Behavior of Genes Directly Injected Into the Rat Heart In Vivo

Peter M. Buttrick, Alyson Kass, Richard N. Kitsis, Matthew L. Kaplan, and Leslie A. Leinwand

Gene transfer can be achieved in the adult rat heart in vivo by direct injection of plasmid DNA. In this report we define the spatial and temporal limits of reporter gene expression after a single intracardiac injection. pRSVcat (100 µg), in which the Rous sarcoma virus long terminal repeat is fused to the chloramphenicol acetyltransferase reporter gene, and pαMHC-luc (100 µg), in which the α-cardiac myosin heavy chain promoter is fused to the luciferase gene, were injected into hearts, and reporter gene activities were assayed at various times. Both chloramphenicol acetyltransferase and luciferase were detectable in 100% of the rats from 1 to 7 days, in 60% of the rats from 17 to 23 days, and in 30% of the rats from 38 to 60 days after injection. Reporter gene activity was largely limited to a 1–2-mm region of the ventricle surrounding the injection site. Closed circular DNA was far more effective than linear DNA in transfecting cells in vivo. The relative strengths of three different promoters, Rous sarcoma virus long terminal repeat, α-myosin heavy chain, and α-antitrypsin, all fused to the luciferase reporter gene were determined. The constitutive viral promoter was ~20-fold more active than the cardiac-specific cellular promoter, and the liver-specific cellular promoter was not active at all in the cardiac environment. Thus, direct injection of genes into the heart offers a simple and powerful tool with which to assess the behavior of genes in vivo. However, the potential of the technique to effect a phenotypic change in the heart is currently limited by the temporal and geographic extent of transfection. (Circulation Research 1992;70:193–198)

It has previously been demonstrated that reporter genes linked to viral and cellular promoters can be expressed after injection of plasmid DNA into the hearts of adult rats in vivo.¹⁻³ Although this technique suggests a simple approach to somatic gene therapy, its utility is dependent on several parameters. One of these is the stability of the transfected gene. Acsadi et al.² recently reported that reporter genes coupled to viral promoters could not be detected in adult cardiac muscle 3 weeks after injection, suggesting that cardiac gene transfer may not be stable. In contrast, Lin et al.³ were able to detect reporter gene activity up to 3–4 months after cardiac injection in a small number of animals. Whether the introduced gene is episomal may affect (either positively or negatively) the stability of muscle gene transfer, and the postmitotic state of adult muscle cells may prevent introduced genes from integrating into chromosomes. In fact, Wolff et al.⁴ have suggested that, both in heart and skeletal muscle, directly transfected genes are episomal.

A second consideration for cardiac gene therapy is the spatial extent of transfection after a single injection. Although this has not been investigated in great depth, the precise number of cells transacted after a single injection appears to be small, based on the extent of β-galactosidase staining in tissue sections from hearts injected with this reporter gene.²³ Acsadi et al.² have shown that no reporter gene activity can be detected in atria after intraventricular injections.

In the present study, we sought to further define some of the parameters of cardiac gene transfer. Specifically, we determined the time course and geographic extent of gene expression after a single intracardiac injection. In addition, we asked whether the state of the injected DNA, either linear or circular, influenced its expression. Finally, we determined the relative strengths of a constitutive viral promoter, a cardiac-specific cellular promoter, and a liver-specific promoter. Transcription from the viral long terminal repeat (LTR) was ~20-fold higher than that derived from the cardiac promoter, but no transcription resulted from the liver-specific promoter.
Materials and Methods

The plasmids used in this study have been previously described: paMHCLuc contains the firefly luciferase coding region coupled to the rat cardiac α-myosin heavy chain 5' flanking sequence, base pairs −613 to +32.5 pRSV-luc contains the chloramphenicol acetyltransferase (CAT) coding sequence spliced to the LTR of the Rous sarcoma virus (RSV), and pRSV-Tluc contains the firefly luciferase coding sequence coupled to the RSV-LTR.6 paATluc contains the luciferase coding region coupled to the 5' flanking sequence, base pairs −522 to +20, of the α,-antitrypsin gene. This construct has significant promoter activity when transfected into cultured hepatocytes.9

Adult female Wistar rats were anesthetized with an intraperitoneal injection of chloral hydrate (0.7 ml/100 g of a 4% solution). Cardiac injections were made directly into the apex of the left ventricle after exteriorization of the heart through a left lateral thoracotomy, after which the heart was replaced in the chest, the rats were briefly hyperventilated, and the incision was closed. Fifty microliters of a DNA solution containing 2 μg/μl of each plasmid in 20% sucrose and 2% Evans blue were injected through a 27-gauge needle.

