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A Chromatin Insulator-Like Element in the Herpes Simplex Virus Type 1 Latency-Associated Transcript Region Binds CCCTC-Binding Factor and Displays Enhancer-Blocking and Silencing Activities

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A previous study demonstrated that the latency-associated transcript (LAT) promoter and the LAT enhancer/reactivation critical region (*rcr*) are enriched in acetyl histone H3 (K9, K14) during herpes simplex virus type 1 (HSV-1) latency, whereas all lytic genes analyzed (ICP0, UL54, ICP4, and DNA polymerase) are not (N. J. Kubat, R. K. Tran, P. McAnany, and D. C. Bloom, J. Virol. 78:1139-1149, 2004). This suggests that the HSV-1 latent genome is organized into histone H3 (K9, K14) hyperacetylated and hypoacetylated regions corresponding to transcriptionally permissive and transcriptionally repressed chromatin domains, respectively. Such an organization implies that chromatin insulators, similar to those of cellular chromosomes, may separate distinct transcriptional domains of the HSV-1 latent genome. In the present study, we sought to identify *cis* elements that could partition the HSV-1 genome into distinct chromatin domains. Sequence analysis coupled with chromatin immunoprecipitation and luciferase reporter assays revealed that (i) the long and short repeats and the unique-short region of the HSV-1 genome contain clustered CTCF (CCCTC-binding factor) motifs, (ii) CTCF motif clusters similar to those in HSV-1 are conserved in other alphaherpesviruses, (iii) CTCF binds to these motifs on latent HSV-1 genomes in vivo, and (iv) a 1.5-kb region containing the CTCF motif cluster in the LAT region possesses insulator activities, specifically, enhancer blocking and silencing. The finding that CTCF, a cellular protein associated with chromatin insulators, binds to motifs on the latent genome and insulates the LAT enhancer suggests that CTCF may facilitate the formation of distinct chromatin boundaries during herpesvirus latency.

Herpes simplex virus type 1 (HSV-1) establishes a lifelong latent infection within sensory neurons. During this time, the latent genomes persist as circular episomes associated with histones (9, 25). Although HSV-1 lytic gene expression is repressed during latency, a latency-associated transcript (LAT) is abundantly expressed in a subset of neurons (20, 26). Previous chromatin immunoprecipitation (ChIP) analyses of latent genomes have shown that several HSV-1 lytic genes are hypoacetylated in acetyl histone H3 (K9, K14), whereas the LAT region is heavily enriched in this transcriptionally permissive histone (15, 16, 27). The region of hyperacetylation within the LAT locus encompasses an enhancer that maps to the LAT 5′ exon, and transcription does not seem to be required to maintain this hyperacetylated state (15). Further, enrichment of acetyl histone H3 (K9, K14) does not include the ICP0 promoter. This suggests that the HSV-1 latent genome is organized into chromatin domains by boundary or insulator elements, similar to those found on cellular chromosomes, which would separate these distinct transcriptional domains (repressed lytic gene regions versus a transcriptionally active LAT region).

CTCF, or "CCCTC-binding factor," is a DNA-binding protein containing 11 zinc fingers that is highly conserved among vertebrates. CTCF is ubiquitously expressed in most cell types and possesses transcriptional activator activity that is regulated by phosphorylation. In addition to the sequence "CCCTC," CTCF binds to several other pentanucleotide motifs (21). While a single DNA-binding motif is sufficient for binding, the binding motifs are often present as clusters of these consensus sequences, which affords higher CTCF-binding affinity (6). CTCF binding results in a number of distinct activities, including gene activation and repression, though it is most often associated with the formation of chromatin insulators (31).

Chromatin insulators are a class of DNA elements found on cellular chromosomes that protect genes in one region of a chromosome from the regulatory influence of another region (for a review, see reference 28). In the simplest example, an insulator separates a region of transcriptionally active euchromatin from a region of transcriptionally repressed heterochromatin (5). Insulator elements are believed to act via protein-protein interactions spanning a chromatin domain, as well as through the recruitment of specific histone-modifying enzymes. For example, several chromatin-modifying proteins have been shown to bind to CTCF at insulator elements, including sin3 and YB-1 (18). There are two main classes of insulators that have been characterized: enhancer-blocking and boundary/barrier elements. Enhancer-blocking insulators have the specific ability to block an enhancer from enhancing gene expression on the distal side of the insulator (32). Boundary elements, on the other hand, act primarily to separate transcriptionally polar regions of DNA and, in many cases, block the "spread" of transcriptionally repressive heterochromatin into regions that are transcriptionally active or permissive (13). In addition, a number of cellular insulators are associated with silencer activity (4, 12). In these cases, CTCF acts as a corepressor with other proteins to recruit transcriptionally repressive histones

