Abstract—Repeated bouts of ischemia in the heart lead to fibrosis and eventually to heart failure. Although certain genes, such as SOD or hemoxygenase and antisense to AT1-R, ACE, and (β1-AR can provide short-term protection of the heart from ischemia, there is no known mechanism for constantly responding to repeated incidences of ischemia. We hypothesized that a “vigilant vector,” designed to be expressed specifically in the heart and switch on therapeutic genes only during hypoxia, would provide cardioprotection. To attain cardiac specificity, we inserted an MLC2v promoter into an adeno-associated virus (AAV) designed to deliver AS to AT1-R and gfp. In vitro experiments in cardiomyocytes (H9C2 cells), the MLC2v-AAV-gfp drove gene expression in all cells at levels comparable to a cytomegalovirus (CMV) promoter. In vivo experiments, the rAAV-MLC2v-gfp was injected intravenously into mice or rats. Green fluorescence protein (GFP) DNA was located in kidney, heart (right and left ventricle), lung, adrenal and spleen. GFP mRNA, however, was expressed only in the heart and absent in other tissues. To switch on the rAAV transgene during ischemia, we inserted a hypoxia response element (HRE). This upregulates transcription when O2 levels are low. Thus, there are 4 components to the vigilant vector; a gene switch (HRE), a heart-specific promoter (MLC2v), a therapeutic gene (AS-AT1-R) and a reporter gene (gfp). To silence or lower basal level of expression while retaining specificity, we have reduced the length of the MLC2v promoter from 3 kb to 1775 bp or 281 bp. The truncated promoter is equally effective in heart specific expression. Preliminary studies with the rAAV-HRE-gfp in vitro show an increased expression in 1% O2 in 4 to 6 hours. By adding additional hypoxia-inducible factor (HIFα) (5 μg), the MLC2v-gfp expression is increased by 4-fold in 1% O2. Further amplification of the gene to 400-fold in 1% O2 can be achieved with a double plasmid. The construct may serve as a prototype “vigilant vector” to switch on therapeutic genes in specific tissue with physiological signals. (Hypertension. 2002;39[part 2]:651-655.)

Key Words: ischemia ■ cardiac function ■ hypoxia ■ myosin ■ gene therapy ■ adeno–associated virus

The human heart can be subject to repeated bouts of hypoxia, which leads to silent or overt myocardial tissue damage.1 Cumulatively, this can lead to heart failure. In an attempt to combat this with gene therapy we are proposing the development of a “vigilant vector,” inactive until switched on by hypoxia, that would protect the heart during ischemia with therapeutic genes. This concept requires the engineering of a stable vector that would contain 4 elements (Figure 1): (1) a safe vector that could reach the heart by systemic injection and show stable expression of the gene in the heart; (2) a therapeutic gene for cardioprotection against ischemia; (3) a tissue-specific promoter to drive the transgene to express mRNA in the heart only; and (4) a gene switch that would switch on the tissue-specific promoter in response to hypoxia and that would switch off in response to normoxia.

For the vector, the adeno-associated virus (AAV) is proving to be a stable, nonpathological vector.2,3 There are several genes that could be considered for protection of the heart during ischemia. In a previous study4 we had found that the angiotensin II type 1 receptor (AT1-R) antisense (AS) protected rat hearts from ischemia-reperfusion. Dzau et al5 have recently shown that transgenic mice with hemoxygenase are protected from cardiac ischemia. Superoxide dismutase protects against super oxide radicals generated during ischemia or reperfusion.6 Thus, these genes are good choices for cardioprotective transgenes in the vector. For tissue-specific expression of AAV in the heart, we have studied the ventricular form of myosin light chain (MLC-2v).7,8 MLC-2v expression is important in the development of the heart during embryogenesis, and alterations in the MLC-2v expression produce cardiac defects.8 In humans, cardiomyopathy is associated with point mutations in MLC-2v.9 MLC-2v seems to be highly specific for hearts, both during embryonic development and in post-natal development and maturity. The MLC-2v promoter is 3.0 kb, but the sequences that give it the property of heart specificity are within 250 bp, close to the TATA box.8,9,11 We tested the specificity of a 1700 bp and a 281 bp MLC-2v promoter in AAV delivered in vitro and in vivo. To switch on the vector, we tested a hypoxia-regulatory element (HRE) which is activated by transactivat-
Vigilant Vector

A vector that responds only to a specific pathological signal, and switches on a therapeutic gene to protect specific or nonspecific tissues.

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<th>Reporter Gene</th>
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**Figure 1.** General design for a “vigilant vector” that could be applied for constant protection against cardiac ischemia, diabetes type 1, stroke, heart attack, cancer, or even bioterrorism. The main elements are (1) a safe, stable vector; (2) a gene switch; (3) a tissue-specific promoter; and (4) therapeutic genes (with a reporter gene to monitor its activity).

ing hypoxia inducible factor (HIF-1) in response to a reduction in oxygen. Under normoxic conditions, the HIF-1α subunit is undetectable because it is degraded by proteosomes, but during hypoxia HIF-1α is no longer degraded; it accumulates exponentially as cellular hypoxia increases. Although we have not completed and tested all components of a vigilant vector, we present the results of a study on the heart specificity of MLC-2v and its interaction with HRE and HIF-1α.

