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ORIGINAL ARTICLE

Thyroid hormone controls the gene expression of HSV-1 LAT and ICP0 in neuronal cells

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Various factors/pathways including hormonal regulation have been suggested to control herpes simplex virus type 1 (HSV-1) latency and reactivation. Our computer analysis identified a DNA repeat containing thyroid hormone-responsive elements (TRE) in the regulatory region of HSV-1 latency-associated transcript (LAT). Thyroid hormone (triiodothyronine, T_3) functions via its receptor TR (thyroid hormone receptor), a transcription factor. Present study investigated the roles of TR and T_3 in HSV-1 gene expression using cultured neuoroblastoma cell lines. We demonstrated that liganded TR activated LAT transcription, but repressed infected cell protein no. 0 (ICP0) transcription in the presence of LAT TRE. Chromatin immunoprecipitation (ChIP) assays showed that TRs were recruited to LAT TREs independently of T_3 and hyperacetylated H4 was associated with the LAT promoter that was transcriptionally active. In addition, ChIP results showed that the chromatin insulator protein CCCTC-binding factor was enriched at the LAT TREs in the presence of TR and T_3. In addition, the BRG1 chromatin remodeling complex is found to participate in the T_3/TR-mediated LAT activation since overexpression of BRG1 enhanced the LAT transcription and the dominant-negative mutant K785R abolished the activation. This is the first report revealing that TR elicits epigenetic regulation on HSV-1 ICP0 expression in neuronal cells and could have a role in the complex processes of HSV-1 latency/reactivation.

***Keywords*:** HSV-1, thyroid hormone, chromatin, transcription, LAT, ICP0, latency
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Introduction

Herpes simplex virus type-1 (HSV-1) causes diseases ranging from mild oral lesions to severe keratitis and lethal encephalitis. HSV-1 infection is composed of four phases: acute infection, establishment of latency, maintenance of latency, and reactivation from latency. During acute infection, HSV-1 targets epithelial cells where the virus undergoes active gene expression in a cascade fashion and generates virion progeny after replication. The expression cascade is characterized by a sequential order with genes grouped into three categories: immediate-early (IE), early (E), and late (L). After the completion of replication and egress, acute infection may be followed by the establishment of latency in the innervating neurons of the peripheral nervous system. In contrast to the acute infection, during latency viral transcriptions are largely void except in one region, which codes for the latency-associated transcript (LAT) [1-4]. The latent virus can survive in the neurons throughout the life of the host and may reactivate in response to various stimuli after the reversal of viral gene silencing [3, 5-8]. A variety of mechanisms have been proposed to describe the establishment of viral latency and reactivation such as altered immune response [9-12], microRNAs-induced gene silencing [13], differential neuronal suppression [14-17], hormonal regulation [18-21], and repressive chromatin [22-28]. Our laboratory identified thyroid hormone-responsive elements (TREs) in several HSV-1 regulatory regions and one of them is located in the LAT regulatory sequence, suggesting that thyroid hormone (triiodothyronine, T_3) could have roles in the transcription of LAT and neighboring regions and therefore modulate the viral latency and reactivation.

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Abbreviations: HSV-1 (herpes simplex virus type 1); IE gene (immediate-early gene); ICP0 (infected cell protein no. 0); HDACs (histone deacetylase complexes); LAT (latency-associated transcript); ChIP (chromatin immunoprecipitation); CTCF (CCCTC-binding factor); BRG1 (brahma-related gene 1); hpi (hours postinfection)

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TRE is the binding site of transcription factor thyroid hormone receptors (TRs). The regulatory activity of TR is dependent on the ligand thyroid hormone. Thyroid hormone is produced by the addition of iodine to tyrosine in the thyroglobulin protein [29]. The primary hormone product is Thyroxine (T_4), but it is converted to the potent T_3 in target tissues [30]. T_3 interacts with TRs to elicit its biological effects [31]. TRs belong to the nuclear hormone receptor superfamily, and vertebrates have two TR genes, TRα and TRβ [32]. The TRE-TR interaction is, nonetheless, independent of T_3, implicating that both unliganded as well as liganded TRs participate in the regulation of gene expression [33].

The present study analyzes the interrelationship of T_3, TRs, and chromatin in HSV-1 gene regulation in neuronal cells. The gene regulation of LAT and infected cell protein no. 0 (ICP0) was investigated in mouse neuroblastoma cells N2a and N2aTRβ, in which the TRβ is constitutively expressed. The effects of TR and T_3 on histone modification and cofactor recruitment to the promoters were also assessed. Our results showed that TR can either activate or repress these genes dependent on the presence of T_3 via recruitment of various co-activators or corepressors for distinct histone modifications and chromatin remodeling.

Results

Location of HSV-1 LAT TREs

We identified putative TREs close to the LAT regulatory region in the HSV-1 genome (Figure 1A). The first sets of TREs were found in the terminal repeat long region from 9 038 to 9 208, coexisting with the reiteration set-1 (RE-1) (Figure 1A). The second sets were located from 117 164 to 117 334, overlapping with the internal repeat long RE-1 (Figure 1A). The positive TRE sequence 5′-GGG AGA-3′ was repeated 21 times in these regions, separated by one or four nucleotides (Figure 1B). The TRE is 1.4 kb upstream of the LAT TATA box. We hypothesize that TR may bind to this TRE and regulate the transcription of LAT and the neighboring gene ICP0.

