FEBS Letters 440 (1998) 370–376 FEBS 21227

Cardiac specific expression of the green fluorescent protein during early murine embryonic development

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Received 6 October 1998

Abstract We demonstrate the establishment of transgenic mice, where the expression of the green fluorescent protein (GFP) is under control of the human cardiac α-actin promoter. These mice display cardiac specific GFP expression already during early embryonic development. Prominent GFP fluorescence was observed at the earliest stage of the murine heart anlage (E8). Cardiomyocytes of different developmental stages proved GFP positive, but the intensity varied between cells. We further show that contractions of single GFP positive cardiomyocytes can be monitored within the intact embryo. At later stages of embryonic development, the skeletal musculature was also GFP positive, in line with the known expression pattern of cardiac α-actin. The tissue specific labeling of organs is a powerful new tool for embryological as well as functional investigations in vivo.

Key words: Cardiac α-actin promoter; Tissue specific green fluorescent protein expression; Transgenic mouse

1. Introduction

The heart is one of the first organs to form during murine development. Between E7.5 and 8 the generation of a single heart tube is initiated and on E 8.5/9 the first contractions of this primitive structure are observed [1]. Due to the small size of the embryo and the relatively difficult isolation procedure, embryological and particularly functional investigations on the whole heart as well as on isolated cardiomyocytes at these very early developmental stages have so far been impossible [2,3]. The discovery of the green fluorescent protein (GFP) originally derived from the jellyfish *Aequorea victoria* has opened a new era for the in vivo labeling of organs and cells [4,5]. GFP has proven an almost ideal in vivo reporter gene, because it does not interfere with cell vitality [6–8], it is highly sensitive and it can be easily detected using fluorescence microscopy [9,10]. The generation of transgenic mice expressing GFP [7] or the enhanced version of GFP (EGFP) [8] under control of the ubiquitously expressed β-actin promoter has proved the feasibility of this approach. Since transcripts of α-cardiac actin are among the earliest detectable in the developing heart of murine embryos [11], we have employed the α-cardiac actin promoter driven GFP reporter gene for the generation of transgenic mice. The construct used was first tested in the ES cell system, where GFP fluorescence was detected specifically in ES cell derived cardiomyocytes already prior to the initiation of spontaneous contractions (E. Kolossov et al., unpublished results); identical results have been reported using Lac-Z as the reporter gene [12].

Here we demonstrate tissue specific GFP expression very early during murine cardiomyogenesis. In later stage embryos and newborn mice, GFP expression was also detected in the skeletal musculature. We propose that this novel approach will prove very helpful for the investigation of embryonic development. In addition, the cardiac GFP expression allows to the best of our knowledge for the first time the easy morphological and functional investigation of the very early embryonic heart in vivo.

2. Materials and methods

2.1. Construction of vector

The vector pPv/B-Act-lacZ containing the (−440 +6) segment of the human cardiac α-actin promoter [13,14] was provided by Dr. McBurney (University of Ottawa, Canada). The pCX-h-EGFP expression vector containing the enhanced version of the GFP coding sequence EGFP (Clontech) under the chicken β-actin promoter [8] was provided by Dr. Okabe (University of Osaka, Japan). The promoter was excised from pPv/B-Act-lacZ by *Sal*I and *Hin*dIII restriction enzymes. The pCX-EGFP was digested with *Sna*BI and *Apa*I restriction endonucleases in order to excise the chicken β-actin promoter which was replaced by the *Sal*I-*Hin*dIII fragment of the cardiac α-actin promoter by blunt-end ligation yielding the pCX-(α-act)-EGFP vector. The CMV enhancer, of the original vector, was left in its position upstream of the specific promoter.

2.2. Production of transgenic mice

Transgenic mice were generated as described before [15]. Briefly, transgenic mouse lines were established by injecting the purified *Bam*HI-*Sal*I fragment of pCX-(α-act)-EGFP into the pronuclei of outbred Him:OF1×outbred Him:OF1 [16] fertilized eggs. In total, 234 DNA injected eggs were transferred into the oviducts of pseudo-pregnant mice, resulting in 23 newborns. The integration of the transgene was investigated by PCR analysis of samples of the mouse tails. PCR was carried out as described elsewhere [17]. The sequences of the primers against the EGFP gene used were 5′ ACA AGT TCA GCG TGT CCG GC-3′ and 5′ TTG TGG CTG TTG TAG TTG TAC TCC AG-3′ for the forward and reverse primers, respectively. The temperature profile of the PCR reaction was 30 s 95°C, 40 s 64°C and 1 min 72°C for 25 cycles after an initial denaturing step of 5 min 95°C.

