

# 一种适于家蚕细胞激素研究的双荧光素酶报告基因系统的内参质粒的构建

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**摘要:** 双荧光素酶报告基因系统能够提供灵敏的读数, 但该系统需要依赖组成型表达的内参对读数进行归一化。然而, 大多数内参并不是在所有条件下都组成型表达。为此, 文中建立了一个有效的方法制备适于家蚕细胞双荧光素酶报告基因系统的内参质粒。首先, 突变 BmVgP78 启动子上的激素应答相关元件, 获得了在家蚕细胞中稳定表达的组成型启动子 BmVgP78M; 然后, 用 BmVgP78M 替换 pRL-SV40 质粒上的 SV40 启动子和嵌合内含子序列, 成功构建了 pRL-VgP78M 内参质粒; 最后, 通过细胞转染实验证实 pRL-VgP78M 内参在家蚕细胞系中稳定表达, 并且 pRL-VgP78M 内参的表达活性不受蜕皮激素、保幼激素及激素相关转录因子的影响。最终, 获得了在家蚕细胞中稳定表达且表达量适中的内参质粒 pRL-VgP78M。该内参可以有效地作为双荧光素酶报告基因系统的内参质粒用于家蚕细胞系中激素的研究。同时, 该内参质粒的构建方法也为构建适于其他物种细胞系的双荧光素酶报告基因系统的内参质粒提供了参考。

**关键词:** 家蚕, 双荧光素酶报告基因系统, 内参质粒, 激素, 方法

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# Establishment of a suitable control reporter plasmid of a dual luciferase reporter gene system for hormone research in silkworm cell lines

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**Abstract:** The dual luciferase reporter gene system provides sensitive readout, while it relies on a constitutively-expressed control gene for readout normalization. However, most standard control reporter genes are not constitutively expressed under all conditions. Here, we report an effective method to construct a control reporter plasmid for the dual luciferase reporter gene system that would be suitable for hormone research in silkworm cell lines. First, we modified BmVgP78M, a stably-expressed constitutive promoter in silkworm cells by mutating its hormone-related element. Then, we constructed the pRL-VgP78M control reporter plasmid by replacing the SV40 promoter and chimeric intron sequences in pRL-SV40 with the BmVgP78M sequence. Finally, we confirmed that the pRL-VgP78M control reporter plasmid could be stably expressed in silkworm cell lines via cell transfection experiments, and it was unresponsive to the induction of ecdysone, juvenile hormone, or their transcription factors. We thus obtained a control reporter plasmid pRL-VgP78M that could be expressed stably and moderately in silkworm cells. It can be readily used as the control reporter plasmid of the dual luciferase reporter gene system for hormone research in silkworm cell lines. It will also provide a reference for construction of control reporter plasmids of dual luciferase reporter gene systems that are adaptable to cell lines isolated from other species.

**Keywords:** silkworm, dual luciferase reporter gene system, control reporter plasmid, hormone, method

The dual luciferase reporter gene system refers to a signaling system in which two luciferase reporter genes are simultaneously expressed whereas their activities can be independently measured<sup>[1-2]</sup>. This system is widely utilized in various fields including the detection of genetic toxicity<sup>[3]</sup>, identification of microRNA target<sup>[4]</sup>, screening of efficient shRNA<sup>[5]</sup>, analysis of signaling pathways<sup>[6]</sup> and transcriptional activity<sup>[7-8]</sup>. In the dual luciferase reporter gene system, the activity change of one reporter gene is associated with specific experimental conditions, whereas the constitutive activity of a second reporter gene provides an internal control to normalize the experimental value<sup>[9]</sup>. Compared with the traditional co-reporter genes *CAT*,  *$\beta$ -Gal* or *GUS*, the dual luciferase reporter system exhibits enhanced sensitivity; in addition, this system can reduce the influence of intrinsic factors that may affect the accuracy of the experimental results, and can shorten the detection time as well.

To obtain valid read-outs, the dual luciferase reporter gene system requires constitutive expression activity of the control reporter plasmid, which in turn needs the promoter of the control reporter plasmid to be expressed constitutively in the system. At present, pRL-CMV, pRL-SV40 and pRL-TK are widely used control reporter plasmid whose *Renilla* luciferase reporter gene is driven by the CMV immediate-early enhancer/promoter, the SV40 enhancer/promoter, and the herpes simplex virus thymidine kinase (HSV-TK) promoter, respectively<sup>[10]</sup>. However, there were some experimental factors affecting the *Renilla* luciferase activity of pRL plasmids<sup>[11-13]</sup>. Meanwhile, nuclear receptor TR4 has been shown to suppress the activity of *Renilla* luciferase of the pRL plasmids<sup>[14]</sup> and the expression activity of pRL-TK plasmid was enhanced by cotransfection with vectors expressing orphan nuclear receptors Nur77 family (Nur77, Nurrl, Nor-1), leading to misinterpretation of the assay results<sup>[15]</sup>. An inappropriate control plasmid

may result in incorrect results<sup>[16]</sup>. Therefore, selecting the appropriate control reporter plasmid has become a key to the accurate application of the dual luciferase reporter gene system in different conditions and different cell lines.