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TABLE 1. PCR primers

DNA target	Sequence	Product size (bp)	Accession no. (nucleotide no.)
Mouse *Tsix* Site A[a]	5′ GGAGCCTAAACCTGTCTGTC 3′ (F)[c] 5′ GTGTGTCATAGCTCAAGAGG 3′ (R)[d]	139	AJ421479 (137291–137430)
Mouse MT498[a]	5′ ACTCAGTCCAAACATATACAAGATGC 3′ (F) 5′ CTATCTACAACAAACTTCTCCTGGG 3′ (R)	185 or 149[b]	NT_039554 (1203018–1203201)
HSV-1 CTRL1	5′ GCATGCGTCGCCCAAC 3′ (F) 5′ CAGTTAGATTGCATGTGATC 3′ (R)	89	NC_001806 (117067–117156)
HSV-1 CTRL2	5′ CTCTGTGGTTAACACCAGAG 3′ (F) 5′ GTCTGTGTTGGATGTATCGC 3′ (R)	204	NC_001806 (120461–120665)
HSV-1 CTRS1/2	5′ CAACGCTACTGCAAAAC 3′ (F) 5′ GACGGGGTGCTGTAAC 3′ (R)	97	NC_001806 (127149–127246)
HSV-1 CTRS3	5′ CACGAACGACGGGAGCG 3′ (F) 5′ CACCCAAGGTGCTTACC 3′ (R)	248	NC_001806 (132140–132388)
HSV-1 CTUS1	5′ GGTGATCGCCTGTCTCC 3′ (F) 5′ CATTGCCAATCGAACCC 3′ (R)	179	NC_001806 (143513–143692)
HSV-1 gC	5′ CCTTGCCGTGGTCCTGTGGA 3′ (F) 5′ GTTGGGGTTTGGGGTCGATG 3′ (R)	186	NC_001806 (96331–96517)

[a] Reference 6.
[b] The MT498 locus is polymorphic within the amplicon.
[c] F, forward primer.
[d] R, reverse primer.

(30). While there is a wide range of transcriptional properties associated with specific insulators characterized to date, all vertebrate elements bind CTCF, which plays an essential role in insulator function (31).

In the present study, we sought to identify the locations of putative insulators that might separate the transcriptionally permissive LAT region from the nearby transcriptionally repressed, hypoacetylated ICP0 region. A previous study suggested that this boundary would be located approximately 5 kb 3′ to the region of LAT that is hyperacetylated during latency (15). We report here the identification of a sequence cluster 3′ of this hyperacetylated LAT region, composed of a repeated motif known to bind the cellular protein CTCF and to have a role in the formation of chromosomal boundaries. This cluster of CTCF motifs encompasses approximately 145 bp in the region encoding the LAT intron. ChIP analysis using an antibody specific for CTCF demonstrated that during a latent infection of murine dorsal root ganglia (DRG), this site is enriched in CTCF. In order to determine if this cluster of CTCF motifs marks the location of a functional insulator element, we analyzed a 1.5-kb fragment that contains this CTCF cluster for insulator functions. Insulator functions were assessed by in vitro analysis using luciferase reporter constructs similar to those used to define cellular insulators (24). These analyses revealed that this CTCF cluster is not only capable of blocking the LAT enhancer from acting on an adjacent promoter, but it also possesses silencing activity. This suggests that the 1.5-kb fragment containing a 135-bp cluster of repeated CTCF motifs possesses cellular-insulator-like properties. This element may therefore contribute to the formation of nucleation sites for the assembly of a functional chromatin boundary, which could play an essential role in insulating the LAT enhancer. Such an arrangement would allow the LAT enhancer to act solely on the LAT promoter during latency and not on surrounding lytic promoters, such as ICP0.

Further analysis of the HSV-1 genome revealed the existence of five other clusters of CTCF motifs. ChIP analysis revealed that during a latent infection of murine DRG, these sites are also enriched in CTCF binding. Interestingly, if all these motifs were to form functional boundaries, the LAT enhancer/*rcr*, as well as each of the five HSV-1 immediate-early (IE) genes, would exist in a separate chromatin domain. Finally, analysis of other alphaherpesvirus genomes reveals that CTCF motifs (flanking IE genes) are conserved, raising the possibility that definitive chromatin domains may be an important regulatory component of alphaherpesviral latent-gene expression and may contribute in a mechanistic way to the control of latency and reactivation.

MATERIALS AND METHODS

Viruses and cells. All ChIP experiments were performed using ganglia from mice infected with a low-passage stock of HSV-1 strain 17*syn*+ prepared from a master stock obtained from J. Stevens. The virus was amplified and titrated on rabbit skin cells using Eagle's minimal essential medium (Life Technologies) supplemented with 5% calf serum, 250 U of penicillin/ml, 250 μg of streptomycin/ml, and 292 μg of L-glutamine/ml (Life Technologies).

Mouse infections. Four- to 6-week old female outbred ND4 Swiss mice (Harlan) were anesthetized by halothane inhalation and pretreated with 0.05 ml of a 10% (wt/vol in water) sterile saline solution injected under each rear footpad. At 3 to 4 h after pretreatment, the mice were anesthetized by intramuscular injection of 0.010 to 0.020 ml of a cocktail of acepromazine (2.5 to 3.75 mg/kg of body weight), xylazine (7.5 to 11.5 mg/kg), and ketamine (30 to 45 mg/kg). The keratinized epithelia of both rear footpads were lightly abraded with an emery board, and the inoculum of 500 PFU of virus was applied to the feet in a volume of 0.05 ml/mouse. The virus was allowed to adsorb for 30 to 45 min. The mice were sacrificed at 76 days postinfection for latency studies, with care taken to ensure that the ganglia were removed and processed as quickly as possible postmortem (between 3 and 5 min per mouse).