**Methods**

**Construction of Plasmid and Recombinant AAV**

The linear, single-stranded AAV-derived vector can be adapted for several genes and promoters between the inverted terminal repeats (ITRs) at each end (Figure 1). We inserted a reporter gene, green fluorescent protein (GFP), and a rat 1.7 kb MLC-2v promoter (pMLC-2v-GFP). Methods to prepare recombinant AAV (rAAV) have been described previously. The pMLC-2v-GFP was packaged (pMLC-2v-GFP). Methods to prepare recombinant AAV (rAAV)

**Rational Expression in Young Animal**

Five-day-old male Sprague Dawley rats (n=3) were obtained accompanied by their dam from Harlan. They were kept with their dam until 21 days of age. At 6 days of age, the pups were anesthetized with Metofane injected intracardiacally with 10⁸ infectious particles of rAAV-MLC-2v-GFP (25 μL) or the same volume of saline as a control. Four weeks later, rats were deeply anesthetized with ketamine, xylazine, and acepromazine (30, 6, and 1 mg/kg, respectively, subcutaneously) and perfused with ice-cold saline via the left ventricle. Samples of spleen, liver, lung, kidney, left ventricle, testes, heart, and brain were dissected and frozen on dry ice.

**Expression of AAV in Vivo**

All animals were kept in a temperature-controlled room on a 12-hour day/night cycle with free access to food and water. The Institutional Animal Care and Use Committee at the University of Florida approved all experimental procedures.

**AAV Expression in Adult Animal**

Adult male BALB/c mice (n=6) were obtained from Harlan (Indianapolis, Ind) and anesthetized with pentobarbital (80 mg/kg). 10⁸ infectious particles of rAAV-MLC-2v-GFP (100 μL) were injected intravenously. After 2 to 8 weeks, animals were deeply anesthetized with pentobarbital (120 mg/kg). Samples of spleen, liver, lung, kidney, left ventricle, testis, heart, and brain were dissected and frozen on dry ice for DNA, RNA, and GFP protein measurements.

**Detection of GFP**

Total RNA and DNA was isolated using TRIZOL reagent. Expression of green fluorescent protein (GFP) was analyzed by nested PCR. The GFP-specific primers used in the first amplification were 5’-CAGCGGAGAGGGTGAAGGTG-3’ (sense) and 5’-CAGGGCAGACTGGGTGGACA-3’ (antisense). The GFP-specific primers used in the second amplification were 5’-GCCACGACGTTGGGCTAC-3’ (antisense). The GFP expression in the adult animal was determined with the same primers used in the young animal.

**RT-PCR**

Twenty μg of total RNA were digested by DNase I in a 40 μL reaction mixture consisting of 40 U DNase I and 33 U RNase inhibitor. Reverse transcription (RT) and first amplification were performed in a single tube. Four μL of the RNA (2 μg) pretreated with DNase I were added to 20 μL of final volume of the PCR reaction. The first amplification was performed in the following conditions: 60 minutes at 37°C (RT); 4 minutes at 94°C; 35 cycles of 1 minute at 94°C; 1 minute at 58°C (annealing); 1 minute at 72°C; and a final extension period of 7 minutes at 72°C in PE DNA Thermal Cycles 480. One μL product from the first amplification was added to 25 μL final volume of the PCR reaction. The conditions of second amplification were the same as the first with the exception of the addition of 30 cycles with annealing at 60°C.

**PCR**

One μg of DNA was amplified by nested PCR to detect GFP expression. The procedures were the same with GFP detection in RNA (RT-PCR) except DNase I digestion and Reverse transcription (RT) were omitted.
Figure 2. The expression of luciferase activity (relative to control) in cardiac (H9c2 cells) versus glioma (C6) cells after treatment with pGL-MLC. Myocardial cells specifically expressed the transgene. Cells were transfected with control pRL-TK (50 ng/well) and pGL-MLC (1 µg/well) plasmids. Duplicate plates were incubated at 20% O2 for 24 hours (mean±SD, n=3 independent experiments).

**Electrophoresis**
Amplification products were analyzed on 1% agarose stained with ethidium bromide. The expected product size was 489 bp.

**Immunofluorescence Staining**
Tissues were incubated in Zamboni’s solution overnight and cryosectioned at 20 µm thickness. The sections were blocked with blocking buffer (10 mmol/L TBS, 1.5% normal goat serum and 1% BSA) for 1 hour and incubated in primary antibody (0.1% anti-GFP, rabbit IgG) overnight at 4°C. After washing with TBS, the sections were incubated with 0.5% anti-rabbit IgG FITC in the dark at room temperature for 1 hour. The sections were washed and put on slides. The slides were covered by slips with fluoromount G when dry. GFP was detected within 3 hours by confocal microscopy.

