Left ventricular targeting of reporter gene expression in vivo by human BNP promoter in an adenoviral vector

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Cardiovascular disease is the leading cause of death in the United States. The primary causes of cardiovascular disease, such as hypertension, coronary artery disease, cardiomyopathy, and diabetes, can be treated, but often the treatment only slows the progression of disease, and heart failure ultimately results (2). With aging of the population and increased mortality from heart failure, great interest has been focused on novel therapies such as gene transfer. For therapeutic efficacy, it is important that a gene be delivered directly to the heart and be expressed at high enough levels over a prescribed period of time to effect a response. In animal models, methods for in vivo cardiac gene transfer include direct injection of DNA into the myocardial wall, perfusion of the heart via the coronary arteries, intrapericardial injection, and catheter-based injection into the heart coupled with cross-clamping of either the aorta alone or both the aorta and pulmonary artery (6).

Presently, adenoviruses represent the best-characterized vector system for expressing recombinant proteins in cardiac myocytes in vitro and in vivo (3, 7, 16, 20). One way to improve the delivery capability of the adenovirus would be inclusion of a cardiac myocyte-specific promoter that would allow expression of the transgene only in myocytes, thus eliminating the problem of inappropriate transgene expression in other organs and avoiding a more widespread inflammatory response. The brain natriuretic peptide (BNP) gene is constitutively expressed in the ventricle and is induced by ischemic injury (21). In fact, BNP gene expression is a good marker for left ventricular (LV) dysfunction (1, 23). We have studied regulation of the human BNP (hBNP) promoter and identified some of the elements involved in cardiac myocyte-specific expression (8, 9, 11). We have also used the proximal hBNP promoter coupled to a luciferase reporter gene to generate transgenic mice and have shown that the proximal promoter confers high-level cardiac-specific expression, primarily in the ventricles. Expression of the reporter gene is virtually absent in all other tissues. In addition, this proximal promoter region responds to ischemic injury caused by coronary artery ligation, resulting in chronic expression of the reporter gene for at least 3 wk in the mouse, reflecting endogenous BNP mRNA (10). On the basis of these studies, we hypothesized that administration of an adenovirus in which the luciferase reporter gene was regulated by the proximal hBNP promoter would allow for 1) cell-specific and inducible regulation of the reporter gene in myocytes in vivo and 2) selective expression in the mouse heart in vivo.

Materials and Methods

Preparation of adenovirus vector. We previously described (18) the construction of the hBNP promoter coupled to a luciferase reporter gene. The proximal promoter from −408 to +100 coupled to firefly luciferase cDNA was subcloned into the HincIII and PvuII sites of the Ad5mcsP shuttle vector.

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A recombinant, replication-defective adenovirus was constructed by homologous recombination in HEK293 cells, resulting in viral particles that we refer to as Ad.hBNPLuc. All steps of the process (construction of the shuttle vector, homologous recombination, and plaque isolation and purification) were carried out by the University of Iowa Gene Vector Core (supported in part by the National Institutes of Health and the Ray J. Carver Foundation). The viral titer was 1 × 10^{12} particles/ml, equivalent to 10^{10} plaque-forming units (PFU)/ml. The virus was diluted in PBS-3% sucrose and was performed, and the heart was exposed. With a 30-gauge syringe, 1 × 10^{12} particles/ml, equivalent to 10^{11} PFU/ml, was injected into the free wall of the LV from the apex upward and distributed among three or four adjacent sites of the LV free wall. Direct injection of Ad.hBNPLuc into the LV free wall was carried out by the University of Iowa Gene Vector Core Facility. The viral titer was 1 × 10^{10} PFU/ml and was stored in 20 mM Tris, 75 mM NaCl, 2 mM MgCl2, 5% trehalose, and 0.0025% Tween 80 at −70°C. A second, replication-defective adenovirus, Ad.CMVLuc, was obtained from the University of Pittsburgh Pre-Clinical Vector Core Facility. The viral titer was 2 × 10^{10} PFU/ml and was stored in 20 mM Tris, 75 mM NaCl, 2 mM MgCl2, 5% trehalose, and 0.0025% Tween 80 at −70°C.

Cell culture. Neonatal rat ventricular myocyte-enriched cultures were generated from Sprague-Dawley rat pups (Charles River) as described previously (17). A mouse embryonic fibroblast (MEF) cell line was obtained from Clontech. Cells were transduced with 10 PFU/cell Ad.hBNPLuc or Ad.CMVLuc and then lysed and assayed for luciferase activity at selected time points.

