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The Cytomegalovirus Enhancer Induces an Immediate Response to the Myosin Light Chain 2v Promoter during P19CL6 Cell Differentiation

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Abstract

The P19CL6 mouse embryonic carcinoma cells efficiently differentiate into cardiac muscle cells in the presence of DMSO. A reporter plasmid for cardiac muscle differentiation was constructed by connecting the CMV enhancer and a 250 bp MLC-2v promoter in front of the GFP gene to further evaluate the role of the CMV enhancer. This plasmid (pCBVenh/MLC-2v$_{pro}$/EGFP) was stably introduced into P19CL6 cells, and the transfectant differentiated into cardiomyocytes with DMSO. Upon DMSO addition, GFP was immediately transcribed (within 2 days) and the amount of the transcript increased with cultivation. Concomitantly, GFP fluorescence was detected in the cells under a microscope. However, native MLC-2v was transcribed later on day 4. This expression time course is different from that of GFP. Clearly the CMV enhancer responded immediately to DMSO. Since GATA DNA-binding proteins play crucial roles in the initiation of cardiomyocyte differentiation, such a response could be ascribed to the presence of multiple GATA motifs in the enhancer sequence but not in the native MLC-2v promoter. Thus the CMV enhancer may be not only useful for gene therapy and monitoring cell differentiation but also the study of the role of GATA transcription factors expressed in P19CL6 cells.

Keywords

Cytomegalovirus Enhancer, Differentiation, GATA Transcription Factor,

Gene Expression, Heart Muscle, MLC-2v, P19CL6 Cells, Promoter

1. Introduction

P19CL6 cells derived from P19 embryonic carcinoma cells can efficiently differentiate into cardiac muscle cells in the presence of 1% dimethyl sulfoxide (DMSO) [1]. Such a property is a good tool for the study of cardiac myocyte differentiation *in vitro*. Before transcriptional activation of the genes for cardiac contractile proteins during the differentiation of P19CL6 cells, the gene for the GATA-4 transcription factor becomes activated [2]. We have also been reported that the GATA-4 gene is immediately transcribed upon addition of DMSO through binding of GATA-6 to the upstream enhancer GATA motif, which is conserved in mammals [3]. Not only the GATA-4 but also the MEF2C and Tbx5 transcription factors play crucial roles in cardiac myocyte differentiation [4]. Furthermore, genetic analysis suggested that NKX2.5 and GATA-6 together with these three transcription factors are essential for cardiac development [5] [6].

As for contractile proteins, transcripts of myosin heavy chains (a-MHC and β-MHC) were detected in parallel in P19CL6 cells at a late stage after the start of cardiac differentiation [1]. Similar to these cardiac MHC isoforms, cardiac myosin light chain 2 (MLC-2v) is also transcribed during the course of differentiation [7]. When the 250 base pair (bp) promoter for the rat cardiac MLC-2v gene was connected upstream of the gene for green fluorescent protein (GFP) together with the enhancer portion of the cytomegalovirus (CMV) immediate early promoter, and the resulting reporter plasmid was stably introduced into P19CL6 cells, expression of GFP was limited to developing cardiac myocytes [8]. Although this system could be advantageous to quantify cardiac differentiation, it has not been addressed as to why the virus enhancer responds to such a differentiation signal. Actually, it has been demonstrated that the SV40 enhancer acted on both heart muscle and non-muscle cells [9].

In this study, we analyzed the MLC-2v promoter driven by the CMV enhancer in more details. Although the enhancer-promoter construct responded to the DMSO signal, the response was immediately before the start of transcription of native MLC-2v. We will discuss the role(s) of GATA motifs in the CMV enhancer sequence from the viewpoint of GATA-4 transcription in P19CL6 cells [3].