Rats were killed 5 days after injection except when indicated. The atria and great vessels were trimmed, and the hearts were washed in iced saline. Except when indicated, the apical two thirds of the heart was homogenized in 1 ml homogenization buffer without Triton X-100, as described,8 with a Tissumizer (Tekmar, Cincinnati, Ohio). Homogenates were then centrifuged at 6,000g for 10 minutes at 4°C.

CAT assays were done on 5% of the supernatant of each homogenate by standard techniques9 with the following modifications. Samples were first heated to 65°C for 10 minutes and then clarified by centrifugation at 10,000g for 5 minutes. Reaction mixtures were incubated for 2 hours at 37°C, and percent CAT conversion was determined by excising the appropriate areas of the thin-layer chromatography plate and measuring the associated radioactivity directly in Econofluor. Luciferase was also measured in 5% of the homogenate as previously described.8 Light production after the addition of d-luciferin to the assay mix was measured for 20 seconds and integrated over time in a Monolight luminometer (model 2010, Analytical Luminescence Laboratory, San Diego, Calif.).

Results

To determine the time course of gene expression after direct intracardiac injection, the rats were injected with 100 μg each of pRSV-luc and paMHC-luc and then killed at various time points. Data for individual rats are shown in Figure 1. No reporter gene activity was detected 2 hours after injection; however, at 1 day all injected hearts showed both CAT and luciferase activities. The levels of expression continued to rise and reached maximal levels 7–10 days after injection. All injected hearts showed significant reporter gene activities up to this point. At subsequent time points, some of the hearts showed no detectable reporter gene activities: four of 10 hearts from 17 to 23 days and seven of 10 hearts from 38 to 60 days. Southern analysis (not shown) of HindIII digests of cellular DNA suggested that the exogenous genes were episomal.

We determined the ratio of luciferase activity to CAT activity in hearts coinjected with pRSV-luc and paMHC-luc as a function of time in order to compare the behavior of these two reporter genes. In general, this ratio was constant over the first 2 weeks after injection: 424 ± 67 in samples from 1 to 2 days versus 484 ± 104 in samples from 7 to 23 days. In the hearts expressing the reporter genes at later time points (from 38 to 60 days), the ratio of luciferase to CAT activity fell significantly to 77 ± 20.

To determine the spatial extent of expression of injected genes in cardiac muscle, we injected the apical portions of individual hearts with a single 50-μl bolus of pRSV-luc, a plasmid containing the luciferase reporter gene coupled to the constitutive RSV-LTR. Five days after injection, the rats were killed, and the hearts were cut into 1-mm sections from apex to base. These sections were then assayed individually for the presence of luciferase activity. Data from two hearts are shown in Figure 2 and indicate that 90% of the expression of the luciferase gene was restricted to two 1-mm sections surrounding the injection site, although very low levels of activity were seen in two other 1-mm sections, so that some minimal activity was detectable over 40% of the long axis of the ventricle. No reporter gene activity was seen in the atria.

To assess whether the state of the injected DNA influenced its expression, we compared luciferase activity after injection with linear versus closed circular DNA. paMHC-Luc was digested to completion with Xmn I, which does not disrupt the promoter and coding regions of the plasmid, and 100 μg of this linear molecule was coinjected with 100 μg closed circular pRSV-luc (as an internal control) in four rats and compared with the uncut circular plasmid, which was also coinjected with pRSV-luc in a similar number of rats. Figure 3 shows the percent CAT conversions, luciferase activities, and luciferase/CAT ratios in these rats. Luciferase/CAT ratios (Figure 3C) were 50–100-fold greater in those rats injected with circular DNA than in rats injected with linear DNA, demonstrating that the linear molecule was poorly expressed relative to the closed circular plasmid. CAT activities tended to be lower in the rats concomitantly injected with the linear molecule (Figure 3B), suggesting that this interfered with pRSV-luc uptake and/or transcription.