In vivo injection of Ad.hBNPLuc and Ad.CMVLuc. C57BL/6J mice (Jackson Laboratory) weighing at least 22 g were anesthetized with pentobarbital sodium (50 mg/kg ip). They were placed supine on a heating pad, intubated, and ventilated with a volume of 0.2 ml at a rate of 95–110 min⁻¹. A thoracotomy was performed, and the heart was exposed. With a 30-gauge 0.5-in. needle attached to a 0.5-ml syringe, 10⁶ PFU of adenovirus was injected into a total volume of 50 μl of PBS-3% sucrose or 50 μl of vehicle was directly injected into the free wall of the LV from the apex upward and distributed among three or four adjacent sites of the LV free wall. Direct injection of Ad.hBNPLuc into the quadriceps muscle served as a control for cardiac muscle-specific expression. Background luciferase activity was measured in vehicle-injected tissue. The LV, right ventricle, septum, atria, lung, liver, kidney, and skeletal muscle were either frozen and subjected to immunocytochemistry. We also introduced either Ad.hBNPLuc or Ad.CMVLuc directly into the LV chamber for systemic distribution throughout the mouse. Four days later, tissues were removed and examined for luciferase activity. These studies were approved by the Henry Ford Hospital Institutional Animal Care and Use Committee in compliance with Public Health Service guidelines.

Luciferase assay. Tissues were homogenized with a Polytron in 10 mM Tris (pH 7.5). After homogenization, one-fifth volume of 5× reporter lysis buffer (Promega) was added. Samples were processed further, and duplicate aliquots of each sample assayed as described previously (10). Luciferase activity (relative light units, RLU) was normalized to milligrams of protein. Data are expressed as means ± SE. Where necessary, statistical significance was determined with Student’s t-test.

Immunocytochemistry. Hearts injected with either Ad.hBNPLuc or vehicle were fixed in formalin, embedded in paraffin, and cut into 5-μm sections. Sections were stained for luciferase protein with a rabbit anti-luciferase antibody (1:250 dilution; Cortex, San Leandro, CA) and an immunalkaline phosphatase staining kit (Biomedia) with fast red (Sigma) as the substrate (10).

RESULTS

Cell-specific and inducible expression of Ad.hBNPLuc. We compared the activity and cell type specificity of the hBNP promoter with the cytomegalovirus (CMV) enhancer/promoter in neonatal ventricular myocytes (NVM) and a MEF cell line (Table 1). The ratio of activity of Ad.CMVLuc in myocytes to that in fibroblasts was ~20 [luciferase activity in NVM: 9.2 ± 0.96 × 10⁶ RLU/mg protein (n = 6); luciferase activity in MEF cells: 5.2 ± 1.5 × 10⁶ RLU/mg protein (n = 6)]. The ratio of activity of Ad.BNPLuc in myocytes to that in fibroblasts was ~1,000 [luciferase activity in NVM: 13.4 ± 7.2 × 10⁶; luciferase activity in MEF cells: 10,000 ± 5,000 (n = 6 for each)]. Thus the CMV enhancer/promoter was more active in both cell types, but it was much less cardiac specific than the hBNP promoter.

In a separate experiment, we examined time-dependent changes in transgene expression in myocytes. Luciferase activity was high 24 h after transduction and persisted for at least 96 h (Fig. 1A). Because expression was high over a 4-day period, we examined whether the proinflammatory cytokine IL-1β and the hypertrophic growth factors phenylephrine (PE, an α-adrenergic agonist) and isoproterenol (Iso, a β-adrenergic agonist) would activate the proximal promoter in the context of the adenoviral expression vector. Myocytes were transduced with virus and 24 h later were treated with IL-1, PE, or Iso for 24 h, resulting in stimulation of luciferase activity by 2.2-, 10.1-, and 2.5-fold versus control, respectively (Fig. 1B).

In vivo injection of Ad.hBNPLuc. To test whether the proximal hBNP promoter would confer cardiac-specific expression on the luciferase reporter gene in an adeno-viral vector in vivo, we directly injected either vehicle alone or 10⁸ PFU of virus in 50 μl PBS-3% sucrose into the apex of the mouse LV. At 1, 4, 7, 14, and 28 days after injection, tissues were removed and assayed for luciferase activity. We averaged the luciferase activity from tissues removed from mice injected with PBS-3% sucrose and determined that background luciferase activity was 300–900 RLU/mg protein. Luciferase activity in the LV increased from 2,000 ± 1,000 RLU/mg protein (n = 5) on day 1 to 126,840 ± 45,181 RLU/mg protein (n = 10) on day 4 (Fig. 2A) and then declined by day 7 to 27,000 ± 5,000 RLU/mg protein (n = 5). Luciferase activity remained stable at 7,000 RLU/mg protein for 14–28 days after injection. For each time point, luciferase activity in virus-injected LVs was significantly higher than in LVs injected with vehicle (P < 0.001 by t-test).