Identification of consensus CTCF-binding motifs. The frequency with which CCCTC or CTCCC motifs are found within the HSV-1 genome was calculated by the following formulas: $R = f_{CCCTC}/1{,}000$ and $R = f_{CTCCC}/1{,}000$, where f is the frequency of the indicated CTCF-binding motif and R is the resulting ratio. The entire viral genome was analyzed in 1,000-bp segments using a Visual Basic program employing the above formulas, and the results were graphed using Microsoft Excel. Regions that exhibited high frequencies of motif occurrence were further analyzed for motif clustering (2). Tandem-repeat analysis was also applied to a group of alphaherpesviruses to screen for similar CTCF motif clusters.

ChIP. ChIP assays were performed as previously described (16) with minor modification. Briefly, the steps were as follows. Six to eight DRG (L4-6) were removed from four mice per ChIP at a minimum of 28 days postinfection The

TABLE 2. Relative enrichment of CTCF at CTCF motif clusters following ChIP with anti-CTCF[a]

Panel[a]	PCR primers	Expt no.[b]	Sample, no. of cycles	Dilution[c]	Fluorescence[d]	IP value[e]	Mean (±SD) IP value
A	*Tsix* site A		Input, 36	0.01	2.319×10^6		
				0.005	1.766×10^6		
				0.0025	1.171×10^6		
		1	IP, 36	0.1	1.575×10^6	0.004	0.003 ± 0.001
		2	IP, 36	0.1	1.174×10^6	0.003	
		3	IP, 36	0.1	6.967×10^5	0.002	
	MT498		Input, 35	0.01	6.124×10^6		
				0.005	4.808×10^6		
				0.0025	4.224×10^6		
		1	IP, 35	0.1	2.917×10^6	0.002	0.001 ± 0.001
		2	IP, 35	0.1	2.293×10^6	0.001	
		3	IP, 35	0.1	2.302×10^6	0.001	
B	CTRL1		Input, 38	0.1	2.998×10^6		
				0.05	1.565×10^6		
				0.025	4.192×10^5		
		1	IP, 38	0.1	1.801×10^6	0.046	0.071 ± 0.060
		2	IP, 38	0.1	5.505×10^5	0.027	
		3	IP, 38	0.1	2.981×10^6	0.139	
	CTRS1/2		Input, 35	0.1	1.1162×10^6		
				0.05	4.7241×10^5		
				0.025	2.4803×10^5		
		1	IP, 35	0.1	6.4805×10^5	0.046	0.056 ± 0.040
		2	IP, 35	0.1	1.9476×10^4	0.022	
		3	IP, 35	0.1	1.7140×10^6	>0.1	
	CTRS3		Input, 38	0.1	6.2552×10^5		
				0.05	3.6653×10^5		
				0.025	1.5552×10^5		
		1	IP, 38	0.1	1.1460×10^6	>0.1	0.075 ± 0.043
		2	IP, 38	0.1	1.5976×10^5	0.026	
		3	IP, 38	0.1	2.0132×10^6	>0.1	
	CTUS1		Input, 33	0.1	7.8063×10^5		
				0.05	2.8792×10^5		
				0.025	2.2306×10^4		
		1	IP, 33	0.1	9.0591×10^5	>0.1	0.080 ± 0.034
		2	IP, 33	0.1	4.2321×10^5	0.041	
		3	IP, 33	0.1	1.5675×10^6	>0.1	
	gC		Input, 33	0.1	4.578×10^5		
				0.05	2.961×10^5		
				0.025	8.640×10^4		
		1	IP, 33	0.1	1.268×10^5	0.028	0.028 ± 0.006
		2	IP, 33	0.1	4.110×10^4	0.023	
		3	IP, 33	0.1	2.129×10^5	0.034	
C	CTRL2		Input, 35	0.1	3.003×10^5		
				0.05	1.869×10^5		
				0.025	1.300×10^5		
		1	IP, 35	0.01	6.408×10^4	0.02	0.025 ± 0.006
		2	IP, 35	0.01	1.175×10^5	0.025	
		3	IP, 35	0.01	1.566×10^5	0.031	
	gC		Input, 34	0.1	8.234×10^5		
				0.05	5.124×10^5		
				0.025	3.956×10^5		
		1	IP, 34	0.01	8.610×10^4	0.016	0.017 ± 0.001
		2	IP, 34	0.01	9.404×10^4	0.017	
		3	IP, 34	0.01	1.727×10^5	0.018	

[a] Samples included either input (mock-immunoprecipitated) or IP (immunoprecipitated with anti-CTCF) samples that were analyzed by PCR.
[b] ChIP analyses were conducted using samples processed from three independent experiments.
[c] Input and IP samples were serially diluted as indicated.
[d] PCR products were resolved by polyacrylamide gel electrophoresis and stained with SYBR green. The band intensities were imaged on a Storm 860 instrument and measured using ImageQuant software.
[e] The data from input dilutions were fitted by linear regression (Kaleidagraph). The IP fluorescence value was calculated from the linear fit of the input dilution data.

