Cell-specific expression of SERCA, the exogenous Ca\(^{2+}\) transport ATPase, in cardiac myocytes

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Ma, Hailun, Carlota M. Sumbilla, Iain K. G. Farrance, Michael G. Klein, and Giuseppe Inesi. Cell-specific expression of SERCA, the exogenous Ca\(^{2+}\) transport ATPase, in cardiac myocytes. Am J Physiol Cell Physiol 286: C556–C564, 2004. First published October 30, 2003; 10.1152/ajpcell.00328.2003.—We evaluated various constructs to obtain cell-specific expression of the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) gene in cardiac myocytes after cDNA delivery by means of transfections or infections with adenovirus vectors. Expression of exogenous enhanced green fluorescent protein (EGFP) and SERCA genes was studied in cultured chicken embryo and neonatal rat cardiac myocytes, skeletal and smooth muscle cells, fibroblasts, and hepatocytes. Whereas the cytomegalovirus (CMV) promoter yielded high levels of protein expression in all cells studied, cardiac troponin T (cTnT) promoter segments demonstrated high specificity for cardiac myocytes. Their efficiency for protein expression was lower than that of the CMV promoter, but higher than that of cardiac myosin light chain or β-myosin heavy chain promoter segments. A double virus system for Cre-dependent expression under control of the CMV promoter and Cre expression under control of a cardiac-specific promoter yielded high protein levels in cardiac myocytes, but only partial cell specificity due to significant Cre expression in hepatocytes. Specific intracellular targeting of gene products was demonstrated in situ by specific immunostaining of exogenous SERCA1 and endogenous SERCA2 and comparative fluorescence microscopy. The −374 cTnT promoter segment was the most advantageous of the promoters studied, producing cell-specific SERCA expression and a definite increase over endogenous Ca\(^{2+}\)-ATPase activity as well as faster removal of cytosolic calcium after membrane excitation. We conclude that analysis of promoter efficiency and cell specificity is of definite advantage when cell-specific expression of exogenous SERCA is wanted in cardiac myocytes after cDNA delivery to mixed cell populations.

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EXPRESSION OF EXOGENOUS sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) gene in cardiac myocytes has acquired an important dimension with the introduction of recombinant adenovirus vectors (7, 12, 15, 25), demonstrating that Ca\(^{2+}\) signaling and homeostasis can be influenced by SERCA expression levels. The use of adenovirus vectors has been extended to whole heart preparations (9) and animal models (16). In fact, adenovirus SERCA gene transfer has been advocated as a therapeutic approach to heart failure (1, 8, 20). Exogenous genes are often placed under control of very strong viral promoters, resulting in high levels of recombinant protein expression. Shortcomings of viral promoters are related to their lack of cell specificity, whereby constitutive expression of the exogenous gene occurs in all cell types in addition to cardiac myocytes. Furthermore, the high transcriptional efficiency of viral promoters can yield excessive levels of protein expression (29). Alternatively, it may be desirable to place the exogenous SERCA gene under control of cell-specific promoters (19). We considered that advantageous features of promoters for exogenous gene expression in cardiac myocytes include 1) small size to facilitate construction of recombinant vectors with the viral cDNA plasmid, 2) cell specificity for selective expression in cardiac myocytes, and 3) appropriate strength to yield SERCA protein levels that are sufficient to influence the cytosolic Ca\(^{2+}\) transient. We report here an evaluation of effective viral titers for exogenous gene expression under control of several promoters, in comparative experiments on expression of enhanced green fluorescent protein (EGFP) and SERCA, under control of constitutive or cell-specific promoters. Furthermore, we also used a helper viral vector to drive cell-specific expression of Cre recombinase and trigger Cre-dependent expression of EGFP cDNA placed in a separate vector under control of the cytomegalovirus (CMV) promoter. We compared all these systems in chicken embryo and neonatal rat cardiac myocytes and in a variety of cells. We found that careful analysis of various promoter segments is required to obtain satisfactory levels of cell-specific expression of exogenous SERCA.

