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Stress-Induced Cellular Transcription Factors Expressed in Trigeminal Ganglionic Neurons Stimulate the Herpes Simplex Virus 1 ICP0 Promoter

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***Alphaherpesvirinae* family members can reactivate from latency following stress. The synthetic corticosteroid dexamethasone induces certain cellular transcription factors in murine and bovine trigeminal ganglionic neurons. Three dexamethasone-induced transcription factors, Krüppel-like factor 15, Slug, and SPDEF, stimulated the herpes simplex virus type 1-infected cell protein 0 (ICP0) promoter more than 150-fold. Conversely, other viral promoters (VP16 and ICP4) were not strongly stimulated, suggesting that the ICP0 promoter is preferentially activated by dexamethasone-simulated stress.**

The primary site of latency for herpes simplex virus type 1 (HSV-1) is sensory neurons in trigeminal ganglia (TG) (1–3). Abundant viral protein expression and infectious virus are not readily detected during latency, in contrast to the expression of the virus-encoded latency-associated transcript that occurs in latently infected sensory neurons (1–3). The ability of herpes simplex virus 1 (HSV-1) to reactivate from latency is crucial for virus transmission and recurrent disease. Increased stress levels correlate with an increased incidence of reactivation from latency (1–3). Dexamethasone (DEX), a synthetic corticosteroid, increases the incidence of reactivation from latency in TG neuronal cultures prepared from latently infected mice (4) and stimulates reactivation from latency in TG organ cultures latently infected with HSV-1 (5). With respect to reactivation from latency, there are at least two important unresolved issues: (i) which viral genes might be involved in the initiation of reactivation and (ii) whether the cascade of viral gene expression during reactivation is the same as productive infection of cultured cells (i.e., immediate early [IE] to early [E] to late [L]). Recent studies have suggested that VP16, a late transcript, stimulates reactivation from latency (6, 7), presumably because VP16 stimulates IE gene expression. The viral IE protein ICP0 also stimulates reactivation from latency (8–10), whereas others have concluded that ICP0 is not required for reactivation from latency (11–13). Several studies have also proposed that E gene expression and DNA replication occur prior to IE gene expression during reactivation from latency (14–17). Finally, viral gene expression is reported to be initially disorganized during explant-induced reactivation from latency (5, 18). Regardless of whether VP16 or ICP0 is involved or required for reactivation from latency, it is reasonable to predict that stimulus-specific cellular transcription factors may stimulate viral gene expression during the early stages of reactivation.

DEX consistently initiates bovine herpesvirus 1 (BHV-1) reactivation from latency in calves and rabbits (1, 2, 19–22), which culminates in lytic cycle viral transcription in neurons within 6 h after treatment (23, 24). We identified DEX-inducible cellular factors in bovine TG neurons during the early phases of reactivation from latency (25). A subset of these cellular genes consists of transcription factors (Fig. 1A gives a summary of the DEX-inducible transcription factors). One of the highly induced transcription factors, promyelocytic leukemia zinc finger (PLZF), stimulated BHV-1 productive infection more than 20-fold. Two other DEX-inducible transcription factors, Krüppel-like factor 15 (KLF15) and KLF4, stimulated BHV-1 productive infection and *trans* activated the bICP0 E promoter approximately 100-fold. In contrast, PLZF and SPDEF (SAM pointed domain containing Ets transcription factor) stimulated the L BHV-1 gC promoter to a higher degree than the bICP0 E promoter and the IE transcription unit 1 promoter. Members of the KLF family, to which PLZF belongs, can repress or activate transcription, and they regulate cell growth, differentiation, apoptosis, and cancer (reviewed in references 26, 27, and 28). KLF and Sp1 family members may be involved in regulation of HSV-1 transcription, including certain aspects of the latency-reactivation cycle, because these family members bind GC-rich sequences (reviewed in references 27 and 29) and many HSV-1 promoters contain Sp1 binding sites and Sp1 activates IE promoters (30).

