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Studying on the 19-bp Palindrome Repeats in Human Cytomegalovirus Immediate Early Enhancer/Promoter Reveals their Diversity in Function for the Promoter Activity

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The human cytomegalovirus major immediate early enhancer/promoter (HCMV MIEP), extending from −588 to +1 relative to the transcription start site, contains a series of reiterated *cis*-acting elements, such as 19-bp repeats, which occur four times in the enhancer/promoter region. However, it is still not clear whether these elements repeat just for backing up or they really execute various indispensable functions. We show here that these reiterated elements are functionally different from each other through serial deletion mutation and site-directed mutation. In addition, we also found that the CG-reverse in the first 19-bp repeat could improve the transcription activity of MIEP in HeLa cells and impair the protein-binding capacity in the EMSA assay. The expression feature of this mutated MIEP in transgenic mice further confirmed its stronger and more universal transcription activity *in vivo*.

Key words: HCMV, transcription regulation, MIEP, P sites, transgenic mice.

Abbreviations: HCMV, human cytomegalovirus; MIEP, major immediate early enhancer/promoter; P sites, 19-bp palindrome repeats; P1 site, the first 19-bp palindrome repeat relative to the transcription start site; 1mP MIEP, the mutated MIEP with a CG-reverse in the sequence of P1 site.

Human cytomegalovirus (HCMV) is a major cause of disease and mortality in immunocompromised individuals and of birth defects in neonates (*1–4*). The majority of the adult population are infected by the virus but only little exhibit illness. However, the virus can persist throughout the lifetime of the individual, and can reactivate to cause viral disease during periods of immunosuppression (*1*, *4*). The HCMV major immediate early gene plays a pivotal role in virus infection and reactivation since its gene products, the key regulators for the viral replication and viral gene expression, are synthesized initially after infection or during reactivation primarily relying on the host factors (*5–9*). Therefore, the investigation into the regulatory mechanisms governing the activity of the HCMV major immediate early enhancer/promoter (HCMV MIEP) contributes not only to our understanding of the cytomegalovirus proliferation mechanism, but also to the study of the transcription regulatory mechanism in eukaryotic organisms.

The HCMV MIEP, extending from −588 to +1 relative to the transcription start site, contains an array of transcriptional regulatory elements (*6*). Previous studies showed that 16-, 17-, 18-, 19-, 21-bp repeat elements could be found in the MIEP region, and that several eukaryotic transcription factors, such as CREB, NFkB and NF-1, can bind to these elements (*10–15*). Among these, the 19-bp repeat, which is structurally remarkable and occurs four times in a completely conservative manner in the MIEP, comprises an 18-bp symmetric palindrome (*16*). Therefore, we named the 19-bp repeats P1, P2, P3 and P4, respectively, corresponding to the transcription start site in this report. Deletion analyses by Boshart *et al*. (*17*) and by Stinski and Roehr (*18*) suggested that the P sites are functionally important in the functioning of HCMV MIEP. Further studies by Ghazal *et al*. (*19*, *20*) showed that synthetic oligonucleotide representing the P sites competed for transcription factors in an *in vitro* assay using HeLa cell nuclear extracts. Subsequently, DNase I footprinting and gel retardation assays by Fickenscher *et al*. indicated sequence-specific protein binding by the P sites (*21*). Subsequently, Chang *et al*. identified that it was a CREB-like factor that bonded to the P sites (*16*). In this study, we asked what the functional significance of the repeated occurrence of these elements is, in particular the 19-bp element. Here, we report that these 19-bp reiterated elements are functionally different from each other. In particular, we also observed that the CG reverse in the P1 site apparently increases the promoter activity measured by the promoter-driven luciferase activity.

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To test this promoter-activity-promoting capability of the CG reverse mutation *in vivo*, we produced two lines of transgenic mice in which wt MIEP and P1 site mutant MIEP (1 mP MIEP), respectively, drive luciferase expression (see Materials and Methods section). Compared to wt MIEP mice, the magnitude of the luciferase activity from mice carrying CG-reverse mutant MIEP is obviously higher, strongly supporting the observation that the CG-reverse mutation increases the MIEP activity *in vivo*. Furthermore, surprisingly we also observed that the luciferase activity could be examined in a broader range of tissues than that from wt MIEP mice, suggesting that the CG-reverse mutation might also broaden the tissue expression pattern. Considering that the activity of the wild type MIEP is actually restricted (*22–29*), our result provides new evidence to construct a more universal promoter to direct the expression of exogenous genes in transgenic mice.