2.3. Observation of the green fluorescence

F1 animals were generated by mating male hemizygous transgenic mice with female wild type animals. Large numbers of F1 animals were produced using standard superovulation protocols. The screening of GFP positive embryos and newborn F1 pups from each transgenic founder mouse was performed using a fluorescence microscope or a lamp with a transcutaneous fiberoptic illumination light source (wavelength optimum, 470 nm) and spectacles with a LP filter (520

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[1]Established the pCX-(α-act)-EGFP vector.

PII: S0014-5793(98)01476-8

nm) (Volpi). For the investigation of the GFP expression at the blastocyst stage, E3.5 blastocysts were collected from the uteri of females mated with hemizygous founder males and placed into microdrops of M 16 medium [18,19]. Photographs (F-601-AF camera, Nikon, Japan) of the blastocysts were taken by placing them under a fluorescent microscope (Diaphot 300, Nikon) and using Nikon excitation (450–490 nm, Nikon) and emission filters (520 nm). The blastocysts were cultured for a period up to 6 days in M 16 medium (37°C, 5% CO_2) and screened every day under fluorescent light for fluorescence.

For the investigation of GFP positive embryos and neonatal pups a Zeiss microscope (Axiophot, Zeiss, Germany) and a Zeiss fluorescence filter set (number 9) were used. Frequently the combination of fluorescence and transmitted light was employed. Pictures were taken immediately after harvesting the embryos or the hearts of postnatal mice (for detail see figure legends). For the morphological analysis, murine embryos and isolated hearts from embryos as well as neonatal mice were immersion fixed in a solution containing 4% paraformaldehyde in 0.1 M PBS buffer, pH 7.4 for 15 min to 4 h. Under these conditions tissue conservation proved good and GFP expression remained stable at 4°C for weeks, although a slight shift of the fluorescence emission towards longer wavelength (yellow) was noticed. After embedding in 18% sucrose for cryoprotection embryos/hearts were cryosected (5–15 μm thickness). GFP negative embryos and neonates were used as controls. Their background fluorescence was negligible and differed strongly from the GFP fluorescence emitted by their transgenic littermates. For the functional analysis embryos were kept in standard PBS solution. At least five GFP positive and GFP negative animals of each developmental stage presented in the manuscript were investigated. Pictures of the whole embryos and the hearts were taken prior to immersion fixation. Live video images of the spontaneously contracting heart were recorded using a three chip color CCD camera (Sony, Japan) and stored on a SVHS video recorder (Panasonic, USA). Single images were imported into a computer based morphometric software program (Optimas, Germany) via an interface. Quantitative morphometric analysis was performed on an array of continuous imaging sequences.

3. Results and discussion

DNA analysis by PCR revealed six transgenic founder animals, where the GFP expression was under the control of the human cardiac α-actin promoter (α-actin EGFP mice). The integration frequency, i.e. transgenic mice/newborn animals, was 26%. Four transgenic founders transmitted the gene construct to their offspring. The transgenic lines #93 and #111 were investigated in greater detail in this study. Hemizygous male F0 animals were mated with wild type females and the offspring were analyzed as described below. GFP positive transgenic embryos and neonatal mice displayed a strong fluorescent signal in the chest upon exposure to a fluorescent transcutaneous illumination light source. About 50% of the littermates of the lines #93 ($n = 54$, four litters) and #111 ($n = 56$, five litters) proved positive for the transgene product as determined by direct screening of the fluorescence of F1 animals. Thus, the gene construct was transmitted according to the Mendelian rules of inheritance for a single integration site. Hemizygous as well as homozygous α-actin EGFP mice of both lines were viable and fertile. In addition, intrauterine fetuses as well as adult hemizygous mice were investigated. No pathophysiological side effects on development, growth performance or body weight were detected.