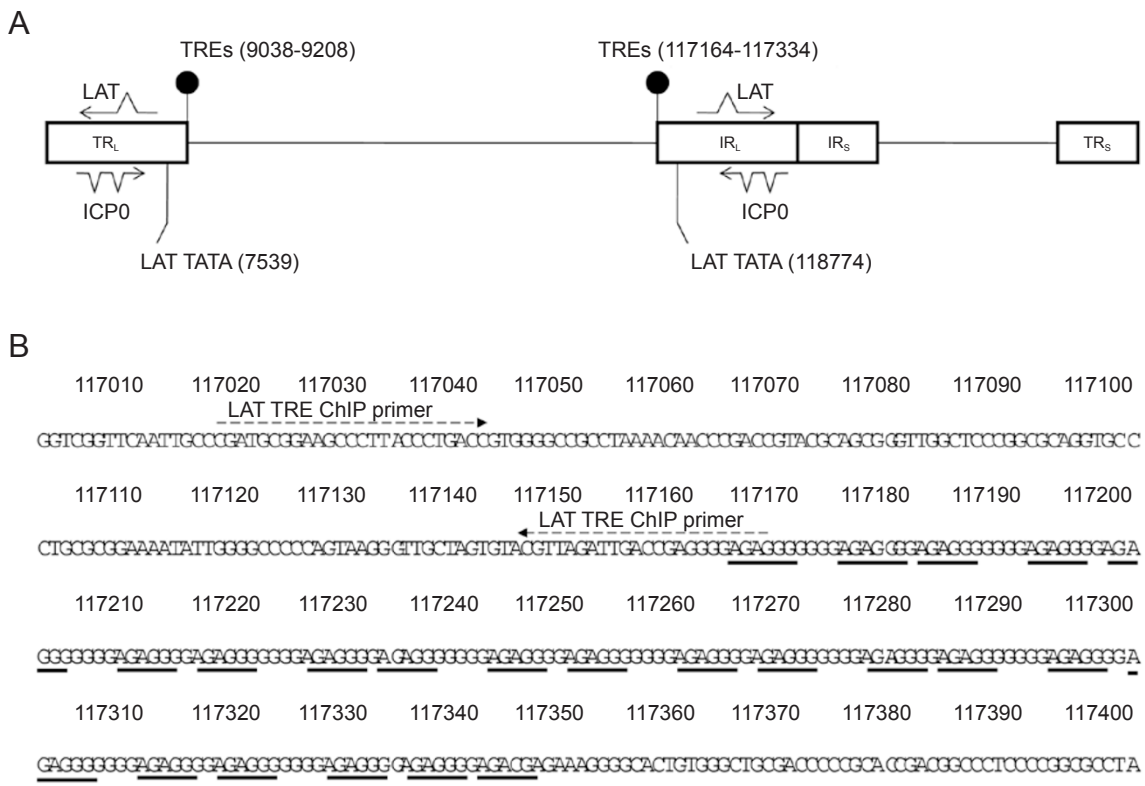

Figure 1 HSV-1 LAT TREs. **(A)** Two sets of positive TREs were identified in HSV-1 genome within LAT regulatory region. The first set was found in the terminal repeat long (TR_L) reiteration set 1 and the second set was located in the internal repeat long (IR_L) reiteration set 1. **(B)** The positive TRE sequence 5′-GGG AGA-3′ was repeated 21 times in these regions separated by one or four nucleotides. These TREs are ~1.4 kb upstream of the LAT TATA box. The figure is based on HSV-1 complete genome sequence (Accession no. X14112). LAT TRE ChIP primers were designed adjacent to one end.

Liganded TR positively regulated LAT but repressed ICP0 transcription

The regulatory effect of TR on LAT and ICP0 was examined by transfecting N2a or N2aTRβ cells with plasmid pSG28, which contains ICP0 and LAT including LAT TREs. RT-PCR assays showed that LAT transcription was up-regulated by TR and T_3 (Figure 2A). ICP0, however, was down-regulated by liganded TR (Figure 2A). To address whether or not ICP0 was directly regulated by TR and T_3, transfections were performed using plasmid pAA3, which contains the ICP0 promoter but not LAT TREs. The results showed no regulation on ICP0 transcription (Figure 2B, compare lanes 4 and 5). Effect of TR on LAT was also investigated, and the RT-PCR assays revealed that liganded TR did not regulate LAT transcription in the absence of LAT TRE (Figure 2B, compare lanes 8 and 9). Transfection of phMGFP followed by fluorescent microscopy revealed similar numbers of cells emitting green fluorescence, suggesting that transfection efficiency was similar between these two cell types (data not shown). Together, these observations indicated that LAT was positively regulated by TR and T_3, while ICP0 was repressed by liganded TR, and this TR-mediated repression requires LAT TREs.

Viral infections were performed to confirm the observation from the transfection experiments. Strain $17Syn^+$ was used at MOI = 10 to infect N2a and N2aTRβ cells with or without 100 nM T_3 followed by RNA isolation and RT-PCR assays. The results showed no TR-mediated regulatory effect on LAT at 8 h postinfection (hpi), probably due to high MOI and IE transactivation (data not shown). After the addition of 50 μg/ml of CHX during infection to inhibit IE protein synthesis, LAT transcription was up-regulated by liganded TR at 8 hpi (Figure 3A). TR and T_3, at the same condition, caused a 60% reduction on ICP0 transcription, as measured by Syngene's molecular biology software GeneTools (Syngene, Frederick, MD, USA) (Figure 3B and 3C). These results dem-