MATERIAL AND METHODS

Plasmid Construction—Serially deleted mutant constructs

To clone the serially deleted MIEP mutants, various upstream primers (1: 5′-gttgacattgattattga-3′; 2: 5′-agta atcaattacggggt-3′; 3: 5′-gcccatatatggagttcc-3′; 4: 5′-ttacgg taaatggcccgc-3′; 5: 5′-ggactttccattgacgtc-3′; 6: 5′-aactgcc cacttggcagt-3′; 7: 5′-ctattgacgtcaatgacg-3′; 8: 5′-tattagtcat cgctatta-3′; 9: 5′-agcggtttgactcacggg-3′; 10: 5′-tgacgtcaatg ggagttt-3′) and the downstream primer R (5′-gagctctgctta tataga-3′) were used to amplify the corresponding fragments using pcDNA3 vector as a template. The PCR production was cloned into T-vector and cut off by restriction enzymes *BamH I* and *Hind III*. Then these fragments were inserted into pGL3-basic luciferase vector. Finally, all resulting clones were verified by sequencing.

Site-directed mutant constructs

Site-directed mutations were introduced to the palindromes on HCMV MIEP using a two-step PCR mutagenesis method with upstream primer (−588 to −570) CGC_*KpnI*-1:5′CGCGGTACCgttgacattgattattga3′ and downstream primer (+1 to −18) CGC_reverse: 5′CGCgagctctgcttatataga3′ (the CGC in both primers are protective bases; the GGTACC in the upstream primer is the *KpnI* restriction site, and the gagctc occurs at the *SacI* restriction site), along with the primers encoding the following sequences in sense or antisense orientation:

For palindrome P1, mP1 sense: 5′-caagtctccaccccatt gagctcaatgggagtttgttttg-3′; mP1 antisense: 5′-cccattgagc tcaatggggtggagacttggaaatccccgt-3′; For palindrome P2, mP2 sense: 5′-caagtacgccccctattgagctcaatgacggtaaatg gcc-3′; mp2 antisense: 5′-cgtcattgagctcaatagggggcgtacttg gcatatgata-3′; For palindrome P3, mP3 sense: 5′-aatag ggactttccattgagctcaatgggtggagtattta-3′; mP3 antisense: 5′-ccattgagctcaatggaaagtccctattggcgttactatg-3′; For palindrome P4, mP4 sense: 5′-aacgacccccgcccattgagctcaata atgacgtatgttc-3′; mP4 antisense: 5′-cattattgagctcaatggg cgggggtcgttgggcggtcag-3′. After getting the spliced mutation promoters, which are about 600 bp, along with pGL3-basic construct, we digested them with *KpnI* and *SacI* and ligated them together using T4 ligase individually. Then, we obtained a series of HCMV MIEP reporter constructs: wt MIEP-luciferase, 1 mP MIEP-luciferase, 2 mP MIEP-luciferase, 3 mP MIEP-luciferase, 4 mP MIEP-luciferase.

Cell Culture, Transfection and Luciferase Assay—HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM; Cellgro) supplemented with 10% fetal bovine serum in a 5% CO_2 humidified atmosphere at 37°C. Cells were seeded 12 h prior to transfection and transfected by the Lipofectamine PLUS method (Invitrogen, Carlsbad, CA, USA). The medium was replaced 12 h after transfection with fresh medium, and cells were lysed after another 12 h. Then the luciferase activities were determined according to the manufacturer's protocol (Dual luciferase assay; Promega Corp.) with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). The data are from at least three independent experiments performed in quadruple with similar results. SEs are indicated.