ganglia were homogenized and chromatin cross-linked, and the cell lysates were sonicated to shear the chromatin into fragments with a median size range of 500 to 1,000 bp, as determined by agarose gel electrophoresis. The sheared chromatin was incubated overnight with shaking with 2 μl of anti-CTCF (Upstate Biotechnology) at a concentration of 2 μg of antibody per ml of precleared chromatin. The chromatin-antibody complexes were collected by incubation with salmon sperm DNA-protein A-agarose beads, the complexes were eluted from the beads and treated with RNase A and proteinase K, and the DNA was purified using a Qiaquick PCR purification kit (QIAGEN).

PCR analysis of ChIP experiments. Following collection of the chromatin-antibody complexes with salmon sperm-protein A-agarose beads, the unbound supernatant (subsequently referred to as "input") was removed and purified in a manner similar to that for the bound ChIP fraction described above. Serial dilutions of input were used as a reference to determine the relative enrichments

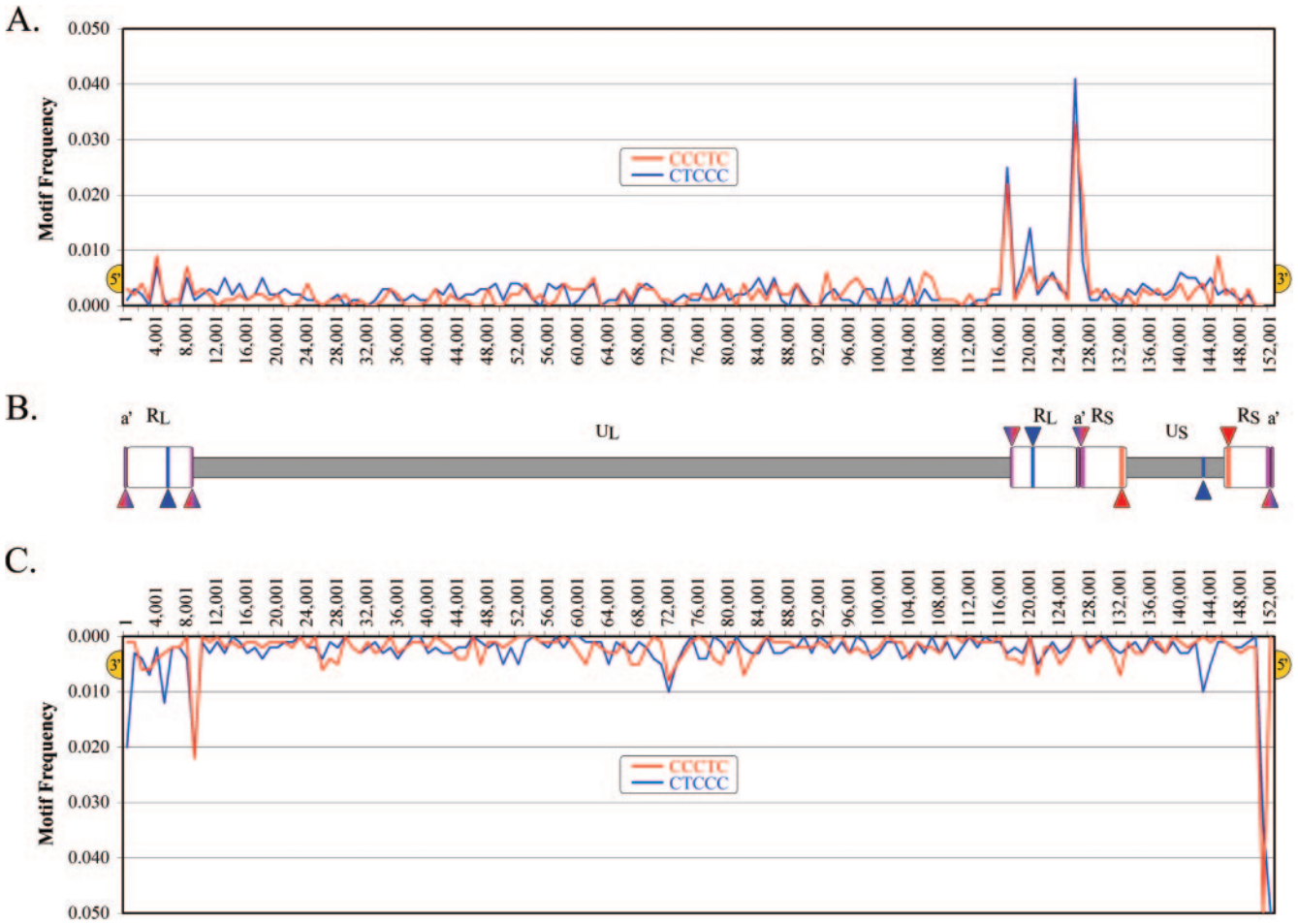

FIG. 1. The HSV-1 genome contains clustered CTCF-binding sites. An algorithm that searched for CCCTC or CTCCC motifs was used (see Materials and Methods) to analyze the HSV-1 strain 17*syn*+ genome (GenBank accession no. NC_001806) in 1,000-bp segments to determine the frequencies with which these CTCF binding sites occur in the (A) positive (direct) and (C) negative (complement) DNA strands. (B) Diagram of the HSV-1 genome illustrating locations of identified CTCF motifs. The shading (blue/red, interleaved CTCCC/CCCTC motifs; blue, CTCCC; red, CCCTC) indicates the regions of the genome where a high frequency of CTCF motifs cluster.