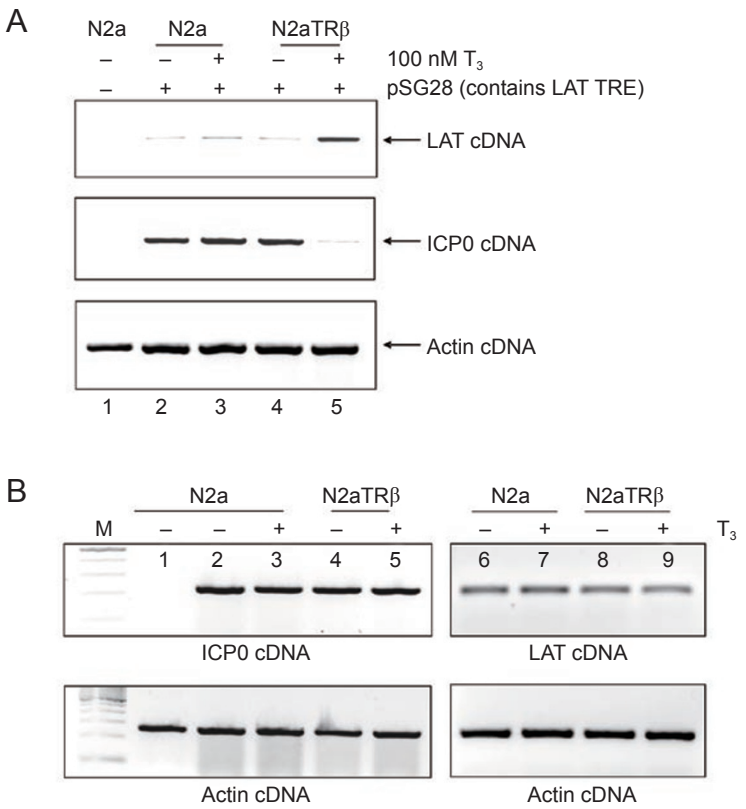

Figure 2 TR/T_3-mediated regulation on HSV-1 LAT and ICP0. **(A)** N2a and N2aTRβ cells were transfected with plasmid containing intact LAT TRE, LAT, and ICP0 to analyze the TR-mediated regulatory effect. Total RNA was purified after 48 h and subjected to RT-PCR assays using primers against LAT and ICP0. Actin primers were used as control. It showed that liganded TR up-regulated LAT but down-regulated ICP0 (compare lanes 4 and 5). **(B)** Transfection of N2a and N2aTRβ cells with plasmids without LAT TREs but with complete ICP0 gene. Total RNA was purified and subjected to RT-PCR assays using primers against LAT and ICP0. Actin primers were used as control. Results showed that neither ICP0 nor LAT was regulated in the absence of LAT TRE.

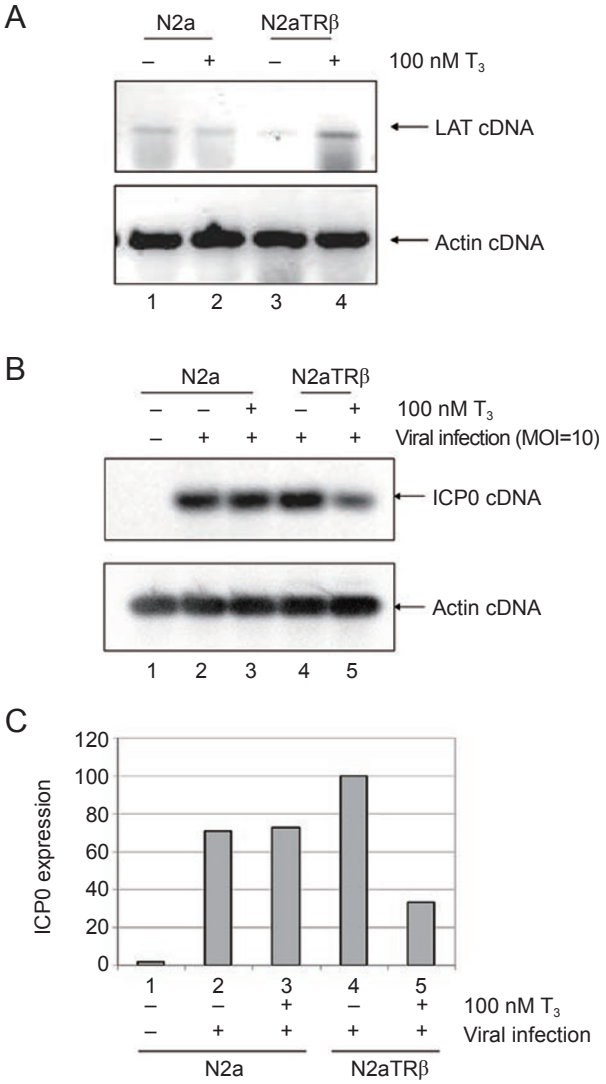

Figure 3 Regulation of LAT and ICP0 by TR and T_3 during infection. **(A)** N2a and N2aTRβ cells were infected with wild-type virus 17Syn$^+$ at MOI = 10 with the addition of 50 μg/ml cycloheximide to prevent the α-transactivation. The total RNA was purified at 8 hpi and subjected to RT-PCR using primers against LAT. Actin primers were used as controls. **(B)** The RNA samples from **A** were used for RT-PCR assays to assess ICP0 transcription. DIG-dNTP was included in the reaction for quantitative purpose. **(C)** The chemiluminescence signal of samples from **B** was captured by GENEgnome camera from Syngene (Fredrick, MD). The quantitative analyses were measured by Syngene's molecular biology software GeneTools.

onstrated that liganded TR up-regulated LAT expression and down-regulated ICP0 during infection.

TRβ was recruited to LAT TRE and CTCF was enriched to the same region in the presence of T_3

Direct interaction of TR and TRE was addressed by electrophoretic mobility shift assay (EMSA). Labeled oligonucleotides (oligos) were incubated with cell extract of N2a and no shifted bands were observed (Figure 4A, lanes 1-8). Labeled LAT TRE oligos generated shifted band in the presence of N2aTRβ extract (Figure 4A, lane 12), and this band was abolished by unlabeled oligo competition (Figure 4A, lane 13). This protein-oligo shift pattern was also observed for the positive control Xenopus TRE (xTRE) (Figure 4A, lanes 14, 15, 16). Mutant LAT TRE (from GGGAGA to GGGATT) failed to produce similar band shift (Figure 4A, lanes 9 and 10), indicating the importance of the sequence context. Collectively, these results demonstrate the *in vitro* interaction of TR/LAT TRE.