Based on the above observations, we hypothesized that DEX-inducible transcription factors identified in bovine TG neurons (25) might stimulate HSV-1 promoters that would be important for mediating stress-induced reactivation from latency. We initially tested the ICP0 promoter because ICP0 expression stimulates reactivation from latency (8–10), the ICP0 promoter is stimulated by hyperthermic stress (31), and BHV-1 IE and E promoters that drive bICP0 expression are *trans* activated by certain DEX-inducible transcription factors (25). For these studies, we used a full-length (FL) ICP0 promoter construct that contains sequences spanning −800 to +150 relative to the ICP0 transcription initiation site (31) (Fig. 1B). Numerous transcription factor-binding sites are located in the FL ICP0 promoter, suggesting that more than one cellular transcription factor may regulate its activ-

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Transcription Factor	Fold induction after DEX (hours)	
Slug (Snail homolog 2)	10.7 (1.5)	15.4 (3)
PLZF	16.6 (3)	9.4 (6)
SPDEF	6.4 (3)	6.4 (6)
KLF15	3.9 (3)	3.0 (6)
KLF4	> 10 fold in one calf	
KLF6	> 10 fold in one calf	
GATA6	6 fold in one animal at 3 and 6 h	

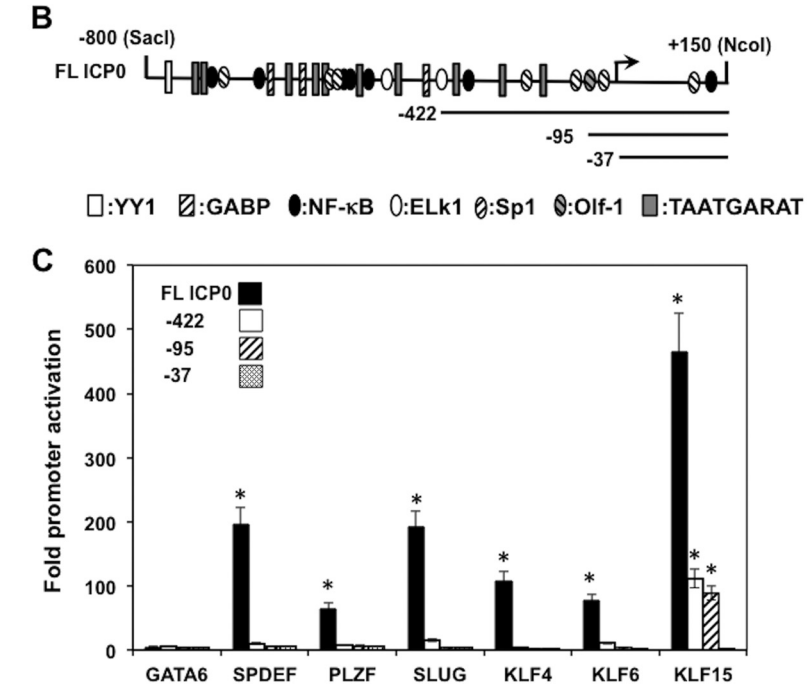

FIG 1 Effect of DEX-inducible transcription factors on ICP0 promoter activity. (A) Cellular transcription factors identified in TG neurons following DEX treatment (25). Fold induction was determined by microarray studies and then confirmed by reverse transcription-PCR (RT-PCR) and immunohistochemistry. KLF4, KLF6, and GATA6 expression was stimulated in 1 of the 3 animals, whereas the other transcription factors were stimulated in 3/3 animals. Plasmids expressing the respective transcription factors were previously described and were shown to express similar levels of protein in transfected Neuro-2A cells (25). (B) The full-length ICP0 promoter (FL ICP0) and the three deletion constructs used in this study. A subset of the known transcription factor binding sites in the FL ICP0 promoter was previously described, and their locations are shown (31). An arrow denotes the start site of ICP0 mRNA. The ICP0 promoter luciferase reporter constructs were obtained from Priscilla Schaffer and were previously described (31). (C) Luciferase activity at 40 h after Neuro-2A cells were cotransfected with the designated ICP0 promoter luciferase reporter construct (1.0 μg DNA) and a DEX-inducible transcription factor (0.5 μg DNA). Transfection of Neuro-2A cells was performed as previously described (25, 48, 49). Luciferase activity was normalized by comparing *Renilla* luciferase levels, which are regulated by a simple TATA box. The results are the average of data from 5 independent experiments. An asterisk denotes significant differences ($P < 0.05$) in cells transfected with the designated ICP0 luciferase plasmid and the designated DEX- inducible transcription factor, as determined by the Student *t* test.