In clear contrast to the chicken β-actin EGFP transgenic mice [8], embryos from wild type mothers and hemizygous males from both lines of our α-actin EGFP mice did not show fluorescence at the morula ($n = 30$) and blastocyst ($n = 84$) stages. Moreover, cultivation of the blastocysts in vitro did not evidence fluorescent signals after 6 days of cultivation ($n = 18$) (data not shown). Starting from the earliest murine heart anlage (E8) cardiac specific GFP expression was observed in murine embryos positive for the EGFP transgene (Fig. 1). At this stage the heart still had the typical tubular structure and the cardiomyocyte wall showed a patchy pattern of GFP content; however, no obvious differences in GFP fluorescence intensity between the atrial and ventricular tubes were noticed (Fig. 1). During the subsequent stages of cardiac differentiation, the heart remained strongly GFP positive. As depicted in Fig. 2A,B combination of transmitted light and blue fluorescence excitation evidenced in an E9 (A) and E10 (B) embryo an intensely fluorescent heart, whereas the remaining embryo displayed almost no or only little

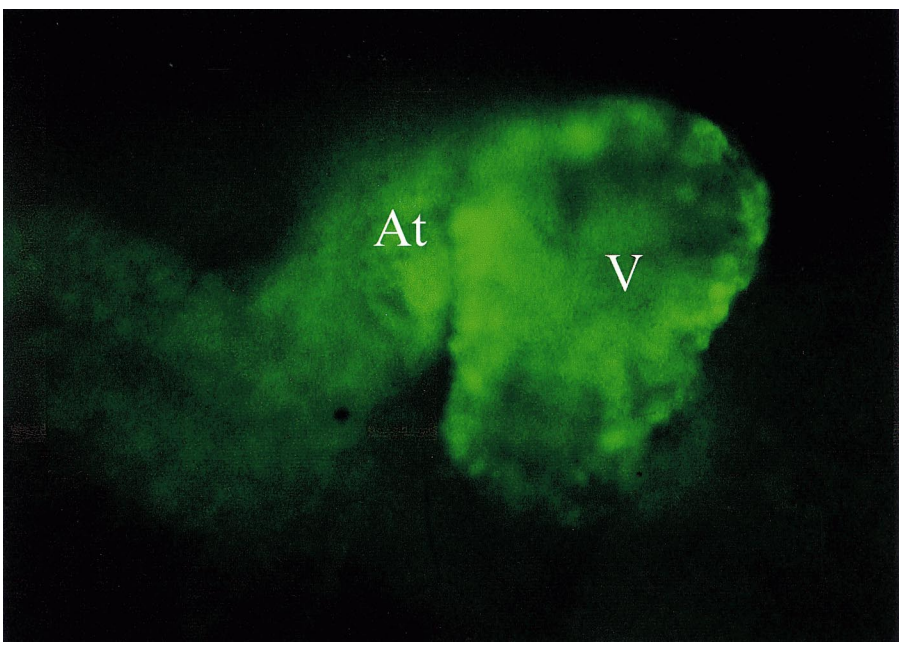

Fig. 1. Fluorescence picture of an E8 α-actin EGFP mouse embryo. The primitive heart tube within the embryo is strongly fluorescent. The heart shows a patchy pattern of GFP expression, since not all cardiomyocytes are GFP positive.