The anti-TRβ antibody (Ab) was used for chromatin immunoprecipitation (ChIP) to confirm the *in vivo* binding of receptor to the LAT TREs. The transfection and ChIP assays showed that TRβ was recruited to the LAT TRE regardless of the ligand status (Figure 4B). In addition, this region was previously described as a chromatin insulator-like element [22]. The potential for TRβ to modulate the binding of CTCF was addressed by ChIP using anti-CTCF Ab. The results demonstrated that liganded TRβ increased the binding of CTCF to the element (Figure 4B). To address the specificity of interaction, ChIP-TRβ and ChIP-CTCF were performed using primers away from the target regions (primers against ICP4 ORF and ICP0 promoter, respectively). These negative controls produced no signal (Figure 4C), demonstrating that TR specifically interacted with LAT TREs and that the liganded TRβ could facilitate the recruitment of CTCF to the HSV-1 chromatin insulator.

Participation of histone modification and chromatin remodeling in the gene regulation of LAT

TR modulates histone modification and recruitment of chromatin remodeling complex at the TRE-containing promoters for gene regulation [34-38]. It is likely that liganded TR regulated LAT expression through these mechanisms. This hypothesis was investigated by ChIP using corresponding Abs. The LAT promoter was found to be hyperacetylated in the presence of TR and T_3 (Figure 5A, compare 1 and 2). In addition, chromatin remodeling complex, BRG1, was enriched at the LAT promoter by liganded TR (Figure 5A, compare lanes 1 and 2) but not at the ICP0 promoter (Figure 5A, lanes 3-6), suggesting that the BRG1 complex may participate in the T_3/TR-mediated regulation. To confirm the role of BRG1 in HSV-1 gene expression, BRG1 over-expression vector pBRG1 and its dominant-negative mutant pK785R were transfected with pSG28 in N2aTRβ cells. RT-PCR assays showed that the overexpression of BRG1 had little ef-

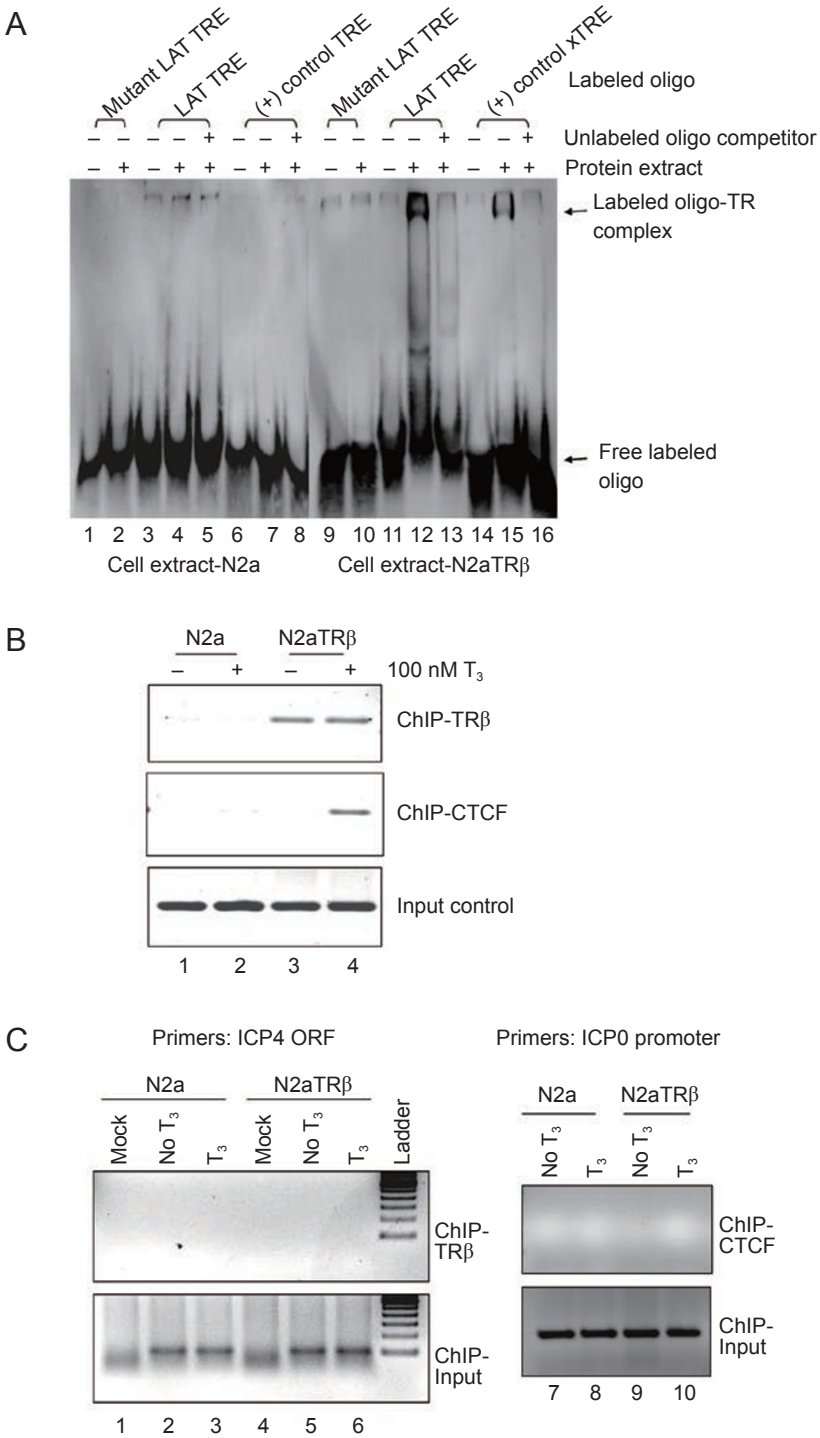

Figure 4 TR bound to LAT TRE and facilitated CTCF enrichment to LAT RE-1 in the presence of T_3. **(A)** EMSA using extract from N2a (lanes 1-8) and N2aTRβ (lanes 9-16). Labeled oligos of LAT TRE, mutant TRE, and positive control Xenopus TRE (xTRE) were used for analyses. Free labeled oligo and labeled oligo-TR complex were marked. Note that extract of N2aTRβ, but not N2a, produced a shifted band while using labeled LAT TRE oligo (lane 12) and positive control (lane 15). Mutant oligo failed to generate the pattern (lane 10). **(B)** Cells were transfected with pSG28 and treated with or without T_3 for 48 h. The cells were then subjected to ChIP using Abs against TRβ or CTCF for immunoprecipitation. The recruitment of TRβ or CTCF was analyzed by PCR. IgG controls showed no signal, indicating the specificity of the assays (data not shown). **(C)** The binding specificity of TR and CTCF to LAT TRE was further analyzed by ChIP using primers against other regions (ICP4 ORF and ICP0 promoter, respectively). Lanes 1-6: ChIP-TRβ using primers against ICP4 ORF; lanes 7-10: ChIP-CTCF using primers against ICP0 promoter.