2. Materials and Methods

2.1. Construction of an Expression Plasmid for GFP under the Control of the MLC-2v Promoter.

pEGFP-N1 (Clonetech) was *Eco*RI-digested, and then its CMV enhancer moiety (nucleotide residue numbers 60 - 465, GenBank Accession No. U55762) was amplified by means of polymerase chain reaction (PCR) [10] with a primer pair,

CMVenh-F/CMVenh-R, and Pyrobest DNA polymerase (TaKaRa) [preheating (94˚C, 5min), followed by 30 cycles of denaturation (94˚C, 15 sec), annealing (55˚C, 30 sec), and extension (72˚C, 30 sec), and then post-heating (72˚C, 5 min)]. The product was cloned into the *Sma*I site of pBluescript II SK(+) (Stratagene). To amplify the MLC-2v promoter [11], rat liver genomic DNA [12] was digested with *Eco*RI and then subjected to PCR with a primer pair, MLC-2v$_{pro}$-F/MLC-2v$_{pro}$-R, and LA Taq DNA polymerase (TaKaRa) [preheating (94˚C, 5 min), followed by 30 cycles of denaturation (94˚C, 15 sec), annealing (52˚C, 30 sec), and extension (72˚C, 30 sec), and then post-heating (72˚C, 5 min)]. The product was treated with T4 DNA polymerase (Toyobo), and then cloned into the *Sma*I site of pBluescript II SK(+). The 406 bp *Ase*I-*Hin*dIII fragment of the CMV enhancer and the 262 bp *Hin*dIII–*Bam*HI fragment of the MLC-2v promoter were inserted into a large fragment of pEGFP-N1 (4079 bp) digested with *Ase*I and *Bam*HI. The resulting plasmid was named pCMVenh/MLC-2v$_{pro}$/GFP (4759 bp), and confirmed by the production of a 406 bp *Hin*dIII-*Ase*I fragment, and a 1008 bp *Not*I and *Hin*dIII fragment (**Figure 1**). The entire nucleotide sequence of pCMVenh/MLC-2v$_{pro}$/GFP was shown in **Figure S1** (Supplementary).

The cloned DNA was sequenced by the dideoxy chain-termination method [13] with a primer (M13 forward or M13 reverse) and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The oligonucleotides used for PCR and sequencing are listed in **Table 1**. The molecular biological techniques were performed by published methods [14].

2.2. Cell Culture

P19CL6 cells [3] were cultured in *α*-Eagle's minimal essential medium (GIBCO

Table 1. Sequences of oligonucleotides used in this study.

	*Ase*I
CMVenh-F	5'-TT**A TTA AT**G CGT TAC ATA ACT TAC GGT AAA-3'
	*Hin*dIII
CMVenh-R	5'-TT**A AGC TT**C AAA ACA AAC TCC CAT TGA CG-3'
	*Hin*dIII
MLC-2v$_{pro}$-F	5'-TT**A AGC TT**G ACC CAG AGC ACA GAG CAT-3'
	*Bam*HI
MLC-2v$_{pro}$-R	5'-TT**G GAT CC**A AGG AGC CTG CTG GCC GG-3'
MLC-2v-S	5'-GCC AAG AAG CGG ATA GAA GG-3'
MLC-2v-A	5'-CTG TGG TTC AGG GCT CAG TC-3'
GFP-F	5'-TGC AGT GCT TCA GCC GCT A-3'
GFP-R	5'-TTG TAC AGC TCG TCC ATG CC-3'
YSactin-S	5'-GCA GGA GAT GGC CAC TGC CGC-3'
YSactin-A	5'-TCT CCT TCT GCA TCC TGT CAG C-3'
--	
Sequence Primer	
M13-F	5'-CGC CAG GGT TTT CCC AGT CAC GAC-3'
M13-R	5'-GAG CGG ATA ACA ATT TCA CAC AGG-3'

