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Quantitative determination of adenovirus-mediated gene delivery to rat cardiac myocytes *in vitro* and *in vivo*

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ABSTRACT **To optimize the use of modified adenoviruses as vectors for gene delivery to the myocardium, we have characterized infection of cultured fetal and adult rat cardiac myocytes *in vitro* and of adult cardiac myocytes *in vivo* by using a replication-defective adenovirus carrying the chloramphenicol acetyltransferase (CAT) reporter gene driven by the cytomegalovirus promoter (AdCMVCATgD). *In vitro*, virtually all fetal or adult cardiocytes express the CAT gene when infected with 1 plaque-forming unit of virus per cell. CAT enzymatic activity can be detected in these cells as early as 4 hr after infection, reaching near-maximal levels at 48 hr. In fetal cells, CAT expression was maintained without a loss in activity for at least 1 week. Using *in vitro* studies as a guide, we introduced the AdCMVCATgD virus directly into adult rat myocardium and compared the expression results obtained from virus injection with those obtained by direct injection of pAdCMVCATgD plasmid DNA. The amount of CAT activity resulting from adenovirus infection of the myocardium was orders of magnitude higher than that seen from DNA injection and was proportional to the amount of input virus. Immunostaining for CAT protein in cardiac tissue sections following adenovirus injection demonstrated large numbers of positive cells, reaching nearly 100% of the myocytes in many regions of the heart. Expression of genes introduced by adenovirus peaked at 5 days but was still detectable 55 days following infection. Adenoviruses are therefore a very useful tool for high-efficiency gene transfer into the cardiovascular system.**

The analysis of elements involved in cardiac myocyte gene regulation would be greatly facilitated by a simple and efficient method of gene transfer. Because there are no permanent cardiac myocyte cell lines, the majority of cardiac myocyte gene expression studies have used transient gene transfer into primary cultures of fetal and neonatal cardiocytes (e.g., ref. 1). Although useful, this methodology has many limitations, including relatively low efficiency, and is restricted to fetal and neonatal stages of development since transient transfection of adult cardiac myocytes has not been reported. As an alternative, *in vivo* studies of cardiac myocyte gene regulation and gene transfer have been successfully carried out in transgenic mice (e.g., refs. 2 and 3). However, the generation of transgenic mouse lines is costly and extremely time consuming. A second approach to cardiac gene transfer *in vivo* has relied on injecting plasmid DNA into the myocardium and measuring reporter gene expression in the cells which have successfully taken up sufficient quantities of DNA (4–6). The major drawback of direct DNA injection is its relative inefficiency, as only ≈0.02% of the myocytes in the adult rat heart take up and express injected DNA (7). For these reasons we sought a gene-transfer vector system which would function effectively with primary cultures of cardiac myocytes and one which would also have application to *in vivo* studies.

Many of the disadvantages of *in vivo* direct plasmid DNA injection and transient transfections of primary cultured cells can be solved through adenovirus (Ad) delivery of genes. Ad expression vectors have been in use for a decade (refs. 8 and 9; for a review see ref. 10) and more recently exploited for gene therapy (e.g., refs. 11–13). Features of Ad-based expression vectors which make them attractive for gene therapy include very efficient uptake into cells which contain the appropriate Ad receptor and uptake pathway and the ability to carry up to 7.5 kb of foreign DNA. Ad vectors allow a reporter gene to be under the control of tissue-specific promoter elements (14, 15) as well as a variety of viral and mammalian constitutive promoter elements (16). Negative aspects of an Ad infection, particularly repression of host cell mRNA translation and shutdown of host normal mRNA production (17–19), have been addressed by using defective Ad vectors which are based on mutations in the dominant regulatory region E1 (20, 21).

A recent report demonstrated efficient gene transfer into adult rat cardiocytes *in vitro* (22). In addition, recent studies using Ad vectors introduced intravenously or intramuscularly into rats and mice indicate that the virus will infect a wide variety of tissue types, including mouse skeletal and cardiac muscle (23, 24). These *in vivo* studies were carried out with the β-galactosidase reporter gene, which is very useful for histochemical analysis. Very few quantitative data are available concerning expression of Ad-mediated gene transfer *in vivo*.