Generation of Transgenic Mice—The plasmid constructs wt MIEP-luciferase and 1mP MIEP-luciferase DNA were linearized by digestion with *ApaII* and *NotI*, and the 3.3 kb fragment, which contains the MIEP linked to luciferase gene, was purified by agarose gel electrophoresis. The transgene was microinjected into pronuclei of fertilized single-cell eggs of C57 mice. Identification of the transgene was determined by PCR analysis of genomic DNA isolated from mouse tails using HCMV MIEP-luciferase specific primers; upstream primer: 5′aat gggcgtggatagcggtttgact3′; downstream primer: 5′actgcat acgacgattctgtgatttgt3′. Transgenic offspring were generated by breeding with wild type C57 mice.

Analysis of Transgenic Mice's Expression—Tissues from adult transgenic mice were cut into small pieces and homogenized with 1 × CCRL buffer (Promega) on ice. After centrifuging, the supernatant fluid was analysed with the luciferase assay mentioned earlier. Both wt HCMV MIEP-luciferase and 1 mP HCMV MIEP-luciferase and both male and female transgenic mice were tested.

Statistical Analysis—Mean values and SE were calculated for each group, and groups were compared using one-way analysis of variance (ANOVA) followed by *t*-test. $P < 0.05$ denotes a statistically significant difference.

RESULTS

Deletion Analysis of HCMV MIEP—To determine the contribution of each individual P site, we constructed a series of deletion mutants focusing on the P sites, and then cloned these mutated promoter fragments into the pGL3-basic vector (Construct 1–10). Transient transfection of all constructs followed by luciferase activity assay in Hela cells was performed and the luciferase activity was measured 24 h later (Fig. 1). The results revealed maximum luciferase activity with Construct 5, in which the P4 site was cut off relative to Construct 1–4, suggesting the strong inhibition of the P4 site to the MIEP activity. The luciferase activity of Construct 6,

in which the P3 site was further cut off, decreased sharply, suggesting that P3 could positively contribute to the activity of MIEP. In addition, we observed a sharp decline on Construct 7, which contains the same number of P sites as Construct 6. This may be ascribed to the destruction of the 47-bp enhancer, which locates in −347 to −300 bp (*30*). Comparing the luciferase activities of Constructs 8 and 9, which only reserve the P1 site, with Construct 7, there is no prominent variation, indicating that the P2 site is not functionally important. We observed a significant increase in the luciferase activity of Construct 10, in which the single P1 site was destroyed, suggesting that P1 may inhibit the activity of MIEP. Viewed together, our results indicated that these four P sites contribute variously to the activity of MIEP. The P1 and P4 sites function negatively; the P2 site might have no significant effect; and the P3 site functions positively.

Site-directed Mutation Analysis of HCMV MIEP—To rule out the possibility that there may be other sequences flanking these P sites that could also contribute to the activity of MIEP, we generated four site-directed MIEP mutants corresponding to each P site, in which the individual P site was mutated with a CG-reverse in the identical 12-bp palindrome core, as the change would abolish the binding of CREB in the DNase I protection assay (*31*, *32*). These four site-directed fragments were also cloned into the luciferase reporter plasmid pGL3-basic (Construct wt, 1 mP, 2 mP, 3 mP and 4 mP MIEP). Similarly, following transient transfection of all constructs into Hela cells, we determined the corresponding luciferase activities 24 h later. Figure 2 summarizes the results of three independent groups of transient transfections that were performed for each of the earlier mentioned constructs. It shows that two mutants of the P sites, 1 mP MIEP and 4 mP MIEP, would lead to an increase in HCMV MIEP transcription activity compared with the wt MIEP, while the other two mutants, 2 mP MIEP and 3 mP MIEP, exhibit no prominent effects. We can conclude that P1 and P4 sites inhibit the activity of MIEP, whereas P2 and P3 sites have no significant influence.

Transgene Analysis of 1mP MIEP—Although the MIEP is known to have a strong constitutive transcription activity in a broad spectrum of cell lines *in vitro*, the transgene expression driven under this promoter in adult transgenic mice is often more restricted (*22–29*, *33*). Here, our site-directed mutation analysis indicated that 1 mP MIEP exhibits a stronger activity in HeLa cells *in vitro*. Next, we'd like to test whether this property could be recapitulated *in vivo*, and to this end we created two lines of transgenic mice, wt MIEP-luciferase and 1 mP MIEP-luciferase transgenic mice. Nine wt MIEP-luciferase founders (female : male = 5 : 4) and eight 1 mP MIEP-luciferase founders (female : male = 5 : 3, two female founders died after giving birth to the babies) were screened by PCR analysis of tail genome DNA.