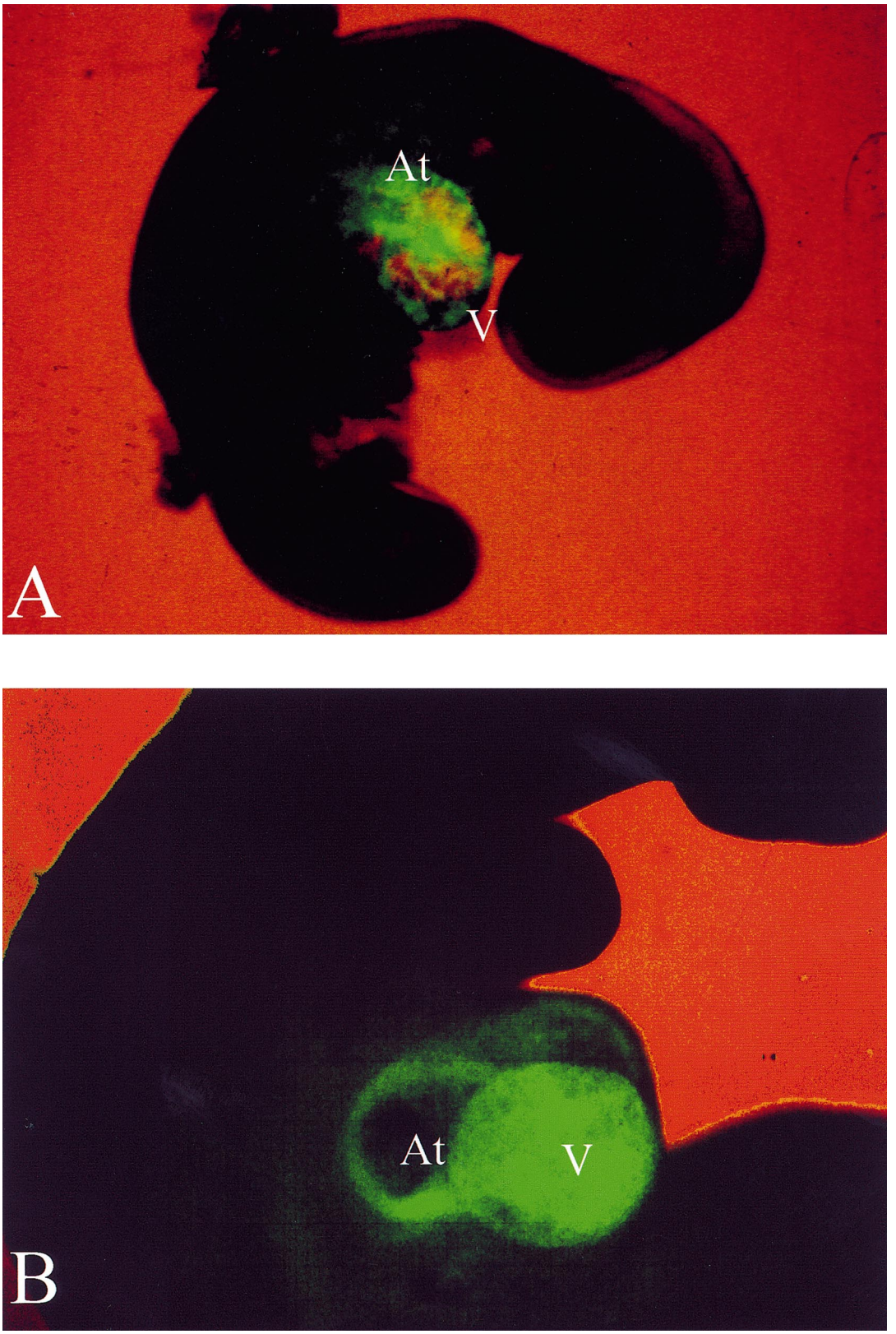

Fig. 2. Combined transmitted and fluorescence excitation light pictures of early stage α-actin EGFP mouse embryos. The GFP fluorescence in an E9 and an E10 embryo is restricted to the heart. A: The GFP positive heart in an E9 embryo displays the tubular-like structure characteristic for this early differentiation stage (magnification ×26). B: The GFP positive heart of an E10 embryo already shows the chamber-like formation as expected for this developmental stage (magnification ×26).