Silkworm (*Bombyx mori*) is an important economic insect that is internationally recognized as a Lepidoptera model insect. The characterization of promoters with tissue or spatiotemporal specificity and the identification of inducible promoters can provide favorable tools and strategies for the research of silkworm bioreactors, genetic breeding, the control of Lepidoptera pests, and the promotion of insect molecular biology research in silkworm. Numerous studies have been performed on the regulation of gene expression in silkworm, mainly related to regulation by hormones including ecdysone and juvenile hormone<sup>[17-18]</sup>. Although the dual luciferase reporter gene system is considered as one of the most effective methods to study gene regulation, the commonly used control reporter plasmids such as pRL-CMV, pRL-SV40 and pRL-TK are expressed at very low levels in silkworm cell lines and their activities are relatively unstable. These may limit the application of this system and concomitantly the study of gene regulation in silkworm. Researchers had attempted to construct a p-IE1-Rlucp control reporter plasmid using the *B. mori* Nucleopolyhedrovirus (immediate early) IE1 promoter to drive the expression of *Renilla* luciferase. However, its expression level was very high, thus it would not be suitable for analyzing promoters with low activity in cell lines because the normalized relative luciferase activity value would be very low. Therefore, there is an urgent need for a suitable control reporter plasmid for silkworm cell lines.

To address this issue, in this study, we used the vitellogenin gene promoter of silkworm itself and mutated its hormone-related elements to obtain the constitutive promoter BmVgP78M, then replaced the promoter and chimeric intron in the pRL plasmid with BmVgP78M. We finally verified whether the control reporter plasmid activity was affected by hormone at the cellular level. In summary, we constructed a control reporter plasmid

suitable for the study of the effect of ecdysone and juvenile hormone on gene regulation in silkworm cell lines.

## 1 Materials and methods

### 1.1 Amplification of the mutated promoter

All primer sequences are shown in Table 1. The upstream regulatory region (from -38 bp to +40 bp) of the *BmVg* gene was amplified from the silkworm genome with the primers BmVgP78-F1 and BmVgP78-R1. The PCR protocol was as follows: initial denaturation was performed at 95 °C for 5 min. This was followed by 30 cycles of varying the temperature in the following manner: 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 10 s. Subsequently, a temperature of 72 °C was maintained for 7 min. The PCR product was inserted into the vector pMD19-T simple (TaKaRa Co., Dalian, China) and sequenced. The mutated promoter termed BmVgP78M was created by PCR with the mutated primers BmVgP78M-F1 and BmVgP78-R1. BmVgP78M was cloned into the pMD19-T simple vector to sequence.

### 1.2 Vector construction

To construct the targeting vector, the BmVgP78M was amplified with the primers BmVgP78M-F2 and BmVgP78M-R1. The PCR product was digested with *Bgl* II/*Nhe* I and cloned into the pRL-SV40 plasmid (Promega, Madison, WI, USA) to generate pRL-VgP78M plasmid. The transfection vector pGL3-VgP1.5K-LUC, over

表 1 文中所用引物

Table 1 Primers used in this study

| Primer name | Primer sequence (5'-3')                                  |
|-------------|--|
| BmVgP78-F1  | CCTATATAAAGGGGGTGACCTG                                   |
| BmVgP78-R1  | TGTACTAGCTCCGCTGTC                                       |
| BmVgP78M-F1 | CCTATATAAAGGAAACAGTTCA<br>GCCAGAGATGTTTCAGAT             |
| BmVgP78M-F2 | GGAAGATCTCCTATATAAAGG<br>AAACAGTTCAGCCAGAGATGT<br>TCAGAT |
| BmVgP78M-R1 | CTAGCTAGCTGTACTAGCTCCG<br>CTGTC                          |
| BmVgP78M-F2 | GGAAGATCTCCTATATAAAG<br>GGGGTGACCTG                      |

expression vector psl1180-A4-BmBrC-Z2-SV40 and psl1180-A4-BmKr-h1 SV40 were stored in our lab, the transfection vector pGL3-BmE93P-LUC was constructed as described in the literature<sup>[19]</sup>.

### 1.3 Cell culture

The *B. mori* embryonic cell line BmE-SWU1, which was originally developed from embryo tissues (stored in our laboratory), was maintained at 27 °C in Grace medium (GIBCO, Invitrogen, Carlsbad, CA, USA) supplemented with 10% (V/V) fetal bovine serum (FBS, Thermo Scientific™ HyClone™, Waltham, MA, USA). The *B. mori* ovarian cell line BmN-SWU1, which was originally developed from ovarian tissues (stored in our laboratory), was cultured in TC-100 insect medium (US Biological, Swampscott, MA, USA) containing 10% (V/V) FBS at 27 °C.