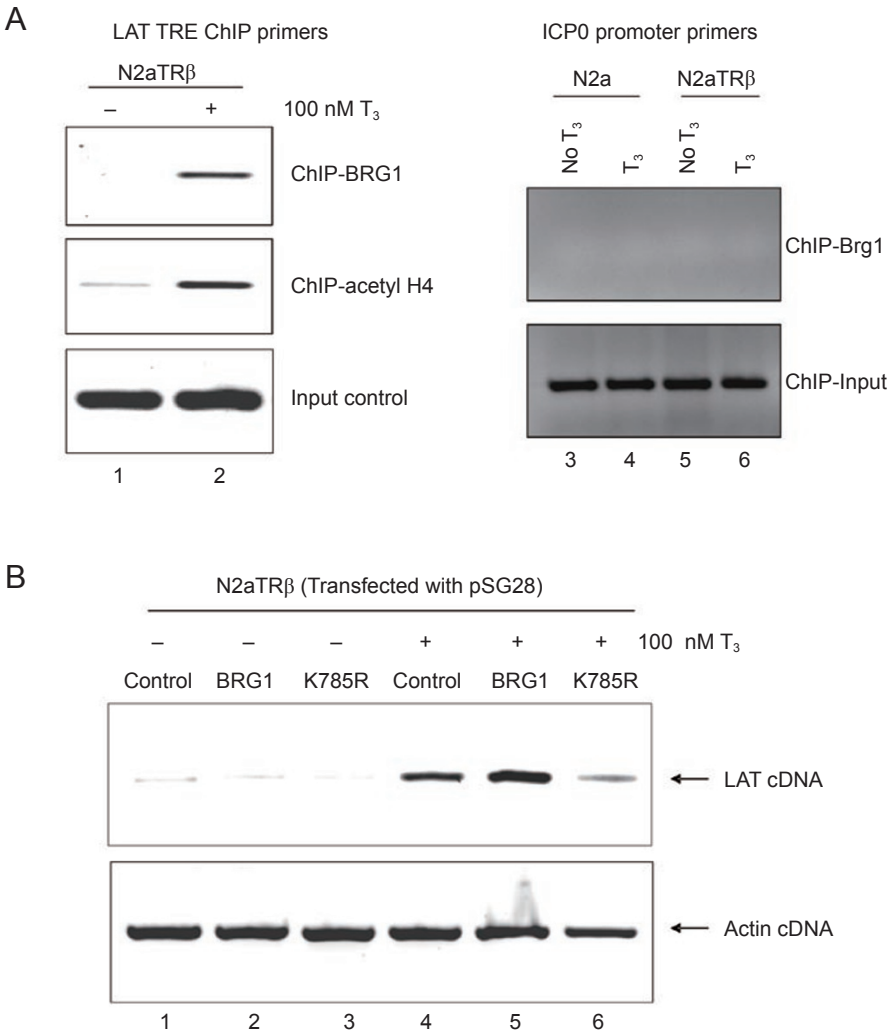

Figure 5 Participation of chromatin remodeling and histone acetylation in regulation of LAT transcription by liganded TR. **(A)** ChIP was performed using anti-BRG1 and anti-acetyl-histone H4 Ab followed by PCR using specific primers. IgG controls showed no signal, indicating the specificity of the assays (data not shown). Lanes 1 and 2: LAT TRE ChIP primers; lanes 3-6: ICP0 promoter primers. **(B)** Cells were cotransfected with pSG28 and with or without expression vectors of BRG1 or K785R specified in the figure. After 48 h, the transfected cells were subjected to RNA isolation and RT-PCR assays using primers recognizing LAT transcript. Actin primers were used as controls.

fect on LAT expression in the absence of TR but further increased the TR-mediated LAT transcription in the presence of T_3 (Figure 5B, compare lanes 1-5). The dominant-negative mutant K785R, on the contrary, abolished the activation by liganded TR (Figure 5B, lane 6). These results suggest that histone modification and chromatin remodeling both contribute to the T_3/TR-mediated LAT regulation.

Histone H4 hypoacetylation at the ICP0 promoter caused by liganded TR in the presence of LAT TRE

The BRG1 remodeling complex appeared to participate in the LAT regulation, but it did not elicit a regulatory effect on ICP0 since overexpression of BRG1 and K786R exhibited no impact on ICP0 expression (data not shown). Nonetheless, the ICP0 promoter was hypoacetylated in the presence of liganded TR, since the ChIP assays showed that histone H4 acetylation was reduced at the ICP0 promoter in the presence of TR and T_3 (Figure 6A). This TR/T_3-mediated hypoacetylation required the presence of LAT TREs, evidenced by the fact that the lack of LAT TRE led to no hypoacetylation at the ICP0 promoter (Figure 6B). Collectively, these results suggest that liganded TR induced hypoacetylation at the ICP0 promoter and it is likely the result of chromatin insulation mediated via LAT TREs.

