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Functional analysis of transcriptional regulation of herpes simplex virus type 1 tegument protein VP22

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The herpes simplex virus type 1 (HSV-1) tegument proteins have important functions in the viral replication process. In order to investigate the role of the HSV-1 tegument protein VP22 in viral replication, its transcriptional regulation of viral promoters was investigated using the chloramphenicol acetyltransferase (CAT) assay. The results indicate that VP22 exerts a dose-dependent transcriptional inhibitory effect on the HSV-1 *α4*, *TK*, and *gC* gene promoters. VP22 had the capacity to repress transcriptional activation of promoters via different viral transcription regulatory factors such as VP16 and ICP0, as evidenced by the specific repression of the *TK* and *gC* gene promoters by ICP0. In addition, VP22 was capable of inhibiting the promotion of ICP0 transcriptional activation in the presence of HAT PCAF, which is even more remarkable than the VP22 repression of ICP0 transcriptional activation. Finally, the transcriptional inhibitory effect of VP22 on other viral promoters was demonstrated by the analysis of β-galactosidase activities in internal controls.

herpes simplex virus type 1, tegument, VP22, transcriptional regulation

As a DNA virus with a rather complex structure, the herpes simplex virus type 1 (HSV-1) has a distinctive feature of having more than 20 tegument proteins between its capsid shell and its envelope[1]. The current data demonstrate that these tegument proteins tend to display multiple biological functions in the viral replication process[2,3]; for example, VP16 plays a major role in transcriptional initiation of the α gene via its interaction with the cytokines Oct1 and HCF in viral gene linear transcriptional replication[4]. Another essential tegument protein, VP22, has received notable attention for its large number of molecules in each polypeptide per virion (2400 molecules/virion)[5]. It has been reported that VP22 from HSV-1 and HSV-2 are highly homologous proteins. During the viral infection process, VP22 interacts with VP16 [6] and locates to the cell nucleus where it binds to chromatin[7], which suggests their potential involvement in the regulation of cell acetylation in other viruses. The bovine herpesvirus 1 (BHV-1) tegument protein VP22, which is highly homologous to HSV-1 tegument protein VP22, was found to have the capacity to bind to histones in cells and decrease the acetylation level of histone H4 [8]. It has been shown that HSV-1 tegument protein VP22 specifically inhibits nucleosome assembly by binding to template-activating factor I (TAF-1)[9]. In the interpretations of all the above data, the assumption is that VP22 functions during viral cell infection, but the fact remains that VP22 may also be involved in the transcriptional regulation of viral genes. However, it is difficult to obtain evidence for the putative function of VP22 in viral transcriptional regulation; an *in vivo* systematic functional analysis of VP22 is

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challenging due to a limited understanding of the complex interactions between the viral protein and its associated cellular molecules. In the present study, a chloramphenicol acetyltransferase (CAT) assay is employed for the general biological characterization of VP22 as a functional protein in the transcriptional system. We analyzed the function of VP22 in the transcriptional regulation of viral α, β and γ gene promoters, focusing on the regulatory effects of VP22 on essential transcriptional regulatory viral proteins such as VP16 and ICP0 in the HSV-1 infection process. Preliminarily studies were also conducted to determine the effects of VP22 on the transcriptional regulatory process via its interactions with histone acetyltransferases (HAT) PCAF. Taken together, out results provide a more detailed understanding of the functions of VP22 in the viral transcriptional regulatory process.

1 Materials and methods

1.1 Cells, viruses and plasmids

African green monkey kidney cells line Vero and HSV-1 were provided in this laboratory. Eukaryotic expression plasmids pcDNA3-VP16, pcDNA3-ICP22 and pcDNA3/HA-PCAF expressing VP16, ICP22 and PCAF, respectively, were constructed in this laboratory. Eukaryotic expression plasmid pDR27 expressing ICP0 cDNA was donated by Prof. Peter O'Hare. Plasmids pCAT3-Basic, pCAT3-Enhancer and pSV-β-Galactosidase were purchased from Promega Company.

1.2 Main reagents

DMEM media, PCR reagents and restriction enzymes, and Lipofectamine 2000 were purchased from GIBCO, Takara, and Invitrogen, respectively. *n*-butyl coenzyme A was obtained from Promega, and [H^3]-chloromycetin was obtained from China isotope. σ-nitrophenyl β–*D*-galactopyranoside (ONPG) and complete/incomplete Freund's adjuvant were purchased from Sigma. PVDF membranes were purchased from Shanghai Huashun, and the secondary sheep anti-mouse Ig-G antibody marked by HRP was purchased from Boster.