Finally, we determined the relative strengths of three promoters linked to the luciferase reporter gene in the heart. The promoters were derived from the RSV-LTR, the rat cardiac myosin heavy chain gene, and the liver-specific mouse α,-antitrypsin gene. In this experiment, 100 μg each pRSV-luc, paMHC-luc, or
Luciferase activity (RLU)  
% CAT Conversion  
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<tr>
<th>Time</th>
<th>Luciferase Activity (RLU)</th>
<th>% CAT Conversion</th>
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<td>2 h</td>
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<td><img src="image2.png" alt="Graph" /></td>
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<td>1 d</td>
<td><img src="image3.png" alt="Graph" /></td>
<td><img src="image4.png" alt="Graph" /></td>
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<tr>
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<td>7 d</td>
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<tr>
<td>60 d</td>
<td><img src="image11.png" alt="Graph" /></td>
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FIGURE 1. Bar graphs showing expression of coinjected genes at six time points (from 2 hours to 60 days) after direct cardiac injection of plasmids pRSVCAT and pαMHCΔluc. Luciferase activity (raw luciferase units [RLU]) and percent chloramphenicol acetyltransferase (CAT) conversion are shown for individual heart homogenates at each time point.

pα1ATluc was coinjected into individual hearts along with 15 μg pRSVCAT. The rats were killed 5 days after injection, and reporter gene activities were assayed. Luciferase activity was normalized to CAT activity. The data are shown in Table 1 and demonstrate that the viral promoter (RSV) was ~20-fold more potent than the cardiac-specific cellular promoter (α-myosin heavy chain) and, in addition, that the liver-specific promoter (α1-antitrypsin) was not active in the cardiac environment.

**Discussion**

This study describes the time course and spatial extent of gene expression in the rat ventricle after direct intracardiac injection of plasmid DNA. One major finding is that reporter gene activities can be reliably detected as early as 1 day and are stable for the first 2 weeks after injection. The proportion of rats expressing injected genes from 17 to 23 days is reduced to 60% and from 38 to 60 days to 30%, but in those rats expressing genes, the level of expression is quite high and appears to depend on the continued presence of the episomal plasmid.

The time course of expression of exogenous genes shown in the present study differs from that previously reported in the heart as well as in other tissues. Acsadi et al. reported no significant activity in nine of 10 normal adult Sprague-Dawley rats 25 days after cardiac injection, although a higher percentage of rats, similar to that seen in our study with Wistar rats, did express reporter genes at this time point if the rats were concomitantly treated with cyclosporin. This suggests that strain differences might contribute to the observed results. Other workers have shown

**Table 1. Relative Promoter Strengths in Cardiac Muscle In Vivo**

<table>
<thead>
<tr>
<th>Promoter</th>
<th>n</th>
<th>Luciferase activity (RLU) Mean±SEM</th>
<th>Range</th>
<th>CAT conversion (%) Mean±SEM</th>
<th>Range</th>
<th>Luciferase/CAT Mean±SEM</th>
<th>Range</th>
</tr>
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<tbody>
<tr>
<td>RSV</td>
<td>5</td>
<td>287,659±82,058</td>
<td>42,546–646,327</td>
<td>3.3±1.0</td>
<td>0.4–5.6</td>
<td>96,841±17,629</td>
<td>56,034–162,124</td>
</tr>
<tr>
<td>α-MHC</td>
<td>6</td>
<td>73,668±13,414*</td>
<td>22,882–114,699</td>
<td>15.0±2.6*</td>
<td>4.0–21.9</td>
<td>5,014±370*</td>
<td>3,651–5,752</td>
</tr>
<tr>
<td>α-AT</td>
<td>6</td>
<td>162±38*</td>
<td>108–349</td>
<td>27.2±4.3*</td>
<td>15.2–42.8</td>
<td>8±3*</td>
<td>3–21</td>
</tr>
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n, Number of rats; RLU, raw luciferase units; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; α-MHC, α-myosin heavy chain; α1-AT, α1-antitrypsin. The three promoters (100 μg each) coupled to the luciferase gene were coinjected with 15 μg pRSVCAT into rat hearts in vivo. Reporter gene activities were measured 5 days later.  

*p<0.05 vs. corresponding value for RSV; †p<0.05 vs. corresponding value for α-MHC.
that transfection of exogenous genes into both skeletal and vascular muscle results in more stable levels of gene expression. Mouse gastrocnemius muscles express exogenous genes 60 days after injection at levels that are similar to those seen at 7–15 days, and pig iliac arteries infected with a modified retroviral vector bearing the β-galactosidase gene show some activity 3–6 months after treatment. This latter result may reflect plasmid integration since some cellular hyperplasia was observed after endothelial injury.

The current study also demonstrates that, after a single injection, expression of reporter genes is localized to a relatively small area of myocardium. The fact that the myocyte is the transected cell derives from other histological studies that have localized reporter gene expression to this cell type and from our previous studies showing tissue-restricted expression of reporter genes driven by a cardiac myocyte-specific cellular promoter. Although the spatial extent of gene transfer after a single injection is limited, it may be possible to achieve gene transfer into a larger area of the myocardium using multiple injections or agents that may improve DNA uptake and delivery.