### 1.4 Transfection assay

Cell transfection and co-transfection assay used X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland). For control reporter plasmid transfection, the cells were seeded at 10<sup>5</sup> cells/mL in 24-well tissue culture plates for 12 h. A mixture of 100 μL containing 0.1 μg control reporter plasmid DNA and 0.3 μL X-tremeGENE HP DNA Transfection Reagent in Grace medium without FBS was used to transfect the BmE-SWU1 cells or in TC-100 medium without FBS for BmN-SWU1. For transfection, a mixture of 100 μL containing 1 μg reporter plasmid DNA, 0.1 μg control reporter plasmid DNA, and 3.3 μL X-tremeGENE HP DNA Transfection Reagent in medium without FBS was used. For co-transfection, a mixture of 100 μL containing 0.5 μg reporter plasmid DNA, 0.5 μg psl1180 over expression plasmid DNA, 0.1 μg control reporter plasmid DNA, and 3.3 μL X-tremeGENE HP DNA Transfection Reagent in medium without FBS was used. After culture for 5–8 h, the transfection mixture was replaced with 500 μL fresh medium with 10% FBS. The cells were cultured further for 24 h or 48 h and then harvested for luciferase activity analysis. Luciferase activity was measured using commercially available kits (Promega) according to the manufacturer's instructions. The

measurement of luciferase activity was as described<sup>[20]</sup>. Transfection and co-transfection were repeated six times independently and the average luciferase activity was expressed as  $\bar{x} \pm s$ . Statistical significance of the regulatory activities was analyzed using Student's *t*-test.

### 1.5 Hormone treatment

20-hydroxyecdysone (20E) and the juvenile hormone analogue (JHA) methoprene (Sigma-Aldrich, St. Louis, MO, USA) were first dissolved in dimethyl sulfoxide (DMSO) and then diluted with water to the test concentrations. The hormones 20E and JHA were added to the medium in different concentration of 10<sup>-8</sup>, 10<sup>-7</sup>, 10<sup>-6</sup> and 10<sup>-5</sup> mol/L when the transfection mixture was replaced by 500 μL fresh medium with 10% FBS.

## 2 Results

### 2.1 Construction of the pRL-VgP 78M control reporter plasmid

Previous studies have shown that the upstream regulatory sequence from -38 to +40 of *BmVg* (BmVgP78) only contained one hormone-related *cis*-response element Estrogen receptor  $\alpha$  (Fig. 1A) and its expression activity was affected by 20E (Fig. 2). In order to avoid the hormones affecting the expression activity of BmVgP78, we mutated the Estrogen receptor  $\alpha$  element. The sequence of Estrogen receptor  $\alpha$  element "GGGTGACCTG" was changed into "AAACAGTTCA" (Fig. 1A), and the mutated sequence being termed BmVgP78M. The BmVgP78M only contained the TATA box but no other predicted elements, which theoretically made it a good candidate for the control plasmid promoter of the dual luciferase reporter gene system for hormones research in silkworm cells. We next confirmed whether BmVgP78M could be used as the constitutive promoter of the control reporter plasmid. Considering that the chimeric intron in the pRL vector contains some transcriptional elements<sup>[14,21]</sup>, we not only replaced the SV40 promoter with the VgP78M promoter, but also removed the intron in pRL-SV40. Finally, the pRL-VgP78M control reporter plasmid was successfully constructed (Fig. 1B).

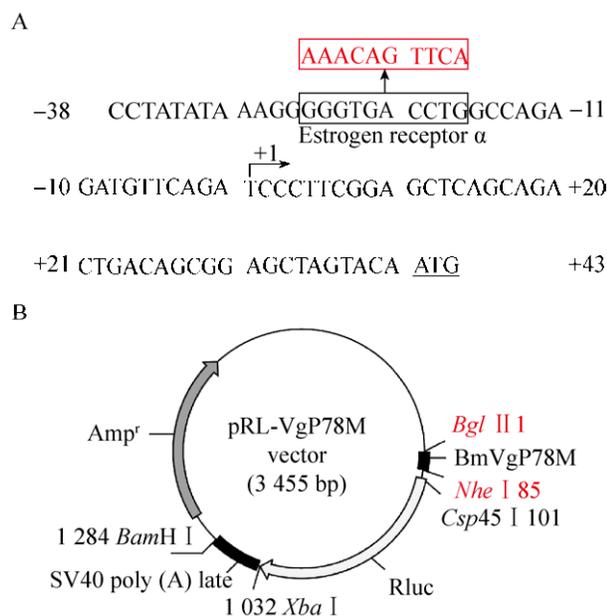


图1 pRL-VgP78M 内参质粒的构建

Fig. 1 The construction of pRL-VgP78M control reporter plasmid. (A) The nucleotide sequence of BmVgP78. The underlined sequence represents the translation initiation site, +1 represents the transcription initiation site, the sequence in the box is the Estrogen receptor $\alpha$  element and the sequence above the box is the mutant sequence. (B) The map of the pRL-VgP78M control reporter plasmid. The BmVgP78M was inserted between *Bgl* II and *Nhe* I.

