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Construction and Identification of Transgenic Mice Carrying Human Cytomegalovirus IE2 Gene

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Abstract: The infection of human cytomegalovirus causes several diseases of congenitally infected neonates and immunocompromised populations. The immediate–early 2 gene of human cytomegalovirus plays an essential role in viral replication and the pathogenic process. Due to the strict species-specific of HCMV infection, any animals besides human beings cannot be infected with HCMV. For this reason, few animal model which is used to explore the effect of the *IE2* gene on the mRNA or protein level has been constructed to. In this study, the cDNA of *IE2* mRNA (1,743 bp) was cloned into the mammalian expression vector pAV.Des1d then identified by polymerase chain reaction and sequencing analysis. Next, the expressed vector was transferred into mouse fertilized eggs from C57BL/6 mice by pronuclear microinjection to obtain the first generation of transgenic mice. In the 59 F0 generation mice, there are only 3 offspring mice were identified as *IE2* positive mice. Furthermore, the positive ratios detected by PCR from F0 to F3 were 53.8%, 43.1%, 62.1%, respectively. The expression of exogenous *IE2* mRNA was detected in kidney, heart, muscle, brain, spleen, and adipose tissue of F1 transgenic mice. These results suggest that we have successfully constructed a stable transgenic mouse line that can be used as a tool to explore the specific effects of *IE2* gene on the growth and development of animals as well as mechanisms of its influence.

Keywords: Human Cytomegalovirus; Immediate Early 2 Gene; Transgenic Mice

INTRODUCTION

Human cytomegalovirus (HCMV), a member of the beta herpes virus family, is a major human pathogen. It infects the majority of the humanity and following primary infection, it can persist as a life-long infection(Griffiths, 2012). Although healthy individuals infected with HCMV are generally asymptomatic, HCMV can lead to severe diseases in immunocompromised individuals such as organ graft recipients and AIDS patients. Moreover, HCMV infection is also a significant cause of infection-related congenital defects and abortions (Griffiths, 2012; Manicklal, Emery, Lazzarotto, Boppana, & Gupta, 2013; Ross & Boppana, 2005). Emerging results have proved that HCMV infection possibly associated with cardiovascular diseases and proliferative diseases such as glioma(Huang et al., 2015; Khan et al., 2014; Soroceanu et al., 2015; X. Wang et al., 2017). Nowadays there is no valuable vaccine to HCMV. And antiviral therapies are limited by low availability, poor toxicity scores, and emergence of resistant virus(Biron, 2006; D. Wang & Fu, 2014). Understanding the role of viral genes in lytic infection is paramount and will yield novel targets for antiviral therapies.

HCMV is a dsDNA virus, which contains ~240kb sequences and 165-250 open reading frames (ORFs)(Davison et al., 2003; Murphy, Rigoutsos, Shibuya, & Shenk, 2003). Replication of HCMV is regulated by a cascade-like manner, which can be divided into immediate-early (IE), early (E), and late (L) phases. *IE1 and IE2* genes shares the same promoter, MIEP.(Stenberg, 1996; Stenberg, Thomsen, & Stinski, 1984; Stenberg, Witte, & Stinski, 1985). The *IE2* gene, encodes the essential multifunctional IE2 protein (IE86) which could provide a suitable cellular environment for viral replication during lytic infection. The IE86 protein of human cytomegalovirus is unique among viral and cellular proteins because it negatively auto-regulates its expression, such as activating the viral promoters and activating or inhibiting cellular promoters(Harris et al., 2010; Marchini, Liu, & Zhu, 2001; Reeves et al., 2006; Stinski & Petrik, 2008). Although the regulation of *IE2* gene expression and the

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function of IE86 have been extensively studied, most of the studies are based on cell-level studies. But the mechanisms how is *IE2* gene regulated is poor-known, especially at the animal's overall level.

Due to the highly species-specific of HCMV, the study of HCMV biology is limited to models in vitro(Kim & Carp, 1972; Lafemina & Hayward, 1988; Tang & Maul, 2006). There are many studies about animal CMVs that coevolved with the animal species. For example infection of rhesus macaques with rhesus CMV (RhCMV) represents an animal model that closely resembles infection of humans with HCMV(Malouli et al., 2014; Yue & Barry, 2008). The mouse homolog of HCMV is the mouse cytomegalovirus (MCMV), and it provides a widely used tool to study the CMV biology in vivo (Reddehase, Podlech, & Grzimek, 2002). However, the study of the function of individual HCMV homologous genes does not fully reflect the molecular interaction of orthologous HCMV-encoded gene products. Therefore, in this study transgenic mice expressing the *IE2* gene product were produced by microinjection with the recombinant adenovirus vector (pAV.ExBi-CMV-IE2-IRES-eGFP). Our results indicate that the *IE2* gene can be expressed stably in transgenic mice.