Restriction enzyme sites are underlined with bold letters.
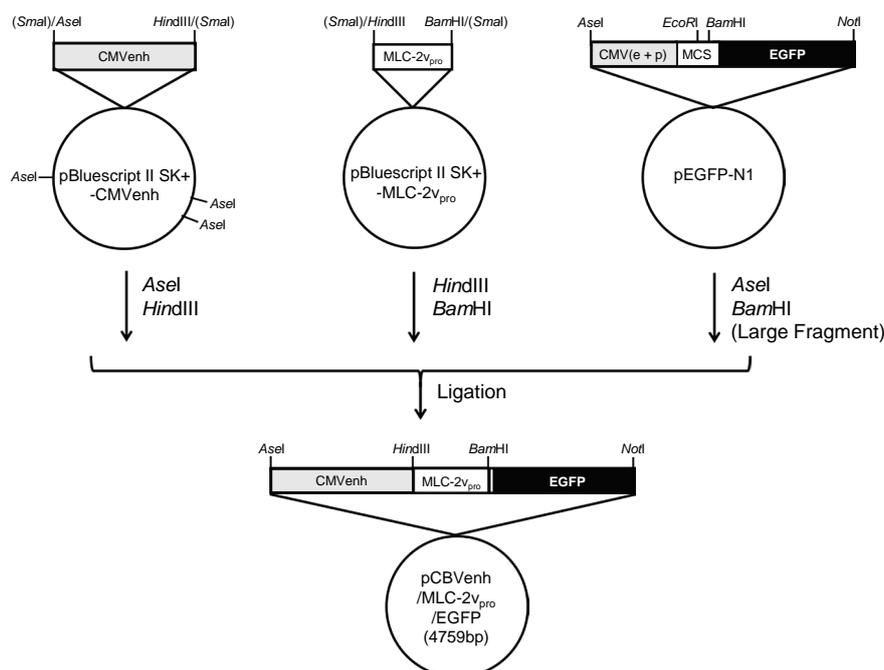
Figure 1. Construction of an expression plasmid for GFP with the CMV enhancer and MLC-2v promoter. The CMV enhancer moiety (CMVenh, 406 bp) derived from pEGFP-N1 and the MLC-2v promoter (MLC-2v$_{pro}$, 262 bp) cloned from rat liver genomic DNA were inserted into the pEGFP-N1 vector from which the human cytomegalovirus (CMV) immediate early enhancer and promoter sequences [CMV(e + p)] corresponding to nucleotide residue numbers 1 - 589 (GenBank Accession No. U55762) had been deleted. Typical restriction enzyme sites used in this study (*Ase*I, *Bam*HI, *Eco*RI, *Hin*dIII, *Not*I and *Sma*I) were indicated in the figure. MCS, multi-cloning site.

BRL) supplemented with 10% (v/v) fetal bovine serum (GIBCO BRL) and antibiotics (100 μg/mL streptomycin sulfate and 100 u/mL benzyl-penicillin) (Wako). An expression plasmid was introduced into the cells (10^5 cells per 6 cm diameter dish) by means of the calcium-phosphate method as described previously [15]. Cells were split into 10 cm diameter dishes after 48 hrs incubation, and then grown in the presence of 1 mg/mL G418 (Nacalai). Among six resistant colonies, one (clone B4) that showed strong green fluorescence in the presence of 1% (v/v) DMSO was analyzed.

The B4 clone (10^4 cells) was seeded into a 6 cm diameter dish. Cells were grown in the medium plus 1% (v/v) DMSO. The medium was changed to fresh medium containing DMSO on the fourth day, and then every two days. The expression of GFP was monitored under a microscope (Olympus IX-70) equipped with an AQUACOSMOS U7501 (HAMAMATSU PHOTONICS).