1.3 Extraction of viral genome

PBS-rinsed Vero cells were infected with HSV-1 at m.o.i. 0.01 for absorption at 37℃ for 30 min. Serum free DMEM media was added for continuous culture until CPE occurred. SDS lytic solution and protease K were added to the harvest and incubated at 37℃ for 2 h. Protein was removed by phenol-chloroform extraction followed by ethanol precipitation for obtaining the viral genome.

1.4 Construction of recombinant plasmids

1.4.1 VP22 eukaryotic expression plasmid. The UL49 gene encoding VP22 was obtained by PCR amplification. The HSV-1 genome was used as a template, and the upstream and downstream primers were 5′-ATAGAA TTCATGACCTCTCGCCGC-3′ and 5′-ATTGAATTCT CACTCGACGGGCCG-3′, respectively. The UL49 gene was subsequently ligated into pcDNA3 via *Eco*RI digestion to construct the VP22 eukaryotic expression plasmid pcDNA3-VP22, whose identity was confirmed by enzymatic digestion and DNA sequencing.

1.4.2 VP22 prokaryotic expression plasmid. The UL49 gene sequences (base pairs 769—906) were amplified by PCR with pcDNA3-VP22 as the template, and 5′-ATCGGATCCAAGAGTTGGTGAATCCA-3′ and 5′-ATTGAATTCTCACTCGACGGGCCG-3′ as the upstream and downstream primers, respectively. This was followed by the ligation of UL49 into pGEX-5X-1 via *Bam*HI and *Eco*RI digestion to construct the prokaryotic expression plasmid pGEX-C45 comprising 45 amino acids of the carboxy terminal of VP22. The identity of the VP22 prokaryotic expression plasmid was confirmed by enzymatic digestion and DNA sequencing.

1.4.3 CAT reporter gene expression plasmid. The immediate-early *α4* gene promoter sequence (332 bp), *TK* gene promoter sequence (249 bp) and late gene *gC* promoter sequence (180 bp) were amplified by PCR with the HSV-1 genome as template, and the respective primer pairs: 5′-AGCGAGCTCGGGCCCCGCCCCCT GC-3′ and 5′-AGCAGATCTGGATCCGTGTCGGCAG CC-3′, 5′-GTTAGATCTTATCTTGTCACCCGGAGG-3′ and 5′-TATAGATCTGAGGCCACACGCGTCAC-3′, and 5′-CGCAGATCTAATAAAAGGCATTAGTCCC-3′ and 5′-TATAGATCTACCTCCACACGGACCACC-3′. Subsequent ligations into the pCAT3-Basic plasmid or pCAT3-Enhancer plasmid produced the CAT reporter gene expression plasmids pCAT-α4, pCAT-TK and pCAT-gC enhancer.

1.5 Cell transfection

Vero cells grown in a six-well plate to 90% confluence were transfected with different combinations of CAT reporter gene expression plasmids and regulatory protein

expression plasmids by Lipofectamine 2000. Different quantities of pcDNA3 were transfected to each well to maintain the same total amount of DNA in every well. The group transfected with only CAT reporter gene expression plasmids and pcDNA3 plasmids were used as background controls. The group transfected with the same quantity of pSV-β-Galactosidase per well were used as internal controls for deviations generated by different transfection efficiencies. Tables 1－3 show the different combinations of plasmids transfected into each well.

1.6 CAT activity analysis and β–galactosidase activity detection

After Vero cells were transfected by plasmids and incubated at 37℃ for 44 h. The CAT and β-galactosidase (β-gal) activities in Vero cell extracts in each group were detected respectively by CAT enzyme assay system and β-gal enzyme assay system, according to standard protocols. With regard to the analysis of the VP22 protein, VP22 repressed the expression of the bacterial LacZ gene in the plasmid when pSV-β-Galactosidase was used as an internal control. This was not surprising, since it is well-known that VP22 generally inhibits transcription. Consequently, it was not possible to useβ-gal activity values as normalization for CAT activity values in this experiment. Instead, the relative CAT activity and β-gal activity values in each experimental group were calculated respectively by defining the CAT activity and β-gal activity values detected by pcDNA3 plasmid and CAT reporter gene expression plasmid transfections as 100. The results presented are the mean values obtained from three independent experiments.