of different DNA targets in the bound ChIP fraction. PCRs on input dilutions and the bound ChIP fraction were performed simultaneously using HotStar *Taq* (QIAGEN) at cycles within the linear range, typically between 30 and 38 cycles. The initial-stage PCR cycle conditions used were as follows: 15 min at 95°C, 3 min at 94°C, 3 min at 55°C, and 3 min at 72°C. Subsequent repeated cycles were as follows: 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C (repeated 30 to 38 times). The PCR primers used for ChIP analysis are listed in Table 1.

All PCR products were resolved on 7.5% polyacrylamide gels, stained with SYBR Green (Molecular Probes), and detected using a Storm 860 Fluorimager (Molecular Dynamics). Band intensities for each PCR product were determined using ImageQuant software version 1.2. For the data shown in Table 2, band intensities for input samples were graphed, a linear regression was applied, and an equation for the line was determined, all using Kaleidagraph software. The equation for the line was used to determine the total relative enrichment of the PCR products generated using the same primer set on DNA from the precipitated (bound) ChIP fraction. The enrichment of one DNA region over another in a given bound ChIP fraction was determined by comparing the relative enrichment quantities obtained for two DNA regions of interest. These comparisons yielded the difference in enrichment of one DNA target over another by dividing the larger relative enrichment value by the smaller relative enrichment value. In all cases, the immunoprecipitated samples were compared with serial dilutions of the input, and mean values and standard deviations were calculated.

Plasmid constructs and enhancer-blocking assays. The HSV-1 LAT enhancer (nucleotides [nt] 118,888 to 119,477) was directionally cloned into the 5′ KpnI and 3′ MluI polylinker sites of the pGL3-simian virus 40 (SV40) promoter control vector (Promega) using PCR-generated KpnI and MluI linkers to create pGL3-enahcer/*rcr*. A putative CTRL2 insulator fragment (nt 120,210 to 120,942) which included the identified cluster of CTCF-binding sites, was cloned into the 5′ NheI and 3′ XhoI polylinker sites of the pGL3-SV40 promoter using PCR-generated NheI and XhoI linkers to create pGL3-Insulator. For the enhancer-blocking test construct, a 2.1-kb fragment (nt 118,888 to 120,942) containing the LAT enhancer/*rcr* and the putative CTRL2 insulator in their native configurations was directionally cloned into the 5′ KpnI and 3′ XhoI polylinker sites of the pGL3-SV40 promoter using PCR-generated KpnI and XhoI linkers to create pGL3-RXI. The CTRL2 deletion construct was generated by PCR amplification of sequences on the left-hand side (nt 118,888 to 120,443) and the right-hand side (nt 120,653 to 120,942) of the CTRL2 cluster tagged with 5′ KpnI and 3′ NheI or tagged with 5′ NheI and 3′ XhoI linkers, respectively. The PCR products were directionally cloned by three-piece ligation into the KpnI and XhoI polylinker sites of the pGL3-SV40 promoter. Transient transfections were set up with 1 μg of luciferase test construct in 24-well dishes seeded 24 h earlier with rabbit skin cells at 2×10^5 cells/ml. We cotransfected 500 ng of phRL-TK *Renilla* construct as a control for transfection efficiency. Each luciferase construct was tested using a minimum of four to six replicates. Transfections were carried out using QIAGEN Superfect reagent following the manufacturer's protocols. Following a 24-h incubation, the cells were lysed and luciferase levels were tested using Promega Dual-Glo reagents following the manufacturer's protocols, and the levels were measured using a Sirius Luminometer (Berthold Detection Systems). Data were graphed in Excel.