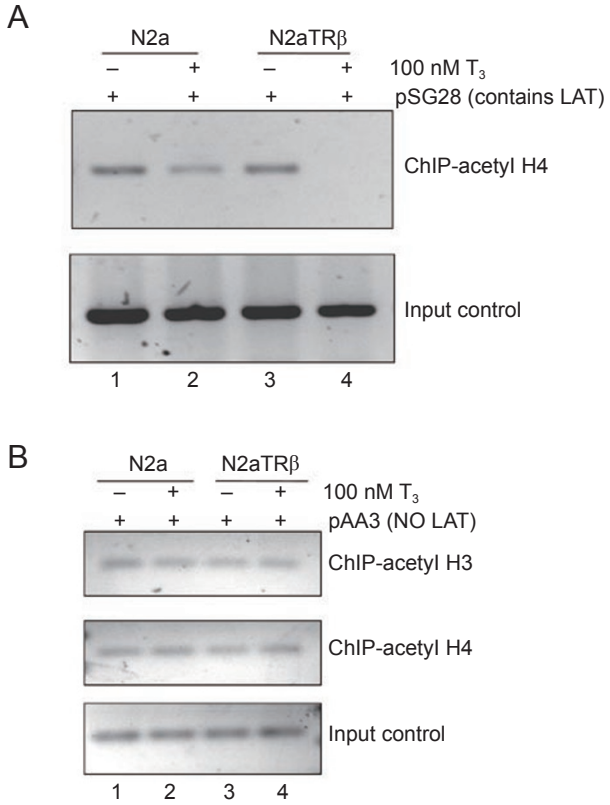

Figure 6 T_3/TR-mediated ICP0 promoter histone modification required the presence of LAT TRE. **(A)** Cells were transfected with pSG28 (containing LAT TRE) and incubated with or without T_3 for 48 h followed by ChIP with anti-acetyl-histone H4 Ab. The IP chromatin solution was purified and the resulting DNA was amplified by primers recognizing ICP0 promoter. **(B)** Parallel experiments to Figure 5A using plasmid pAA3 (containing no LAT TRE) were carried out with the anti-acetyl-histone H4 Ab as well as acetyl-histone H3 Ab for IP. The histone modification was analyzed by the same ICP0 primers.

T_3 washout derepressed ICP0 and increased infectious virus release

To investigate the effect of T_3 on TR-mediated regulation during HSV-1 replication and the release of infectious virus, N2a and N2aTRβ cells were pretreated with T_3 for 5 days followed by infection at MOI of 1 with or without T_3 washout (Figure 7A). RT-PCR assays showed that the expression of ICP0 was de-repressed upon washout of T_3 at 48 hpi in N2aTRβ but not N2a cells (Figure 7B). The plaque assays indicated that the T_3 washout increased the virus release by more than 60-fold compared to the cells being treated with T_3 in N2aTRβ (Figure 7C). There was no change in N2a cells upon T_3 removal (Figure 7C). These results suggest that subtraction of hormone reversed the ICP0 repression and this viral protein may play an active role during viral replication in resting cells, leading to enhanced viral gene expression and virus release.

Discussion

This study focused on the roles of TR and its ligand T_3 in the regulation of HSV-1 gene expression in neuronal cells. Our data showed that the transcription factor TR utilized its ligand to elicit differential regulatory effect on HSV-1 genes, LAT and ICP0, in neuronal cells. The results, for the first time, showed that liganded TR bound LAT TRE and activated LAT promoter via histone modification as well as BRG1-mediated chromatin remodeling. In addition, liganded TR mediated down-regulation of ICP0, at least in part, by histone hypoacetylation which requires the LAT TRE. The current model using T_3-treated N2aTRβ cells was particularly useful for initial *in vitro* studies since N2aTRβ cells were differentiated after T_3 incubation, mimicking the sensory neurons *in vivo*. Investigations using animal models are underway and the preliminary data indicated that T_3 treatment decreased and delayed the virus release in TG explant assays, supporting the hypothesis that T_3 participated in the regulation of HSV-1 latency and reactivation (data not shown).

The BRG1 complex disrupts histone-DNA interactions and has been implicated in the regulation of transcriptional activation [39]. The role of the BRG1 chromatin remodeling complex in gene regulation via nuclear hormone receptors was shown to involve modification of the nucleosomal structure to allow transcriptional activation or repression [40]. *In vitro* assays with several nuclear hormone receptors, including the estrogen receptor and the glucocorticoid receptor, have shown that BRG1 can modify the pattern of nucleosomal positioning and aid local chromatin structure disruption within preassembled nucleosomal display [41]. Our results showed that BRG1 was recruited to the LAT promoter by liganded TR and its dominant-negative mutant eliminated the TR-mediated LAT activation. BRG1 contributes to HSV-1 IE gene expression through the VP16-HCF-Oct1 complex [42]. Intact VP16 complexes may not be able to enter the nucleus of sensory neurons but BRG1, on the other hand, is present in neurons. It is likely that BRG1 is recruited to HSV-1 regulatory sequences via T_3 and TR to regulate LAT transcription. More experiments are necessary to analyze the enrichment of this chromatin remodeling complex at the HSV-1 genome in neurons.

The functions of LAT and its role in ICP0 expression during HSV latency and reactivation are elusive. In neuronal cells, LAT was shown to reduce viral gene expression and replication during productive infection [43]. *In*