To examine the expression pattern of the luciferase gene and its activity, we prepared a variety of tissues from the cerebrum, cerebellum, esophagus, stomach, duodenum, liver, pancreas, kidney, bladder, testes,

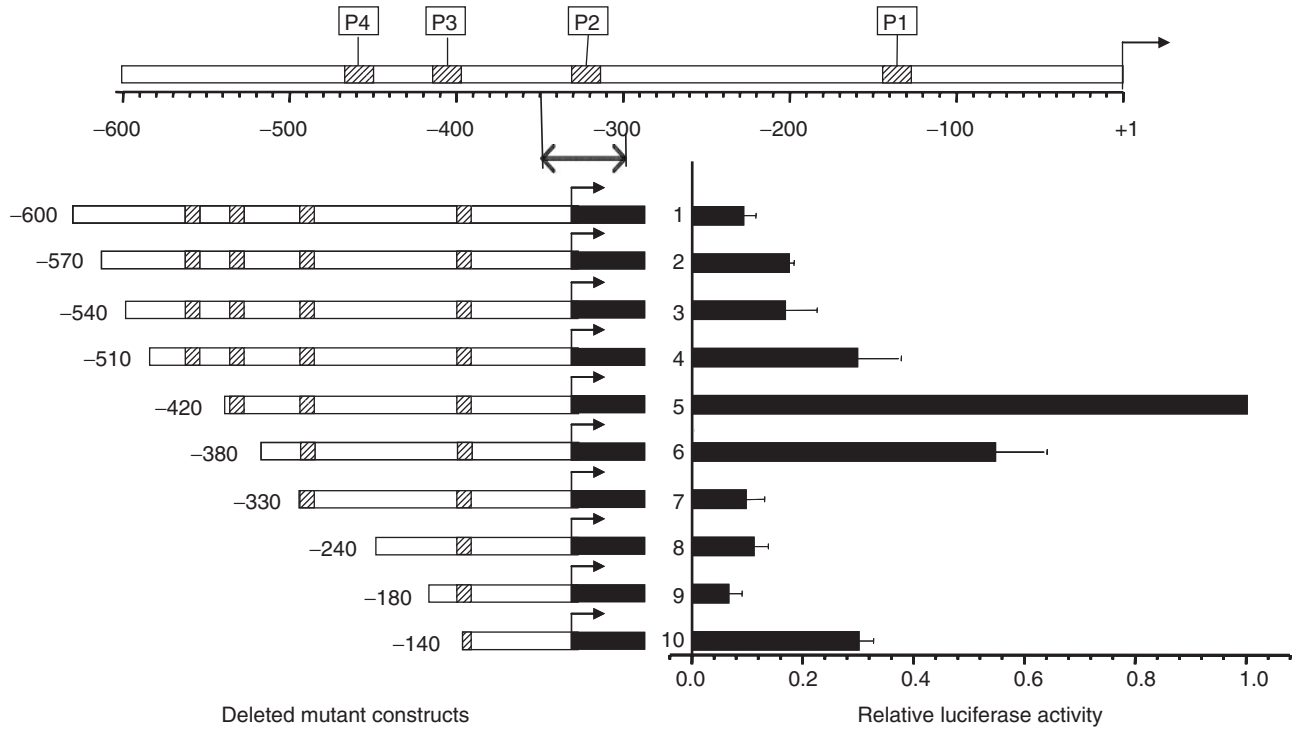
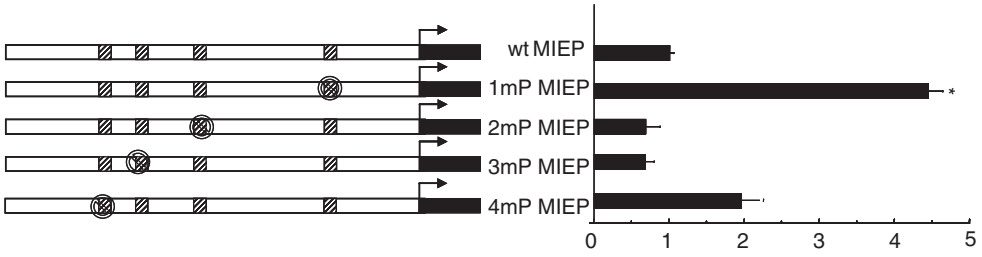