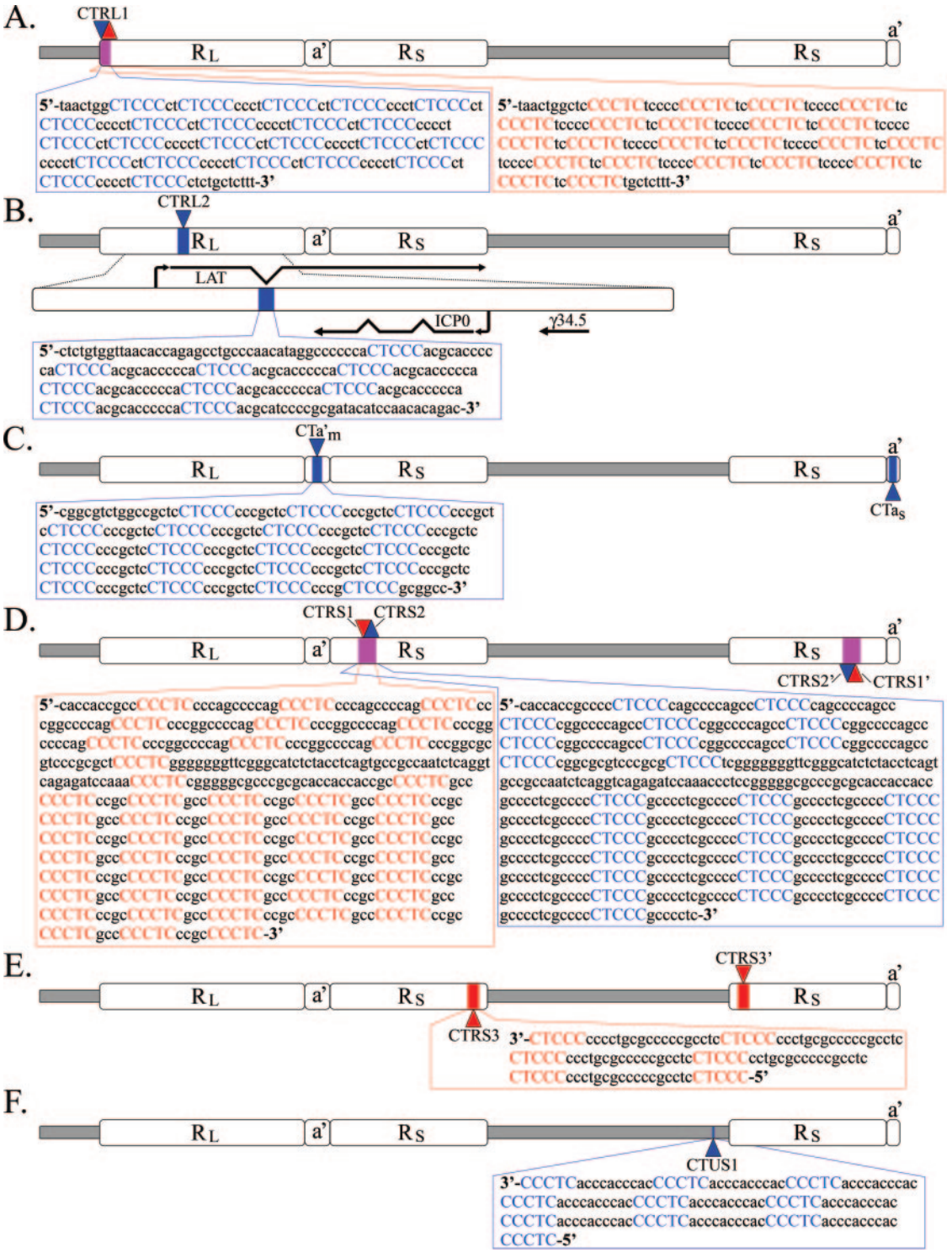

FIG. 2. Genome segments containing a high frequency of motifs reveal tandem CTCF motif clustering. Shown is an expanded view of a portion of the HSV-1 U_L, internal R_L and R_S (IR_L and IR_S), U_S, and terminal R_S regions illustrating the locations of clustered CTCF motifs that occur in tandem. The corresponding positive (direct)- or negative (complement)-strand DNA sequence is shown with 5′-CTCCC-3′ motifs highlighted in blue and 5′-CCCTC-3′ motifs highlighted in red. The CTCF motifs are shown in capital letters. (A) CTCCC/CCCTC cluster on the positive (direct) DNA strand that maps to the U_L/IR_L junction (nt 117,158 to 117,342). Since both CTCCC and CCCTC motifs cluster at this site, two copies of identical sequences are shown, but one motif type is selectively highlighted for viewing simplicity. (B) CTCCC cluster on the positive (direct) DNA strand that maps to the LAT intron region (nt 120,503 to 120,635). (C) CTCCC cluster on the positive (direct) DNA strand that maps to the a′ sequence (nt 126,057 to 126,274). (D) CCCTC/CTCCC cluster on the positive (direct) DNA strand that maps near the a′ sequence/IR_S junction (nt 126,571 to 127,141). The adjacent blocks of sequence are identical, but one motif is selectively highlighted for viewing simplicity. (E) CCCTC cluster on the negative (complement) strand that maps near the IR_S/U_S junction (nt 132,396 to 132,507). (F) CTCCC cluster on the negative (complement) strand that maps to the U_S region (nt 143,722 to 143,861). (G) Linear diagram of a portion of the genome labeled with relative locations of CTCF motif clusters and immediate-early genes. (H) Circular diagram of the entire genome labeled with relative locations of CTCF motif clusters and immediate-early genes.