ity, either independently or in a synergistic manner. Three additional deletion mutants, the −422, −95, and −37 mutants, were also examined. Mouse neuroblastoma cells (Neuro-2A) were used for these studies because they are of neuronal origin, they are readily transfected, DEX-inducible transcription factors are not abundantly expressed in Neuro-2A cells, and plasmids expressing the DEX-inducible transcription factors are active in these cells (25). KLF15 stimulated FL ICP0 promoter activity more than 400-fold in Neuro-2A cells (Fig. 1C). The −95 ICP0 construct was activated nearly 100-fold by KLF15, whereas the −37 ICP0 construct was not *trans* activated. SLUG and SPDEF stimulated the FL ICP0 promoter nearly 200-fold, while PLZF, KLF6, and KLF4 but not GATA6 *trans* activated the FL ICP0 promoter at least 50-fold. In contrast to KLF15, the ICP0 deletion constructs were not responsive to these factors. Titration studies demonstrated that the concentrations of the respective DEX inducible transcription factors used for this study were optimal for activating the FL ICP0 promoter (data not shown). In summary, these studies demonstrated that DNA sequences located between −800 and −37 were responsive to KLF15 while sequences between −800 and −422 were responsive to SLUG, SPDEF, PLZF, KLF6, and KLF4.

The ICP0 promoter appeared to contain 2 separate KLF15 response elements: the first between −800 and −422 and the second between −95 and −37. Deleting DNA sequences between −800 and −422 reduced promoter activity 4- to 5-fold, whereas deletion of DNA sequences between −95 and −37 reduced promoter activity 80-fold. An ICP0 promoter deletion mutant virus that lacks −70 to −420 (32) does not reactivate from latency following hyperthermic stress (13), suggesting that KLF15-mediated *trans* activation of the ICP0 promoter via sequences in the −95 ICP0 construct are important for stress-induced reactivation from latency. These observations led us to hypothesize that KLF15-mediated *trans* activation of sequences in the −95 ICP0 construct are important for certain aspects of reactivation from latency. DNA