Fig. 1. **Activity of the MIEP serially deleted constructs 1–10.** HeLa cells were transfected with equal amount of plasmids containing Constructs 1–10. Luciferase activity was measured 24 h after transfection. Error bars indicate SD of triplicate values.

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mutated P sites:
1mP MIEP(-145 -128) cccattgagctcaatggg
2mP MIEP(-331 -314) cctattgagctcaatgac
3mP MIEP(-414 -397) tccattgagctcaatggg
4mP MIEP(-467 -450) cccattgagctcaataat
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Fig. 2. **Activity of the various P site-directed MIEP mutants.** Luciferase activity was measured as description for serially deleted constructs.

Table 1. **Luciferase expression in various tissues of transgenic mice.**

	wt HCMV MIEP						1mP HCMV MIEP					
	Female			Male			Female			Male		
System and organ	05	19	21	03	09	24	11	12	13	14	15	23
Brain												
Cerebrum	–	–	+	++	–	++	–	+	–	–	–	–
Cerebellum	–	–	–	+	–	++	+	++	–	–	–	–
Gastrointestinal												
Esophagus	–	–	++	+	–	+	++	+++	+	–	+	–
Stomach	–	++	++	++	–	+	++	+++	+	+	+	–
Duodenum	–	–	–	–	–	–		++	–	–	–	–
Liver	–	+	+	+	–	++	+++	+++	–	++	+	–
Pancreas	+	+	+	+	+	++	N	+++	++	++	+++	+
Genitourinary												
Kidney	–	++	+	+	–	+	++	+++	+	++	–	–
Bladder	–	–	–	–	–	+	+	++	–	–	–	–
Testes				+	+++	+++				+	++	+++
Ovary	+	–	–				++	++	+			
Oviduct	–	+	–				+++	+++	+			
Respiratory												
Lung	–	+	++	+	–	++	+++	+++	++	+	+	–
Circulatory												
Heart	+	+	++	++	++	+++	++	+++	++	++	+	–
Lymphoid and Blood												
Spleen	–	+	–	–	–	+	++	++	+	–	–	–
Thymus	–	+	+	+	+	+++	++	+++	++	–	–	+
Blood	–	–	–	–	–	–	–	–	–	–	–	–
Others												
Skeletal muscle	+	++	++	+++	+++	+++	++	+++	+	++	++	++
Skin	–	++	+++	+	+++	+++	++	+++	+++	++	++	++

–, No expression; +, low level of expression; ++, moderate level of expression; +++, high level of expression. N, none defined.

ovary, oviduct, lung, heart, spleen, thymus, blood, skeletal muscle and skin, and then examined them for luciferase activity assay. Meanwhile, we analysed two females and two males wild type adult C57 mice using the same method. Since there is no sex difference between wild type C57 mice, we set the means of luciferase activity of these four mice as the negative control. Table1 shows the comparative luciferase activity of each individual founder. Two independent female founder mice (#11, #12) carrying 1 mP MIEP-luciferase transgene exhibited much higher luciferase activities and a more universal expression pattern than those of wt MIEP-luciferase founder mice, and the other founder (#13) exhibited a moderate expression level. Taking three lines each together after normalized by the control, Fig. 3 shows the general expression patterns of each individual cluster of the transgenic animals. Consistent with previous studies, we found similar and restricted expression patterns among wt MIEP-luciferase transgenic mice (Fig. 3A). Almost, no luciferase activities were