We have used an Ad reporter vector (AdCMVCATgD) which contains the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of the cytomegalovirus (CMV) promoter and eukaryotic RNA-processing elements to characterize quantitative aspects of cardiocyte infection by Ad. Using an anti-CAT antibody, we have identified conditions where virtually 100% of cultured fetal and adult cardiac myocytes express the reporter gene and have determined the dose–response and duration of expression of virus injected directly into adult heart. CAT enzymatic activity was approximately linear over a viral input dose of four orders of magnitude both *in vitro* and *in vivo*. Quantitative and immunohistochemical analyses of Ad-injected hearts indicate that the use of the AdCMVCATgD virus *in vivo* is substantially more efficient and reproducible than plasmid injections and may produce sufficient quantities of an introduced gene to result in phenotypic modification.

METHODS AND MATERIALS

Isolation and Culture of Rat Cardiac Myocytes. Primary fetal cardiac myocytes were prepared from Sprague–Dawley

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Abbreviations: Ad, adenovirus; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; pfu, plaque-forming unit(s).

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rats at fetal day 20 (Taconic Farms) by modification of a published protocol (25). Cardiac cells were preplated for 1 hr to remove fibroblasts. Cells (1.8×10^6 per 25-mm Corning tissue culture dish) were then plated in heart medium [Hanks' salt solution supplemented with minimal essential medium (MEM) vitamin stock, amino acids, and nonessential amino acids, L-Glutamine (2 mM), glycine (0.5 mg/ml), hypoxanthine (0.125 mg/ml), penicillin (5 units/ml), streptomycin (5 μg/ml), and $NaHCO_3$ (1.65 mg/ml)] with 10% fetal bovine serum (HyClone). Primary adult cardiac myocytes were prepared from the hearts of 200-g female Sprague–Dawley rats (Taconic Farms) by a standard protocol (26). Cells [2.4×10^5 per 60-mm dish coated with laminin (20 μg/ml; Boehringer Mannheim)] were plated in heart medium. Cells were maintained in culture at 37°C, 5% CO_2. Cell culture medium was changed every other day for the duration of the assay.

Virus Production. Virus plaquing and the preparation of viral stocks were performed on human embryonic kidney 293 monolayer cells (27).

Infection of Cardiac Myocytes. Forty-eight hours after plating, fetal myocytes were infected with AdCMVCATgD at 0.01, 0.1, 1, and 10 plaque-forming units (pfu) per cell. The adult cells were infected with the same doses immediately after plating. AdCMVCATgD (10^{10} pfu/ml) was diluted in heart medium without added serum. One milliliter of medium plus virus was added to each 60-mm dish. The dishes were incubated at 37°C with gentle swirling every 15 min for 90 min, after which 1 ml of heart medium (supplemented with a final concentration of 10% fetal bovine serum) was added to each dish.

Immunohistochemistry. Cells were fixed on coverslips in 3.7% formaldehyde in phosphate-buffered saline (PBS: 137 mM NaCl/2.7 mM KCl/10 mM Na_2HPO_4/2 mM KH_2PO_4, pH 7.4) for 10 min at room temperature. Coverslips were then washed in PBS. Cells were blocked in 10% normal goat serum (NGS) (Jackson ImmunoResearch) for 2 hr at 37°C. The coverslips were then incubated for 2 hr at 37°C with a commercially available unconjugated rabbit polyclonal antibody which recognizes CAT (5 Prime → 3 Prime, Inc.) at a 1:1000 dilution in PBS/0.1% Triton X-100/1% NGS. After three 5-min washes in PBS, the coverslips were incubated for 1 hr at 37°C with a peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad) at a 1:200 dilution in PBS/0.1% Triton X-100/1% NGS. After three 5-min washes in PBS, the peroxidase reaction was developed with Vectastain DAB (Vector Laboratories). For tissue sections, 5 days after injection, hearts were removed and the distal quarter of the heart was placed in 3.7% formaldehyde at 4°C overnight. The samples were embedded in paraffin (Paraplast; Oxford Labware, St. Louis) (28). Tissue sections (4–10 μm) were cut, placed on slides coated with 0.05% poly(L-lysine) (Sigma), and dried overnight at room temperature. The sections were then ethanol-dehydrated and deparaffinized in xylenes. After rehydration, sections were placed in PBS/0.1% Triton X-100 for 5 min. The endogenous peroxidase activity was blocked by placing the sections in 0.3% hydrogen peroxide in methanol for 30 min. The antibody staining procedure was carried out as above. Following the peroxidase developing reaction the slides were washed in distilled water and the heart sections were counterstained with hematoxylin for 12 sec. The slides were then washed in distilled water and mounted with gelvatol (Airvol, Air Products and Chemicals, Allentown, PA).