MATERIALS AND METHODS

DNA constructs and vectors. Enhanced green fluorescent protein (EGFP) or chicken SERCA1 (23) cDNA was subcloned into pAdlox (17) or pAE1sp1A (13) plasmid. These plasmids were preceded by various promoters, including a 250-bp segment of chicken ventricle-specific cardiac myosin light chain-2v (MLC-2v) promoter (14), a 215-bp segment of rat β-myosin heavy chain (β-MHC) promoter (24), the −268−, −374−, and −550-bp segments of chicken cardiac troponin T (cTnT) promoters (28), the −303-bp segment of the rat troponin T (cTnT) promoter (36), and the CMV promoter, followed by simian virus polyadenylation signal (Fig. 1A).

Recombinant adeno-EGFP cDNA was obtained by cotransfection of CRE8 cells with pAdlox plasmids, in conjunction with the replication-defective Δ5 adenovirus genome, according to the method described by Hardy et al. (17). The CRE8 cells are derived by transformation of the HEK-293 line and constitutively express Cre recombinase for recombination between loxP sites in the Δ5 genome and in the pAdlox vector to yield recombinant adenovirus vectors. An alternative method for generation of recombinant adeno-EGFP cDNA was obtained by homologous recombination in HEK-293 cells with pAE1sp1A plasmid and pm17 (Microbix). The pAdlox and pAE1sp1A plasmids were constructed
such that homologous recombination resulted in antisense direction of the gene of interest with respect to the adenovirus E1 gene promoter. The recombinant products were selected by plaque purification in HEK-293 cells and band purified by centrifugation in cesium chloride gradients to yield concentrations of the order of 10^9–10^11 plaque-forming units (PFU)/ml.

For the double virus system, Cre recombinase (21, 30) cDNA was subcloned into pADE1sp1A plasmid under control of the cardiac cell-specific 268 or 374 cTnT promoter. In addition, EGFP cDNA was subcloned into pADE1sp1A plasmid under control of the CMV promoter. In these latter construct, however, CMV promoter and reporter gene were separated by a neo-polyA “stuffer” flank of two loxP sites. In the helper virus (pJCVMVloxPEGFP), the CMV promoter and the EGFP cDNA are under the control of the cell-specific 268cTnT or 374cTnT promoters.

**Fig. 1.** Diagram of promoter and cDNA elements used to control expression of exogenous genes directly (A) or through Cre recombinase expression (B). A: Constitutive promoter of cytomegalovirus (CMV), −550, −374, and −268 segments of the chicken cardiac troponin T (cTnT) promoter (28), −303-bp segment of the rat cTnT promoter (36), −250 segment of the venticle-specific cardiac myosin light chain (MLC-2v) promoter (14), and −215 segment of the β-myosin heavy chain (β-MHC) promoter (24), were used with enhanced green fluorescent protein (EGFP) or fast skeletal muscle sarco(end)plasmic reticulum Ca^{2+}-ATPase isoform 1 (SERCA1) cDNA. B: In the main vector (pJCVMVloxPEGFP), the CMV promoter and the EGFP cDNA are separated by a neo-polyA “stuffer,” flanked by two loxP sites. In the helper virus (pJCVMVloxPEGFP), the CMV promoter and the EGFP cDNA are under the control of the cell-specific 268cTnT or 374cTnT promoters.

**Cell cultures, transfections, and infections.** Cultures of HEK-293 cells and CRE8 cells were maintained as described by Graham and Prevec (13) and Hardy et al. (17), respectively. Stable lines of rat smooth muscle myocytes (A7r5) and rat liver cells (K-9) were maintained as specified by their supplier (American Type Culture Collection). Harvesting of tissue to prepare primary cell cultures was described previously (4). Cardiac myocyte cultures were obtained from day 8 chicken embryos; skeletal myocytes and skin fibroblasts were obtained from day 11 chicken embryos as previously described (19). Cardiac myocytes were also obtained from neonatal rats as described by Simpson (31). Transfections were performed with the LipofectAMINE Plus kit (GIBCO). The cells were plated (650 cells mm^{-2} for embryonic chicken cardiomyocytes and 350 cells mm^{-2} for neonatal rat cardiomyocytes) so that they were 50–80% confluent on the day (day 2) of transfection. Solutions of plasmid DNA and PLUS reagent, and LipofectAMINE only, were diluted with serum-free and antibiotic-free medium. After incubation at room temperature for 15 min, the two solutions were mixed and incubated at room temperature for additional 15 min. The medium in the cell culture plates was then replaced with serum-free and antibiotic-free medium. After a 3-h incubation at 37°C in the presence of 5% CO_{2} (chicken myocytes) or 1% CO_{2} (rat myocytes), complete medium was added to obtain normal volume and serum concentration. Two to three days after transfection, the cells were harvested for microscopic or biochemical tests.

