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Regulation of herpes simplex virus type 1 thymidine kinase gene expression by thyroid hormone receptor in cultured neuronal cells

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Herpes simplex virus type 1 (HSV-1) undergoes acute infection in epithelial cells followed by establishment of latency in the neurons of trigeminal ganglia. The latent virus maintains a dormant state and can recurs spontaneously, suggesting transcriptional silencing and reactivation occur in neurons. Computer data mining identified a nuclear hormone response element (NRE), the binding site for the thyroid hormone receptor (TR) or other nuclear hormone receptor, in the promoter of HSV-1 thymidine kinase (TK). TRs are transcription factors whose activity is dependent on their ligand thyroid hormone (T_3; triiodothyronine). We hypothesize that TR and T_3 exert regulation on HSV-1 gene expression in neurons. A neuroblastoma cell line expressing the TR isoform β (N2aTRβ) was utilized for *in vitro* investigation. Results showed that liganded TR repressed TK promoter activity but unliganded TR relieved the inhibition. The mutagenesis study demonstrated that one nucleotide mutation at the NRE abolished the T_3/TR-mediated regulation. N2aTRβ cells treated with T_3 were suppressive to TK expression and virus release but the removal of T_3 de-repressed TK expression and increased virus release, confirmed by reverse transcriptase–polymerase chain reaction (RT-PCR) and plaque assays, respectively. Chromatin immunoprecipitation (ChIP) assays showed that TRs were enriched at TK NRE in the presence of T_3. Additional results demonstrated that hyper acetylated histone H4 and monomethylated H3 modified at lysine 9 (H3K9me1) were enriched at transcriptionally active TK promoters but were dissociated from the NRE by T_3/TR. These results suggest that T_3 could regulate HSV-1 gene expression through its receptor via histone modification in cultured neuronal cells. *Journal of NeuroVirology* (2010) 16, 13–24.

Keywords: gene regulation; herpes simplex virus type-1; histone acetylation; histone methylation; neuronal cell; thymidine kinase; thyroid hormone receptor

Introduction

Herpes simplex virus type 1 (HSV-1) primary infection initiates when the virus invades epithelia and starts active gene expression and replication to produce progeny. The virus may subsequently establish life-long latency in the sensory neurons of trigeminal ganglia (TG). The reactivation may occur

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spontaneously. The recurrent infections can attack the anterior buccal mucosa, lips, or perioral area of the face and eyes (Fatahzadeh and Schwartz, 2007). HSV-1 gene expression in the lytic phase follows a cascade and is characterized by a sequential order. However, viral gene expression during latency differs completely from what is observed in acute infection and significant levels of transcription are detected from only one region of the viral genome, the latency-associated transcripts (LAT) (Javier *et al*, 1988; Jones, 2003; Bloom, 2004). In addition, the profile of viral gene expression during reactivation is likely to be different, in which the cascade is disrupted. For example, thymidine kinase (TK), a β gene, could be expressed concurrently or before the expression of α genes (Kosz-Vnenchak *et al*, 1993; Tal-Singer *et al*, 1995; Nichol *et al*, 1996).

A variety of mechanisms have been proposed to describe the establishment of viral latency and reactivation, such as altered immune response (Bystricka and Russ, 2005; Koelle and Corey, 2008), LAT-mediated antiapoptosis (Perng *et al*, 2000; Bloom, 2004; Peng *et al*, 2004; Branco and Fraser, 2005; Carpenter *et al*, 2007), microRNA-induced gene silencing (Cui *et al*, 2006; Umbach *et al*, 2008) differential neuronal suppression (Block *et al*, 1994; Su *et al*, 2000, 2002; Moxley *et al*, 2002), hormonal regulation (Garza and Hill, 1997; Hardwicke and Schaffer, 1997; Noisakran *et al*, 1998; Marquart *et al*, 2003), and repressive chromatin (Kubat *et al*, 2004a; Amelio *et al*, 2006; Bedadala *et al*, 2007; Chen *et al*, 2007; Pinnoji *et al*, 2007; Knipe and Cliffe, 2008). In an effort to discover new transcription factor binding sites, our sequence analysis revealed two nuclear hormone response elements (NREs) located in the promoter regions of TK, suggesting that thyroid hormone and its receptor (TR) and/or other nuclear hormone receptors may have a role in HSV-1 TK regulation and could control viral latency and reactivation.

The thyroid gland produces two hormone products, thyroxine (T_4) and triiodothyronine (T_3), from thyroglobulin protein (Deme *et al*, 1976). Although triiodothyronine (T_3) is produced less, it is more potent and responsible for the majority of biological effects. T_3 exerts its action via its nuclear receptors (TRs). They are transcription factors belonging to the superfamily of nuclear hormone receptors, usually dimerized with RXR (Tsai and O'Malley, 1994). It has been shown that both liganded and unliganded TRs are involved in gene regulation.

The present studies target the interrelationship of T_3, TRs, and chromatin structure during HSV-1 gene regulation in neuronal cells. The gene regulation of TK was analyzed in neuroblastoma cells N2a and N2aTRβ (N2a cell constitutively expressing TRβ). The effect of TR and T_3 on histone modification and cofactor recruitment to the TK promoter was analyzed. Our results indicated that TR can control the TK promoter activity and this regulatory effect is determined by its ligand via distinct histone modifications. This is the first *in vitro* evidence showing that TR exerted epigenetic regulation on HSV-1 gene expression in neuronal cells and could play a role in the complex processes of establishing latency and reactivation of HSV-1.