CAT Assays from Myocytes. At each time point, infected cardiac myocytes were harvested (28) and the amount of protein in the supernatant was measured by Bradford assay using bovine serum albumin as the standard (Bio-Rad). CAT assays were performed on 10 μg of total protein. When the CAT activity was >70% and out of the linear range, supernatants were diluted in 1% bovine serum albumin. CAT assays were done by TLC (7) with incubation for 2 hr at 37°C.

DNA and Virus Injections *in Vivo*. Ten micrograms of CMV–CAT plasmid DNA in 50 μl of PBS was injected into the apex of the left ventricle of 200-g female Sprague–Dawley rats as described (7). For Ad injections, 6×10^6 to 6×10^8 pfu in 50 μl of PBS were injected; 2×10^9 pfu were injected undiluted in a volume of 50 μl.

CAT Assays on Tissue. At indicated times following injection, hearts were removed, rinsed in PBS, and weighed. For the spatial-distribution experiment, the hearts were then sectioned into seven roughly equivalent slices. Each slice was then homogenized with a Tissumizer (Tekmar, Cincinnati) in 0.5 ml of 25 mM glycylglycine, pH 7.8/15 mM $MgSO_4$/4 mM EGTA, pH 8.0/1 mM dithiothreitol for 20 sec. For the dosage and time course experiments the hearts were homogenized the same way but in a volume equal to 0.5 g of wet tissue weight per milliliter of buffer. The homogenates were centrifuged for 25 min at $4640 \times g$. Supernatants were removed, heated at 65°C, and clarified in a microcentrifuge for 5 min. Supernatant volumes were measured and CAT assays were done on 5% of the lysate or on dilutions of lysate in 1% bovine serum albumin. Assays were done as above for 2 hr at 37°C.

RESULTS

A recently generated replication-defective recombinant Ad reporter vector, AdCMVCATgD (Fig. 1), consisting of a combination of strong eukaryotic promoter (CMV-1) and splicing elements, has proven to be a very sensitive vector for gene expression studies in human cell lines (E.F.-P. and M.A., unpublished work). Because of the high level of CAT expression seen with this vector, we have used it to characterize Ad-mediated gene transfer into cardiac myocytes *in vitro* and *in vivo*. Fig. 2 *Left* demonstrates the dose–response and time course of AdCMVCATgD infection of primary fetal rat cardiocytes. In these studies we assessed infection both by quantitating CAT reporter gene expression and by determining the percentage of cells expressing the CAT reporter gene by immunostaining. Because of the extremely high levels of CAT activity obtained, dilutions of cell extracts were made to maintain assays in the linear range of the CAT assay. We found that CAT activity was easily detected at the earliest measured time point (4 hr), was near maximal by 48 hr, and was maintained at stable levels through the remainder of the experiment (a total of 167 hr). A dose-dependent increase was maintained over a range of four orders of magnitude of virus input throughout much of the time course. The same basic extent and level of infection and expression was found in adult cardiocytes (Fig. 2 *Right*) when infected

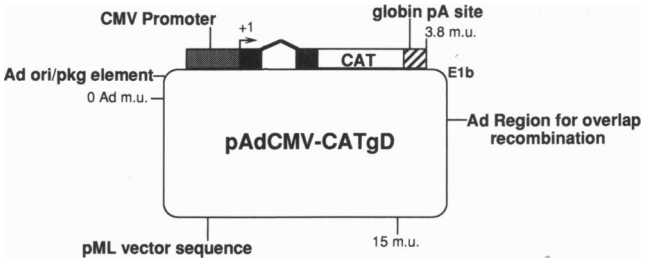

FIG. 1. Plasmid pAdCMVCATgD, used in construction of Ad-CMVCATgD virus. The left end of Ad DNA [0–1 map unit (m.u.)] contains the origin of replication (ori) as well as the viral packaging sequence (pkg). Ad sequence from 1.0 to 3.8 m.u. was deleted and replaced with the sequence elements for the CMV-1 promoter, the bacterial CAT sequence, and the mouse β^{maj}-globin poly(A) site. Ad sequence from 3.8 to 15.0 m.u. provides DNA sequence for overlap recombination.