MATERIALS AND METHODS

Construction of pAV.ExBi-CMV-IE2-IRES-eGFP eukaryotic expression vector

The pEGFP-N3-IE2 plasmid which contains the cDNA of HCMV *IE2* mRNA (1,743bp) was constructed by our lab before. The pAV.Des1d was bought from Cyagen Biosciences Inc. (Guangzhou, China). The pAV.Des1d and pEGFP-N3-IE2 plasmids were first digested with *BamHI* and *HindIII* (Takara, Dalian, China), respectively, then the IE2-eGFP fragment was inserted into the pAV.Des1d to construct the pAV.ExBi-CMV-IE2-IRES-eGFP plasmid (Figure 1A). Then plasmid target fragment was confirmed by PCR using pAV.ExBi-CMV-IE2-IRES-eGFP vector as template and primer pair F: CAAGTTTGTACAAAAAAGCAGGCT and R: CACTTTGTACAAGAAAGCTGG. Clone 6 was picked and further sequenced to demonstrate that the sequence was correct and complete.

Generation of *IE2* transgenic mice

The pAV.ExBi-CMV-IE2-IRES-eGFP cassette was digested with *Nhe I* (Takara, Dalian, China) and purified by gel extraction (Qiagen, USA). The purified linear expression vectors were then microinjected into the pronuclear of 100 fertilized C57 mice eggs, which were implanted into pseudopregnant C57 females to obtain the founder mice (F0). All the founder mice were housed at the transgenic mouse facility of Cyagen Biosciences Inc. (Guangzhou, China).

PCR amplification of the genomic DNA from transgenic mice

Genomic DNA of tails was isolated from two weeks old transgenic mice using CWBIO Universal Genomic DNA Kit (CWBIO, China). The concentration of genomic DNA was measured in a UV–VIS spectrophotometer (Shanghai Precision Instrument Inc., China) at 260 nm. 400 ng of each sample was used as template in PCR reactions of with primer pair F: GGAGATACCAAAGCACCGTCAA and R: CATAGGTCACCAGCTCAGCACA, respectively, such that amplification would yield a PCR product of 335 bp. The internal control PCR that targets the endogenous mouse Actb (beta-actin) and Rgs7(G protein signaling 7) locus was identified by PCR with primer pair F: TCTTAGCTCTGCTCTCCGGT and R: CACTGGCTG AGGAAGGAGAC, and the PCR product was 632 bp. A positive control (plasmid pAV.ExBi-CMV-IE2-IRES-eGFP) and a wild type control (DNA from non-transformed mice) were included with each experiment. Analysis of PCR product was carried out by loading 5 µl of each sample onto a 1.2% agarose gel containing ethidium bromide, followed by electrophoresis and visualization via ultraviolet transillumination. A molecular weight standard was included with each experiment.

RNA extraction and RT-PCR

RNA was extracted from each tissue of three F1 transgenic mice using CWBIO RNApure Tissue Cell Kit (*DNase I*) (CWBIO, China) according to the manufacturer's instructions. And 1 µg RNA was reverse-transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) to produce cDNAs according to the manufacturer's instructions. Primers *IE2*-F: CCGCAAGAAGAAGAGCAAACG and *IE2*-R: CACCTGGTGCATACTGGGAAT were used to amplify the *IE2* gene with 217 bp product. And primers *actin*-F: AGATCGTGCGGGACATCAAG and *actin*-R: GCGGCAGTGGCCATCTC were used as a reference gene with 93 bp product. Semi-quantitative PCR were used for the relative expression of *IE2* gene. The band intensity was obtained using Image J software. Statistical analyses of relative band intensity was performed using GraphPad Prism 5. The p values were determined using one-way ANOVA test; $p < 0.01$ was considered significant.