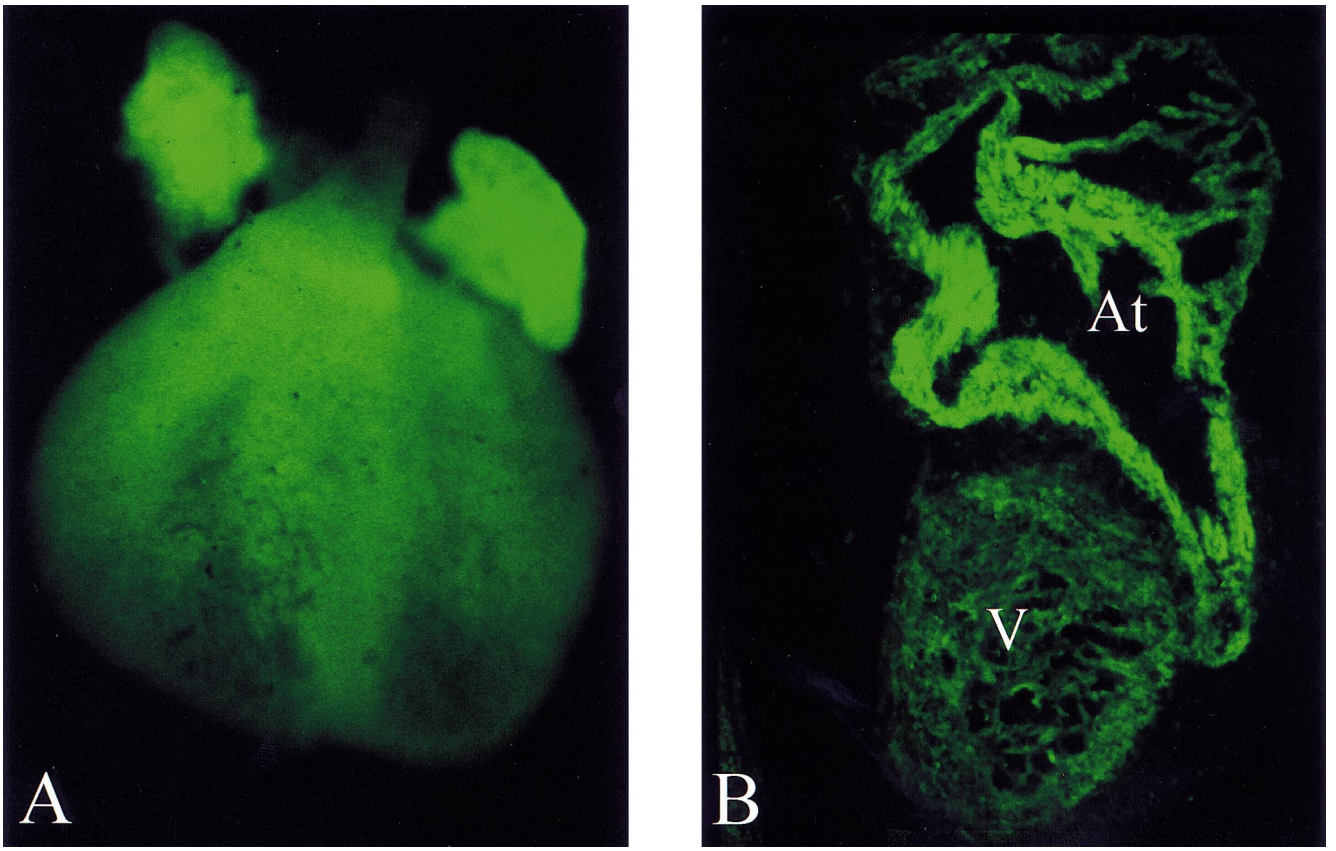

Fig. 3. Fluorescence pictures taken of an E15 α-actin EGFP mouse embryo. A: The dissected heart is photographed from ventral. A clear difference in the fluorescence intensity between the auricola and the ventricles is visible (magnification ×28). B: The sagittary cross section of this heart evidences further the clear difference in the GFP expression between the atrial and the ventricular walls (magnification ×18).