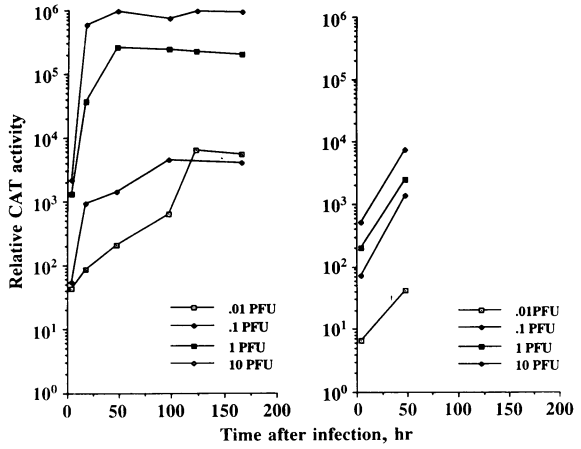

FIG. 2. Dosage- and time-dependent expression of Ad in fetal (*Left*) and adult (*Right*) cardiocytes. Relative CAT activity refers to the percent of acetylated chloramphenicol relative to 10 μg of total protein multiplied by the dilution factor of the cell lysate in order to keep the assays within the linear range. The duration of study in adult cells was shortened due to reduced cell viability regardless of the presence of Ad.

under similar conditions. However, in this case the study was shortened to 48 hr due to the difficulty in maintaining healthy differentiated adult cardiac myocytes in culture, independent of virus infection. Based on these assays, the sensitivity of the AdCMVCATgD CAT assay, and the levels of activity resulting from these infections, we predict that CAT expression could be reliably detected in as few as 10 infected cells.

At each dose of virus, we determined the percentage of fetal cells which were expressing CAT by immunostaining coverslips of infected fetal cardiocytes 18 and 48 hr after infection. Mock-infected cells showed no staining, but cells infected with increasing doses of virus showed a proportional increase in the number of cells infected, with 1 pfu/cell (100 particles) resulting in virtually 100% of the cells being stained (data not shown). The virus infection included both myocytes and the small proportion of nonmyocyte fibroblasts (<5%) which remained in the culture following initial myocyte purification (data not shown). Similar results were obtained with adult cardiac myocytes. At an infection of ≥1 pfu, 100% of the rod-shaped adult myocytes stained positive with an anti-CAT antibody. This was true at both 4 and 48 hr.

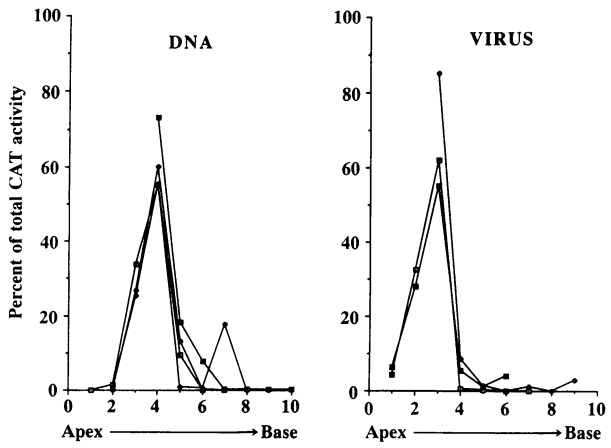

FIG. 3. Distribution of CAT activity in DNA- or Ad-injected hearts. Total CAT activity from DNA-injected hearts in relative units was 2799 ± 1353. Total CAT activity for Ad-injected hearts in relative units was 117,501 ± 15,944. The fold difference in activity was calculated based on 75 ng of CAT DNA in 6×10^7 pfu of virus. Each line corresponds to a different animal.