Table 1. Ad.BNPLuc and Ad.CMVLuc activity in vitro

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<th>Ad.BNPLuc, RLU/mg protein</th>
<th>Ad.CMVLuc, RLU/mg protein</th>
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<tr>
<td>Neonatal ventricular myocytes</td>
<td>13.4 ± 7.2 × 10⁶</td>
<td>9.2 ± 1 × 10⁶</td>
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<tr>
<td>MEF cells</td>
<td>10 ± 5 × 10⁵</td>
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Values represent mean ± SE for n = 6 experiments. Cells were cultured for 24 h with 10 plaque-forming units/cell of virus and then lysed and assayed for luciferase activity. MEF, mouse embryonic fibroblast; RLU, relative light units.
We examined all areas of the mouse heart for luciferase expression and found no luciferase activity above background level in the septum, right ventricle, or atria (Fig. 2B) at 4 days after injection. Because we were injecting the virus into the LV free wall, we considered the possibility that some virus could enter the circulation and be distributed to other tissues. We assayed luciferase activity in the lung, liver, kidney, and skeletal muscle and found no activity above background levels at the 4 day time point (Fig. 2B). Of all of these extracardiac sites, luciferase activity in the liver is of greatest importance, because the liver is a major site of adenovirus uptake and transgene expression, especially with vectors that use viral enhancers/promoters like CMV. Nonetheless, when Ad.hBNPLuc was injected into the LV free wall, luciferase activity in the liver was $573 \pm 187$ RLU/mg protein ($n = 10$), a value not statistically different from background ($400 \pm 121$ RLU/mg protein; $n = 5$).

We next determined whether a higher titer of Ad.hBNPLuc ($10^9$ PFU in $100 \mu$l of PBS-sucrose) would result in expression of the luciferase transgene in the liver. We injected Ad.hBNPLuc into the LV free wall and then measured luciferase activity in all regions of the heart and in the liver. Luciferase activity was $2.1 \pm 0.6 \times 10^5$ RLU/mg protein in the LV free wall, whereas it was only $719 \pm 265$ RLU/mg in the liver. Activity in the atria, right ventricle, and septum was $11,434 \pm 7,861$, $10,678 \pm 4,539$, and $25,720 \pm 11,260$ RLU/mg protein, respectively. Thus luciferase activity is restricted to the heart, even when a high titer of Ad.hBNPLuc is used.

To further confirm luciferase gene expression in the heart, we performed immunocytochemistry on sections...
of mouse hearts taken 4 days after injection of either Ad.hBNPLuc or vehicle. Luciferase protein (red staining) was evident in the LV free wall in the area where the virus was injected but was absent in the control hearts (Fig. 3).

Finally, Ad.hBNPLuc or vehicle was injected directly into the skeletal muscle of the mouse. Four days later, luciferase activity in virus-injected skeletal muscle was not different from muscle injected with vehicle (Fig. 4). There was only background luciferase activity in the LV of mice injected with virus in the skeletal muscle (329 ± 74 RLU/mg protein, n = 5).

In vivo injection of Ad.CMVLuc. To confirm the tissue specificity of Ad.BNPLuc, we repeated the experiments described above using Ad.CMVLuc, which contains a very strong viral enhancer/promoter that is expressed in all cell types. Injection of Ad.CMVLuc into the LV free wall resulted in very high luciferase activity in the LV (10^8 RLU/mg protein), ~1,000 times the activity of the hBNP promoter. In other tissues, luciferase activity ranged from 10% (in the liver, septum and RV) to 0.1% (in the atria, lung, kidney, and skeletal muscle) of that of the LV (Fig. 5A). Moreover, Ad.CMVLuc injected into the skeletal muscle also demonstrated widespread luciferase activity in tissues throughout the mouse, with activity in the liver being ~1% that of the skeletal muscle (Fig. 5B). When Ad.CMVLuc was injected into the LV cavity to distribute it to the general circulation, the highest activity was detected in the liver (Fig. 6A). In contrast, when Ad.hBNPLuc was injected into the circulation through the LV cavity, low-level activity was detected only in the heart (Fig. 6B). Thus the CMV enhancer/promoter results in widespread expression of reporter gene activity, in contrast to the cardiac-specific nature of the hBNP promoter.