Results

Identification of HSV-1 TK NRE

We identified NREs in the HSV-1 genome located from 47915 to 47932 (5′-tatta **AGGTCA**cgcgtg-**TGGCCT**-3′; Figure 1A) based on the HSV-1 complete genome sequence (accession no.: X14112). The sequence is represented as inverted repeats with six-nucleotide spacing (IR6). These IR6 NREs are located between the TATA box (47933–47937) and the transcription initiation site (47911). The sequence in this context was suggested to function as negative NRE (TR response element) in neural cells (Park *et al*, 1993). Negative NRE is defined as that liganded TR exerted transcription repression and unliganded TR reversed the inhibition. To investigate this ligand/receptor-mediated regulation, a point mutation was introduced adjacent to TK TATA box to disrupt the IR6 TK NRE. The resulting plasmid was named pRL-mTK (Figure 1B).

HSV-1 TK promoter was repressed by TR in the presence of T_3

To determine the regulatory effect of TR on HSV-1 TK promoter, N2a and N2aTRβ cells were cotransfected with pRL-TK and pEGFP in the absence or presence of T_3. The Luc assays showed that TK promoter activity from pRL-TK was reduced to 34% by T_3 in N2aTRβ cells but exhibited no significant difference in N2a cells (Figure 2A). In addition, pRL-mTK showed no significant repression by T_3/TR compared to its wild-type counterpart (Figure 2A). Control experiments using reporter plasmids containing HSV-1 ICP4 promoters showed no regulatory effect by T_3 and TR (Figure 2B), confirming that TR/T_3- mediated regulation required NRE. Fluorescent microscopy demonstrated that the transfection efficiency was similar between N2a and N2aTRβ cells, suggesting that these two cells are equivalent in this respect (Figure 2C). These results indicated that HSV-1 TK NRE behaved as negative TR response element and exert down-regulation on the promoter by TR and T_3. Unliganded TR, in contrast, maintained TK promoter activity.

Viral infections were performed to investigate TR-mediated regulation. N2a and N2aTRβ cells were infected with strain 17Syn$^+$ EGFP at multiplicity of infection (m.o.i.) = 10 with or without T_3. Reverse transcriptase–polymerase chain reaction (RT-PCR) assays revealed that at high m.o.i. of 10, no regulatory

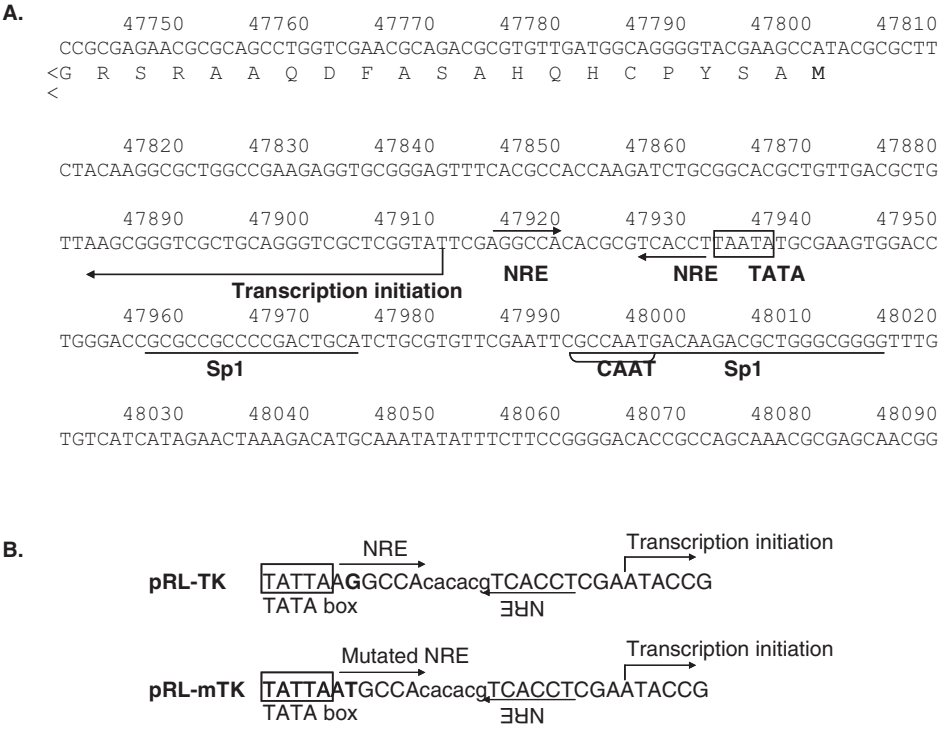

Figure 1 HSV-1 TK Promoter, NREs, and mutagenesis. (**A**) Negative NREs were identified in the HSV-1 TK promoter. The NREs were organized as inverted repeats with six-nucleotide spacing (IR6) and located immediately after the TATA box. Transcription initiation site, Sp1 elements, and CAAT box were shown. (**B**) A point mutation was introduced into TK NRE by PCR mutagenesis using plasmid pRL-TK as template. The NRE AGGCCA adjacent to TK TATA box was altered to ATGCCA to disrupt NREs. The resulting plasmid was named pRL-mTK.

effect was observed (Figure 3A). However at low m.o.i. of 0.1, TK promoter activity, but not ICP4, was found to be repressed by liganded TR at 48 h post infection (p.i.) (Figure 3B). Semiquantitative analyses showed that TK transcription was reduced 60% by liganded TR (Figure 3C). Fluorescent microscopy showed that the efficiency of infections was similar between N2a and N2aTRβ cells, indicating again that these two cell lines were comparable and equivalent in this matter (Figure 3D). Taken together, these findings demonstrated that HSV-1 TK was negatively regulated by liganded TR in the neuronal cells.

Removal of T_3 activated TK expression and induced the virus release

N2aTRβ exhibits neuronal-like differentiation upon T_3 treatment (Lebel *et al*, 1994), providing a biological relevance to the current study. To address the regulatory effect of T_3/TR on viral replication, N2a and N2aTRβ cells were treated for 5 days with T_3 followed by infection at m.o.i. = 0.1. RT-PCR assays showed that the T_3/TR-mediated TK repression was reversed upon washout of T_3 at 48 h p.i. (Figure 4A, lane 6). The plaque assays indicated that the removal of T_3 increased the virus release 60-fold more than the cells being treated with T_3 (Figure 4B). The *P* value measured by a Student's paired *t* test with a two-tailed distribution was .0015, indicating a significant increase. There was no significant change of virus release in N2a cells upon T_3 removal (Figure 4B). These results suggested that subtraction of hormone abolished the TK repression and the enzyme presumably induced viral replication in resting cells and enhanced the replication and virus release.