## 2.2 pRL-VgP78M control reporter plasmid was expressed stably in silkworm cell lines

To analyze the expression stability of pRL-VgP78M in silkworm cell lines, pRL-VgP78M was transfected into BmE-SWU1 cells and the activity of *Renilla* luciferase was measured at 24 h and 48 h after transfection. The standard deviation analysis showed that the activity of *Renilla* luciferase was relatively stable at 24 h (Fig. 3A) and 48 h (Fig. 3B) after transfection. These results indicated that *Renilla* luciferase driven by VgP78M could be constitutively expressed in silkworm cells, suggesting it could be used as the control reporter plasmid of the dual luciferase reporter gene system in silkworm cell lines.

To determine whether pRL-VgP78M was more suitable as the control reporter plasmid compared

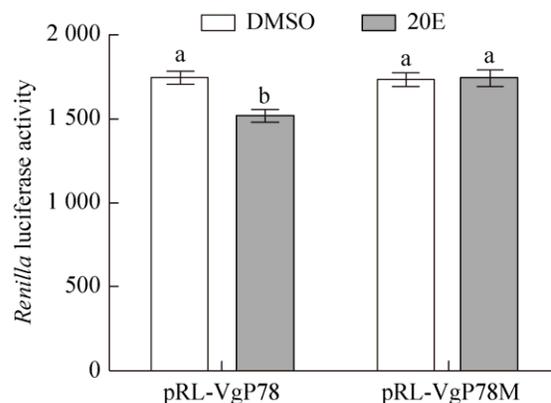


图2 pRL-VgP78 和 pRL-VgP78M 两个载体荧光素酶活性的比较

Fig. 2 The comparison of luciferase activity between pRL-VgP78 and pRL-VgP78M. *Renilla* luciferase activity of pRL-VgP78 and pRL-VgP78M control reporter plasmid was detected at 24 h after transfection, the treatment concentrations of ecdysone was  $10^{-6}$  mol/L; DMSO concentration was 0.1%. The error bars represent  $\bar{x} \pm s$  ( $n=6$ ). The significance of the difference between data sets was calculated using a two-tailed Student's *t*-text; Bars with the different letters are significantly different ( $P<0.05$ ).

with other control plasmids of the dual luciferase reporter gene system in silkworm cell lines, we transfected pRL-VgP78M, pRL-CMV, pRL-SV40, pRL-TK and p-IE1-Rlucp into BmE-SWU1 cells and measured the activity of *Renilla* luciferase at 24 h after transfection. The expression activity of pRL-CMV, pRL-SV40 and pRL-TK were very low, close to that of the blank control, indicating that these were not suitable as the control reporter plasmid of the dual luciferase reporter gene system in silkworm cell lines. The *Renilla* luciferase activity of p-IE1-Rlucp was extremely high, showing activity up to  $10^4$ – $10^6$  when transfected with only 0.1  $\mu$ g plasmid in a 24-well plate. Therefore it was not suitable for the study of low activity promoters in silkworm cells because it could result in a relatively low value of luciferase activity, making the results inaccurate. However, the *Renilla* luciferase activity of pRL-VgP78M was moderate; thus pRL-VgP78M was considered most suitable as the control reporter plasmid of the dual luciferase reporter gene system in silkworm cells (Fig. 3C).