RESULTS

Identification of pAV.ExBi-CMV-IE2-IRES-eGFP eukaryotic expression vector

The pAV.ExBi-CMV-IE2-IRES-eGFP eukaryotic expression vector was constructed. The CMV promoter was inserted into the 5′UTR of the *IE2* gene, and the *eGFP* gene was inserted into the 3′UTR (Figure 1A). The inserted sequence (3,000 bp) which contains *IE2* and *eGFP* fragments was verified by PCR (Figure 1B). Clone 6 was picked for further sequencing (the data not shown). PCR bands have proved that vector was correct. The sequence of inserted *IE2* fragment was shown in figure 1C.

Generation and detection of transgenic mice

The vector containing *IE2* gene was microinjected into

the male pronucleus of 200 fertilized oocytes of C57BL/6 mice. The injected eggs were implanted into the oviducts of 24 pseudopregnant foster mothers, of which 11 mice became pregnant and gave birth to 59 offspring. Three offspring carried the *IE2* gene detected by PCR analysis (Figure 2). We numbered them NO.1 (femal), NO.2 (male) and NO.3(male). The *IE2* positive ratio of transgenic mice was 5.1% (Table 1). Each positive F0 mouse needs to be propagated as an independent lineage. F0 generation mice were mated with wild type mice to obtain F1 mice. In order to get more transgenic mice, F1 generation mice mated with wild-type mice obtaining F2. Similarly, *IE2* positive mice and wild-type mice gave birth to F3 generation. The number of F1, F2 and F3 were 39, 51 and 29 respectively. The *IE2* positive ratios were 53.8%, 43.1% and 62.1%, respectively. Moreover, PCR analysis also proved that the mice carried *IE2* gene (data not shown).

Analysis of target gene expression in in transgenic mice

To further characterize the inserted gene, organs of F1 transgenic mice (three positive mice, random selection) were separated to analyze *IE2* expression by the semi-quantitative PCR. The results of semi-quantitative PCR showed that *IE2* mRNA are expressed in kidney, heart, muscle, brain, spleen, and adipose tissue (Figure 3 A). The relative band intensity in different tissue is significantly different, $p<0.01$ (Figure 3 B). Together these data demonstrated that the transgenic mice were successfully constructed.

Discussion

Constructing of a recombinant expression plasmid contained a cDNA sequence with an exogenous promoter binding fragment is one of the ways to establish a transgenic mouse(Brenin, Talamonti, & Iannaccone, 1997). In this study, we cloned the cDNA of *IE2* gene which encoded IE86 protein and promoted by the CMV promoter was extensively expressed in transgenic mice (Figure 1). The *IE2* mRNA was detected in several tissues such as brain, heart, muscle, spleen, kidney and adipose (Figure 3). PCR were used to identify positive transgenic mice. Figure 2 showed that the primers were well designed. As shown in Table 1, the positive rate was increased by the nest-positive mice mating. In the next course of the mice's reproduction, the homozygous positive mice will be screened. Of course, the premise is that homozygous transgene does not cause death of mice.

A number of studies have shown that the transcriptional responding of immune-related cytokines are required for HCMV gene expression. HCMV immediately early 2 gene product (IE86) can effectively block the expression of proinflammatory chemokines and cytokines during HCMV infection. It demonstrates that IE86 attenuates the expression of a number of chemokines, such as RANTES (regulated upon activation normal T cell expressed and secreted), MIG (monokine induced by interferon-γ), monocyte chemotactic protein-1 and -2 (MCP-1 and -2), macrophage inflammatory protein-1 alpha (MIP-1α), and interleukin-8 (IL-8)(Taylor & Bresnahan, 2006b). Moreover, IE86 is also identified as an IFN-β antagonist. The study have shown that IE86 could effectively block the induction of IFN-β by inhibiting virus-induced binding of NFκB to the *IFN-β* promoter. Not only HCMV infection could block the effect of *IFN-β* but also Sendai virus infection could(Taylor & Bresnahan, 2005, 2006a). Previous studies have also demonstrated that IE86 regulates the expression of *IL-1β*, *IL-2*, *IL-6*(Gealy, Humphreys, Dickinson, Stinski, & Caswell, 2007; Geist & Dai, 2000; Geist, Monick, Stinski, & Hunninghake, 1991, 1992; Listman, Race, Walker-Kopp, Unlu, & Auron, 2008). In our study, the results have shown that IE86 is extensive expression in transgenic mice. But whether it will affect immune microenvironment and the resistance of transgenic mice to disease, we need further exploration.