TRβ as well as RXR was recruited to the TK promoter

TRβ overexpression was previously verified by Northern blot analysis (Lebel *et al*, 1994) but the protein level was not assessed. To confirm the protein expression, extracts from N2a and N2aTRβ cells were subjected to Western blot analyses. The results supported the Northern blot analysis that TRβ is overexpressed in N2aTRβ but not N2a cells (data not shown). Earlier studies showed that TRβ was bound to TK promoter *in vitro* (Park *et al*, 1993). The *in vivo* interaction was therefore analyzed by chromatin immunoprecipitation (ChIP) using specific TRβ antibody (Ab) and the results demonstarted that TRβ was enriched at the TK NRE in the presence of ligand (Figure 5A, compare lanes 2 and 3), suggesting that liganded TR occupancy is preferred at the negative NRE of HSV-1 TK promoter. These observations also raised the hypothesis that TR dimerization may play a role in the occupancy and regulation. Recruitment of TR partner RXR to

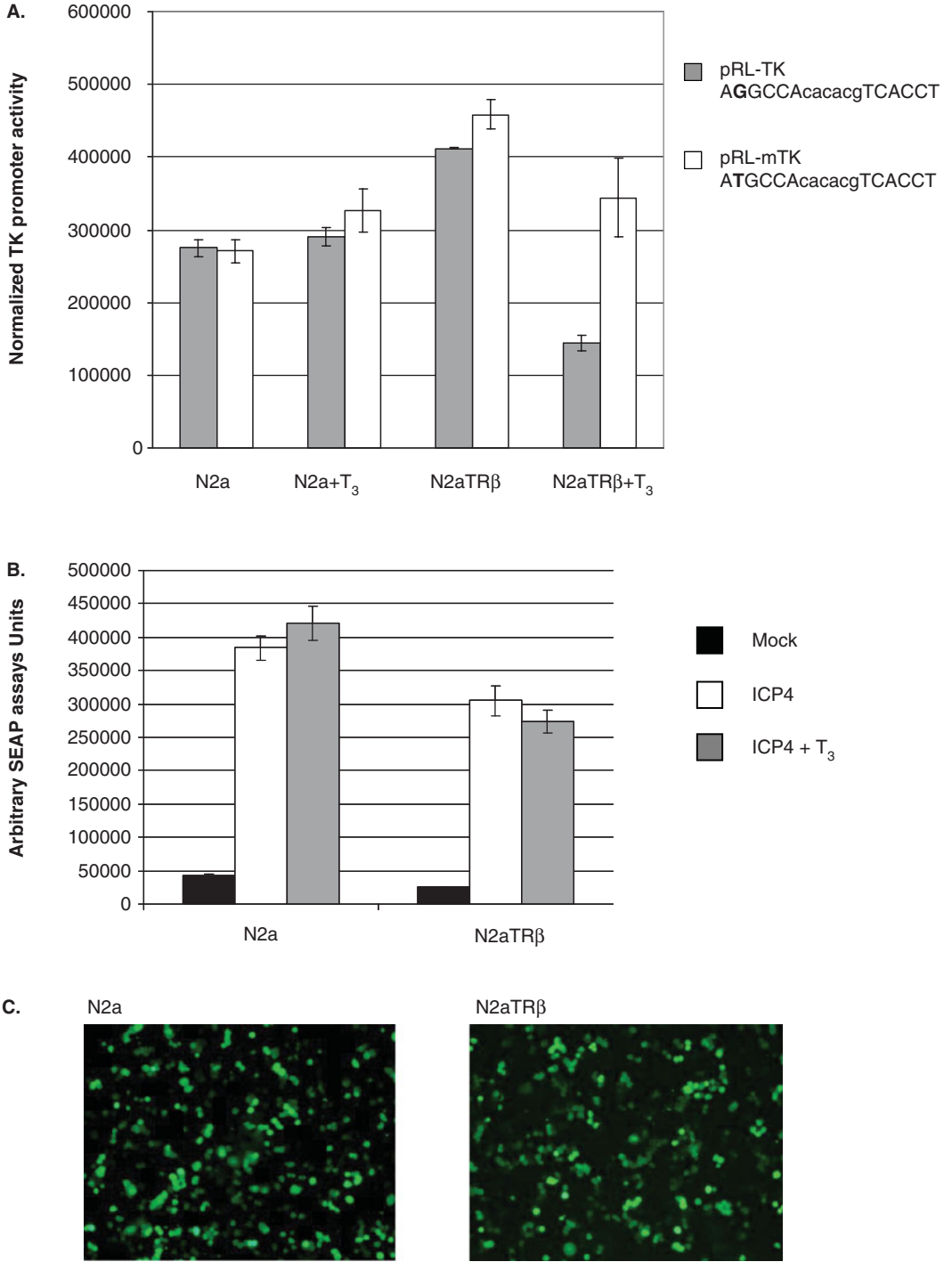

Figure 2 HSV-1 TK transcription was negatively regulated by TR and T_3. (**A**) N2a and N2aTRβ cells were cotransfected with plasmids by Nucleofector II with triplication to analyze the regulatory effects of TR and T_3 on TK promoter. Cells were subjected to luciferase assay using Promega Luciferase Assay Kit at 48 h post transfection. The signal was measured by luminometer. The results showed that T_3/TR repressed the TK promoter activity from pRL-TK (*gray bar*) to 34% but exhibited no significant difference if the TK NREs were mutated (*white bar*: pRL-mTK). Error bars represent the mean plus or minus the standard deviation. (**B**) Cells were cotransfected as described in **A** with pICP4-SEAP followed by SEAP reporter assays (Materials and Methods). ICP4 promoter activity was not changed upon T_3 addition. (**C**) Plasmid phMGFP was cotransfected as efficiency control. The similarity of green cells indicated that these two cells were equivalent in the respect of transfection.