fluorescent signal. As expected for the early embryonic stage E9, the heart still maintained the primitive tubular structure (Fig. 2A). In contrast, the heart of an E10 embryo already showed the organization into chambers (Fig. 2B). At these early stages of embryonic development, whole embryos were dissociated using collagenase treatment and single cardiomyocytes were identified based on their fluorescence (data not shown). As depicted in Fig. 3A,B in an E15 embryo, both atria and ventricles were fluorescent upon exposure to blue excitation light. The GFP expression at this stage was inhomogeneous with the atria being more strongly fluorescent than the ventricles. All cardiomyocytes of both atria and ventricles were GFP positive, but the individual degree of GFP expression varied considerably even among cells of the same region (Fig. 3B), most likely related to various degrees of cardiac α-actin synthesis in cardiomyocytes (Fig. 3B). In line with our findings in middle to late embryonic stages, the early postnatal heart was characterized by a similar pattern of GFP expression. As shown in Fig. 4 the difference in fluorescence intensity between atria and ventricles at 4 days after birth was less pronounced as compared to E15 (Fig. 4A,B). At a higher magnification the pattern of the GFP expression in individual ventricular cardiomyocytes was cross striation-like (Fig. 4D). Since the coronary vessels lack any fluorescence, they can be easily identified on the whole heart (Fig. 4A). This can be explained by the absence of the transgene product in erythrocytes and the prominent fluorescence quench by the Fe^{2+} containing hemoglobin in these cells. This finding is in line with the β-actin EGFP transgenic mice, where the spleen was not fluorescent due to the high content in erythrocytes [8]. Histological cross sections of the murine heart demonstrated that the coronary vessels (Fig. 4B), the endocardium as well as the epicardium were GFP negative, underlining the tissue specific GFP expression in these transgenic mice. In accordance with previous findings using in situ hybridization techniques [20], where cardiac α-actin was expressed in both cardiac and skeletal muscle, our mice displayed GFP expression in the skeletal musculature at the embryonic as well as postnatal stages. The first GFP fluorescence in skeletal muscle was detected with somite formation on E10–E11. However, the heart was the only organ prior to E11 to express large amounts of GFP. In clear contrast to the β-actin (E)GFP transgenic mice [7,8], the GFP expression in our EGFP α-actin mouse was almost restricted to cardiac and skeletal musculature. Faint fluorescence was observed in other tissues. It is currently under investigation whether this background is due to promoter activity or GFP metabolism.

Since the embryo at the E8–E10 stage is transparent and the heart strongly fluorescent, the beating heart could be observed in the embryo. The hearts continued to spontaneously contract for hours after harvesting of the embryos. As can be seen in Fig. 5, single GFP positive cardiomyocytes in an E9 embryo were easily distinguishable and the contractile activity during one contraction cycle was monitored within the intact embryo. The degree of contraction of this myocyte amounted to a 20% reduction in cell length. These experiments prove the feasibility in the future of monitoring chronotropy, degree of contraction as well as inotropy (estimation of shortening ve-