2.3. Determination of the Expression Levels of mRNAs

Cells (10^4 cells) were seeded into a 6 cm diameter dish. Total RNA was extracted with Isogen (Nippon Gene), and an aliquot (5 μg) was reverse transcribed with M-MLV reverse transcriptase (TaKaRa) and an oligo (dT)$_{15}$ primer. After RNaseH (TaKaRa) treatment, cDNA was subjected to semi-quantitative PCR with

Go Taq® (Promega) and a primer pair, MLC-2v-S/MLC-2v-A, GFP-F/GFP-R or YSactin-S/YSactin-A (Table 1): the PCR conditions comprised preheating (94˚C, 3 min), followed by denaturation (94˚C, 0.5 min), annealing [(60˚C for MLC-2v and GFP, and 55˚C for β-actin), 0.5 min] and extension (72˚C, 0.5 min), and then post-incubation (72˚C, 5 min). The PCR products were size-separated on an agarose gel and DNA bands were visualized with ethidium bromide. Images were recorded with a FAS-III UV-imaging system (Toyobo).

2.4. Chemicals

Restriction enzymes were obtained from NEB and Toyobo. Agarose LO3 and a DNA ligation kit ver. 2.0 were purchased from TaKaRa. A GENECLEAN® III KIT and oligonucleotides were provided by MP Biomedicals and Gene Design Inc., respectively. All other chemicals used were of the highest grade commercially available.

3. Results

3.1. Expression of GFP in the Stable Transfectant Cells

We constructed an expression plasmid for GFP under the control of the CMV enhancer and MLC-2v promoter (Figure 1). This expression plasmid was stably introduced into P19CL6 cells and a G418-resistant clone carrying the reporter gene was isolated. The clone was cultured in the presence and absence of 1% DMSO, and green fluorescence derived from GFP was monitored under a microscope. As shown in Figure 2, weak fluorescence was detected on day 4 in the presence of DMSO. The fluorescence increased gradually and was much stronger on day 12 under the differentiation conditions. However, without DMSO only a background level of fluorescence was detected on day 12. Previous study also showed that very little but detectable amounts of GFP was expressed in undifferentiated cells [8].
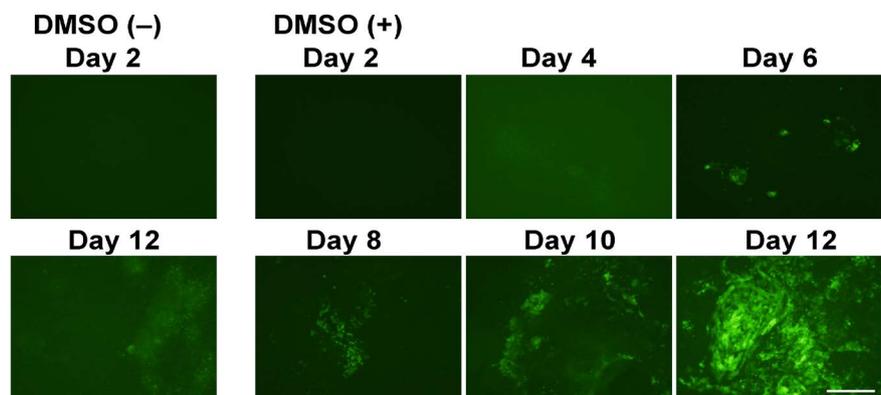
Figure 2. Expression of GFP in the stable transfectant clone derived from P19CL6 cells. The expression plasmid (pCBVenh/MLC-2v$_{pro}$/EGFP) constructed as in Figure 1 was stably introduced into P19CL6 cells. The G-418 resistant clone was cultured in the presence [DMSO (+)] or absence [DMSO (−)] of 1% (v/v) DMSO. Fluorescence images on the indicated days after the start of the experiment are shown. Bar, 200 μm.