**In Vitro**
The pGL-MLC was specifically expressed in cardiomyocytes. Figure 2 shows the luciferase activity of pGL-MLC-2v in cardiomyocytes (H9c2) and a lack of expression in a nonmyocardial cell line (C6). The relative luciferase activity ratio of H9c2 cells to C6 cells was 29.38±13.11. The uptake efficiency in both cell types was 90%.

**In Vivo**
PCR of DNA showed the transduction of rAAV-MLC-2v-GFP in many tissues at 4 weeks after a systemic injection. The tissue-specific expression of GFP under MLC-2v promoter was examined by RT-PCR of RNA in the adult mouse tissues and young rats (Figure 3). GFP DNA was detected in the spleen, liver, lung, kidney, and heart. However, GFP mRNA was detected only in the heart.

Four weeks after intracardiac injection of rAAV-MLC-2v-GFP, the presence of GFP in various tissues of rats was further examined by immunofluorescence staining (Figure 4). The green epifluorescence of the protein was clearly apparent in the heart and absent in the control (no GFP). GFP was undetectable in the kidney and liver of the same animals and undetectable in controls.

Hypoxia did not induce an increase in transgene expression of the pGL-HRE/MLC (Figure 5). However, hypoxia induces a 3 to 4-fold increase in transgene expression when the HRE-MLC-2v enhancer/promoter complex is in the presence of 0.5 to 4 µg of HIF-1α in H9c2 cells (Figure 5).

**Discussion**
The results demonstrate that the MLC-2v promoter incorporated into a rAAV vector can drive a reporter gene specifically in the heart. rAAV-MLC-2v-GFP was injected systemically, either through direct injection into the heart or via the jugular vein. Measurements of DNA showed that the vector was taken up into multiple tissues, including liver, lung, kidney, heart, and spleen. Although the rAAV-MLC-2v-GFP was taken up into many tissues after a single injection, the transgene (gfp) was only expressed in heart tissue. This was found in both mice and in rats. In two animals we found low-level expression in the liver but not in the kidney or other tissues. As AAV has limited loading capacity, we tested two truncated forms of MLC-2v. We used the 1700 bp length for the promoter in vivo and 250 bp in vitro to attempt to reduce basal levels without losing specificity. Both lengths contain the heart-specific cis regulatory elements that endow the MLC-2v with its heart-specific responsiveness. In glioma cells (C6) there was no expression of luciferase, although there was comparable uptake efficiency with or without the MLC-2v promoter.

Attaching HRE to the MLC-2v with luciferase (Luc), as the transgene, did not alter basal expression in vitro at 20% O2. However, the HRE plus MLC-2v did not respond to 1% O2. With an HRE-SV40 promoter-Luc plasmid in heart cells, we have shown elsewhere that HRE will drive the promoter up to 7-fold under hypoxia within 4 to 6 hours. When we cotransfected a plasmid containing HIF-1α cDNA with pGL-HRE/MLC and exposed the cells to 1% oxygen we noted a 4-fold increase in Luc expression. Thus, the results indicate that MLC-2v can be used as a specific promoter for heart tissue, and HIF-1α plus HRE (but not HRE alone) will cause the MLC-2v to increase transgene expression in vitro by at least 4-fold in response to hypoxia. This is not a major limitation because dual vectors overcome the vector size limitation and increase gene
expression. We are not yet satisfied that a 4-fold increase is 
sufficient to provide a cardioprotective effect with a therapeu
tic gene. A double plasmid approach that produces a 
powerful chimeric transcription factor consisting of the yeast 
transactivator factor GAL4 DNA binding domain and the p65 
transactivation domain is being tested. Incorporating the 
HRE in this double plasmid system with SV40 promoter 
increased Luc gene expression by 400-fold when activated by 
hypoxia.

The concept of a vigilant vector for cardioprotection can be 
applied generally to a number of other disease states. For 
example, in diabetes type 1, glucose would be the gene switch 
and insulin and its necessary enzymes would be the trans
genes. The tissue specificity could be limited to the pancreas 
or to muscle. In cancer, tumor markers could be the gene 
switch, and the transgenes could be tumor suppressors. In 
heart attacks the switch would again be hypoxia or a protein 
marker and the transgene tPA. Similarly in stroke, hypoxia 
could be the switch and GFAP the tissue-specific promoter 
with hemoxygenase or superoxide dismutase or AT1-R-
antisense as the therapeutic genes. For the vector, the rAAV 
seems to have the most desirable qualities of being safe and 
stable for a very long time. Obviously each vigilant vector 
has to be designed and thoroughly tested, both in vivo and in 
vitro. Basal levels times of response, tissue specificity and 
amplification of signals are all challenges to be met. The 
present results represent promising new data for the develop
dent of a vigilant vector for long-term protection of cardiac 
performance during exposure to hypoxia.

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Vigilant Vector: Heart-Specific Promoter in an Adeno-Associated Virus Vector for Cardioprotection
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