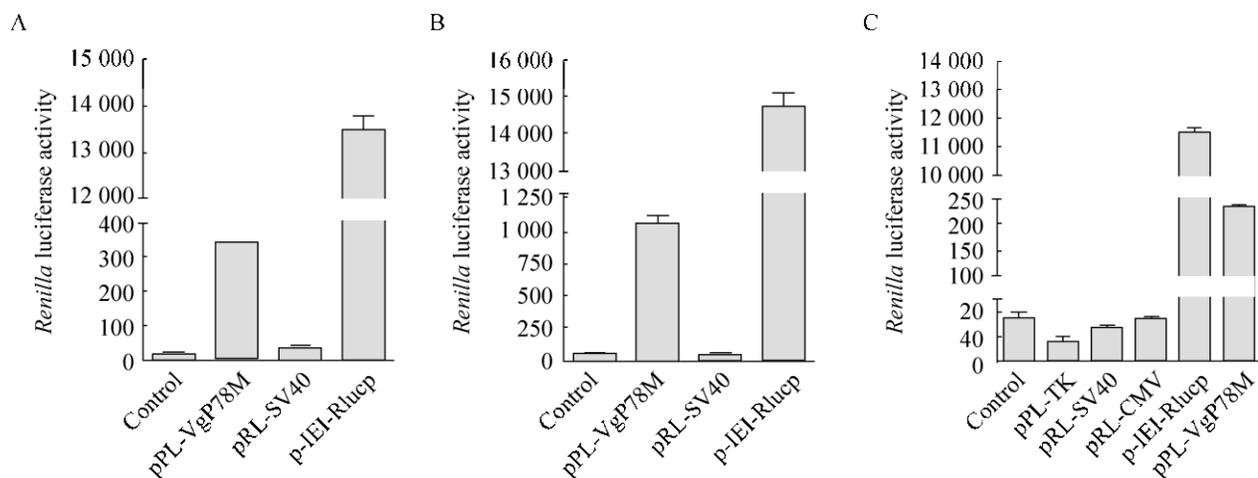


图3 pRL-VgP78M 内参质粒在家蚕胚胎细胞系 BmE-SWU1 中稳定表达

Fig. 3 The control reporter plasmid pRL-VgP78M was expressed stably in BmE-SWU1 cells. *Renilla* luciferase activity of pRL-VgP78M control reporter plasmid was detected at 24 h (A) and 48 h (B) after transfection; pRL-SV40 and the commonly used control reporter plasmid p-IEI-Rlucp in silkworm cells were used as positive control and the non-transfected cells were used as the negative control. (C) Comparison of *Renilla* luciferase activity of pRL-VgP78M, pRL-TK, pRL-SV40, pRL-CMV and p-IEI-Rlucp in BmE-SWU1 cells. The error bars represent the  $\bar{x} \pm s$  ( $n=6$ ).

### 2.3 The expression activity of pRL-VgP78M control reporter plasmid was not induced by 20E or its transcription factor

The study of hormones is very extensive in silkworm. To confirm that pRL-VgP78M control reporter plasmid could be used for ecdysone regulation research in silkworm cell lines, we first analyzed whether ecdysone itself affected the expression activity of pRL-VgP78M. The transfection vector pGL3-VgP1.5K-LUC, whose expression activity had been reported to be upregulated by 20E<sup>[22]</sup>, and pRL-VgP78M were co-transfected into BmE-SWU1 cells among with different concentrations of hormone treatment. The luciferase activity analysis showed that pGL3-VgP1.5K-LUC was induced by 20E (Fig. 4A), which was consistent with the reported results<sup>[22]</sup>, whereas pRL-VgP78M was not induced by 20E (Fig. 4B). In comparison, pGL3-VgP1.5K-LUC and pRL-VgP78M were transfected into BmN-SWU1 cells which was also a cell line commonly utilized to study the effect of 20E in silkworm. After treatment with 20E, the activity of pGL3-VgP1.5K-LUC was also induced (Fig. 4E),

whereas pRL-VgP78M was not (Fig. 4F).

To confirm that the key genes of the ecdysone signaling pathway would not affect the activity of pRL-VgP78M, we used the early ecdysone response gene BmBrC-Z2 for subsequent experiments. The over expression vector psl1180-A4-BmBrC-Z2 SV40<sup>[22]</sup> was co-transfected with pRL-VgP78M and pGL3-VgP1.5K-LUC into BmE-SWU1 cells. The results showed that the luciferase activity of pGL3-VgP1.5K-LUC was induced by BmBrC-Z2 transcription factor (Fig. 4C), whereas the luciferase activity of pRL-VgP78M was not (Fig. 4D). In addition, the same experiment was conducted in BmN-SWU1 cells. Similarly, BmBrC-Z2 transcription factor induced the luciferase activity of pGL3-VgP1.5K-LUC (Fig. 4G) but not the luciferase activity of pRL-VgP78M (Fig. 4H). The BmBrC-Z2 transcription factor is an early response gene in the ecdysone signal pathway, which further confirmed that pRL-VgP78M was not induced by ecdysone. These results confirmed that pRL-VgP78M could be used as the control reporter plasmid for studies of ecdysone regulation in silkworm cell lines.