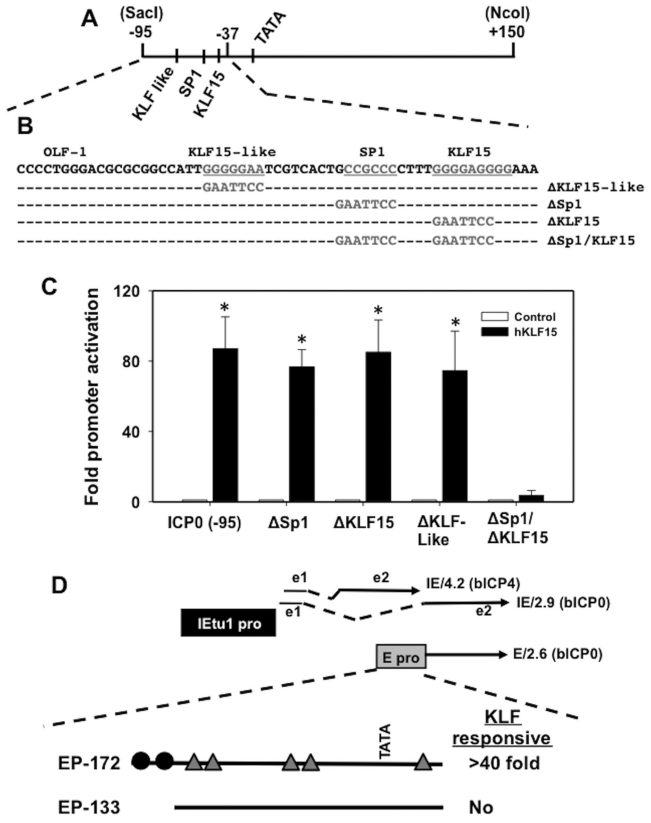

FIG 2 Identification of KLF15-responsive regions in the −95 ICP0 promoter construct. (A) Schematic of the −95 ICP0 promoter deletion and location of potential *cis*-acting motifs between −37 and −95 that might be important for KLF15 mediated *trans* activation. (B) ICP0 promoter DNA sequences that are located between −37 and −95. Locations of KLF15-like motif, Sp1, and KLF15 sites are denoted by underlined gray nucleotides. The respective mutant constructs contain an EcoRI linker located in the KLF15 site or the KLF15-like site. In addition, a construct containing EcoRI linker insertions in the Sp1 and KLF15 site was synthesized. Integrated DNA Technology (Iowa) synthesized the respective mutant −95 ICP0 promoters. The respective ICP0 mutant −95 promoter constructs were then cloned into the promoterless luciferase reporter construct (pGL3-Basic; Promega) using unique SacI and NcoI restriction enzyme sites. The ΔSp1 binding site mutant was previously described (31). (C) Neuro-2A cells were cotransfected with the designated −95 ICP0 mutant constructs and KLF15 or a control empty vector. At 40 h after transfection, luciferase activity was measured. Luciferase activity was normalized by comparing *Renilla* luciferase levels, which are regulated by a simple TATA box. The results are presented as fold induction relative to results for the empty vector control and are the averages of data from 3 independent experiments. An asterisk denotes significant differences ($P < 0.05$) in cells transfected with the designated ICP0 luciferase plasmid and the DEX-inducible transcription factor KLF15, as determined by the Student *t* test. (D) The positions of BHV-1 transcripts that encode bICP4 and bICP0 are shown. The immediate early transcription unit 1 (IEtu1) encodes bICP4 (IE/4.2) and bICP0 (IE/2.9) (50, 51). The IEtu1 promoter (denoted by the black rectangle) activates IE expression of IE/4.2 and IE/2.9. E/2.6 is the early transcript that encodes bICP0, and an early promoter (denoted by the gray rectangle) activates expression of the early bICP0 transcript (E/2.6) (36). Exon 2 (e2) of bICP0 contains all of the protein coding sequences of bICP0. The dashed lines are intron sequences. The ability of KLF4 and KLF15 to *trans* activate the bICP0 promoter constructs (EP-172 and EP-133) is also included and was summarized from a previous study (25).