The mechanism whereby DNA is taken up by striated muscle is unknown. The demonstration that linear DNA does not result in efficient gene transfer in vivo may not be surprising, given its inefficient uptake by cultured cells in transfection experiments. However, these two gene transfer systems may be distinct, and given the results of Wolff et al., who reported that linear RNA is effective in skeletal muscle gene transfer, the finding is an important one.

An additional piece of information provided in the present study is the first quantitative assessment of the relative strengths of promoters in vivo. Our data indicate that the constitutive RSV-LTR is ~20-fold more active than a cardiac-specific cellular promoter. The absence of any discernible promoter activity after injection of a liver-specific cellular promoter in the heart confirms our previous demonstration that directly injected cellular genes behave in a tissue-restricted fashion and also establishes that the heart is not a promiscuous environment for gene expression via this technique. An additional finding from this experiment is that competition for transcription factors likely occurs when two strong promoters are co-injected into cardiac muscle. This is striking when contrasting CAT activities in the rats injected with pRSVluc/pRSVcat (3.3% conversion) and pMHCcat/pRSVcat (15.0% conversion) but is also evident when comparing these two groups with the rats injected with pATluc/pRSVcat (27.2% conversion). Similar competition among promoters has been observed in cultured cells (e.g., see Reference 11). These findings are important when considering the utility of this approach for in vivo promoter mapping.

There are several potential applications of this technology. One is to alter cardiac phenotype through somatic cell gene therapy and a second is to identify regulatory regions of genes that respond to stimuli that can only be modeled in vivo, such as hypertension or pressure overload. On the basis of the current data, the first of these may have limited feasibility. Gene expression after a single injection using the current approach is temporally variable. The spatial restriction of cells expressing injected

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**FIGURE 2.** Graph showing the geographic extent of gene expression after direct cardiac injection of the plasmid pRSVluc in two individual hearts. The entire heart was sectioned 5 days after the injection of DNA. The distance from the left ventricular (LV) apex of the heart (mm) is indicated on the abscissa, and the luciferase (LUC) activity (raw luciferase units [RLU]) in individual heart slices is indicated on the ordinate.
genes makes it unlikely that a given gene product will effect a prolonged, global phenotypic change, with the possible exception of transfection with genes such as angiotensin, which may function in an autocrine or paracrine fashion. The second application, identification of regulatory elements of genes in vivo, appears to be possible, and the current data define caveats that ought to be observed for such an experimental approach. First, a constitutively expressed control expression vector must be used as an internal control. The variability in the expression of an individual reporter gene can be quite significant, which likely reflects injection technique. This is most striking in Figures 3A and 3B, which show significant interanimal variability in CAT and luciferase expression after a single injection. However, when the expression of a cellular promoter (α-cardiac myosin heavy chain) is normalized to that of a constitutive promoter (RSV-LTR) as in Figure 3C, the data are remarkably consistent. The standard error of these four measurements is <15%. Similar experimental variability can be seen in the time course data shown in Figure 1. Second, comparisons of reporter genes should be done during periods of stable gene expression. In the case of the two reporter gene constructs used in the present study, this period is 2–7 days after injection, during which time expression is consistent in all injected hearts and the ratio of CAT/luciferase is constant. At later times, the ratio of the two reporter genes decreases, which may reflect a longer half-life of the CAT protein or preferential transcription of the RSV-LTR-driven reporter gene. The half-life of these reporter genes in mammalian tissue is not known, although the half-life of luciferase expression after injection of RNA expressing the luciferase coding region in skeletal muscle is ~12 hours and that of CAT in mammalian cells is <2 days. This suggests that expression of the reporter genes at later time points is the result of ongoing transcription and translation. Finally, potential interactions between plasmids must be considered. For example, competition between strong promoters probably occurs in vivo, and in the experiment comparing linear and closed circular DNA, the linear molecule appeared to inhibit either the uptake and/or the expression of the coinjected pRSVCAT.

If these caveats are acknowledged, direct injection of DNA appears to offer a simple and powerful tool with which to assess gene behavior in vivo. Future studies, aimed at extending the geographic limits of expression and maintaining the integrity of an injected plasmid, may allow the development of a strategy for somatic cell gene therapy in the heart.

References
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KEY WORDS • in vivo gene transfer • cardiac myosin heavy chain • firefly luciferase • chloramphenicol acetyltransferase
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