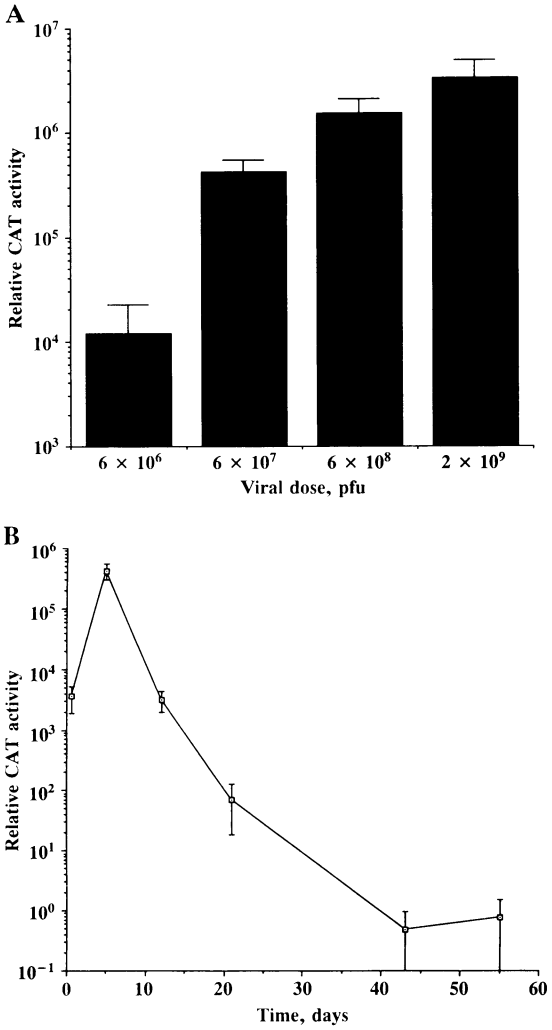

FIG. 4. (*A*) CAT expression in the left ventricle 5 days after intracardiac injection of AdCMVCATgD at 6×10^6 ($n = 4$), 6×10^7 ($n = 4$), 6×10^8 ($n = 3$), or 2×10^9 ($n = 2$) pfu. (*B*) CAT expression over time in the left ventricle following injection of 6×10^7 pfu of AdCMVCATgD. Animals were sacrificed and CAT activity in the left ventricle was measured 15 hr, 5 days, 12 days, 21 days, 43 days, and 55 days after injection ($n = 4$, except for the 43- and 55-day time points, where $n = 2$).

Myocytes which were rounded up also stained positive for CAT and sarcomeric myosin heavy chain, and they excluded trypan blue (data not shown).

Since Ad-mediated gene transfer clearly offers certain advantages over transient transfection assays when cultured myocytes are used, we next asked whether the quantitative advantages of using AdCMVCATgD *in vitro* could be extended to *in vivo* studies. AdCMVCATgD virus (6×10^7 pfu in 50 μl) was injected into adult rat hearts. A parallel injection of the plasmid pAdCMVCATgD (10 μg) was carried out for quantitative comparison. Five days after injection of virus or DNA, hearts were sliced into seven 1.5-mm sections perpendicular to the long axis of the heart. The amount of CAT activity was quantitated in each section. When either plasmid DNA or AdCMVCATgD was injected into rat heart, expression of the reporter gene was localized predominantly to the vicinity of the injection site (Fig. 3). Although the virus infection proved to be at least 5000-fold more efficient than the plasmid DNA injection on the basis of input DNA, the distributions of CAT activity from DNA and virus administration were essentially identical. The highest level of expression was observed at the area of injection, with a gradient of CAT activity extending toward the base of the heart.