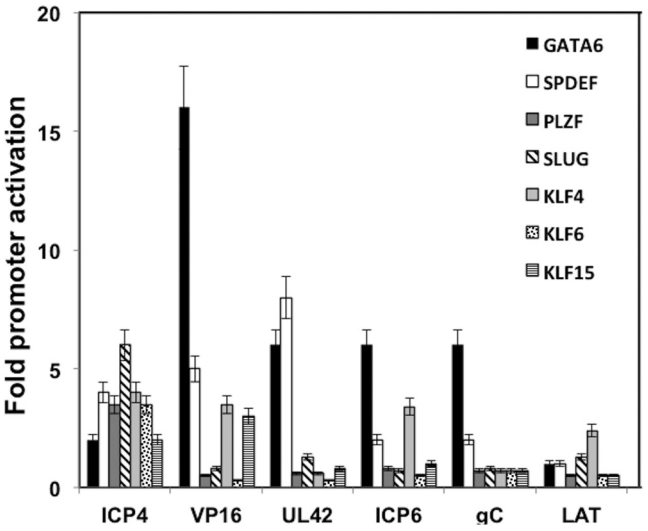

FIG 3 Effect of DEX-inducible transcription factors on additional HSV-1 promoters. The ICP4, VP16, UL42, ICP6, gC, and LAT promoter luciferase reporter constructs were previously described (31). Neuro-2A cells were cotransfected with 1 μg of the designated reporter plasmid and 0.5 μg of a plasmid expressing the designated DEX-inducible transcription factor. Levels of DNA were made the same in each transfection by adding the pcDNA3.1 empty vector. Transfection of Neuro-2A cells was performed as previously described (25, 48, 49). At 40 h after transfection, luciferase activity was measured. The numbers represent fold induction relative to results for the empty vector control. The results are the average of data from 4 independent experiments.

sequences between −37 and −95 contain three distinct motifs for KLF15-induced *trans* activation (Fig. 2A). These include a KLF binding site analogous to that located in the promoter of the gene encoding an interphotoreceptor binding protein (33), a KLF-like binding site, and a consensus Sp1 binding site. To identify DNA sequences between −95 and −37 that are responsive to KLF15, the core sequence of each potential KLF15-responsive motif was mutated and an EcoRI linker inserted (see Fig. 2B for a schematic of the respective mutants). In several independent experiments, the ΔSp1/KLF15 mutant was not *trans* activated by KLF15 (Fig. 2C). As expected, the wild-type (wt) −95 ICP0 construct was *trans* activated by KLF15 at least 80-fold. KLF15 also *trans* activated the ΔKLF15-like, ΔSp1, and ΔKLF15 promoter constructs approximately 80-fold. It is interesting to note that KLF15 and Sp1 have been shown to synergistically activate cellular promoters (34, 35), and synergistic activation correlates with the ability of KLF15 to stably interact with Sp1 (35). Since the KLF15 binding site in the ICP0 promoter is 4 bp from the 3′ end of the Sp1 binding site, it is possible that an interaction between SP1 and KLF15 is important for *trans* activation of the ICP0 promoter.

bICP0 RNA expression is regulated by an IE promoter (IEtu1) and a separate E promoter (36) (Fig. 2D). The IEtu1 and E bICP0 promoters were *trans* activated approximately 100-fold by KLF4 and KLF15 (25). In contrast to the ICP0 promoter, KLF4 *trans* activated both bICP0 promoters more efficiently than KLF15 (25). Although the KLF-responsive region of the IEtu1 promoter was not precisely localized, there are numerous SP1 binding sites and potential KLF binding sites in this promoter. The KLF15-responsive region present in the −95 ICP0 promoter is not present in the smallest IETu1 promoter *trans* activated by KLF4 or KLF15 (data not shown). Comparison of the sequences of the E bICP0 promoter construct that was not responsive to KLF4 and KLF15 (EP-133) to the sequence of the minimal bICP0 promoter construct responsive to KLF4 and KLF15 (EP-172) revealed that there is no