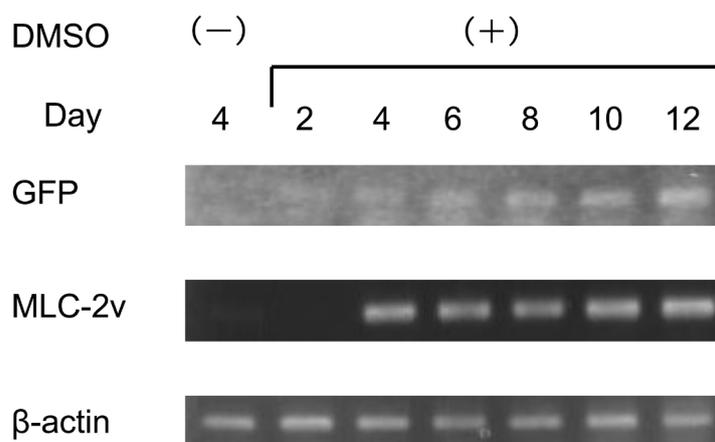To determine the start of transcription of the GFP gene, total RNA was extracted and subjected to semi-quantitative RT-PCR. The results of the cDNA amplification indicated that the gene for GFP had already been transcribed on day 2 after the addition of DMSO, whereas it was not detected in the absence of DMSO (compare the results on days 2(+) and 4 (+) with those of day 4(-), Figure 3, upper panel). Furthermore, the amount of the transcript of GFP clearly increased up to day 12(+). Although we did not determine the GFP protein by means of Western blotting, the transcript of GFP was translated as shown in Figure 2.

3.2. Comparison of the Expression of Native MLC-2v and GFP

The expression pattern of GFP was compared with that of native MLC-2v. As shown in Figure 3 (middle panel), the start of transcription of the native MLC-2v was clearly retarded in contrast to the appearance of the transcript of GFP. The transcript of MLC-2v appeared abruptly on day 4, and its amount was maintained constantly. Since the rat and mouse MLC-2v prompter sequences are essentially the same (Figure 4(a)), it is suggested that the immediate transcription of the GFP reporter gene could not be ascribed to the MLC-2v prompter moiety but rather to the CMV enhancer.

3.3. Presence of GATA Motifs in the CMV Enhancer

Since the CMV enhancer started to operate immediately after DMSO addition (Figure 3), it seems likely that the potential master regulator(s) that directs P19CL6 cells toward cardiomyocytes may instantaneously bind to and activate the enhancer in the presence of DMSO. Our previous study demonstrated that the binding of pre-existing GATA-6 to an upstream GATA motif is essential for the immediate activation of the GATA-4 gene upon DMSO addition to P19CL6

Figure 3. Detection of transcripts of GFP and native MLC-2v. Total RNA was prepared on the indicated days after DMSO addition, and then subjected to RT-PCR to detect transcripts of GFP and native MLC-2v. *β*-actin mRNA was used as a positive control. Amplified fragments were analyzed by 2 % (w/v) agarose gel-electrophoresis.

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 60  GCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTAT  139

140  GTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCC CACTTGGCAGT  219

220  ACATCAAGTG TATC ATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGT  299

300  ACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCA TGGTGA TGCGGTTTTGG  379

380  CAGTACATCAATGGGCGTG GATA GCGGTTTGACTCACGGG GATT TCCAAGTCT CCACCC CATTGACGTCAATGGGAGTTT  459

460  GTTTTG                                                                          465
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(a)


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                                                    HF-3
Human   -225  ccgcagagagcgcgcagcatctctccccgtgaccatga-cccagctactgcctctttaaccttgaatgccttttttggggg  -147
Rat     -251  ggacccagagcacagagcatc-gttcccagg-ccaggc-cccagccactgtctctttaaccttgaaggcatttttgggt-  -176
Mouse   -259  gggaagagagcacaccccatca-t-cccaggccaggc-cccagccactgactctttaaccttgaaggcatttttgggt-  -184
Chicken -238  tctgcccactcga-gtgtagctcgtgggagctgcagggcctcctggtgtg-ct-gctaaccttgaaagcctctgt--gt-  -282
                   *        *         ** *  * ** **  ********* ** * * *

               E-box                                        HF-2
Human   -146  ct cacgtg t-cacccagtggcgagt--------gag ccaccc ttacttcagaagaacggcatg-------- gggtgg gg-  -85
Rat     -175  ct cacgtg tccaccc aggcgggtgtcggactttgaacggctcttacttcagaagaacggcatg------- gggtgg gg-  -105
Mouse   -183  ct cacgtg tccaccc aggcgggtggccgcctttgagcggctcttacttcagaagaacggcatggagtgg-- gggtggg -  -107
Chicken -164  ctcgcatgt-cacgtcggccagtgac-aac----agcaag-cttactgcagattagaggaggagaaagtcagagacaaga  -92
              *** * *** ***    *     * *       * *   ****** ****  *  **          * *      *