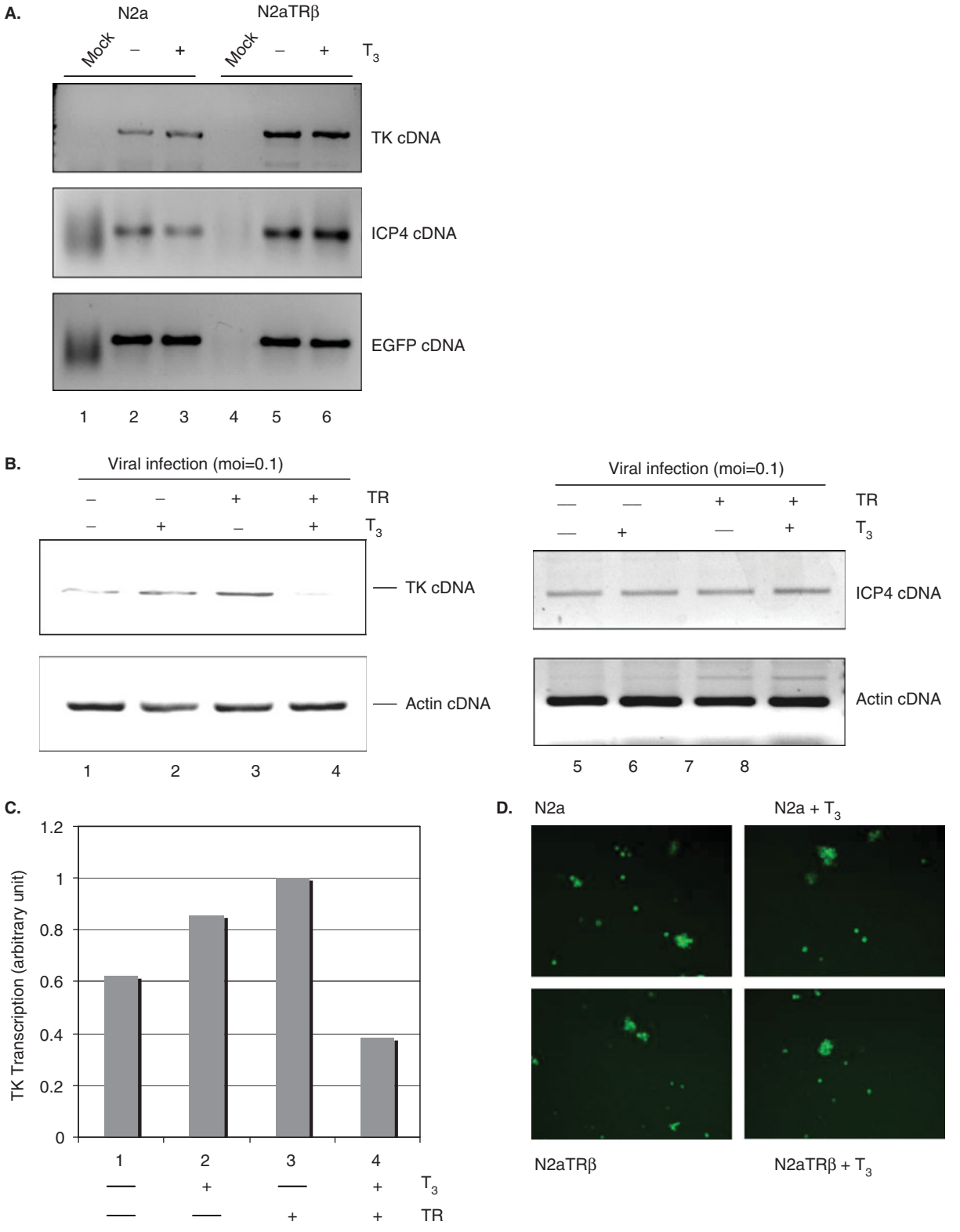

Figure 3 HSV-1 TK was repressed by liganded TR during infections. (**A**) N2a and N2aTRβ cells were infected with wild-type virus 17Syn$^+$ EGFP at m.o.i. = 10 in the absence or presence of T_3. The total RNA was purified and subjected to RT-PCR by primers against TK and ICP4. EGFP primers were used as controls. The assays showed that T_3/TR had no effect on transcription of TK and ICP4 at m.o.i. of 10. (**B**) N2aTRβ cells were infected with wild-type virus 17Syn$^+$ EGFP at m.o.i. = 0.1. The total RNA was purified at 48 h p.i. then subjected to RT-PCR using primers against TK, ICP4, and actin. The results showed that TK transcription was repressed by the addition of T_3 (compare lanes 3 and 4). ICP4 was not regulated by liganded TR. (**C**) Semiquantitative analysis demonstrated that TK transcription was reduced to 40% by liganded TR when the cells were infected at m.o.i. = 0.1. (**D**) Infection of cells by 17Syn$^+$-EGFP was examined by fluorescent microscopy and revealed no difference of infection efficiency between N2a and N2aTRβ cells.