Infections of cultured cells with recombinant adenoviral vectors were performed 1 day after initial plating, as described by O’Donnell et al. (29). Briefly, the cells were washed and treated with the viral preparations (1–20 PFU/seeded cell) by incubating for 60 min in half-volume of serum-free medium in the presence of CO_{2} at 37°C. The medium was then diluted with half-volume of medium containing serum and antibiotic solution (2× concentration) and allowed to incubate for another 48 h before cell harvesting.

**Visualization and immunostaining of cells.** In situ immunofluorescence staining of exogenous SERCA1, endogenous SERCA2, myosin, and α-actinin were performed as previously described (32).

Primary monoclonal antibodies were CaF3-5C3 for chick SERCA1 (23), CaS-3H2 for chick SERCA2 (22), anti-myosin (Developmental Studies Hybridoma Bank, MF 20), anti-α-actinin (Sigma Clone EA-53), and a polyclonal antibody for SERCA2 (Novus Biologicals Novus NB 100–327A). Secondary antibodies used for fluorescence imaging were biotinylated horse anti-mouse or anti-rabbit IgG (Vector Labs), followed by incubation with fluorescein-streptavidin (American).

Phase contrast and fluorescence microscopy were performed using either a Zeiss axioplan or a Nikon Eclipse TE 200 equipped with a mercury lamp and the appropriate fluorescence filter attachments. The enlarged fluorescent images were obtained with a Bio-Rad Radiance confocal scanner.

**Cell harvesting, fluorescence, and protein analysis.** The cells were harvested 48 h after infection or 72 h after transfection. Levels of EGFP expression were determined as previously described (34).

Primary monoclonal antibodies were CaF3-5C3 for chick SERCA1 (23), CaS-3H2 for chick SERCA2 (22), anti-myosin (Developmental Studies Hybridoma Bank, MF 20), anti-α-actinin (Sigma Clone EA-53), and a polyclonal antibody for SERCA2 (Novus Biologicals Novus NB 100–327A). Secondary antibodies used for fluorescence imaging were biotinylated horse anti-mouse or anti-rabbit IgG (Vector Labs), followed by incubation with fluorescein-streptavidin (American).

Western blotting. The Western blots were performed as previously described (34). Cellular proteins were separated in 7–12% polyacrylamide gels, transferred onto nitrocellulose paper, and probed with the monoclonal antibody for SERCA1 (23). The reactive bands were visualized by using an enhanced chemiluminescence-linked Western blotting detection system (Pierce).

**ATPase activity measurements.** The assay for ATPase activity of the protein suspensions were performed as previously described (34).

**Extracellular calcium transients.** The myocytes in culture were loaded with the Ca^{2+} indicator fluo 4 (Molecular Probes), mounted on an Olympus 1 × 70 inverted microscope, and superfused with Ringer solution at 30°C temperature. The myocytes were field stimulated, and cell fluorescence was recorded and corrected for background signal as described previously (4).
RESULTS

Efficiency of adenovirus vectors in various cell types. The efficiency of recombinant adenovirus vectors carrying EGFP cDNA under control of the CMV promoter is different in various cell types, although it is always higher than that of shuttle plasmids carrying the same EGFP construct and transfected by the LipofectAMINE method. It is shown in Fig. 2 that only a small number of cells exhibit the green fluorescence signal after transfection of shuttle plasmid by the LipofectAMINE method. On the other hand, infection with adenovirus vector yields expression in nearly 100% of cardiac myocytes, fibroblasts, and liver cells in culture. We find, however, that effective viral titers vary between 1 and 50 PFU/cell and depend on the cell type. Gene transfer to 100% of neonatal rat cardiac myocytes in culture is obtained with viral titers as low as 2 PFU/seeded cell, as originally observed by Kass-Eisler et al. (25). The variable efficiency of adenovirus vector-mediated gene transfer is related to different expression levels of coxsackie adenovirus receptor and integrins (2, 6, 34, 35).