1.7 Expression and purification of proteins

The BL21 bacterial strain transfected with the pGEX-C45 plasmid expressed the GST-C45 fusion protein by IPTG induction. The purified protein was obtained by SDS-PAGE electrophoresis and electroelution from an excised gel band that contained a protein with the corresponding molecular weight.

1.8 Preparation of VP22 antibody

The same volumes of the purified GST-C45 fusion protein and complete Freund's adjuvant were emulsified and mixed for multiple abdominal subcutaneous injections into Kunming mice. The mice were boosted with incomplete Freund's adjuvant emulsifications in weeks 2

Table 1 Different combinations of plasmids for detecting transcriptional effects of VP22 on HSV-1 promoters

Plasmids (μg)	Group 1	Group 2	Group 3	Group 4
pcDNA3	1.5	1	0.5	
pcDNA3-VP22		0.5	1	1.5
pCAT-α4 (or pCAT-TK or pCAT-gC enhancer)	1	1	1	1
pSV-β-Galactosidase	1	1	1	1

Table 2 Different combinations of plasmids for detecting VP22 effects on transcriptional function of different HSV-1 transcriptional regulators on different promoters

Plasmids (μg)	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
pcDNA3	2	1		1		1	
pcDNA3-VP22			1		1		1
pcDNA3-VP16		1	1				
pDR27				1	1		
pcDNA3-ICP22						1	1
pCAT-α4 (or pCAT-TK or pCAT-gC enhancer)	1	1	1	1	1	1	1

Table 3 Different combinations of plasmids for detecting VP22 effects on promotion of ICP0 transcriptional activation by PCAF

Plasmids (μg)	Group 1	Group 2	Group 3	Group 4	Group 5
pcDNA3	3	2	2	1	
pcDNA3-VP22					1
pDR27		1		1	1
pcDNA3/HA-PCAF			1	1	1
pCAT-TK or pCAT-gC enhancer	1	1	1	1	1

and 4. Blood was taken from the eyeball 2 weeks after the 3rd immunization for preparation of the VP22 anti-sera.

1.9 Western blot analysis

Vero cells incubated for 44 h after pcDNA3 or pcDNA3-VP22 plasmid transfections were rinsed thrice in PBS, then scraped and treated with RIPA lytic solution and incubated in an ice bath for 30 min. Protein supernatants were collected by centrifugation. Equal volumes of protein supernatants were subjected to SDS- PAGE electrophoresis and transferred to a PVDF membrane followed by a 5% BSA-TBST block. Western blot analysis was performed using VP22 anti-sera as the primary antibody, and sheep anti-mouse IgG-HRP as the secondary antibody for the ECL reaction.

1.10 Statistical analysis

The experimental data are presented as mean values ± standard deviations.

2 Results

2.1 Construction of eukaryotic expression plasmids

The eukaryotic expression plasmids pcDNA3-VP22, pcDNA3-VP16, pDR27, pcDNA3-ICP22 and pcDNA3/HA-PCAF expressing VP22, VP16, ICP0, ICP22 and PCAF, respectively, were constructed with the CMV promoter. Our previous findings demonstrated that HSV-1 regulatory proteins with transcriptional inhibitory functions, such as ICP22, had no effect on the CMV promoter[10]. Therefore, the target genes are assumed to be efficiently expressed in these constructed eukaryotic expression plasmids.

2.2 Expression of VP22 in Vero cells

Western blot analysis of transfected Vero cell extracts using VP22 anti-sera showed the expression of VP22 in pcDNA3-VP22-transfected cells (Figure 1), which suggests that VP22 has no transcriptional inhibitory effects on the CMV promoter. As a result, CAT assays were performed on each experimental group for further analysis.

2.3 Transcriptional inhibitory effects of VP22 on viral promoters

The potential transcriptional regulatory function of VP22 was investigated by CAT activity assays. The VP22 expression plasmid pcDNA3-VP22 was co-transfected into Vero cells with the CAT reporter gene expression plasmids pCAT-α4, pCAT-TK and pCAT-gC enhancer constructed with the HSV-1 α, β and γ gene promoters, respectively. The results indicated that VP22 not only produced a transcriptional regulatory effect on these three promoters, but that this effect was dose- dependent to a certain extent (Figure 2). In order to control for the deviations created by different transfection efficiencies in this experiment, pSV-β-Galactosidase was used as an internal control for normalizing CAT activity values to transfected β-gal activity values attained under the same conditions. The results corresponded to previous observations: VP22 had a transcriptional regulatory effect on pSV-β-Galactosidase SV40 early gene promoter, and exhibited a remarkable dose-effect relationship to some extent (data not shown). Therefore, we abandoned this approach and attempted instead to correct the deviations by controlling experimental conditions and performing statistical analysis on our results.