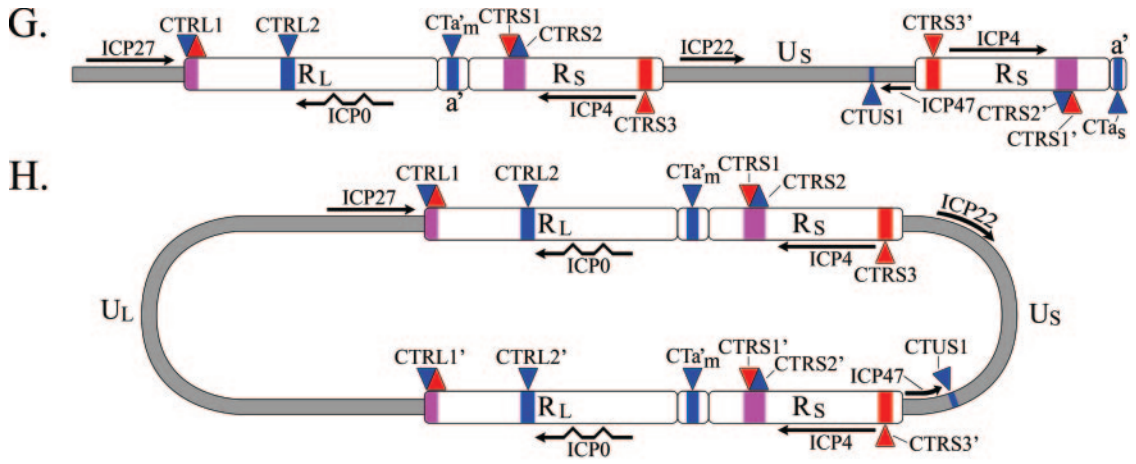

FIG. 2—*Continued.*

RESULTS

The HSV-1 genome contains clusters of binding motifs for the cellular protein CTCF. Previous studies indicated that a region of the latent HSV-1 genome encompassing the LAT promoter and extending the LAT 5′ exon is significantly enriched in acetylated histone H3 (K9 and K14), a histone modification associated with transcriptional permissiveness, whereas the ICP0 promoter and UL54 are transcriptionally repressed and underenriched in this histone (15, 16). This arrangement suggests that chromatin boundaries may separate these regions of differing transcriptional permissiveness and histone compositions. The regions analyzed in the previous study focused attention on a 5-kb region upstream of the LAT promoter and a 5-kb region downstream of the LAT 5′ exon. Examination of the sequences in these regions revealed clusters of two different consensus motifs (5′-CTCCC-3′ and 5′-CCCTC-3′) for the cellular protein CTCF (Fig. 1). A striking feature of these clusters is that they contain multiple copies of the CTCF motifs, which are periodically separated by intervening sequences (Fig. 2A and B). For example, the cluster CTRL2, located within the region encoding the LAT intron (Fig. 2B), contains nine copies of the CTCF motif "CTC CC" separated by eight reiterations of the sequence "ACGCAC CCCCA." The cluster CTRL1 located upstream of the LAT promoter near the unique-long/repeat-long (U_L/R_L) junction possesses a slightly different arrangement (Fig. 2A), with 23 copies of the CTCF motif "CTCCC" interspersed by alternating reiterations of "CT" and "CCCT." In addition, the CTRL1 cluster also contains 22 copies of the alternate CTCF motif "CCCTC" that are interleaved within the same sequence containing the CTCCC motif.

Additional analyses of both strands of the HSV-1 genome using the motif-searching algorithm as described in Materials and Methods identified four other significant clusters of the CTCF motifs in the HSV-1 genome (Fig. 1 and 2). One cluster was located in the "a" sequences (Cta'_m and Cta_1a_n), three clusters within the repeat short (R_S) (CTRS1, CTRS2, and CTRS3), and one cluster in the unique-short [U_S] region (CTUS1). As depicted in Fig. 2, some of these CT clusters are similar to CTRL2 and contain reiterations of only a single type of CTCF motif (Cta'_m, Cta_1a_n, CTRS3, and CTUS1), while CTRS1 and CTRS2 contain 51 reiterations of the CCCTC motif and 29 copies of the CTCCC motif interleaved. Note that CTRS1 and CTRS2 are two separate motif clusters separated by an intervening stretch of 87 nt of nonrepeated sequence (Fig. 2D).

The clustered motifs are present on both strands of the genome and possess a striking symmetry when viewed on a linear depiction of the genome (Fig. 2G). When the HSV-1 genome is depicted as a circular episome, it can be seen that these CT clusters have the potential to organize, or partition, the genome into 11 separate domains (Fig. 2H). In this arrangement, each of the IE genes, as well as the LAT enhancer/*rcr*, is located within separate domain compartments bounded by CTCF motif clusters.