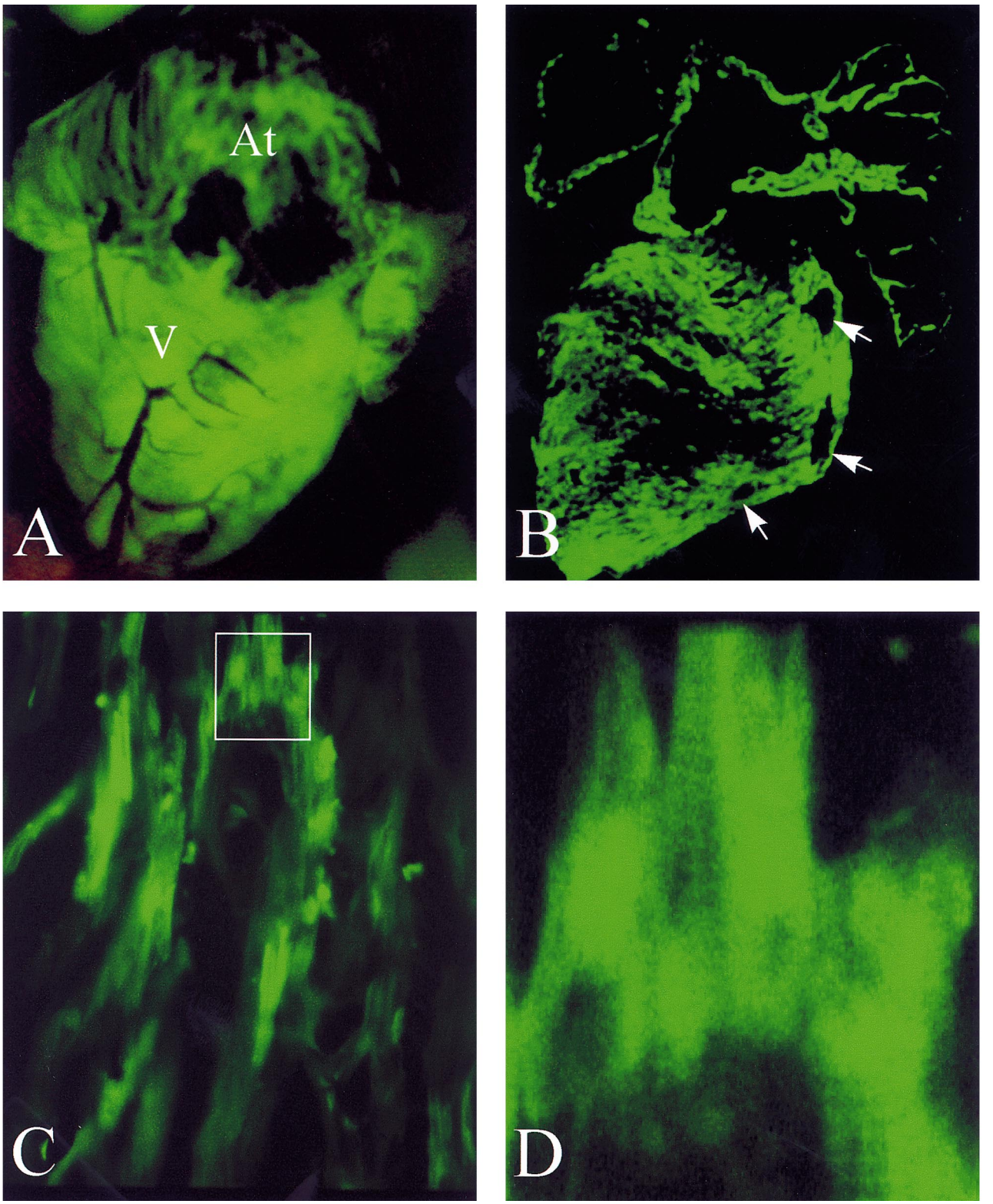

Fig. 4. Fluorescence pictures of the heart from a 4 day old α-actin EGFP mouse. A: The whole heart is photographed in the open chest. Atrial and ventricular musculature display an intense GFP fluorescence. No fluorescence signal is detected in parts of the atrium and in coronary vessels (magnification ×20). B: Sagittal cryosection of the heart displayed in A. Atria and ventricles display comparable fluorescence intensities and expression patterns for GFP. Crossection of the coronary vessels demonstrated the absence of GFP fluorescence (arrows). The vessel wall is not visible due to the absence of GFP expression (magnification ×20). C: A representative segment of the ventricle, where the distinct differences of the GFP expression between cardiomyocytes can be observed (magnification ×39). D: 1940× magnification of the framed segment in C. Single ventricular cardiomyocytes evidence a specific pattern of GFP fluorescence similar to cross striation.