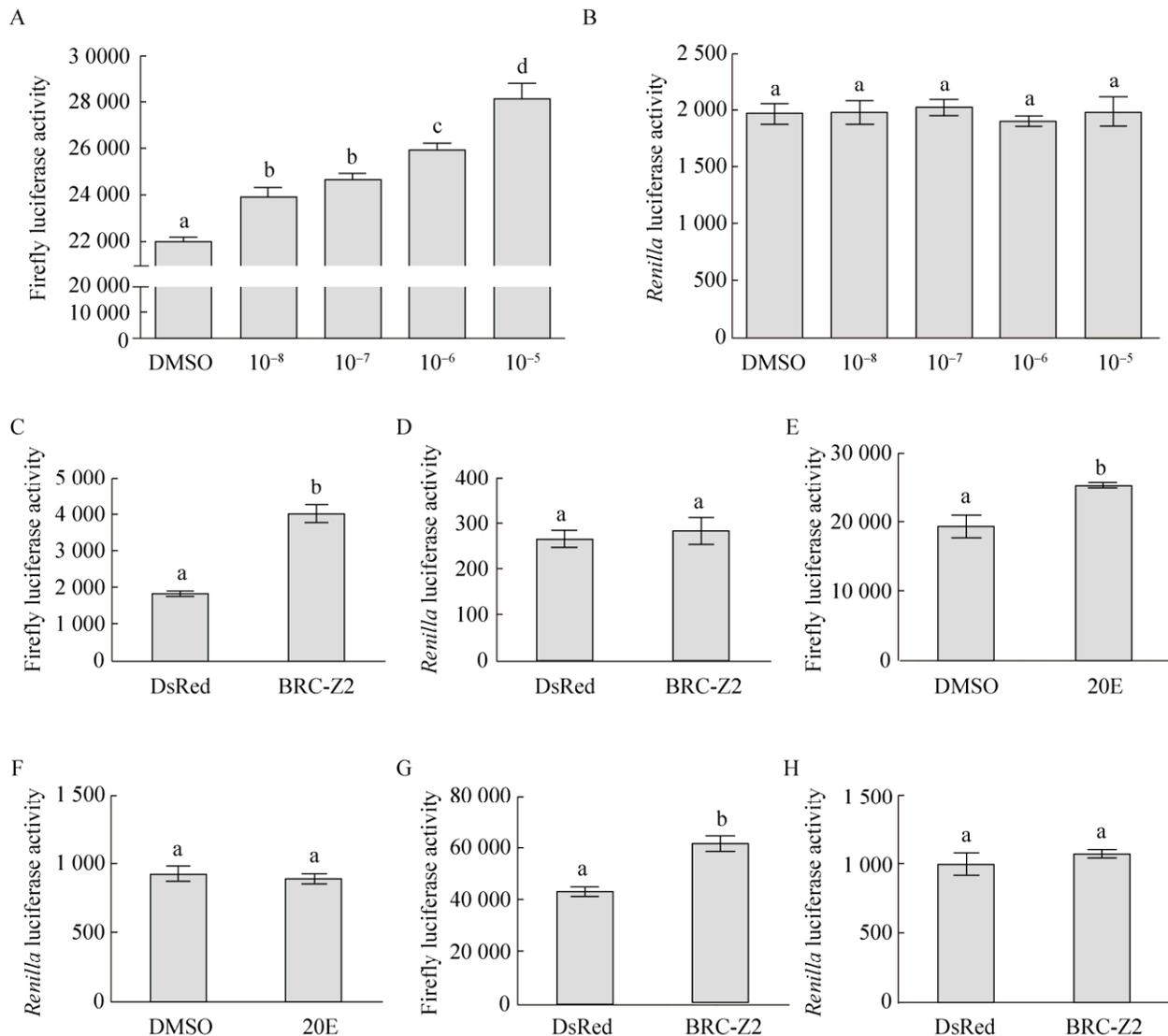


图 4 蜕皮激素及蜕皮激素早期应答基因 **BrC-Z2** 对 **pRL-VgP78M** 内参质粒荧光素酶活性的影响

Fig. 4 Effects of ecdysone and its early response gene BrC-Z2 on the luciferase activity of pRL-VgP78M. (A, B): Effects of different concentrations of 20E on the luciferase activity of pGL3-VgP1.5K-LUC (A) and pRL-VgP78M (B) in BmE-SWU1 cells. (C, D): Effects of the over expression vector psl1180-A4-BmBrC-Z2 SV40 on the luciferase activity of pGL3-VgP1.5K-LUC (C) and pRL-VgP78M (D) in BmE-SWU1 cells. (E, F): Effects of 20E on the luciferase activity of pGL3-VgP1.5K-LUC (E) and pRL-VgP78M (F) in BmN-SWU1 cells. (G, H): Effects of the over expression vector psl1180-A4-BmBrC-Z2 SV40 on the luciferase activity of pGL3-VgP1.5K-LUC (G) and pRL-VgP78M (H) in BmN-SWU1 cells. The treatment concentrations of ecdysone were  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$  and  $10^{-5}$  mol/L; DMSO concentration was 0.1%. The error bars represent the  $\bar{x} \pm s$  ( $n=6$ ). The significance of the difference between data sets was calculated using a two-tailed Student's *t*-text; Bars with the different letters are significantly different ( $P < 0.05$ ).

#### 2.4 The expression activity of pRL-VgP78M control reporter plasmid was not induced by JHA

In order to confirm that pRL-VgP78M control reporter plasmid could be used for juvenile hormone regulation research in silkworm cell lines, we

constructed the transfection vector pGL3-BmE93P-LUC, whose activity was dependent on 20E and was repressed by JHA<sup>[19]</sup>. pGL3-BmE93P-LUC and pRL-VgP78M were transfected into BmE-SWU1 cells with 20E treatment. We then treated the cells

with different concentrations of JHA and determined the luciferase activity at 24 h after transfection. We found that the 20E-induced activity of pGL3-BmE93P-LUC was inhibited by JHA (Fig. 5A), which was consistent with the reported results<sup>[19]</sup>, whereas the activity of pRL-VgP78M was not induced by JHA (Fig. 5B).