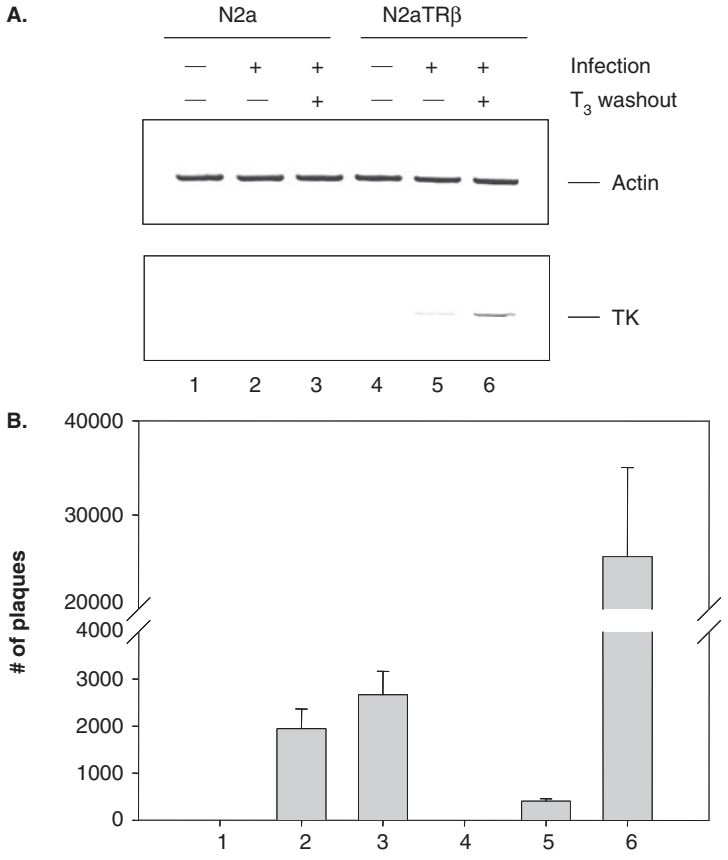

Figure 4 Long-term T_3 treatment prevented the TK expression and the washout of T_3 reversed the TK repression and increased virus release. (**A**) Cells were pretreated with T_3 for 5 days and subsequently infected with $17Syn^+$-EGFP viruses at m.o.i. of 0.1. Total RNA was isolated at 48 h p.i. and subjected to RT-PCR assays using TK primers. RT-PCR assays using actin primers were performed as internal control. (**B**) The media of infected cells were collected 48 h p.i. and subjected to plaque assays using CV-1 cells to investigate the release of infectious viruses.

TK NRE was therefore analyzed and the results showed that RXR exhibited strong binding to the TK NRE, presumably as RXR/RXR homodimers, while the TR was absent (Figure 5A, compare lanes 2–3 and lanes 5–6). When the TR was present, RXR was enriched at TK NRE as TR/RXR heterodimers when T_3 was available (Figure 5A, compare lanes 2 and 3), suggesting that RXR can be recruited to TK NRE by liganded TR to exert T_3-mediated regulation. Unliganded TR reduced the RXR occupancy to TK NRE, possibly by interacting with RXR (Figure 5A, compare lanes 2 and 5). Together, these results showed that liganded TR formed heterodimer with RXR and exerted T_3-mediated regulation. Unliganded TR exhibited much less binding to TK NRE, which may be responsible for the formation of open chromatin or decreased steric hindrance.

Active histones were dissociated from the TK promoter by liganded TR

The T_3/TRβ-mediated histone modification was investigated by ChIP using antibodies against acetyl histone H4, a chromatin associated with active promoter. The results indicated that histone H4 acetylation was reduced by liganded TR (Figure 5B, lanes 2 and 3). Immunoglobulin (Ig) control showed that the results were specific because ChIP using IgG revealed little signal (Figure 5B). The liganded TR–mediated H4 hypoacetylation required intact TK NRE because alteration of NRE exhibited no change (Figure 5B and C). Another active chromatin monomethylated histone H3K9 (H3K9me1) was also analyzed by ChIP and the results indicated that it was enriched at the TK promoter with correct IR6 NRE in the absence of T_3 but reduced when T_3 is present (Figure 5D, lane 3). Altogether, these results demonstrated that liganded TR decreased the association of active histone at the TK promoter and contribute to, at least in part, the gene regulation by forming closed chromatin.

Discussion

This study focused on the roles of TR and its ligand T_3 in the regulation of HSV-1 TK. Our results demonstrated that the transcription factor TR utilized its ligand to exert repression on TK in neuronal cells.