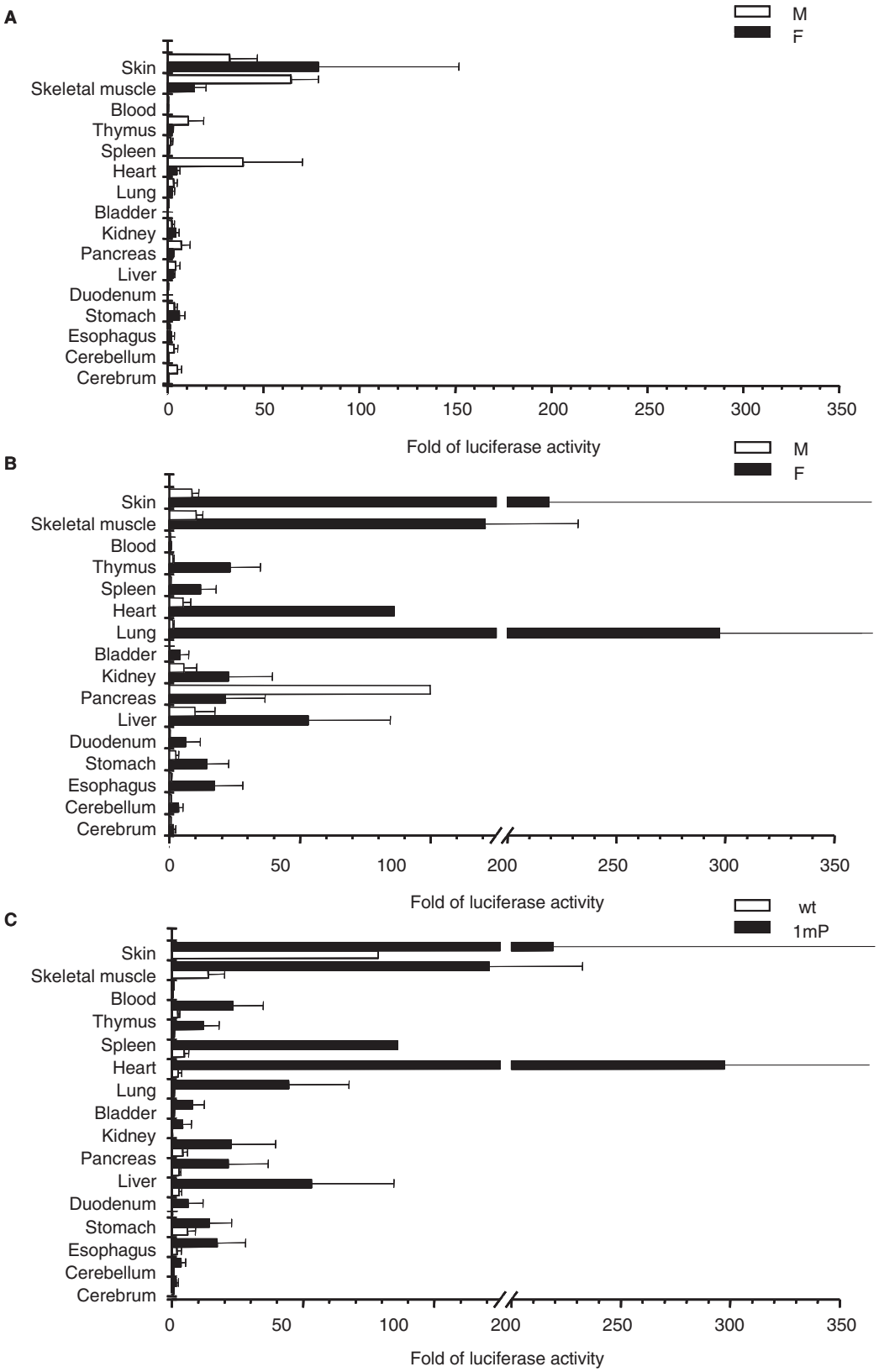

Fig. 3. **Comparative analysis of the luciferase expression between transgenic mice.** (A) Comparing the luciferase expression between the male and female belonging to the wt MIEP-luciferase transgenic mice, it suggests no difference there. (B) Comparing the luciferase expression between the male and female belonging to the 1 mP MIEP-luciferase transgenic mice, female mice have a general higher expression than male ones. (C) Comparing the female 1 mP MIEP and wt MIEP-luciferase transgenic mice, the 1 mP MIEP ones exhibit a universal higher expression than the wt MIEP ones.