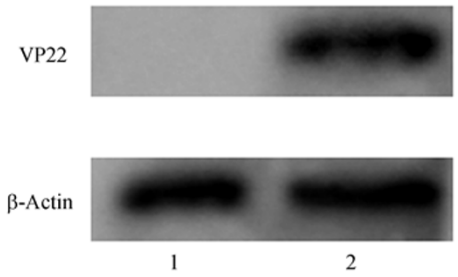

Figure 1 Western blot analysis. 1, Vero cells transfected by pcDNA3; 2, Vero cells transfected by pcDNA3-VP22.

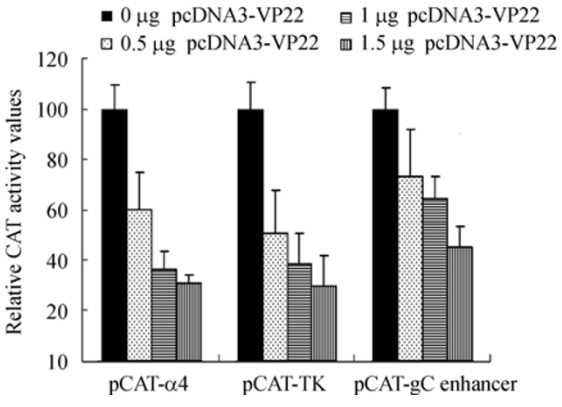

Figure 2 Dose-response curve for the transcriptional repression of VP22 on HSV-1 promoters. 1 μg CAT reporter gene expression plasmid was cotransfected into Vero cells with different amounts pcDNA3-VP22. The amount of transfected DNA was kept constant by addition of pcDNA3 in every well. The relative CAT activity values in each experimental group were calculated respectively by assigning 100 to CAT activity values detected by 1 μg CAT reporter gene expression plasmids and 1.5 μg pcDNA3 transfections.

2.4 VP22 inhibits transcriptional activation of different viral transcriptional regulatory factors on different viral promoters

The transcription of HSV-1 genome is initiated via the cooperation of many transcriptional regulatory proteins in the virus during cell infection. This includes the transcriptional activation of VP16 on the α gene[11], transcriptional promotion of VP16 and ICP0 on the β gene[12] and transcriptional enhancement of ICP0 on the γ gene[13], during which many viral transcriptional regulatory factors and cytokines work cooperatively to create optimal regulation. Consequently, investigating the transcriptional inhibitory effects of VP22 in cooperation with other viral regulatory factors would directly facilitate our understanding of the mechanisms of HSV-1 infection. By using CAT assays, the comprehensive effects of VP22 on three viral promoters were studied in the presence of VP16, ICP0 and ICP22. Taken together, our results indicate that VP22 can reduce, to some extent, the transcriptional activation effect of VP16 on the *α4* promoter, and slightly reduce the basic transcriptional level of ICP0. However, VP22 had no effect on the transcriptional function of ICP22 (Figure 3(a)). With regard to the analysis of the HSV-1 β gene *TK* promoter, VP22 appears to exert some effect on the transcriptional activation of VP16 with a lower inhibition (Figure 3(b)). As concerns ICP0, which has an essential function in β gene transcription, VP22 was observed to significantly decrease the efficiency of its transcriptional initiation (over 50% decrease, see Figure 3(b)). In the analysis of the γ gene *gC* promoter, VP22 inhibition of the transcriptional activation of ICP0 is further evidenced (a decrease of over 60%, see Figure 3(c)).

2.5 VP22 is likely to inhibit promotion of ICP0 transcriptional activation by HAT PCAF

The acetylation of histone and some transcriptional regulatory factors in cell lines is recognized to be an important component in cellular transcriptional regula-