In conclusion, we successfully generated a mouse model expressing *IE2* gene by pronuclear microinjection. This model will provide basis for exploring the influence of *IE2* expression on *IE2*-effected signaling pathway and animal development. We also could explore toxicity experiments on the transgenic model. In the following study, we could then foster a larger number of transgenic mice and focus on connection between *IE2* expression and immune microenvironment.

Table and Figures

Table1 Characteristics of the mice in different generations

	Number of male mice	Number of female mice	Number of offspring	Number of positive offspring	Positive rate (%)
F0	28	31	59	3	5.1
F1	16	11	39	21	53.8
F2	32	19	51	22	43.1
F3	13	16	29	18	62.1

Figure 1

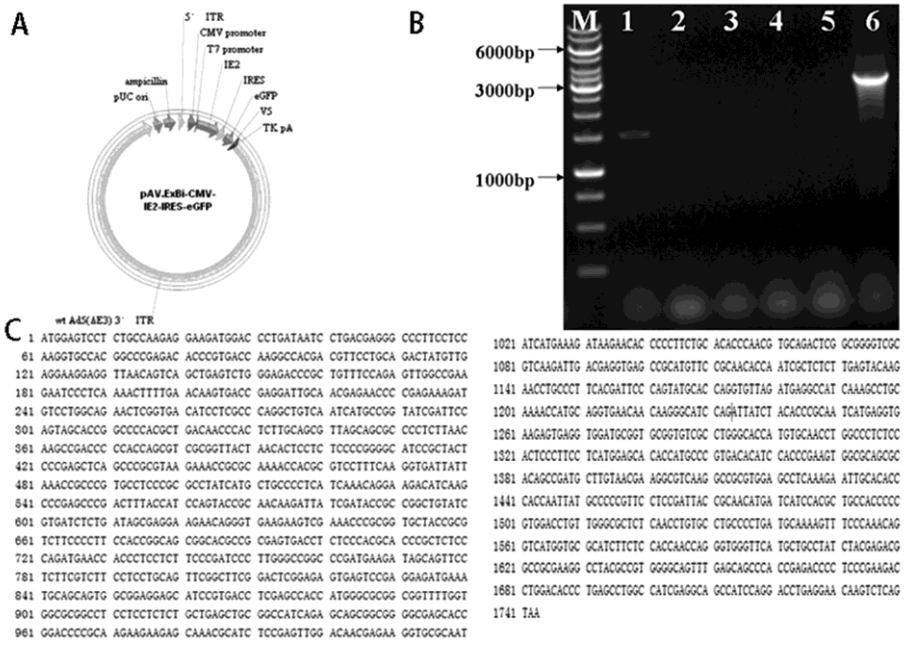

Figure 1 A. vector map of pAV.ExBi-CMV-IE2-IRES-eGFP; B. PCR results for *IE2* gene in pAV.ExBi-CMV-IE2-IRES-eGFP vector. The length of target fragment is 3000 bp. M: 1 kb marker; Lane1- Lane6: pAV.ExBi-CMV-IE2-IRES-eGFP clones from number 1 to number 6; C. the sequence of inserted *IE2* fragment.