DISCUSSION

Our findings demonstrate that the hBNP promoter in the context of an adenoviral expression vector confers cardiac-specific transgene expression both in vitro and in vivo. When luciferase activity was measured in vitro in both cardiac myocytes and fibroblasts, the CMV enhancer/promoter generated much more luciferase activity than the BNP promoter; however, CMV was almost as active in fibroblasts as in myocytes (ratio of activity in myocytes to fibroblasts was 20), whereas the hBNP promoter was preferentially active in myocytes (ratio of activity in myocytes to fibroblasts was 1,000).

The hBNP promoter in the adenoviral vector was also inducible in cardiac myocytes by hypertrophic and proinflammatory stimuli shown to affect BNP pro-
moter activity in transient transfection studies. Ad. BNPLuc responded to the hypertrophic stimuli PE and Iso as well as the proinflammatory cytokine IL-1β. Stimulation of the −408hBNP promoter in the present studies was similar in magnitude to that in transient transfection studies using the −1818hBNP promoter (8, 11) and the −408hBNP promoter (Q. He and M. C. LaPointe, unpublished observations) in plasmid expression vectors. These data suggest that regulatory elements in the adenoviral DNA sequences are not influencing hBNP promoter activity. Because the hBNP promoter in the vector can respond to pathophysiological stimuli, we believe it could be used to deliver genes to the diseased heart in vivo, where these stimuli would foster expression of therapeutic transgenes. Given a viral delivery system with long-term expression, such as adeno-associated virus, it is conceivable that the hBNP promoter would foster low-level, constitutive expression of a transgene. Changes in the structure/function of the heart, such as in hypertrophy or heart failure, would induce the promoter to be expressed at even higher levels. A transgene with the ability to oppose the signals stimulated by hypertrophy and heart failure thus could elicit a therapeutic response in the heart. We have indirect evidence of the inducibility of the hBNP promoter in vivo. In our studies (10) of 408hBNPLuc transgenic mice, ischemic injury caused persistent upregulation of the luciferase transgene for at least 3 wk.

In our in vivo studies, the CMV enhancer/promoter was much more active than the hBNP promoter, as expected. When Ad.CMVLuc was injected into the LV free wall, its activity was 1,000 times greater than the hBNP promoter, similar to the difference in transduced myocytes in vitro. However, using the CMV promoter/enhancer, we detected luciferase activity in every other tissue tested, with the highest extracardiac activity in the liver (10% of the activity of the heart), presumably because at least 10% of the virus leaked out of the heart into the general circulation. The difference in liver expression between Ad.CMVLuc and Ad.hBNPLuc did not result from the difference in promoter strengths or problems with detecting low-level luciferase activity in tissues in Ad.hBNPLuc-injected mice. When Ad.hBNPLuc was injected into the heart, luciferase activity at day 4 was 1.3 × 10^5 RLU/mg. Given that we can expect the same amount of virus leakage for Ad.BNP as Ad.CMV and that in other similar experiments (15), liver expression was ≥10% of heart expression, we would expect to find at least 10^4 RLU/mg in the liver if the hBNP promoter were non-specific. Instead, the value was 573 ± 187 RLU/mg (n = 10), which is not different from background. When Ad.CMV was introduced into the circulation by injection into the LV cavity, activity in the liver was higher than in the heart (20 × 10^6 vs. 10 × 10^6 RLU/mg). In contrast, when Ad.hBNP was injected into the circulation, activity in the heart was 6 × 10^3 RLU/mg. Again assuming the same scenario as with Ad.CMV, we would expect to find at least 6 × 10^3 RLU in the liver if the hBNP promoter were non-specific. Instead, only background levels of activity were detected, suggesting

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Fig. 5. Expression of Ad.CMVLuc in mouse tissues after direct injection into LV free wall and skeletal muscle. Axes are identical to Fig. 2B. A: luciferase activity after injection into LV free wall; n = 5. B: luciferase activity after injection into skeletal muscle; n = 5.