To verify that key genes in the juvenile hormone signaling pathway, such as BmKr-h1 transcription factor, would not affect the activity of pRL-VgP78M, the over expression vector psl1180-A4-BmKr-h1 SV40 was co-transfected with pRL-VgP78M and pGL3-BmE93P-LUC into BmE-SWU1 cells. Then, cells were treated with 20E and the luciferase activity was detected at 48h after transfection. The results showed that BmKr-h1

inhibited 20E-induced activity of pGL3-BmE93P-LUC (Fig. 5C). On the contrary, the expression activity of pRL-VgP78M was not downregulated by the BmKr-h1 transcription factor (Fig. 5D). These results further verified that the expression activity of pRL-VgP78M control reporter plasmid was not affected by JHA. Therefore pRL-VgP78M could be used as a control reporter plasmid of the dual luciferase report system for JHA research in silkworm cell lines.

### 3 Discussion

In this study, the mutated form of BmVg78 promoter, BmVgP78M was constructed, which only contained the TATA box and but no other predicted elements, to be used as the control reporter plasmid

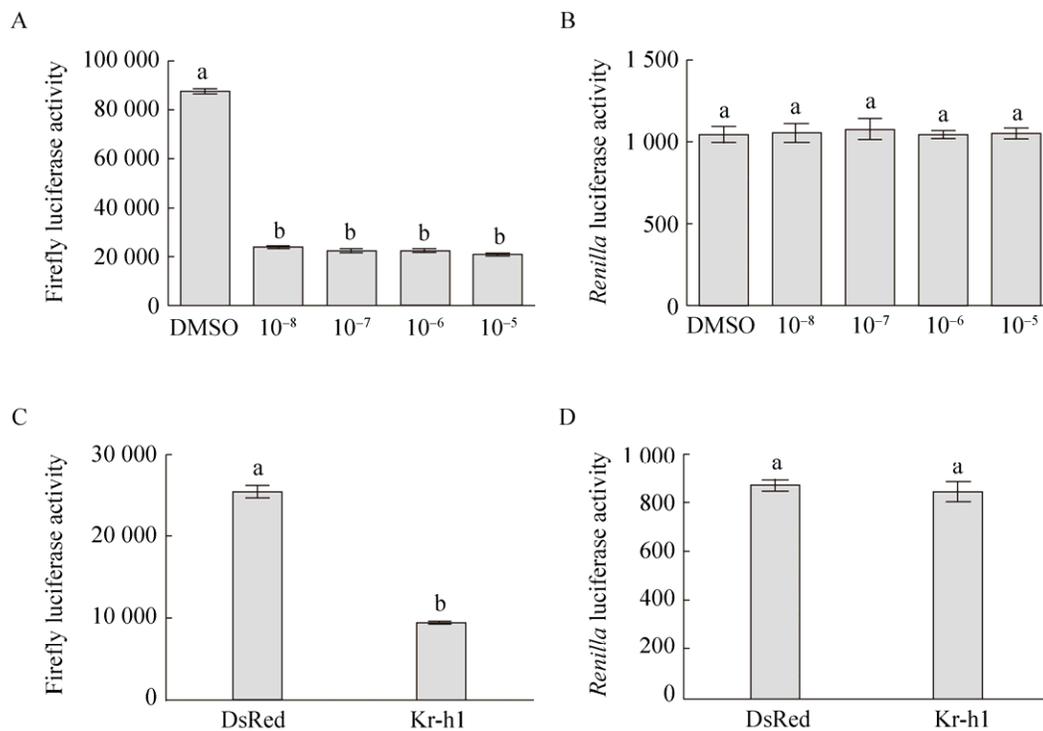


图5 保幼激素及保幼激素信号通路中关键基因 Kr-h1 对 pRL-VgP78M 内参质粒荧光素酶活性的影响

Fig. 5 Effects of JHA and the key gene of the JH pathway, Kr-h1 on the luciferase activity of pRL-VgP78M. (A, B): Effects of different concentrations of JHA on the luciferase activity of pGL3-BmE93P-LUC (A) and pRL-VgP78M (B) in BmE-SWU1 cells. (C, D): Effects of the over expression vector psl1180-A4-BmKr-h1 SV40 on the luciferase activity of pGL3-BmE93P-LUC (C) and pRL-VgP78M (D) in BmE-SWU1 cells. The treatment concentrations of JHA were 10<sup>-8</sup>, 10<sup>-7</sup>, 10<sup>-6</sup> and 10<sup>-5</sup> mol/L, and DMSO concentration was 0.1%. The error bars represent the  $\bar{x} \pm s$  (n=6). The significance of the difference between data sets was calculated using a two-tailed Student's *t*-text; Bars with the different letters are significantly different ( $P < 0.05$ ).

of the dual luciferase reporter gene system for hormones research in silkworm cells. To generate pRL-VgP78M, we replaced the promoter and intron sequences of the pRL control reporter plasmid with the VgP78M promoter. Although the intron sequence enhanced the activity of the control reporter plasmid, the intron sequence contained some transcriptional elements<sup>[13,19]</sup>, which might affect the activity of the promoter. We analyzed the expression stability of pRL-VgP78M by single luciferase activity in the silkworm cells, and found the results of the single luciferase system were not as accurate as the dual luciferase reporter gene system. In order to minimize the inconsistency between each experiment, the assay was repeated by six wells in each experiment to guarantee the reliability of the results.