observed in samples of tissue taken from the blood, spleen, bladder, duodenum and esophagus; samples from lung, kidney and liver tissue exhibit detectable but very low luciferase activity; in samples of skin and skeletal muscle tissue high luciferase activity level could be established. Moreover, data from male and female wt MIEP-luciferase mice showed that there is no remarkable difference in the expression patterns of the tissue samples examined, although different luciferase activity levels could be obtained in several tissue samples, including the cerebrum. Unexpectedly, data from the 1 mP MIEP-luciferase mice indicate an obvious distinction in the levels and expression patterns of the luciferase gene, with a higher and broader expression in female mice (Fig. 3B). Except for samples from blood, 17 of the 18 examined types of tissue exhibit higher levels of luciferase expression in female mice, including those taken from the lung, liver, muscle and duodenum, for which in previous studies only trace amounts were measured (*22*, *29*). However, in the male 1 mP MIEP mice the luciferase activities from different tissue samples showed lower levels and more restricted expression patterns. To summarize, consistent with the results with HeLa cells, the mutant MIEP showed a tendency for higher and more universal expression in transgenic mice (Fig. 3C).

DISCUSSION

In the present study using *in vitro* and *in vivo* assays, we have shown that the four P sites are functionally different from each other: P1 and P4 sites inhibit the activity of HCMV MIEP, while P2 and P3 sites have no obvious effect. In addition, we identified a mutant promoter, 1mP MIEP, with stronger and more universal transcription activity than wt MIEP in female transgenic mice. These results revealed that the P1 site can be viewed as a negative regulatory element both *in vitro* and *in vivo*, which was not clear before. Furthermore, this mutant promoter could also be viewed as a candidate for a universal promoter to lead a ubiquitous expression of exogenous genes in transgenic mice.

Several previous reports, using synthesized decoy oligonucleotide perturbation assay or deletion analysis, have elucidated the general contribution of P sites to the constitutive activity of the HCMV MIEP (*21*). Although the scenario in which each individual P site could function in a unique manner was considered, it had not been experimentally confirmed. The serial deletion mutation analysis in this report, especially the site-directed mutation focused on every P site, conceivably showed that these P sites do function in the distinct manner. It is unclear whether this difference is due to the different location of these P sites on the HCMV MIEP or the sequence diversity existing in their flanking sequence around the absolutely identical 12-bp core. The importance of the flanking sequence results from two possibilities. (i) The individual flanks could influence the binding capacity of multiplex CREB isoforms onto the given P site. As established in several reports, the P site is readily bound *in vitro* by cellular CREB and ATF-1, which are found in most human tissues. The CREB family consists of CREB, ATF-1, CREM and their respective isoforms. Among these proteins, CREB2 has been described as a repressor of CREB and CRE-mediated transcription. (ii) The individual flanks could determine the given P site interaction with additional *trans*-acting elements rather than CREB.

It is something of a surprise that the activity of the 1 mP MIEP with only a CG-reverse in the P1 site is evidently stronger than the wild type MIEP. Nowadays, transgenic technology is widely used in gain-of-function experiments in animals. But there is still less ideal promiscuous promoter to lead strong expression in multiple tissues and organs. Even though wild type MIEP can drive the strongly exogenous gene expressed in virtually all cell lines from different species, it became evident that the wild type MIEP-directed expression of a variety of transgenes in adult transgenic mice was more often restricted to certain tissues. We used the wt MIEP and the 1 mP MIEP to obtain transgene expression in multiple tissues and were surprised to find that the patterns of expression in three lines of female 1 mP MIEP-luciferase transgenic mice were universal, indicating that it is possible to develop 1 mP MIEP into a truly universal promoter *in vivo*. Additionally, contrasting with the expression patterns driven by wild type MIEP, which shows no significant difference between the male and female mice, 1 mP MIEP directs an evident female-favorable universal expression. The observation suggests that the repressive regulation mechanism on HCMV MIEP in male mice is different from that in female mice.

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