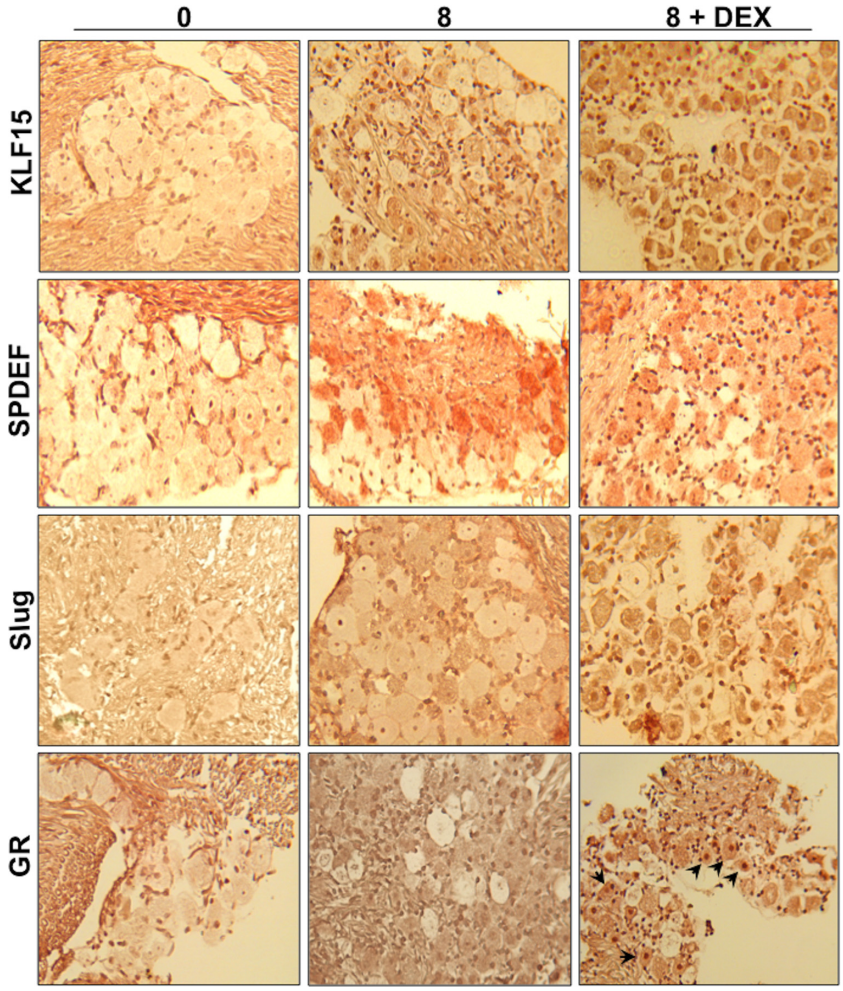

FIG 4 Examination of transcription factors that *trans* activated the ICP0 promoter in explanted mouse TG. TG from adult female Swiss Webster mice (Charles River Labs) were minced into four pieces/TG and either explanted in Eagle minimal essential medium (EMEM) containing 2% charcoal-stripped fetal bovine serum (FBS) or immediately formalin fixed. Normal FBS but not charcoal-stripped FBS contains glucocorticoids that activate the GR, which masks the effect of glucocorticoids (52). As indicated, certain TG samples were also incubated with DEX (100 μM) for 8 h. Samples marked "0" were TG extracted from mice and then immediately fixed (no explant). All samples were fixed in neutral buffered formalin, paraffin embedded, and thin section prepared. IHC was performed as previously described (25, 48, 52). The goat anti-KLF15 antibody (sc-34826), rabbit anti-Slug antibody (sc-67022), and rabbit anti-SPDEF antibody (sc-67022) were purchased from Santa Cruz Biotechnology. The rabbit anti-GR antibody (36605) was purchased from Cell Signaling. All antibodies were diluted 1:1,000. Vectastain ABC kits containing biotinylated goat anti-rabbit IgG (PK-6101; Vector Laboratories) or biotinylated rabbit anti-goat IgG (PK-6105; Vector Laboratories) were incubated with sections to allow visualization of TG neurons that were recognized by the respective antibody. Magnification, ×200.

Sp1 binding site or consensus KLF15 binding site present in the −95 ICP0 promoter (Fig. 2D) (25). However, there are several potential KLF binding sites (Fig. 2D, closed circles), and two Sp1 binding sites are 13 bp from the 5′ terminus of the EP133 promoter (denoted by triangles). Since KLF family members bind to multiple GC-rich and related CACCC sequences in DNA, reviewed in reference 29, it is not surprising that the KLF15-responsive sequences in the HSV-1 ICP0 promoter are not identical to KLF-responsive sequences in the BHV-1 ICP0 promoters.