Comparative evaluation of promoters. A preliminary evaluation of promoter cell specificity was performed by LipofectAMINE method or infected with adenovirus vector carrying EGFP cDNA under control of \(-374\) cTNT promoter segment or the CMV promoter (see MATERIALS AND METHODS). Although all transfections were performed under identical conditions, various cell types required different adenovirus titers: 10 PFU/chicken cell (cardiac myocytes, skeletal muscle myocytes, and fibroblasts) and 2 PFU/cell (rat cardiac myocytes and liver cells) or 10 PFU/cell (rat smooth muscle myocytes) for both promoters. Comparison of the \(-374\) cTNT and CMV promoters was performed with identical adenovirus titer for each cell type. In all cases, phase contrast microscopy revealed confluent cultures. Therefore, the visible number of fluorescent cells represents transfection or infection efficiency.

Fig. 2. Expression of EGFP under the control of \(-374\) cTNT promoter segment or constitutive (CMV) promoter in various cell types. Various chicken embryo and neonatal rat cells were transfected by the LipofectAMINE method or infected with adenovirus vector carrying EGFP cDNA under control of \(-374\) cTNT promoter segment or the CMV promoter (see MATERIALS AND METHODS). Although all transfections were performed under identical conditions, various cell types required different adenovirus titers: 10 PFU/chicken cell (cardiac myocytes, skeletal muscle myocytes, and fibroblasts) and 2 PFU/cell (rat cardiac myocytes and liver cells) or 10 PFU/cell (rat smooth muscle myocytes) for both promoters. Comparison of the \(-374\) cTNT and CMV promoters was performed with identical adenovirus titer for each cell type. In all cases, phase contrast microscopy revealed confluent cultures. Therefore, the visible number of fluorescent cells represents transfection or infection efficiency.
fectAMINE-assisted transfections in primary cultures of chicken embryo cardiac myocytes, skeletal myocytes, and fibroblasts, as well as on neonatal rat cardiac myocytes and on established lines of rat smooth muscle myocytes and hepatocytes. Although the percentage of cells effectively transfected is rather low, this method provides a gene transfer modality alternative to adenovirus infection and is independent of the efficiency of adenovirus entry. We found that when the EGFP vector was under control of cTnT promoter segments, fluorescent cells appeared only in chicken or rat cardiac myocyte cultures (Fig. 2), whereas fluorescence appeared in all cell types when the EGFP was under control of the CMV promoter.

The cell specificity of the promoters was also observed using adenovirus vectors (Fig. 2) in experiments in which the adenovirus vector titer was adjusted for optimal gene transfer in adenovirus vectors (Fig. 2) in experiments in which the adenovirus vector titer was adjusted for optimal gene transfer in

Table 1. Expression of enhanced green fluorescent protein in various cell types under control of various promoters