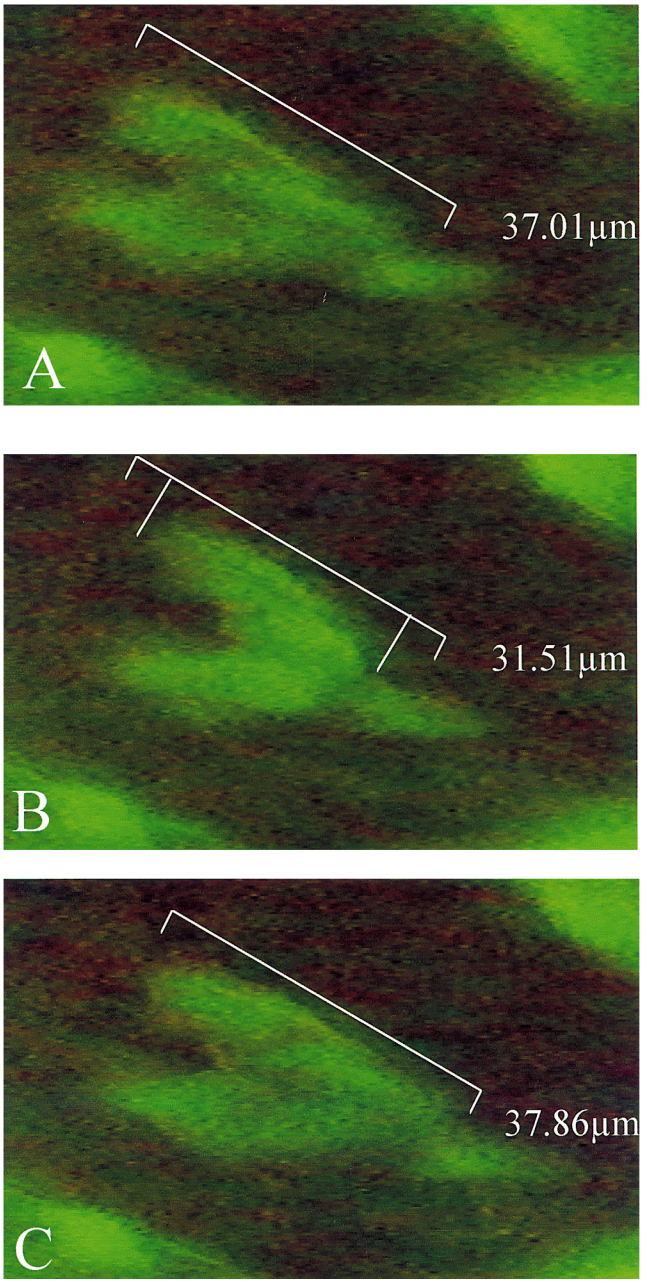

Fig. 5. Fluorescence video images taken of single spontaneously contracting cardiomyocytes during a contraction cycle in the whole heart of an E9 α-actin EGFP embryo. A small cluster of strongly fluorescent cardiomyocytes is depicted. The full bar to the right corresponds to the individual cell length before contraction. The cell length (see numbers on figure) is estimated by morphometric analysis of the video image. A: Cardiomyocytes before contraction. B: Cardiomyocytes during maximal contraction. The longer cross bars indicate the actual length, the shorter ones the original cell length. C: Cardiomyocytes after contraction.

locity) of the very early embryonic heart at the single cell level under control conditions and after application of various pharmacological agents.

In conclusion, the overall GFP expression pattern in our α-actin EGFP transgenic mice differs strongly from that detected in the β-actin GFP and EGFP transgenic mice [7,8], as would be expected for a tissue specific promoter. While blastocysts and morulae are GFP negative, prominent GFP expression is already detected in the initial heart anlage (E8). This is in line with in situ hybridization experiments, where the first transcripts for cardiac α-actin were detected at E7.5 [11,20]. During later developmental stages the heart remains GFP positive, but also the skeletal musculature expresses GFP. Tissue specific GFP expression under various promoters has been reported for *Drosophila* [21] and zebrafish [22]. However, in contrast to ubiquitous [7,8] or cell specific [23,24] GFP expression in transgenic mice, we here show to the best of our knowledge for the first time the generation of transgenic mice with organ/tissue specific GFP expression.

Transgenic mice with GFP as a reporter of gene expression provide a new powerful tool to address a variety of relevant biological questions. The high sensitivity of GFP [25] and its easy mode of detection make this reporter gene far superior to the currently used ones. We demonstrate in the present manuscript that the strong GFP expression in the very early murine embryonic heart allows (i) easy identification of the heart, (ii) investigation of morphological and functional characteristics in the intact embryo, (iii) functional analysis of single cardiomyocytes in the spontaneously beating heart, and (iv) dissection and enzymatic dissociation of the whole embryo for molecular biological and functional studies on single cardiomyocytes. In addition, our mice will prove helpful for the investigation of KO mice with defects of early cardiomyogenesis. Thus, the generation of transgenic mice where the GFP expression is under control of tissue specific promoters is a powerful approach to address a variety of biological questions.

Acknowledgements: We thank Dr. M. Okabe (University of Osaka, Japan) and Dr. J. Miyazaki (Tohoku University, Japan) for kindly providing the expression vector (pCX-h-EGFP), and Dr. M.W. McBurney (University of Ottawa, Canada) for supplying us with the pHCActLacZ (−440–+6) segment of the human cardiac α-actin promoter.

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