrast, Anf-positive cells were never observed on EB sections, even not when the staining time was considerably extended (Fig. 3a). Mlc2v on the other hand displays a stronger reaction on
EB sections compared to cTnl as shown in Figure 3d. In addition, different intracellular staining intensities were observed suggesting that the EB comprises a heterogeneous population of cells that express high and low levels of Mlc2v mRNA. No significant staining was observed in control sections using hybridisation mix without probe and tyramide signal amplification. On embryonic sections the tyramide procedure did not enhance the signal but resulted in an increase of background as reported previously (Moorman et al. 2001).

Relative Expression Levels of Cardiac mRNAs

To determine the relation between signals obtained with non-RA ISH and the actual levels we have performed an RT-PCR analysis. To this end, we determined the absolute amounts of the mRNAs encoding Anf, cTnl and Mlc2v in EBs at 3+14 days of differentiation, and embryonic day 16.5 and adult mouse heart (Table 3). Expression of
In situ mRNA detection in embryoid bodies

### TABLE 3. mRNA Levels Expressed Relative to Anf in EB 3+14 (± Standard Deviation)

<table>
<thead>
<tr>
<th></th>
<th>Anf</th>
<th>cTnl</th>
<th>Mlc2v</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB 3+14</td>
<td>1 ± 0.2</td>
<td>4.7 ± 0.8</td>
<td>183 ± 32</td>
</tr>
<tr>
<td>E16.5 heart</td>
<td>308 ± 28</td>
<td>203 ± 23</td>
<td>4035 ± 894</td>
</tr>
<tr>
<td>Adult heart</td>
<td>575 ± 19</td>
<td>157 ± 8</td>
<td>5292 ± 192</td>
</tr>
</tbody>
</table>

Figure 3. Application of the tyramide mediated amplification procedure after non-RA ISH on EB sections that were allowed to differentiate for 3+14 days. Serial sections were hybridised to probes against Anf (a), cTnl (b), blank (c) and Mlc2v (d). Whereas cTnl and Mlc2v mRNAs are well detectable, Anf mRNA cannot be detected. Blank means hybridisation without probe. Bar indicates 0.1 mm.

All cardiac mRNAs analysed are much lower in differentiated ES cells than in embryonic and adult hearts. Anf mRNA is approximately 440 (average of 308 and 575) times lower, cTnl approximately 77 times lower and Mlc2v approximately 26 times lower in EBs compared to mouse hearts. Furthermore, from the three mRNAs measured Anf was expressed at the lowest level in EBs: 4.7 and 183 times lower than cTnl and Mlc2v, respectively. The differences in expression level between EBs and mouse heart as well as between Anf, cTnl and Mlc2v one to another, are in agreement with what was observed on the in situ hybridised sections. Most likely, using the non-radioactive ISH method applied with tyramide mediated amplification, cTnl is just detectable, but Anf is not.

**Non-Radioactive In Situ Hybridisation on Single ES Cell-Derived Cardiomyocytes**

Analysis of single cells has the advantage of investigating sub-cellular mRNA expression. Detection of mRNA at the single cell level requires a very sensitive ISH technique as no additional gain can be obtained from increased section thickness, as is the case with tissue sections (Moorman et al. 2001). Therefore, we investigated the applicability of non-RA ISH applied with tyramide-mediated amplification on single cells obtained after ES cell
differentiation. It turned out that proteinase K treatment resulted in detachment of the cells from the microscope slide; therefore this step has been omitted from the protocol when applied on single cells. Representative examples for cTnI and Mlc2v are shown in Figure 4a and b, respectively. As is indicated in Figure 4, some cells do not show any staining, functioning as an internal control. To evaluate the specificity we performed hybridisation without probe, which did not show any signal (not shown).

DISCUSSION

Cardiac development is subjected to a strict and complex program of gene expression. It comprises the transition from a peristaltic-contracting linear tube to a synchronous-contracting four-chamber pump that simultaneously drives two separate blood flows. Besides morphological remodelling, the physiological specialisation of the cardiac components involves a molecular adaptation of the initial embryonic cardiomyocytes. Each compartment can be discriminated by its own functionally tailored expression program (Christoffels et al. 2000; Moorman et al. 2000b; Franco et al. 1998). Therefore, expression patterns can be used to molecularly define and demarcate developmental stages and to understand the subsequent processes involved. However, expression of a gene acknowledged as a marker for a specific compartment in the adult heart like, e.g., Mlc2a for the atria and Mlc2v for the ventricles, does not necessarily identify these compartments in the embryo as well. The expression of these markers becomes confined to their corresponding chambers only late during development (reviewed by Franco et al. (1998). This illustrates the intrinsic difficulty of revealing cellular phenotypes in an EB. Interpretation of expression patterns becomes even more complicated by the absence of any fixed morphological (organ) structure in the EB.