Figure 2

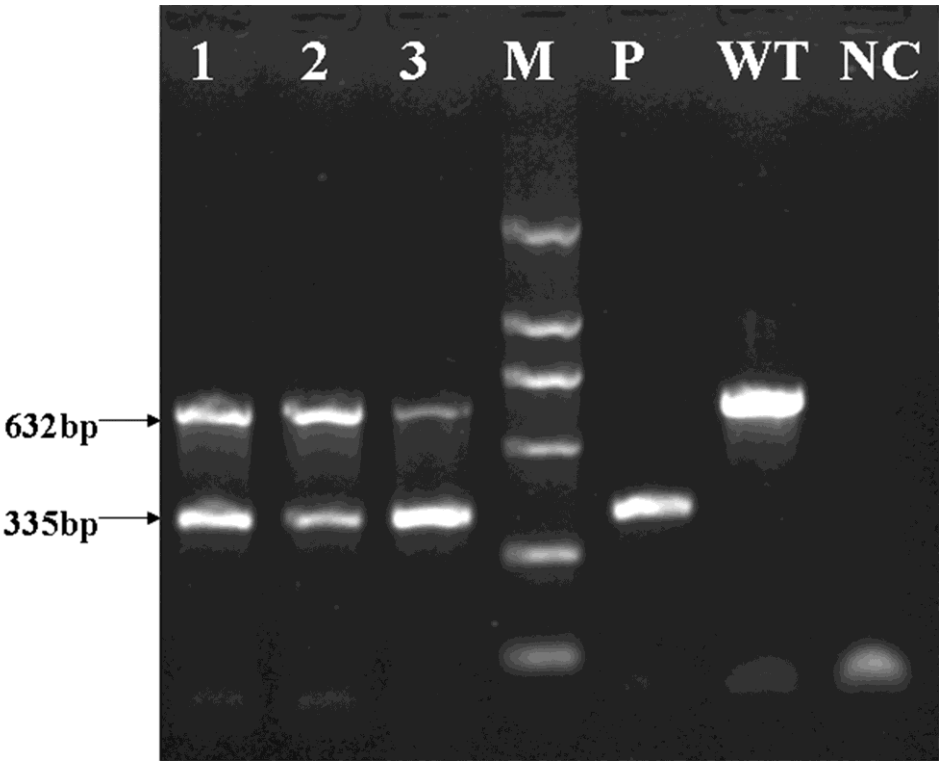

Figure 2 Three positive transgenic mice of founder mice were identified by PCR. The positive fragment is 335 bp and the internal control fragment is 632 bp. Lane 1-3: the three positive mice NO.1, NO.2, NO.3; M: 100 bp marker; p: positive plasmid control; WT: wild type mouse DNA control; NC: water control

Figure 3

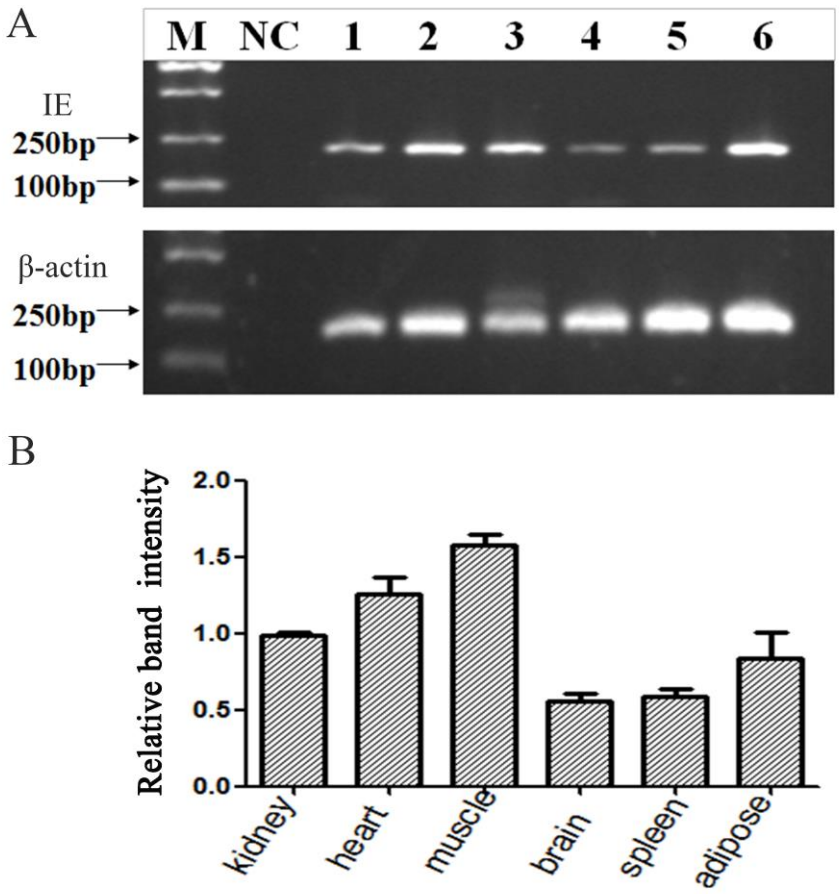

Figure 3 RT-PCR of *IE2* expression in various tissues of the transgenic mouse. A, M: 100 bp marker; NC: water control; Lane 1-6: different tissues, namely kidney, heart, muscle, brain, spleen, adipose tissue; B. The relative band intensity was calculated by the intensity of the IE gene band against the intensity of β-actin gene. The relative band intensity was significantly different in various tissues, $p < 0.01$.

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