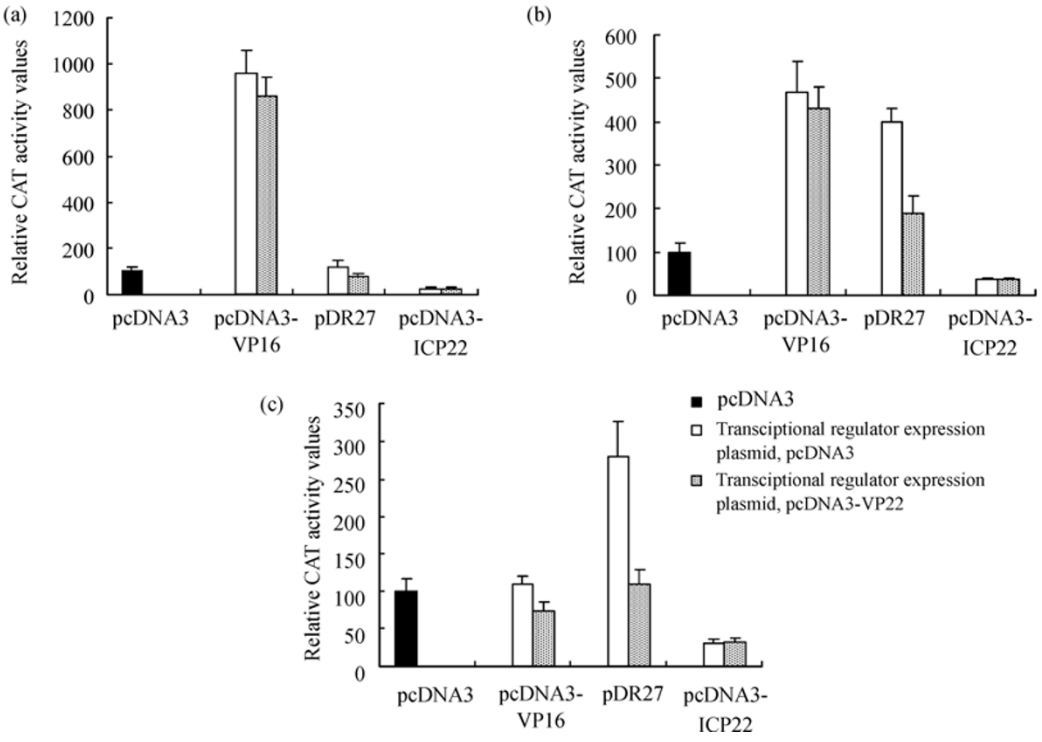

Figure 3 The VP22 effects on transcriptional function of different viral transcriptional regulators. (a) 1 μg pCAT-α4 and 1 μg transcriptional regulator expression plasmids were cotransfected into Vero cells with 1 μg pcDNA3 or pcDNA3-VP22. The relative CAT activity values in each experimental group were calculated respectively by assigning 100 to CAT activity values detected by 1 μg pCAT-α4 and 2 μ g pcDNA3 transfections. (b) 1 μg pCAT-TK and 1 μg transcriptional regulator expression plasmids were cotransfected into Vero cells with 1 μg pcDNA3 or pcDNA3-VP22. The relative CAT activity values in each experimental group were calculated respectively by assigning 100 to CAT activity values detected by 1 μg pCAT-TK and 2 μg pcDNA3 transfections. (c) 1 μg pCAT-gC enhancer and 1 μg transcriptional regulator expression plasmids were cotransfected into Vero cells with 1 μg pcDNA3 or pcDNA3-VP22. The relative CAT activity values in each experimental group were calculated respectively by assigning 100 to CAT activity values detected by 1 μg pCAT- gC enhancer and 2 μg pcDNA3 transfections.

tion[14]. The potential involvement of VP22 and ICP0 in acetylation[15–17] provided the impetus for investigating the putative functions of PCAF with VP22 in promoting ICP0 transcriptional activities. In this experiment, the effects of VP22 were further studied by the analysis of PCAF on the transcriptional activation of ICP0 on *TK* and *gC* genes. The results showed an enhanced promotion of ICP0 transcriptional activation on *TK* and *gC* promoters in the presence of PCAF (Figure 4). Remarkably, this promotion was eliminated in the presence of VP22 (Figure 4).