The majority of genes in silkworm, a model of Lepidoptera insects, are regulated by hormones, which mainly include 20E and JHA<sup>[23]</sup>. Thus, we first studied the stability of the control reporter plasmid pRL-VgP78M, and then analyzed the effect of 20E and JHA on the expression activity of pRL-VgP78M. We demonstrated that the control reporter plasmid pRL-VgP78M was expressed stably, and its expression activity was not induced by hormones. Based on these results, we believed it could be used as a control reporter plasmid for gene expression regulation study in common silkworm cell lines. The p-IE1-Rlucp control reporter plasmid could also be used in the study of silkworm cells, however the activity of its promoter was relatively high, which is not suitable for the study of low-expressing promoters. Meanwhile, there is an ecdysone response element in the promoter of IE1, a baculovirus immediate-early gene<sup>[24]</sup>. An IE2 promoter was also constructed in another control reporter plasmid, pIZT-rluc<sup>[25]</sup>; however, the activity of the IE2 promoter was 5–10 times stronger than that of IE1, rendering it less suitable for low-expressing promoter study. In comparison, the activity of control reporter plasmid pRL-VgP78M we constructed was moderate and considered to be more suitable for the gene regulation research in silkworm cells.

Vitellogenin exists widely in insects, raising the possibility that the control reporter plasmid pRL-VgP78M we constructed might be widely applicable in other insect cells. At present, there are no other elements in the sequence of the promoter VgP78M, it can be expressed stably in BmE-SWU1 and BmN-SWU1 cells and its expression activity was not induced by 20E and JHA. Notably, pRL-VgP78M also exhibited stable expression in the *Spodoptera frugiperda* cell line Sf9 (Fig. 6).

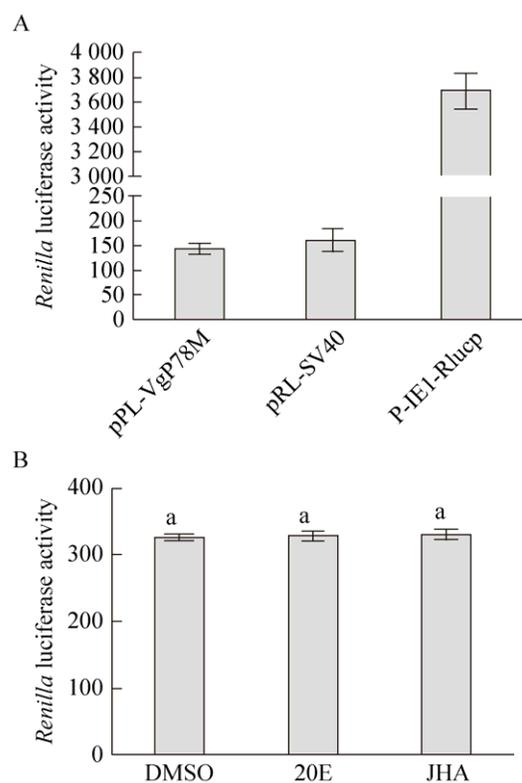


图6 pRL-VgP78M内参质粒在sf9细胞中稳定表达

Fig 6 The control reporter plasmid pRL-VgP78M was expressed stably in sf9 cells. (A) *Renilla* luciferase activity of pRL-VgP78M control reporter plasmid was detected at 24 h after transfection, pRL-SV40 and the commonly used silkworm control reporter plasmid p-IE1-Rlucp were used as positive control. (B) The expression activity of pRL-VgP78M was not affected by 20E or JHA, the treatment concentrations of 20E and JHA was  $10^{-6}$  mol/L, DMSO concentration was 0.1%. The error bars represent  $\bar{x} \pm s$  ( $n=6$ ). The significance of the difference between data sets was calculated using a two-tailed Student's *t*-test; Bars with the different letters are significantly different ( $P<0.05$ ).

Thus pRL-VgP78M may be expressed stably in other insect cell lines. However, Whether pRL-VgP78M could be used as a common control reporter plasmid of the dual luciferase reporter gene system across insect cell lines requires further confirmation.

The construction strategy and method of the control reporter plasmid pRL-VgP78M used in this study can provide good reference for the construction of the required control reporter plasmid. First, the gene promoter of the species itself would be predicted. Secondly, the potential transcription factor binding sites would be mutated to obtain constitutively-expressed promoter. Finally, the promoter of original control reporter plasmid would be replaced with the modified promoter. This method is relatively simple and can be applied to the construction of a control reporter plasmid of the dual luciferase reporter gene system in most species. Because the promoter is from the species itself, it has high potential to be constitutively expressed in its native cell type even after modification.

## 4 Conclusion

In conclusion, pRL-VgP78M was expressed stably in the common cell lines of silkworm but was not induced by 20E and JHA. It could be used as the control reporter plasmid of the dual luciferase reporter gene system in silkworm cell lines, especially for hormone research. Furthermore, the construction method of the control reporter plasmid pRL-VgP78M could be referenced for the construction of other control reporter plasmids.

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