                                                    HF-1
Human    -84  gggcctt aggtgg tgcccgcc tcacct atgactgccaaaagcggtcatgggggttattttaaacatggggaggaagtatt  -5
Rat     -104  gggc-tt aggtgg cctctgcc tcacct acaactgccaaaagtggtcatgggggttatttttaacccagggaggaggtatt  -26
Mouse   -106  gggc-tt aggtgg cctccgcc tcacct acaactgccaaaagtggtcatgggggttatttttaacccagggagaggtatt  -28
Chicken  -91  ggg---tcggggtggctggtttacctattacagccaaaagtggacatgggggttattttagcctggaatggggtgtatt  -15
              ***    * ** **   * *  * **   ** ********** *************** *        *     * *****

Human     -4  tatt gttcctgggctgcagag--agctgggcggagtgtggaattcttctcgggaggcagtgctgggtcctttccacc ATG  +74
Rat      -25  tatt gttccacagcaggggcc--ggcagcaggctccttgaattcttc---agaggcagcagccagcctcagacacc ATG  +50
Mouse    -27  tatt gttccacagcaggggcagagagcagcaggctcctcgaactctcc---agaggtggcaactggcctcagacacc ATG  +50
Chicken  -14  tatt gtttcagcccagggaggcagagggactgcctctgagatccctcctgcagcaccaccagcacctctgcgaagac ATG  +66
              ********   * **             * *       *     ** *    *          *           ****
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(b)

Figure 4. GATA motifs in the CMV enhancer, and comparison of vertebrate MLC-2v promoter sequences. (a) The CMV enhancer sequence (nucleotide residues 60 - 465) in pEGFP-N1 (GenBank Accession No. U55762) is shown. The positions of GAT(A/T) motifs are boxed. Similar sequences of the canonical NKX2.5 and Tbx5 binding sites [23] [24] (5'-TYAAGTG-3' and 5'-(A/G) GGTGT-3', respectively) are double- and single-underlined, respectively; (b) Upstream sequences of the genes for human, rat and mouse MLC-2v [NCBI Reference Sequences NG_007554 and NM_001035252, and GenBank Accession No. AF326769, respectively], and chicken MLC$_{2A}$ [29] were aligned. The transcribed sequences are shown in italics. Initiation codons are indicated by capital letters. Vertical arrowheads indicate the transcriptional start sites. Residues conserved in all four vertebrates are denoted by asterisks. Two arrows indicate the sequence primers for amplifying the rat promoter sequence (262 bp) between nucleotide residues -250 and +12. Thick overlines labeled HF-1-HF-3 are conserved regions that were characterized previously [27]. A typical E-box sequence (CANNTG) [27] and closely related Tbx5 binding sites [5'-(A/G)GGTGT-3'] [24] are boxed.

cells [3]. Consistent with this observation, the CMV enhancer has multiple GATA-6 binding motifs, **5'-GAT(A/T)-3'** [16]; two GATA and one GATT sequence are present in the downstream half of the 406 bp enhancer sequence (**Figure 4(a)**). However, such a motif could not be found in the 262 bp MLC-2v promoter sequence (**Figure 4(b)**). Since GATA-6 could activate reporter genes through GAT(A/T) sequences [17] [18], GATA-6 may bind to the CMV enhance in response to a cardiac differentiation signal.