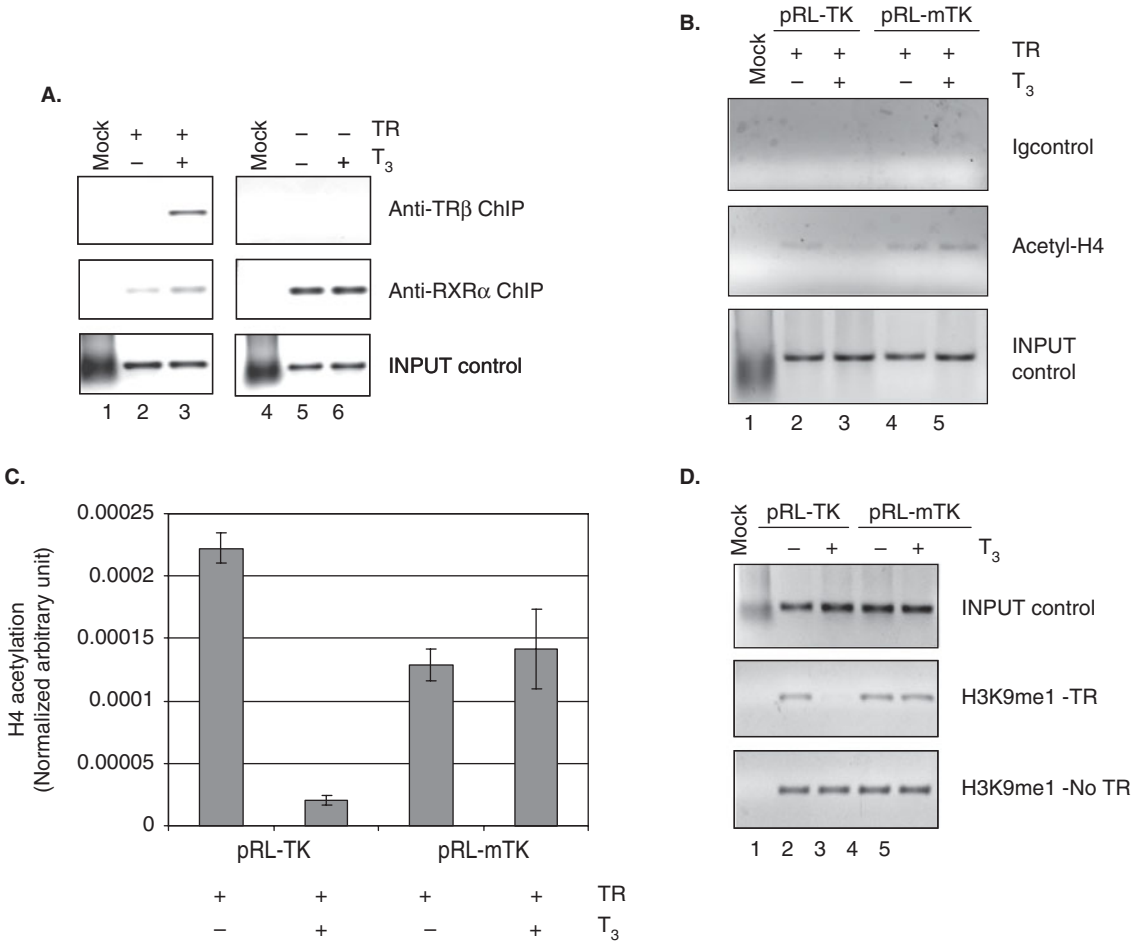

Figure 5 Liganded TRβ was recruited to the TK promoter and active chromatin was released by T_3/TR at TK promoter. (**A**) N2a and N2aTRβ cells were transfected with pRL-TK and treated as noted. The transfected cells were subjected to ChIP after 48 h of transfection. Anti-TRβ or -RXRα Abs were used for investigation. (**B**) ChIP using Ab against acetylhistone H4 was performed to investigate histone modification at TK promoter (original and mutated versions). Anti-goat IgG Ab was used as control indicating the specificity. (**C**) Semiquantitative analyses by comparing chemiluminescent signals showed that liganded TR reduced the histone H4 hyperacetylation at original TK promoter (pRL-TK). One nucleotide alteration within TK NREs abolished this regulation (pRL-mTK). (**D**) ChIP using Ab specifically against monomethylated H3K9 (H3K9me1) demonstrated that H3K9me1 was enriched at TK promoter without T_3 but reduced by the addition of ligand. TK promoter containing NRE alteration exhibited no change.

Liganded TR interacted with the TK NREs and induced histone deacetylation and released monomethylated H3K9 from the promoter for transcription repression. The NRE sequence context is critical, since one nucleotide alteration from AGGCCA to ATGCCA abolished this T_3-mediated regulation. In addition, the removal of T_3 can de-repress HSV-1 TK transcription and enhanced the virus release from the cells. These results provide evidence suggesting that the binding of TR/T_3 to HSV-1 NREs played roles in HSV-1 gene regulation and replication in neuronal cells.

T_3 participates in development and homeostasis in adults (Evans, 1988). Both sensory neurons and peripheral glial cells in dorsal root ganglia have been shown to possess specific T_3 binding sites (Walter and Droz, 1995). The presence of TR in sensory neurons indicates that the feedback regulation of circulating T_3 could occur by binding to the receptors (Glauser and Barakat Walter, 1997). T_3 can affect different processes involved in the survival, differentiation, and maturation of neurons (Walter, 1996). In a physiological concentration, T_3 enhances neurite outgrowth of primary sensory neurons in cultures, possibly in combination with nerve growth factor, to regulate the expression of cytoplasmic dynein, a protein that is involved in retrograde axonal transport (Barakat-Walter and Riederer, 1996). Many procedures that were routinely used to induce HSV-1 reactivation also reduced the level of T_3. For example, whole-body hyperthermia was reported to decrease the level of serum T_3 by 50%, probably due to the suppression of thyroid stimulating hormone release, monodeiodination alteration of T_4 from T_3 to reverse T_4, or enhanced T_3 clearance (Shafer *et al*, 1980).

Hyperthermia can be used to trigger HSV-1 reactivation in the mouse latency model (Sawtell and Thompson, 1992). Dexamethasone, which initiated HSV-1 reactivation (Higaki *et al*, 2002), also reduced T_3 level (Vigneri *et al*, 1975). Therefore, T_3 is likely to participate in the regulation and maintenance of viral latency and reactivation.

The role of T_3 in HSV-1 gene regulation has not been extensively investigated. Park *et al* reported that the addition of T_3 induced the HSV-1 TK promoter by 4- to 5-fold in pituitary rat cells by transient transfection (Park *et al*, 1993). Nevertheless, the authors indicated that similar NREs are found in several genes that were characterized to mediate negative regulation in brain via transcription complex occlusion (Chatterjee *et al*, 1989). Our transfection assays indicated that HSV-1 TK was repressed by T_3 in neuroblastoma cells stably expressing TR. This result was further supported by the infection experiments showing that TK, a β gene, was inhibited by liganded TR at m.o.i. of 0.1. TR-mediated negative regulation was not as clear as the positive regulation. Many hypotheses have been proposed such as the binding to transcription factor II D (TFIID) (Crone *et al*, 1990), inhibition of Sp1 stimulation (Xu *et al*, 1993), hetero- to homodimer conformational change (Bendik and Pfahl, 1995), recruitment of histone deacetylases (HDAC) (Sasaki *et al*, 1999), recruitment of chromatin insulator protein (Burke, Zhang *et al*, 2002), cAMP response element binding (CREB) competition (Mendez-Pertuz, Sanchez-Pacheco *et al*, 2003), TATA binding protein (TBP) recruitment (Sanchez-Pacheco and Aranda, 2003), repression of S-phase genes (Nygard, Wahlstrom *et al*, 2003), Sp1 competition (Villa *et al*, 2004), interaction of GATA2 and TRAP220 dissociation (Matsushita *et al*, 2007), and weak binding of unliganded TR to negative NRE (Berghagen *et al*, 2002; Shibusawa *et al*, 2003). We tested these hypotheses and showed that weak occupancy of unliganded TR that led to open chromatin in the vicinity of TATA box and transcription initiation site may be responsible for the regulation. Our results further indicated that acetylated histone H4 and H3K9me1, a histone associated with the active promoter (Barski *et al*, 2007), were released at TK promoter by liganded TR. It is likely that liganded TR released active histones and recruited repressive chromatin/histones, thereby induced closed chromatin to occlude the access of transcription complex.