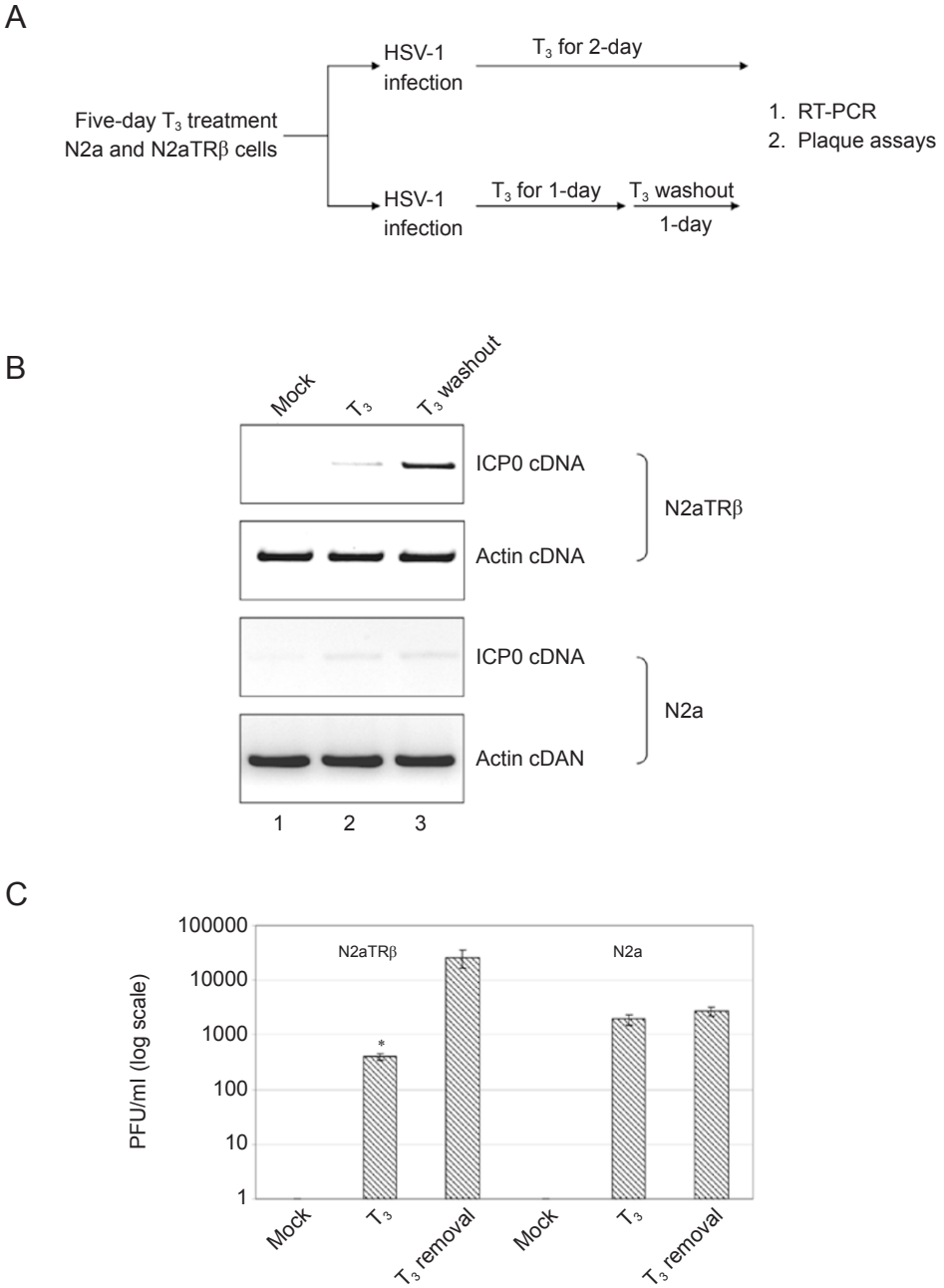

Figure 7 Long-term T_3 treatment prevented the ICP0 expression and the washout of T_3 derepressed the ICP0 expression and increased virus release. **(A)** Cells were pretreated with T_3 for 5 days and subsequently infected with 17Syn$^+$-EGFP viruses at MOI of 1 with T_3. The washout was carried out a day after the infection and the assays were performed at 48 hpi. **(B)** Total RNA was isolated at 48 hpi and subjected to RT-PCR assays using ICP0 primers to study TR-mediated regulation. **(C)** The media of infected cells were collected at 48 hpi and subjected to plaque assays using CV1 cells to investigate the release of infectious viruses. The *P*-value measured by a Student's paired *t*-test with a two-tailed distribution was 0.0015, indicating a significant increase.

vivo, LAT mutant virus showed enhanced gene expression in sensory neurons during lytic and latent infection [44]. A more recent report showed that LAT augments the transcription of several lytic genes during the latent stage in rabbits, possibly by chromatin modifications [45]. Chromatin insulator motifs were identified within the LAT regulatory regions [22, 24]. Our results showed that TR could bind to the LAT TREs and facilitate the

enrichment of the chromatin insulator-binding protein CTCF to the region in the presence of T_3. Under the same condition, liganded TR caused hypoacetylation of the ICP0 promoter. TREs and CTCF motifs were often adjacent and possessed overlapping functions [46-48]. In addition, ICP22 intron contains reiterative repeats similar in position and composition to the reiterative elements but different in their actual sequences. It is likely that the liganded TR facilitates the binding of CTCF to the elements or directly recruits CTCF to the sites, thus forming a boundary element (between LAT TRE/RE-1 and ICP22 intron) to regulate gene expression. Studies using mutant viruses with specific deletions are underway to investigate the effects of T_3/TR-mediated chromatin changes on viral gene expression and silencing in animal models.

Our results further indicated that TR and T_3 repressed HSV-1 TK expression and modulated viral replication/release in neuronal cells (unpublished data). TK is required to provide dNTP for viral replication in resting cells such as neurons, and viral replication is required for efficient α and β expression in neurons during reactivation [49]. TK was further suggested to initiate α transcription and subsequent replication during reactivation [50], and the mutant virus lacking TK exhibited greatly reduced α and β expression during reactivation, suggesting the importance of TK [51]. Based on these results, T_3 and TR may have additional regulating roles in controlling HSV-1 replication and gene expression during latency/reactivation.

Together, our studies suggest that thyroid hormone and its receptor are able to recruit different cofactors to control critical transcription of various HSV-1 genes in neuronal cells, which may have implications in regulating latency and reactivation. More experiments, especially those with mutant viruses in cell culture and animal models, are necessary to identify key mechanisms involved in HSV-1 latency and viral reactivation through the TR/T_3-mediated regulations.

Materials and Methods

Viruses, cell lines, and culture conditions

HSV-1 strain 17Syn$^+$ was obtained from Dr James Hill's laboratory at Louisiana State University Health Science Center (New Orleans, LA, USA). The N2a cell line was purchased from the American Type Culture Collection (ATCC). N2aTRβ cell is a gift from Dr Robert Denver (University of Michigan, Ann Arbor, MI, USA) and maintained in DMEM/F12 supplemented with 10% charcoal-treated fetal bovine serum. All cells were grown in an incubator at 37 °C with 5% CO_2. T_3 was purchased from Sigma (St Louis, MO, USA).