Fig. 6. Luciferase activity after systemic administration of virus. Axes are identical to Fig. 2B. A: luciferase activity in tissues after injection of Ad.CMVLuc into LV chamber; n = 4. B: luciferase activity after injection of Ad.hBNPLuc into LV chamber; n = 6.
cardiac specificity. Finally, when Ad.CMVLuc was directly injected into skeletal muscle, luciferase activity was $5 \times 10^6$ RLU. Given that the CMV promoter is 1,000 times stronger in vitro and in vivo than the hBNP promoter, one might expect that we would detect $5 \times 10^6$ RLU in skeletal muscle injected with Ad.hBNP if the difference in activity were solely based on promoter strength. Instead, we detected fewer than 300 RLU. In all of our protocols, our assay would have allowed us to detect Ad.hBNPLuc activity in the liver and skeletal muscle if the promoter were nonspecific, but because of its cardiac specificity, the promoter was essentially inactive and luciferase activity was background. Thus the higher activity of the CMV enhancer/promoter in vivo is likely a combination of two factors, 1) high-level constitutive activity of the promoter and 2) the ability of the promoter to function in many different cell types in the heart, such as fibroblasts, endothelial cells, and smooth muscle cells, and many cell types in other tissues.

Kass-Eisler et al. (14) showed that direct injection of an adenoviral vector into adult rat hearts resulted in at least 5,000-fold higher transgene expression than injection of plasmid DNA. Expression was highest at the injection site and persisted for at least 55 days, but because they used a CMV viral enhancer/promoter, expression was seen in nonmyocytes as well as myocytes. In a separate study, they found that there was widespread transgene expression in all tissues tested when virus was administered either directly to the LV free wall or to the LV chamber (15). Thus our results with Ad.CMVLuc in the mouse confirm these studies in the rat and point to the utility of a cardiac-specific promoter to localize gene expression to the heart.

Rothmann et al. (24) showed that 800 bp of the myosin light chain-2v (MLC-2v) promoter allowed for heart-specific transgene expression after virus was injected into the cardiac cavity of neonatal rats. This method of delivery resulted in spillover of the virus into the circulation, likely reducing expression in the heart. In contrast to our studies, they did not test inducible regulation of the MLC-2v promoter in the adenoviral expression vector either in vitro or in vivo. In other studies using the MLC-2v promoter (either 2,100 or 250 bp) to drive expression of a luciferase transgene in mice, ventricle-specific expression was shown; however, the promoter was not tested to see whether it responded to pathophysiological stimuli in vivo (5, 12, 19). Our studies extend these previous observations, indicating that the hBNP promoter can produce localized transgene expression in cardiac myocytes for at least 28 days while at the same time minimizing expression in other tissues. In light of this finding, coupled with our transgenic mouse data, we believe that the hBNP promoter would be inducible in vivo when included in an adenoviral vector.

There are several advantages to viral delivery by direct injection into the LV free wall, including high-level, localized expression and reduced viral spillover. A potential drawback to this method is that most of the myocardium would not be affected, which might limit the ability of a transgene to impact global myocardial function. However, Okubo et al. (22) indicated that direct injection of an adenovirus expressing heat shock protein 70 into the rabbit heart reduced infarct size in an ischemia-reperfusion injury model. Also, direct injection of adenoviruses encoding two therapeutic genes [sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase 1 and Kir2.1] into a normal guinea pig heart was able to modulate cardiac function in vivo (4). In addition, phase I clinical trials with adenoviruses encoding secreted angiogenic factors have used direct intramyocardial injection for ischemic heart disease and direct skeletal muscle injection for peripheral vascular disease. Although most of these trials were uncontrolled studies to test the safety of the viral vector, improvement in the function of the ischemic tissue was noted (13), thus establishing the efficacy of this delivery method.

Other means of effecting global transgene expression in the heart have been developed, most notably catheterization of the LV coupled with cross-clamping of vessels, allowing for modification of cardiac function as a result of transgene overexpression. Despite the enhanced degree of expression of transgenes in the heart, a substantial amount of virus is localized and expressed in the liver (7, 20). Percutaneous delivery of adenovirus through the left circumflex artery has also been effective (25–27). With this method, genes have been transferred to the infarcted and failing heart to improve cardiac function. Use of either delivery method coupled with an adenoviral vector employing the hBNP promoter or another cardiac-specific promoter could conceivably have even greater beneficial effects, especially in light of the inducible regulation of the promoter in the heart during ischemia.

In conclusion, our studies indicate that the proximal hBNP promoter results in inducible and cardiac-specific transgene expression. Because some forms of cardiovascular disease resulting from ischemic injury are amenable to short-term treatment by gene transfer, the hBNP promoter may prove effective in regulating expression of selected therapeutic gene products.

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REFERENCES