<table>
<thead>
<tr>
<th></th>
<th>Rat MLC-2v-250</th>
<th>Rat MHC-215</th>
<th>Chicken cTnT-268</th>
<th>Rat cTnT-303</th>
<th>Chicken cTnT-374</th>
<th>Chicken cTnT-550</th>
<th>CMV</th>
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<tr>
<td>Chicken cardiac myocytes</td>
<td>0.17 ± 0.04</td>
<td>1.25 ± 0.16</td>
<td>15.10 ± 0.20</td>
<td>8.30 ± 0.20</td>
<td>31.01 ± 1.22</td>
<td>20.35 ± 2.16</td>
<td>100</td>
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<tr>
<td>Chicken skeletal muscle</td>
<td>0.36 ± 0.12</td>
<td>0.83 ± 0.10</td>
<td>1.21 ± 0.19</td>
<td>ND</td>
<td>0.91 ± 0.56</td>
<td>1.12 ± 0.21</td>
<td>100</td>
</tr>
<tr>
<td>Chicken skin fibroblast</td>
<td>0.21 ± 0.08</td>
<td>0.21 ± 0.09</td>
<td>0.66 ± 0.07</td>
<td>ND</td>
<td>0.31 ± 0.08</td>
<td>0.49 ± 0.15</td>
<td>100</td>
</tr>
<tr>
<td>Rat cardiac myocytes</td>
<td>0.59 ± 0.10</td>
<td>0.62 ± 0.18</td>
<td>5.80 ± 0.35</td>
<td>11.92 ± 0.40</td>
<td>32.80 ± 2.10</td>
<td>21.26 ± 1.20</td>
<td>100</td>
</tr>
<tr>
<td>Rat smooth muscle</td>
<td>1.75 ± 0.09</td>
<td>2.25 ± 0.31</td>
<td>1.36 ± 0.21</td>
<td>ND</td>
<td>1.36 ± 0.12</td>
<td>1.72 ± 0.37</td>
<td>100</td>
</tr>
<tr>
<td>Rat liver cells</td>
<td>0.28 ± 0.08</td>
<td>0.44 ± 0.08</td>
<td>0.69 ± 0.18</td>
<td>ND</td>
<td>0.51 ± 0.12</td>
<td>0.67 ± 0.05</td>
<td>100</td>
</tr>
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Cells were infected with enhanced green fluorescent protein (EGFP) containing adenovirus vectors under the following conditions: chicken embryo-derived cells and rat skeletal smooth muscle cells, 10 pfu per cell; rat cardiac muscle and liver cells, 2 pfu per cell (see Fig. 2 legend also). The values given are as percentages of the expression obtained under control of the cytomegalovirus (CMV) promoter in each cell type. Each value ± SD is the average of 5 determinations.

In a separate series of experiments, we obtained further evidence of cell-specific expression in mixed cultures of cardiac fibroblasts and myocytes, after infection with adenovirus vector carrying EGFP cDNA under control of the −374 cTnT promoter. The cells were immunostained specifically for endogenous SERCA2, myosin, or α-actinin. It is clearly shown in Fig. 3 that only the cardiac myocytes are stained, whereas the fibroblasts, visualized by phase images, are not.

**Activation of exogenous gene expression by Cre recombinase.** An alternative method for cell-specific exogenous gene expression is based on cell-specific Cre recombinase expression to trigger CMV-driven transcription of the gene of interest (21). This method takes advantage of the fact that, normally, Cre recombinase is not present in mammalian cells. Accordingly, we performed our experiments with two recombinant adenovirus vectors: 1) the −374 or −269 cTnTCre helper virus, containing Cre cDNA under control of the cardiac-specific −268 cTnT promoter, and 2) the main virus (CMV-loxPEGFP), containing EGFP cDNA under control of the CMV promoter (Fig. 1). In this latter virus, the CMV promoter and the EGFP reporter gene were separated by a neo-polyA "stuffer" sequence flanked by a pair of loxP sites, so that effective transcription would be permitted only after excision of the loxP sites in cells expressing Cre recombinase.

We found that infection with CMVloxPEGFP does not yield any EGFP expression in the absence of Cre recombinase. On the other hand, coinfection of cardiac myocytes with helper virus carrying Cre cDNA under control of a cardiac-specific promoter yields EGFP expression levels comparable to those obtained with CMV-EGFP virus without loxP stuffer (Fig. 4). EGFP expression is much lower in fibroblasts infected with the double virus system (Fig. 4), due to the very low activity of the cardiac-specific cTnT promoter (controlling Cre expression) in the fibroblasts. Comparatively low EGFP expression was also obtained after double infection of skeletal muscle and smooth muscle myocytes (results not shown), consistent with low activity of cTnTCre in these cells. We found, however, that significant EGFP expression is produced by the double infection in liver cells (Fig. 4). It should be pointed out that these infections were performed with the lowest cTnT Cre viral titer possible, i.e., 1 PFU per cell. This indicates that even though the cTnT promoter is minimally active in cells other than cardiac myocytes, it does nevertheless sustain sufficient (although very low) expression of Cre recombinase in liver cells.