Non-RA ISH perfectly meets the demands of high sensitivity and high resolution in the cellular context of the tissue. An additional advantage of the non-RA ISH is that the course of the chromogenic reaction to visualise hybridisation can be followed by eye. The signal to background ratio can be evaluated and accordingly adjusted during the reaction process.

In this study the sensitivity of non-RA ISH has been challenged and enhanced. Non-RA ISH proved to be an ideal detection method for the detection of low abundant gene expression as observed in cardiomyocytes derived from embryonic stem cells by in vitro differentiation.
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Figure 4. Application of the tyramide signal amplification procedure after non-RA ISH on ES cell-derived single cells. Probes against Mlc2v (a) and cTnl (b) were used. Arrows indicate cells that have not been stained in the procedure. Bar indicates 0.01 mm.

In sections of embryos, our protocol was able to detect the mRNAs of cTnl, Anf and Mlc2v without any difficulty. In EBs expression levels were shown to be relatively low compared to embryonic and adult tissue, as became clear from the RT-PCR analysis. This has hampered proper detection. Visualisation of mRNA sequences using ISH depends on several aspects, among them the level of expression of the relevant gene, length of the probe, secondary structure of the mRNA, and the number of digoxigenin groups per molecule of probe. Since Dig-labelled DTP is incorporated opposite to adenosine during probe synthesis, the amount of adenosine residues in the template is indicative for specific labelling, as has been indicated in Table 1. cTnl contains 1.9 times more A-residues in its cDNA template than Anf. Since 4.7 times more cTnl than Anf mRNA molecules were observed by RT-PCR analysis, cTnl mRNAs are 9 times easier to detect than Anf mRNAs. This makes it conceivable that Anf mRNA remained undetected by non-RA ISH.

Different from radioactive ISH, the length of the probe seems not to limit penetration of the tissue (Moorman et al. 2001) and alkaline hydrolysis is not necessary. Increasing the concentration of the probe most often leads to a concomitant increase in background. Therefore we decided to improve the signal intensity using an amplification method mediated by deposition of tyramide residues.

Tyramide signal amplification is based on amplification by peroxidase-mediated deposition of biotinylated tyramide residues at the immediate surrounding of the peroxidase group (Fig. 5). Due to this proximal deposition, the resolution remains high. Final localisation is visualised by alkaline phosphatase driven substrate transition that results in a blue precipitate. In this way, low concentrations of mRNA could be visualised. The surplus value of tyramide amplification when applied on EB sections probably does not primarily originate from an increase of secondary epitopes, but from the continuous
Figure 5. Cartoon of the tyramide signal amplification reaction as has been used in this study, based on a figure in the manufacturer’s protocol. Digoxigenin-labeled probes are hybridised to the mRNA molecules. The digoxigenin epitope is recognised by a specific antibody conjugated to horseradish peroxidase. Peroxidase activates biotinylated tyramide groups that deposit in the surrounding of the probe. Biotin is bound by streptavidin that carries an alkaline phosphatase group, responsible for chromogenic substrate transition that results in final detection.

Accumulation of the precipitate in time: Replacing the alkaline phosphatase conjugated tyramide groups by tyramide groups carrying a (fluorescent) chromatophore does not result in any detectable signal (data not shown). Only when a chromogenic substrate was used that gave a fluorescent precipitate after enzymatic transition at the tyramide residue, a clearly detectable signal was obtained (data not shown). On embryonic sections, amplification by tyramide residues does not lead to enhanced signal intensity, but resulted in an increase of background for reasons that are not completely clear yet. One might hypothesise that the concentration of mRNA present in embryonic and adult tissue is relatively high, increasing the possibility of non-specific hybridisation. Therefore, in more mature tissue, tyramide signal amplification may result in relatively more amplification of non-specific signal, increasing the background. On sections of EBs, however, application of tyramide signal amplification greatly improved reliable signal intensity and even proved indispensable, depending on the probe that was used and the experiment performed. The protocol worked on single cells as well, which has the additional advantage that the subcellular localisation of mRNAs can be examined. Taken together, non-radioactive ISH supplemented with tyramide signal amplification is the method of choice to detect low abundant mRNA sequences in EBs. The method has a high resolution, is sensitive and fast, without concomitant increase in background staining. Therefore, it has the great advantage that it permits assessment of the relation between the morphological, functional and molecular phenotype of the in vitro developing cardiac muscle cells in a cellular and developmental context.
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In situ mRNA detection in embryoid bodies