The ability of the DEX-inducible transcription factors to *trans* activate additional HSV-1 promoter reporter constructs was also examined. These promoter constructs contain representative IE, E, or L promoters. The VP16 promoter (L) was stimulated by GATA6 (>15-fold), SPDEF (~5-fold), and KLF4 or KLF15 (4-fold) (Fig. 3). GATA6 also stimulated the UL42 (E), ICP6 (E), and gC (L) promoters (~5-fold). SLUG was the only transcription factor that stimulated the ICP4 (IE) promoter more than 5-fold. In contrast, LAT promoter activity was not *trans* activated by any of the DEX-inducible transcription factors more than 3-fold. Compared to the ICP0 promoter, the other HSV-1 promoters examined in this study were not efficiently *trans* activated by the DEX-inducible transcription factors.

Immunohistochemistry (IHC) studies were performed to determine whether explanted mouse TG expressed any of the transcription factors that strongly *trans* activated the ICP0 promoter (KLF15, Slug, and SPDEF) and whether DEX stimulated their expression. TG were excised from adult female Swiss Webster mice, and each TG was minced into 4 pieces (37, 38). At 8 h after explantation, more neurons were $KLF15^+$ and $SPDEF^+$ than at time zero, where explanted TG were immediately formalin fixed (Fig. 4). Many TG neurons were $KLF15^+$ and $SPDEF^+$ when explanted for 8 h in the presence of 100 μM DEX. $Slug^+$ neurons

were not readily detected at time zero or following explant for 8 h: however, Slug$^+$ neurons were readily detected in TG after explant for 8 h in the presence of DEX (Fig. 4). At 2 and 4 h after explant, lower numbers of KLF15$^+$, SPDEF$^+$, and Slug$^+$ neurons were detected even when incubated with DEX (data not shown). As expected, more neurons were glucocorticoid receptor positive (GR$^+$) at 8 h after explant, and many of these neurons exhibited nuclear GR staining when explanted TG were incubated with medium containing DEX (Fig. 4, GR panel, arrows). This result is expected because DEX activates the GR, resulting in nuclear localization (39, 40). A subset of TG neurons were weakly GR$^+$ at time zero, which was not surprising because approximately 30% of rat TG neurons express the GR (41). KLF15 expression is induced by DEX in human airway smooth muscle cells (42), suggesting that KLF15 expression is stimulated by corticosteroids in multiple tissues. In summary, these studies were consistent with the findings observed in bovine TG (25). However, it took longer for DEX to induce expression of these transcription factors in sensory neurons of explanted mouse TG.

Exogenous expression of ICP0, independent of other viral gene products, can initiate HSV-1 (8) or HSV-2 (43) reactivation from latency using an *in vitro* neuronal culture system. Furthermore, in the absence of VP16, ICP0 enhances the ability of transfected viral DNA to initiate productive infection in cell culture (44). These observations suggest that reactivation stimuli activate the ICP0 promoter as an important event during reactivation from latency. Consequently, it is not surprising that ICP0 promoter activity is stimulated by hyperthermic stress (31). In transgenic mice, the ICP0 promoter but not the ICP4 promoter is active in a subset of neurons at certain times after birth (45, 46), providing additional evidence that specific neuronal signaling pathways regulate ICP0 promoter activity. VP16, the *trans* activator of IE promoters (47), has also been proposed to initiate reactivation from latency (6, 7). Although the VP16 promoter was *trans* activated by GATA6 15-fold, stimulation by DEX-inducible transcription factors was in general minimal relative to findings for the ICP0 promoter. It remains possible that other specific cellular factors may *trans* activate the VP16 promoter during reactivation from latency or that low levels of VP16 in certain "permissive" neurons may promote reactivation from latency. It is also possible that certain reactivation stimuli, DEX for example, directly stimulate ICP0, leading to induction of viral gene expression, including that of VP16, and further amplification of viral gene expression by this viral activator. Irrespective of this, it seems unlikely that successful reactivation from latency (shedding of infectious virus following a reactivation stimulus) occurs in the absence of ICP0. In summary, our studies provide evidence that certain cellular transcription factors induced by DEX in bovine or mouse TG neurons, including KLF15, preferentially activate the ICP0 promoter and mediate certain aspects of the HSV-1 latency-reactivation cycle.

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