Table 1. Expression of enhanced green fluorescent protein in various cell types under control of various promoters
(and to some extent fibroblasts) to catalyze cleavage of neo-polyA stuffer in the CMVloxPEGFP vector. Exogenous SERCA expression under control of constitutive and cell-specific promoters. Exogenous SERCA expression has been obtained in cardiac myocytes under control of viral promoters, reaching levels threefold higher than that of endogenous enzyme and resulting in acceleration of cytosolic Ca$^{2+}$ transients (4, 5, 12, 15). Considering the importance of obtaining exogenous SERCA expression specifically in cardiac myocytes, even when heterogeneous cell systems are infected, we then performed a series of experiments for comparative evaluation of cell-specific promoters. We used SERCA1 as the exogenous gene because it provides a convenient method to distinguish the exogenous gene product from the endogenous SERCA2 by means of specific antibodies. In addition, a greater functional effect can be obtained due to its higher turnover compared with SERCA2 (26, 33).

The expression levels shown in Fig. 5 were determined by Western blotting using antibodies specific for SERCA1 that do not react with the endogenous SERCA2. Therefore the levels shown reflect only expression of exogenous SERCA1 gene. These levels are significantly higher with the CMV promoter, within a range of viral titers yielding maximal expression. On the other hand, approximately one third of the expression level obtained under control of the CMV promoter can be obtained under control of the −374 cTnT promoter segment, at a viral titer of 5–10 PFU/cell. As demonstrated with the EGFP reporter gene (Figs. 3 and 4), we found that this cTnT promoter segment sustains negligible SERCA expression in cells other than cardiac myocytes, whereas the CMV promoter sustains constitutive protein expression in all cell types. Therefore, the −374 cTnT promoter segment offers the advantage of cell-specific and relatively high SERCA expression in cardiac myocytes.

Fig. 3. Cell-specific expression in a mixed population of cardiac fibroblasts and myocytes after infection with adenovirus vector carrying EGFP cDNA under control of the −374 cTnT promoter segment. Mixed populations of embryonic chicken cardiac fibroblasts and myocytes were infected at a multiplicity of infection (MOI) of 10 PFU −374 cTnT-EGFP virus per cell. Seventy-two hours after infection, the cells were immunostained with primary monoclonal antibodies for endogenous chick SERCA2 (CaS-3H2; Ref. 22), myosin (Developmental Studies Hybridoma Bank, MF-20), or α-actinin (Sigma, Clone EA-53) in the presence of saponin. The secondary antibody used was Texas red-conjugated goat antimeouse IgG (Molecular Probes). The cells were then observed by fluorescence microscopy using either a Zeiss axioskope or a Nikon Eclipse TE 200 equipped with a mercury lamp and the appropriate fluorescence filter attachments. Top: phase contrast images of the mixed cardiac fibroblasts and myocytes; middle: cells infected with EGFP; bottom: cells stained with 3 different antibodies specific for myocytes. A: endogenous SERCA2; B: alpha actinin; C: myosin. The figure demonstrates that exogenous EGFP under control of −374 cTnT promoter segment is expressed only in cardiac myocytes.
It should be noted that the chicken −374 cTnT promoter segment is equally effective in chicken and rat myocytes, whereas the −268 segment is efficient in chicken myocytes, but less so in rat myocytes (Table 1). The rat cTnT promoter appears to be equally effective in chicken and in rat myocytes but yields a somewhat lower expression (Table 1).

It is noteworthy that the SERCA expression shown in Fig. 5 represents asymptotic levels of expression. In fact, we found...
that under control of either the CMV or the −374 cTnT promoter segment, the level of exogenous SERCA increases for 2 days after infection and, by the third day, reaches asymptotic levels (Fig. 5, C and D) that differ depending on the promoter used.

Targeting of the exogenous SERCA gene product. The functional significance of exogenous gene expression is dependent on appropriate intracellular targeting. We have therefore examined myocytes expressing exogenous SERCA1 after in situ immunostaining with specific antibodies for exogenous SERCA1 and endogenous SERCA2, each followed by the appropriate secondary antibody. It is shown in Fig. 6 that intracellular targeting is identical for exogenous and endogenous SERCA. These observations reveal specificity of targeting and constitute a favorable premise for the functional contribution of these exogenous gene products to overall Ca\(^{2+}\) signaling.