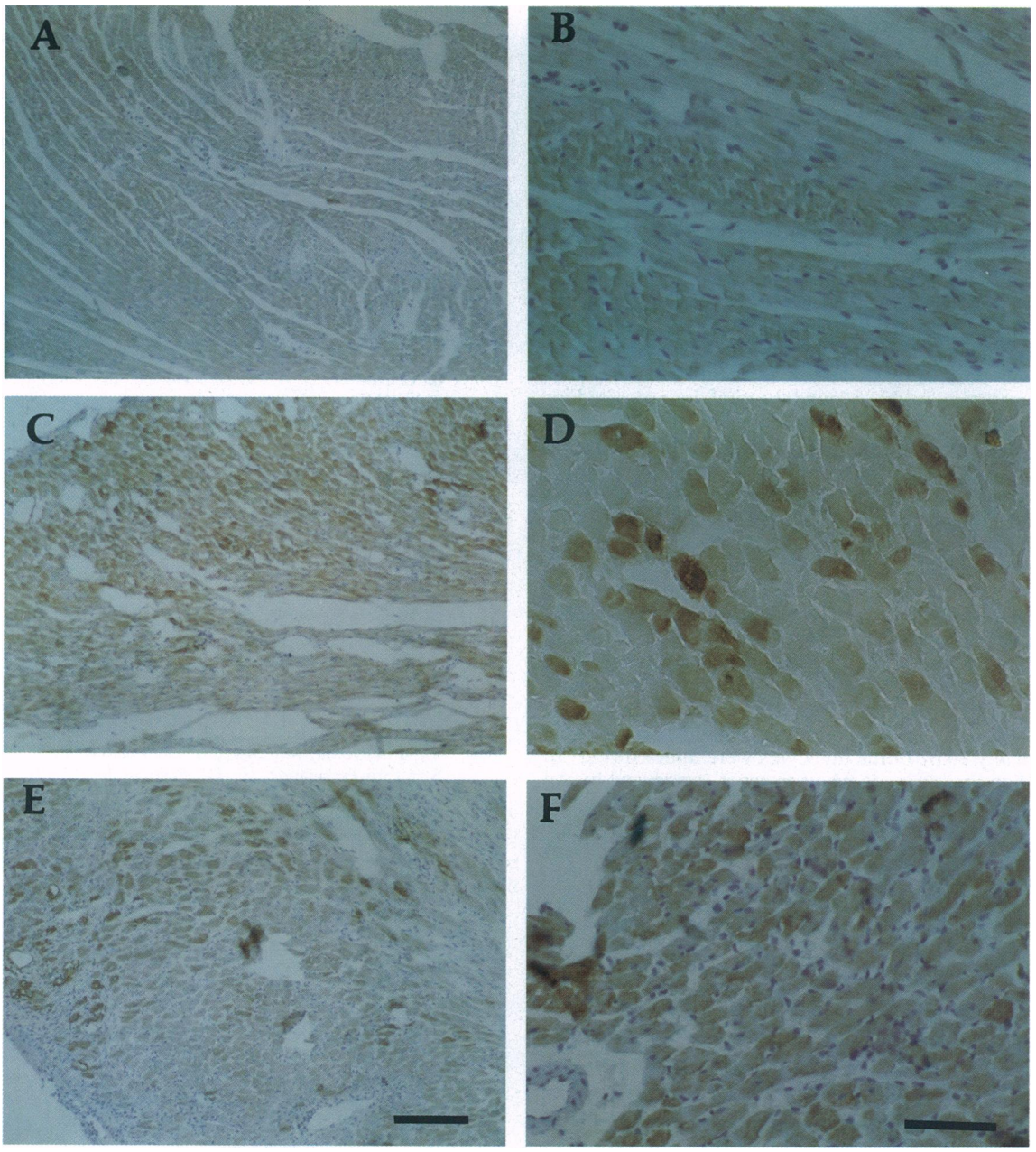

FIG. 5. Immunohistochemical staining for CAT protein in Ad-infected hearts. Viral doses were 6×10^6 pfu (*A* and *B*), 6×10^7 pfu (*C* and *D*), and 2×10^9 pfu (*E* and *F*). Photographs of tissue sections were taken under differential interference contrast microscopy. CAT-positive cells were stained brown by the peroxidase reaction. All sections were counterstained with hematoxylin. [Bar = 1 mm (*A*, *C*, and *E*) or 0.05 mm (*B*, *D*, and *F*).]

Given the high levels of CAT activity that were obtained from virus injection, we examined the dose responsiveness of a range of virus from 6×10^6 pfu up to 2×10^9 pfu per injection. Five days after injection, hearts were homogenized and assayed for CAT activity (Fig. 4*A*). Increasing CAT activity correlated with increasing virus, although not in an entirely linear fashion. Fig. 4*B* shows the duration of CAT activity following a single injection of 6×10^7 pfu of AdCMVCATgD. CAT activity was detected as early as 15 hr after infection, reaching maximal levels at ≈5 days. Although CAT activity was still easily detectable 43 and 55 days following injection, expression levels were 5–6 orders of magnitude lower than the peak activity. To determine the number and type of cells in the heart which expressed CAT, tissue sections were stained with an anti-CAT antibody. A very high proportion of cells in many regions of the myocardium expressed CAT antigen at all doses of virus (Fig. 5). In many regions, virtually 100% of myocytes stained positive. Positive cells included both myocytes and nonmyocytes, although it appears that the proportion of myocytes infected exceeded that of nonmyocytes. It should also be noted that a substantial number of inflammatory cells were seen (see Fig. 5 *D* and *E*). The nature of this inflammatory response did not appear to correlate with the amount of introduced virus. Within the limits of our ability to make quantitative statements of peroxidase staining, the intensity appeared to increase with increasing viral dose. The lowest dose of virus (6×10^6) resulted in a lower intensity of CAT antigen per cell as well as a reduced number of infected cells. At higher doses of virus, both an increased number of cells and an increased amount of CAT per cell were obtained.