4. Discussion

P19CL6 cells are used as a model of differentiation of cardiac myoblasts into myocytes [1] [8]. In this study, we found that the MLC-2v promoter harboring the CMV enhancer responded immediately in P19CL6 cells upon the addition of DMSO, which is an induction reagent for cardiac differentiation [1]. However, the intrinsic MLC-2v promoter was activated rather later during the differentiation (Figure 3). Thus, the transcription of GFP under the control of the CMV enhancer is not a result of cardiac myocyte differentiation, but rather parallel to activation of the start of differentiation. It should be noted that such comparison between native and hybrid MLC-2v promoters focused on the CMV enhancer has not been carried out previously [8].

As for GATA-4 gene expression, binding of GATA-6 to the distal enhancer GATA motif was obligatory for transcriptional activation under the differentiation conditions for P19CL6 cells [3]. Since GATA-4 and GATA-6 function in transcription through a GAT(A/T) motif [17] [18], it seems likely that the CMV enhancer with GATA motifs (Figure 4(b)) immediately responds to pre-existing GATA-6 in P19CL6 cells on DMSO treatment. Actually, the CMV enhancer induces efficient cell-type specific expression of genes such as those of atrial natriuretic factor and MLC-2v in cardiomyocyte cell line HL-1 [19], which expresses GATA-4 and GATA-6 [20]. Consistent with this finding, the SV40 enhancer composed of the 72 bp tandem repeat [21] without GATA motifs (NCBI Reference Sequence NC_001669) abolished the cardiac specificity [9].

The gradual increases in the amounts of GFP transcripts shown in Figure 3 could be explained by the participation of the pre-existing GATA-6, and increased amounts of GATA-4 and GATA-5 arising through transcription and translation [3]. It is also suggested that factors like GATA-4 that potentiate transcription during development are inherently capable of initiating chromatin opening through binding to an enhancer [22]. Thus, the CMV enhancer with GATA motifs may become open and activated in an environment in which GATA factors are expressed although modulation and/or a trigger such as DNA methylation and protein modification could be needed by DMSO.

In the CMV enhancer (Figure 4(a)), there are sequence motifs similar to the canonical binding sites for NKX2.5 and Tbx5, **5'-TYAAGTG-3'** and **5'-(A/G) GGTGT-3'**, respectively [23] [24]. Since these transcription factors also participate in cardiac development [25] and interact with GATA-4 specifically [5] [26], they may facilitate the role of GATA-4 in cardiac differentiation. Although three *cis*-elements (HF-1-HF-3) located in the MLC-2v promoter were analyzed (Figure 4(b)) [27], the first intronic region further participates in the up-regulation of the MLC-2v gene in cardiac hypertrophy [28]. Such an additional regulatory element may explain the different responses of native and hybrid MLC-2v promoters to DMSO (Figure 3).

Although roles of GATA factors expressed in P19CL6 cells and GATA motifs in the CMV enhancer should be further examined in detail, the specific and in-

ducible gene expression by means of the CMV enhancer in the cardiac environment may be useful for gene therapy as well as the tracing of differentiated cells [19].

Acknowledgements

Conflict of Interest

The authors have no conflict of interest.

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Supplementary

Figure S1. Nucleotide sequence of pCBVenh/MLC-2v_{pro}/EGFP.

Nucleotide sequence of pCBVenh/MLC-2v$_{pro}$/EGFP (4759 bp) was shown. Orange, blue, green and black letters indicate the sequences for CMV enhancer, MLC-2v promoter, a coding region of GFP and pEGFP-N1, respectively. Restriction enzyme sites for *Ase*I, *Hin*dIII, *Bam*HI and *Not*I were shown in red bold letters (ordered from 5' side).

tagtt**ATTAAT**GCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAAC
GACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGA
CTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACA
TCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCC
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AGTTTGTTTTG**AAGCTT**GACCCAGAGCACAGAGCATCGTTCCCAGGCCAGGCCCCAGC
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TCGGACTTTGAACGGCTCTTACTTCAGAAGAACGGCATGGGGTGGGGGGGCTTAGGTG
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AAGAGGTATTTATTGTTCCACAGCAGGGGCCGGCCAGCAGGCTCCTT**GGATCC**accgg
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