Functional competence of the exogenous SERCA gene product. Evaluation of the relative contributions of exogenous SERCA1 (expressed under control of various promoters) to the total Ca\(^{2+}\) ATPase of the infected myocytes was obtained by direct measurements of ATPase activity of myocyte homogenates. It is shown in Fig. 7 that the maximal activity obtained in rat cardiac myocytes after exogenous gene expression represents a 2.0-, 5.0-, or 10.0-fold increase over the endogenous ATPase activity when exogenous expression was under control of the −268 cTnT segment, the −374 cTnT segment, or the CMV promoter, respectively. These ATPase measurements indicate that the exogenous gene product is functional and also reflect the higher turnover of the exogenous SERCA1 over the endogenous SERCA2 ATPase (33).

We have previously shown that SERCA overexpression is not accompanied by overexpression of phospholamban (4),
which is known to affect the Ca$^{2+}$ dependence of endogenous SERCA (27).

An advantage of SERCA overexpression under control of strong viral promoters is its influence on the kinetics of cytosolic Ca$^{2+}$ uptake by SR, thereby shortening the decay constant of cytosolic Ca$^{2+}$ transients after membrane excitation. It was then necessary in our experiments to find out whether the various levels of exogenous expression obtained with cell-specific promoters is sufficient to influence the Ca$^{2+}$ transients. It is shown in Fig. 8 that no significant effect is observed with the expression level obtained under control of the −268 promoter segment in neonatal rat cardiac myocytes, whereas a significant effect was obtained in chicken myocytes (19). On the other hand, a full effect is observed with the expression level obtained under control of the −374 cTnT promoter segment. In fact, the latter is approximately of the same magnitude as that observed with the expression level obtained under control of the CMV promoter, suggesting that no further acceleration of the transients is produced by excess levels of SERCA expression, likely due to limiting factors related to Ca$^{2+}$ dissociation from binding proteins and diffusion to the SERCA pump.

**DISCUSSION**

SERCA plays a major role in relaxation of skeletal and cardiac muscle, pumping cytosolic Ca$^{2+}$ into the sarcoplasmic reticulum to induce relaxation and providing stored Ca$^{2+}$ to trigger subsequent contractions (3, 10). In fact, modulation of endogenous SERCA may play a role in cardiac pathology (11, 18), and expression of exogenous SERCA in cardiac myocytes from failing heart improves contractile function (8). Therefore, expression of endogenous SERCA in cardiac myocytes is of interest in studies of its basic physiological role, as well as of its possible relevance to pathological states.

SERCA gene transfer by means of adenovirus vectors, under control of constitutive viral promoters, has been a prevalent and successful modality, although the effective viral titer is variable and depends on receptor density. On the other hand, should gene transfer be performed in heterogeneous cell system, such as whole organs or animals in vivo, restriction of exogenous gene expression to cardiac myocytes would be desirable (19). For this reason, we evaluated several cell-specific promoters, of suitable length for adenovirus vector construction, and compared their promoter activity and myocardial cell specificity with the constitutive CMV promoter. The cell-specific promoters evaluated in our experiments include the −250-bp segment (14) of the ventricle-specific cardiac MLC-2v promoter, the −215-bp segment (24) of the β-MHC promoter, the −268-, −374-, and −550-bp segments of the cTnT promoter (28), and the −303-bp segment of the rat cTnT promoter (36). As the exogenous SERCA gene, we used SERCA1 because this isoform has a higher turnover than the SERCA2 (33) and because its targeting to the sarcoplasmic reticulum of transgenic animals results in improvement of cardiac Ca$^{2+}$ handling capacity (26). In addition, the immunological specificity of SERCA1 and SERCA2 allows distinctive expression and targeting of endogenous and exogenous SERCA expression levels and targeting.

Our experiments indicate that although the constitutive CMV promoter yields higher expression levels, the cTnT promoter fragments can be used advantageously for cell-specific expression of exogenous SERCA in cardiac myocytes. We demonstrated the expression of exogenous SERCA1 in rat cardiac myocytes by immunohistochemistry in situ and Western blotting, determinations of enzyme activity, and effects on cytosolic Ca$^{2+}$ transients. We conclude that the −374 cTnT segment is particularly advantageous in neonatal rat cardiac myocytes.
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