The roles of TK in neurons were studied for HSV-1 infection. TK is required to provide dNTP for viral replication in resting cells such as neurons. Furthermore, viral replication is required for efficient α and β expressions in neurons during reactivation (Nichol *et al*, 1996). Based on these observations, TK was suggested to initiate α transcription and subsequent replication during reactivation (Tal-Singer *et al*, 1995). Moreover, Tal-Singer *et al* (1997) reported that during *in vivo* reactivation in TG, TK was one of the first genes to be expressed. Another report showed that a TK mutant exhibited greatly reduced α and β expression during reactivation (Kosz-Vnenchak *et al*, 1993). Based on these results, TK was suggested to play a critical role during viral reactivation. However, another report indicated that TK as well as α genes were expressed at the same time (Pesola *et al*, 2005). Although the role of TK during reactivation is ambiguous, it is likely that T_3 has the potential to control TK expression and control viral transcription. To test the hypothesis, we utilized a unique cell culture model, N2aTRβ. T_3 treatment has been shown to induce these cells to differentiate, mimicking the state of neurons in brain (Lebel *et al*, 1994). The results supported our hypothesis that removal of T_3 by washout derepressed the TK expression and significantly increased virus release, presumably by producing dNTP and inducing viral replication. It is noted that TK transcript was more abundant in the N2aTRβ than N2a cells of T_3 washout experiments (Figure 4A). It is further determined that viruses were released from the T_3-treated N2aTRβ cells (Figure 4B). Further infection experiments with appropriate mutations in TK promoter are underway to investigate the T_3-mediated regulation of viral replication/transcription.

The current studies provide evidence that strongly implicates the roles of T_3 and TR in the regulation of HSV-1 gene expression in cultured neuronal cells. The current hypothesis is that the liganded TR inhibited viral replication by repressing the expression of TK and other critical viral genes through the recruitment of repressive chromatin (Figure 6) and these outcomes led to the maintenance of latency. Nonetheless, while the T_3 concentration was low, unliganded TR relieved the TK repressions and promoted the viral gene expression and replication. The active replication and increased transcription of α genes would lead to the subsequent reactivation. More experiments, especially using specific mutants and animal models, are necessary to identify key mechanisms involved in the establishment of latency and reactivation of HSV-1.

Materials and methods

Viruses, cell lines, and culture conditions

The N2a cell, a mouse neuroblastoma cell line, was purchased from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% charcoal-treated fetal bovine serum (FBS). N2aTRβ cell was a gift from Dr. Robert Denver (University of Michigan, Ann Arbor, MI) and was grown under the same conditions. CV-1 cells were cultured in 10% FBS supplemented with DMEM. All cells were maintained in an incubator at 37°C with 5% CO_2. The titers of HSV-1 strains $17Syn^+$, $17Syn^+$-EGFP (Foster *et al*, 1998), and McKrae were

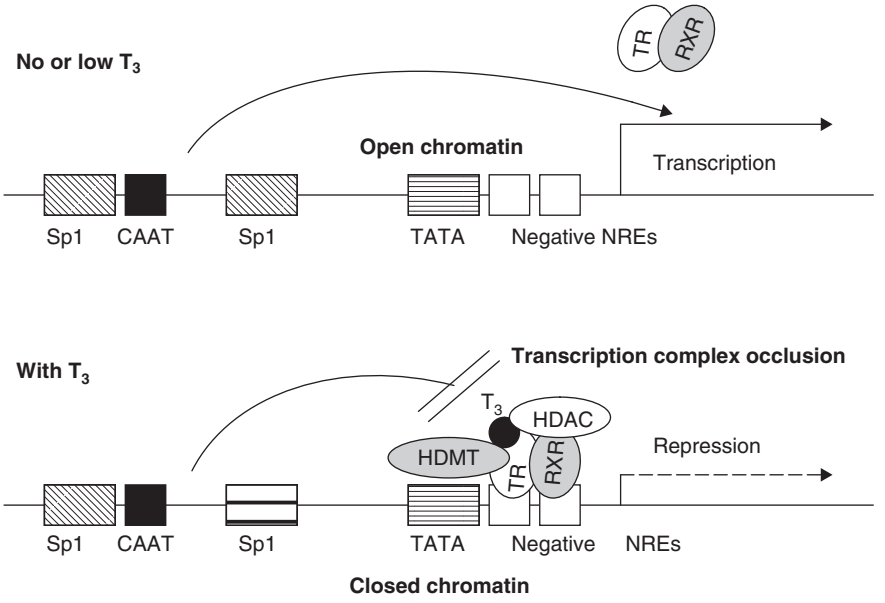

Figure 6 A model for T_3/TR-mediated TK regulation. In the absence of T_3, open chromatin allows the occupancy of transcription complex to TK promoter for active transcription. T_3 presence recruits TR/RXR to the TK NREs and facilitates histone modification, resulting in the establishment of closed chromatin/steric hindrance and therefore leading to transcription repression.

determined on CV-1 cells. T_3 was purchased from Sigma (St. Louis, MO).