Plasmids

Plasmid pSG28 contains HSV-1 sequence from 106 785 to 131 534. It covers the LAT TRE and open reading frames such as ICP4, LAT, ICP0, etc. Plasmid pAA3 possesses the HSV-1 sequence from 122 713 to 134 792. It contains partial deletion of LAT and does not have LAT TRE. Plasmid phMGFP (Promega, cat no. E6421) was used as transfection efficiency control. Plasmid pBRG1 containing the complete ORF of BRG1 was a gift from Dr Keji Zhao (NHLBI, NIH). The plasmid pK785R, another gift from Dr Zhao, contains a mutation in the BRG1 ORF with amino acid change from K to R at position 785.

Transfection

Nucleofector II (cat no. AAD-1001S) from Amaxa (Gaithersburg, MD, USA) was used for high efficiency of transfection. The protocol was essentially described by the manufacturer. The experiments were performed using Kit V (cat no. VCA-1003) and the protocol number was T-024.

Antibodies

The Abs used for ChIP are listed in Table 1. The dilution was according to the manufacturer's suggestions.

RT-PCR

Total RNA from cells was isolated by Trizol reagent (Invitrogen, Carlsbad, CA, USA). RT-PCRs were performed using Superscript One-Step RT-PCR (Invitrogen) with 0.2 μg of total RNA and correspondent primers. Their sequences are shown as follows: *Actin*: 5′-ATT CCT ATG TGG GCG ACG AG-3′ and 5′-TGG ATA GCA ACG TAC ATG GC-3′; *LAT*: 5′-CGG CGA CAT CCT CCC CCT AAG C -3′ and 5′-GAC AGA CGA ACG AAA CGT TCC G -3′; *ICP0*: 5′-TTC GGT CTC CGC CTG AGA GT-3′ and 5′-GAC CCT CCA GCC GCA TAC GA-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 results were documented by Kodak Gel-Logic 100 imaging system. To increase the sensitivity of ICP0 assays, DIG-dNTP was included in the reaction for quantitative purpose. The gel was transferred to a nylon

Table 1 The sources of antibodies

Antibody	Species	Clonality	Source
TRβ1	Rabbit	Polyclonal	Millipore/Upstate (cat no. 06-539)
CTCF	Rabbit	Polyclonal	Millipore/Upstate (cat no. 07-729)
BRG-1	Rabbit	Polyclonal	Abcam (cat no. ab4081)
Acetyl-histone H3	Rabbit	Polyclonal	Millipore/Upstate (cat no. 17-245)
Acetyl-histone H4	Rabbit	Polyclonal	Millipore/Upstate (cat no. 06-866)

membrane, developed by CDP-star, and measured by Syngene G:BOX lightly cooled CCD camera and molecular biology software GeneTools described previously [27].

Electrophoretic mobility shift assay

EMSA was performed using a DIG Gel shift Kit 2nd generation (Roche applied science, Indianapolis, IN, USA) essentially as described in the manufacturer's protocol. Briefly, single-stranded oligos for LAT TRE: 5′-GGG GAG AGG GGA GAG GGG GGGAGA GGG GAG AG-3′; xTRE (positive control): 5′-GAT CGC AGG TCA TTT CAG GAC AGC-3′ [52]; Mutant TRE: 5′-GGG GAG AGG GGA TTG GGG GGG ATT GGG GAG AG-3′ and their complementary oligos were synthesized (Invitrogen, San Diego, CA, USA) and annealed (95 °C for 10 min followed by slow cooling) to make double-stranded (ds) oligos. The cell extracts of N2a or N2aTRβ were isolated by first washing the cells twice with cold PBS followed by lysis with NP40 buffer (Invitrogen, cat no. FNN0021) containing protease inhibitor and PMSF. The ds oligos were terminally labeled with non-radioactive DIG-11-ddUTP by terminal transferase and incubated with the cell extracts of N2a or N2aTRβ for 30 min at room temperature. For competition, 10× of unlabeled oligos were added to the labeled oligos for specificity analysis. The samples were electrophoresed on a 6% DNA Retardation Gel (Invitrogen) at 70 V for 1 h followed by electrophoretic transfer (Novex X Cell II Blot module) to positive-charged nylon membrane (Roche). The DIG-labeled oligos were visualized by an enzymatic immunoassay using anti-digoxigenin-alkaline phosphatase, Fab-fragments, and chemiluminiscent substrate CSPD (Roche). The chemiluminiscent signal was captured using GeneGenome HR imaging system (Syngene).

Chromatin immunoprecipitation

The protocol was described previously [27]. The sequences of PCR primers: ICP0 (promoter) ChIP: 5′-TAA TGG GGT TCT TTG GGG GAC ACC-3′ and 5′-TGC AAA TGC GAC CAG ACT GTC-3′; LAT ChIP: 5′-GCT ACG CCT TCG GGA ATG G-3′ and 5′-AGA GGG GAG CCA GTT AGA TTG C-3′; ICP4 ORF: 5′-CGA CAC GGA TCC ACG ACC C-3′ and 5′-GAT CCC CCT CCC GCG CTT CGT CCG-3′. The location of LAT ChIP primers (117 017-117 165) is immediately upstream of LAT TRE (117 161-117 334) since LAT TRE is highly GC rich and not suitable for PCR (Figure 1B).

T_3 removal assays

N2a and N2aTRβ cells were plated on a six-well plate with 40-50% confluency with the addition of T_3 (10 nM) into the media refreshed daily. On day 6, viral infection was performed for 1 h at MOI of 1. The inoculum was then removed and the cells were washed twice with 1 ml of PBS followed by the addition of 1 ml of fresh media containing T_3 for 1 day. The T_3 incubation was stopped after 1 day 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 at 48 hpi and subjected to RT-PCR and plaque assays. The paradigm was shown in Figure 7A.

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