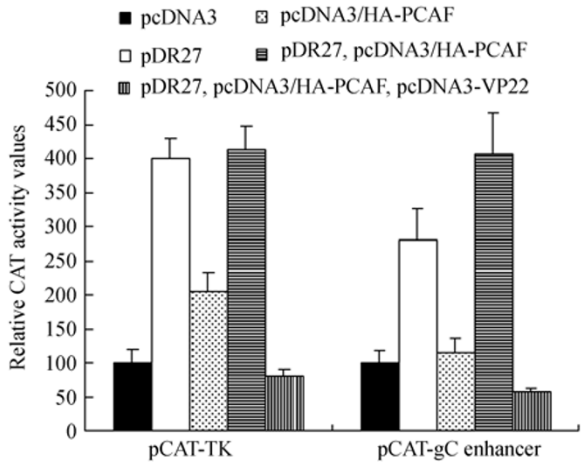

Figure 4 Inhibitive effects of VP22 on promotion of ICP0 transcriptional activation by PCAF. 1 μg pCAT-TK or pCAT-gC enhancer was cotransfected into Vero cells with different combinations of 1 μg pDR27, 1 μg pcDNA3/HA-PCAF and 1 μg pcDNA3-VP22. The amount of transfected DNA was kept constant by addition of pcDNA3 in every well. The relative CAT activity values in each experimental group were calculated respectively by assigning 100 to CAT activity values detected by 1 μg CAT reporter gene expression plasmids and 3 μg pcDNA3 transfections.

3 Discussion

As essential proteins in the HSV-1 infection process, tegument proteins tend to have multiple functions in viral replication. Although there is little systematic knowledge regarding these more than 20 different kinds of tegument proteins, previous findings on their associated proteins, in particular VP16, have revealed their multiple functions in HSV-1 biological events. Previous studies on tegument protein VP22 indicate that VP22 is highly phosphorylated during HSV-1 infection[18], is correlated in its function to certain links during virion assembly[19], and has the capacity to stabilize the microtubule[20]. These observations imply that VP22 is involved in certain biological events in virus-directed intercellular transport and assembly. In addition, its phosphorylation and localization in the nucleus in the late infectious stage, association with cellular chromatin, and special relationship with the important viral transcriptional regulatory factor ICP0 [21] further suggest that VP22 is involved in the transcriptional regulatory process of viral genes. In light of the observations mentioned above, the involvement of VP22 in viral transcriptional regulation is the most likely interpretation of our results, despite the fact that the CAT activity assays in this study only revealed the VP22-mediated transcriptional inhibition from the standpoint of molecular biology. On the other hand, the functional diversity displayed by many HSV-1 viral proteins, and the dose-dependent relationship of transcriptional inhibition exhibited exclusively by VP22 on different viral gene promoters in this study (Figure 2) suggest that such a transcriptional inhibitory function has profound biological significance. This was further confirmed by the analysis of internal controls of β-gal activities transcribed by the SV40 early gene promoter.

A functional activity displayed by the VP22 protein alone in the cellular environment does not necessarily reflect its true function in the virus during cell infection. Therefore, studying the functions of VP22 in association with VP16, ICP0 and ICP22 in the transcriptional system is necessary for confirming the distinct transcriptional inhibitory effect exhibited by VP22. Despite the capacity of VP22 to bind VP16 [6], this binding fails to have any significant influence on the *α4* gene- and *TK* gene-related transcription promoted by VP16. The slight alteration generated by this binding cannot be addressed by the current data. Intriguingly, VP22 obviously affects the transcriptional activation, but eliminates the transcriptional promotion targeted exclusively at the *TK* and *gC* genes by ICP0. Interestingly, ICP0 displays a transcriptional activation function in a normal cellular environment (Figure 3), and markedly improves the promotion of transcription when the HAT PCAF level is increased (in comparison to cellular PCAF), which is evidenced by a more specific inhibitory effect promoted by VP22 on PCAF (Figure 4).

To summarize our results, we can first speculate that ICP0 has the capacity to utilize certain acetylation systems in its interaction with viral genes, mainly the *β*- and *γ* gene promoters, and is an essential transcriptional regulatory factor in the HSV-1 infection process, con-

sistent with published data[15–17] and our previous findings. Secondly, the mechanism by which VP22 blocks transcriptional activation of the *TK* and *gC* gene promoters by ICP0 via HAT PCAF remains unclear. Nevertheless, the CAT activity inhibitory effects observed in VP22, PCAF and ICP0 co-transfections are more distinct than that in the VP22 and ICP0 co-transfection (Figures 3 and 4), which most likely reveals certain associations between the inhibitory effects of VP22 and the expression of PCAF. Indeed, VP22 has been shown to bind non-specifically to DNA[9], although there has been no report on the binding of ICP0 to associated viral gene promoter sequences. Hence, it is reasonable to conclude that the transcriptional inhibitory effects of VP22 exhibited in the CAT activity assays are mediated by interactions with PCAF or ICP0 protein molecules, or are the modified effects resulting from these interactions. The details of the mechanisms involved require further investigation.

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