DISCUSSION

Cardiac myocytes appear to be ideally suited for the use of Ad-mediated gene transfer. Transient transfection of fetal

cardiocytes under optimized conditions traditionally results in 10–20% of the cells being transfected. Ad can infect virtually 100% of the cells and does not require the use of damaging treatments such as electroporation, which generally kills a large number of the cells in the culture. Clearly, fetal cardiocytes possess viral receptors in numbers that do not present a limitation to use of Ad vectors in rat cardiocytes. With Ad infection, there is no apparent effect on cell viability or morphology at the multiplicities of infection tested here. In addition, Ad infections provide an efficient means of gene transfer into adult cells, which has not been possible with conventional transfection strategies (this report and ref. 22). A recent report of Ad infection of adult rat cardiocytes (22) reported 90% infection at a dose of 10^3 pfu per cell. Based on our observations it is not necessary to use such a high dose of virus. Because of the efficient CAT expression system, we have found the viral dose required for infection of virtually all cells is in the vicinity of 1 pfu per cell (100 particles). In addition, due to the ability to accurately and reproducibly assay the reporter gene activity within the first 24 hr of infection, studies on primary cell cultures can be accomplished at times when host expression functions may not have been grossly altered, which may not be the case with more conventional transfection techniques.

Given the high level of enzymatic activity obtained from infection of cardiocytes *in vitro*, we anticipated a high level of activity from injection of virus into the myocardium *in vivo*. In fact, the activity observed was exceedingly high, and although the activity was not strictly proportional to the amount of input virus, it is conceivable that even greater activity could be obtained with a higher input of virus. In many regions of the heart, virtually 100% of the myocytes were infected (Fig. 5). One question that arises is whether genes introduced by Ad can produce enough protein to functionally modify the phenotype or physiology of a target organ or animal. At least $\approx$150 μg of CAT protein can be expressed in a single rat heart following administration of 2×10^9 pfu of virus (A.K.-E., unpublished observation), suggesting that the quantity of a foreign gene product is not likely to be a limitation. Of course, whether similar quantities of a bioactive mammalian gene product can be obtained remains to be determined.

When tissue sections were stained with an anti-CAT antibody, both the number of positive cells and the amount of CAT protein per cell increased with increasing virus dose. This was most apparent at the two lowest doses of virus (6×10^6 and 6×10^7 pfu). This difference was not as apparent among the three highest doses of virus, probably because of the nonquantitative nature of the peroxidase stain. The adult rat heart has been estimated to have $\approx 2 \times 10^7$ myocytes, which represent about 80% of the cells in the intact heart. If Ad infection *in vivo* is as efficient as it is *in vitro*, then the three highest doses of virus would theoretically result in infection of all myocytes in the heart. It is difficult to estimate the total number of positive cells because of the unknown sensitivity of the antibody in paraffin and the variation in the staining intensity. However, we can demonstrate many regions in any one heart that appear to be 100% positive, and other regions with somewhat less CAT antigen, as well as some regions that do not show any apparent staining. However, visual inspection leaves no doubt that a vastly greater number of cells are infected than when plasmid DNA is introduced by injection (7).

One of the issues currently under debate concerning the use of Ad as a gene transfer vector is duration of expression of introduced genes. Our results and those of Lemarchand *et al.* (29) demonstrate a rather transient pattern of expression. It may be that in order to generate long-term expression it will be necessary to introduce the virus into neonates, as has been suggested by Strattford-Perricaudet *et al.* (24). However, it is apparent that Ad-mediated gene transfer in the heart is extremely efficient and should be a very useful tool for the introduction of genes into cardiac myocytes.

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