Plasmids and construction of plasmid with mutated NRE

The pRL-TK vector was purchased from Promega (Madison, WI; catalog no.: TB240). It contains 722 bp of the HSV-1 TK promoter (Figure 1A). Monster Green Fluorescent Protein phMGFP Vector (Promega, catalog no.: E6421) was used as a transfection efficiency control. Plasmid pICP4-SEAP contains reporter secreted alkaline phosphatase (SEAP) driven by ICP4 promoter (Pinnoji *et al*, 2007) and was used for normalization in Figure 2. Plasmid pRL-TK was used as a template for PCR mutagenesis. Primers were designed in such a way that the one of the primer contains the mutated NRE site. Mutation was created by changing only one nucleotide from G to T (Figure 1B). The forward primer used was 5′-GCG TCA GGT ACC GGA AGG AAC GAA ACA CTC TCA TTC-3′. The reverse primer containing the mutated NRE was 5′-GCC GTC CTC GAG GAT GCC ACA CGC GTC ACT-3′. The PCR condition started by heating to 94°C for 10 min followed by 30 cycles of 94°C for 1 min, 57°C for 30 s, 74°C for 3 min. The amplification was concluded by a final cycle of 74°C for 10 min. The mutant PCR product was digested with restriction endonucleases Mlu1 and Nco1 and subcloned back into the original plasmid pRL-TK to obtain the mutant version pRL-mTK. Plasmid pICP4-SEAP was constructed by inserting HSV-1 ICP4 promoter into pREP-SEAP so the reporter SEAP (secreted alkaline phosphatase) was driven by ICP4 promoter (Pinnoji *et al*, 2007).

Transfection

N2a and N2aTRβ cells were transfected by electroporation using Nucleofector II from Amaxa (Gaithersburg, MD; catalog no.: AAD-1001S) for high efficiency of transfection (protocol essentially described by the manufacturer). The experiments were performed using Kit V (Amaxa, catalog no.: VCA-1005) and the protocol number was T-024. Plasmid phMGFP was cotransfected as a control.

Antibodies

Anti-TRβ1 is a rabbit polyclonal Ab purchased from Millipore/Upstate (catalog no.: 06-539). Anti-monomethylhistoneH3K9 was obtained from Millipore/Upstate (catalog no.: 07-395). Anti-acetylhistone H4 Ab also came from Millipore/Upstate (catalog no.: 06-866). IgG Ab is an anti-goat polyclonal Ab purchased from Santa Cruz (catalog no.: sc-2020) used as Ig control.

All these antibodies were used for ChIP and the dilution was based on the manufacturer's suggestions.

Reporter assays

The cells were harvested for the luciferase assay after 48 h of transfection, essentially as described by the manufacturer. Luciferase activity was measured by a luminometer using the Luciferase reporter assay system (Promega). Luminescence was measured over a 10-s interval with a 2-s delay on the, 20/20

Luminometer (Turner Biosystem, Sunnyvale, CA). The results were presented as the fold induction of the reporter plasmid in the presence or absence of T_3 (100 nM). The SEAP (secreted alkaline phosphtase) assay was described previously (Bedadala *et al*, 2007).

RT-PCR

Total RNA was isolated by Trizol reagent (Invitrogen, Carlsbad, CA). RT-PCRs were performed using Superscript One-Step RT-PCR (Invitrogen) with 0.5 μg of total RNA and primer set. Their sequences are as follows: Actin: 5′-ATT CCT ATG TGG GCG ACG AG-3′ and 5′-TGG ATA GCA ACG TAC ATG GC-3′; TK: 5′-ATG GCT TCG TAC CCC TGC CAT-3′ and 5′-GGT ATC GCG CGG CCG GGT A-3′; EGFP: 5′-GCA GAA GAA CGG CAT CAA GGT G-3′ and 5′-TGG GTG CTC AGG TAG TGG TTG TC-3′; ICP4: 5′-CGA CAC GGA TCC ACG ACC C-3′ and 5′-GAT CCC CCT CCC GCG CTT CGT CCG-3′. The RT-PCR reaction was carried out at 45°C for 20 min followed by 25 cycles of 94°C for 30 s, 57°C for 30 s, and 68°C for 30 s. The RT-PCR products were analyzed using 2% agarose gel electrophoresis. The Kodak Gel-Logic 100 imaging system was used for documentation and quantification.

ChIP

The protocol was described previously (Pinnoji *et al*, 2007). PCR primers were: 5′-ATG GCT TCG TAC CCC TGC CAT-3′ and 5′-GGT ATC GCG CGG CCG GGT A-3′. Quantitative analyses were performed by adding DIG-dNTP into reaction and the chemiluminescent signal of PCR products were captured by Syngene GeneGnome HR Bioimaging system (Frederick, MD). The protocol was essentially as described by the manufacturer.

T_3 removal experiments

N2a and N2aTRβ cells were plated on a 6-well plate with 40% to 50% confluency with the addition of T_3 (100 nM). Media were replaced daily with fresh T_3. On day 6, viral infection was performed for 1 h at m.o.i. of 0.1. The inoculum was then removed and the cells were washed twice with 1 ml of phosphate-buffered saline (PBS) followed by the addition of 1 ml of fresh media containing T_3 for 8 h. The T_3 incubation was stopped after 8 h by removing the medium completely and washing the cells twice with PBS. New medium was added in each well with or without T_3. The media were collected 48 h post infection (p.i.) and subjected to RT-PCR or plaque assays.

Plaque assays

Media collected from mock and infected N2aTRβ cells at 48 h p.i. were subjected to plaque assays by infecting CV-1 cells. Monolayers of CV-1 with 90% to 100% confluency in 24-well plates were incubated with 200 μl of medium for 45 min on a rocking platform at different dilutions followed by addition of 1 ml fresh medium to each well for 48 h. At the end of the incubation, the infected cells were washed with PBS and treated with crystal violet (PML Microbiologicals, Wilsonville, OR) for 20 min followed by washing with water. Plaques were counted in each well and the probability was measured by a Student's paired *t* test with a two-tailed distribution using Microsoft Excel.

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