ChIP analysis reveals that the CT clusters are enriched in CTCF during latency. Since sequence analysis revealed that the HSV-1 genome contains clustered CTCF motifs, we sought to determine whether the cellular protein CTCF binds these clusters during latency. ChIP analysis was performed on chromatin extracted from DRG of mice latently infected with HSV-1 strain 17*syn*+. Each primer set (Table 1) that was used to amplify the ChIP bound fraction was also used to amplify serial dilutions of the input fraction as controls for relative primer efficiencies. These input dilutions also served as references for determining the relative enrichment of CTCF within the IP samples at the various DNA clusters. The results of three independent ChIP experiments (Fig. 3A) revealed significant enrichment of CTCF at the *Tsix* locus (positive cellular control) compared to the MT498 (negative control), consistent with a previous report (6). PCR primers specific for the HSV-1 CTCF motif clusters were then used to analyze the same three IP samples for CTCF binding (Fig. 3B and C). The viral CTCF clusters show significant enrichment of CTCF compared to the glycoprotein C (gC) region, which does not contain CTCF motif clusters. It should be noted that the PCR analysis of the CTRS1/2 region may not be distinguishable from enrichment at the Cta'_m region, since there are fewer than 700 bp between the clusters and the distribution of sonicated fragments in the ChIPs is 500 to 1,000 bp. Nevertheless, the enrichment of CTCF at the motif clusters observed within the HSV-1 latent genome was on the order of two- to three-

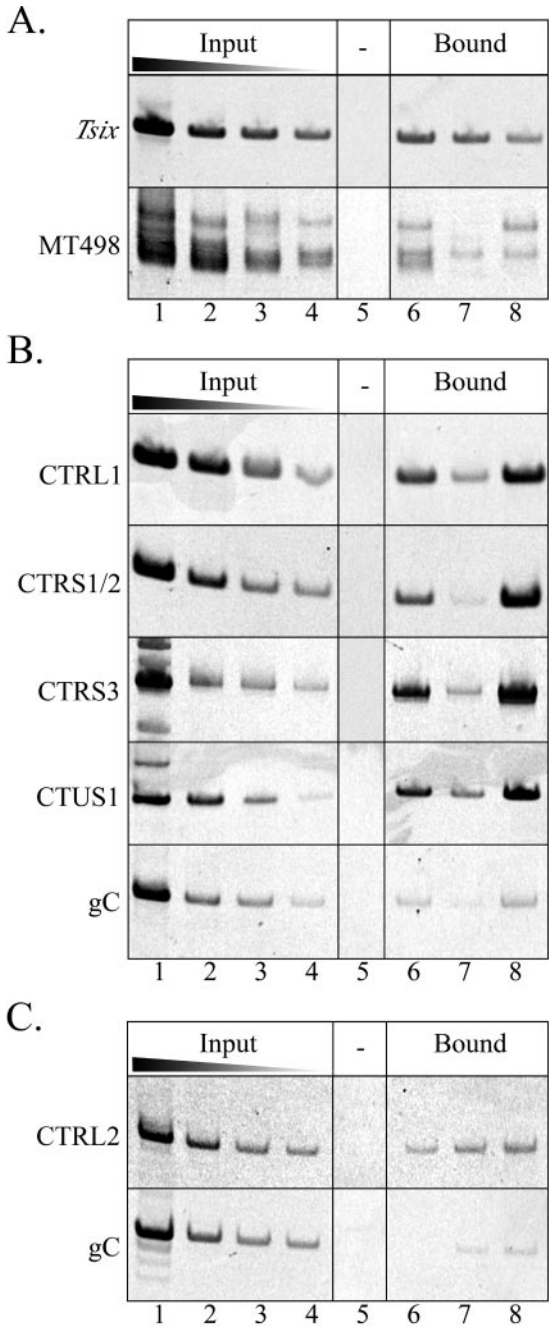

FIG. 3. ChIP analysis of the CTCF motif clusters within latent HSV-1 DNA using antiserum specific for anti-CTCF. DRG from mice latently infected with HSV-1 strain 17*syn*+ were processed and subjected to ChIP analysis as described in Materials and Methods. The relative enrichment of CTCF at each motif cluster was determined by PCR analysis of the ChIP (bound) fraction. Lanes 6 to 8 represent three independent ChIP experiments, and lanes 1 to 4 represent dilutions of the input material for comparison. Lanes 5 are the no-input controls. (A) ChIPs were validated by serial dilutions of input and a 1/10 dilution of bound ChIP sample using primers to the cellular target *Tsix* imprinting/choice center CTCF site A (positive control) and MT498 (negative control) (6). (B) PCRs performed with the same titrated input and bound ChIP sample with primers to the CTRL1, CTRS1 and -2, CTRS3, CTUS1, and gC viral targets. (C) PCRs performed with titrated input and 1/100 dilution of bound ChIP sample with primers to the CTRL2 and gC viral targets. Band intensities of PCR products generated with ChIP-precipitated DNA were quantitated with respect to twofold dilutions of input and used to demonstrate enrichments.

fold and is comparable to the difference in enrichment between the cellular controls (Table 2).

A larger fragment containing the CTRL2 cluster displays enhancer-blocking and silencing activities, common features of cellular insulators. The 135-bp cluster of repeated CTCF motifs (CTRL2) is located downstream of the LAT enhancer/*rcr* and upstream of the lytic gene ICP0 transcription termination site, effectively separating the enhancer/*rcr* from the ICP0 promoter. Since enhancer-blocking activity represents one of two well-known functions of cellular insulators, we set out to determine if the region encompassing CTRL2 is capable of blocking the LAT enhancer. A 1.5-kb fragment containing sequence flanking the core 135-bp CTRL2 CTCF cluster was examined, because the defining functional properties of cellular-insulator elements often reside 5′ and 3′ to the CTCF elements (28). To define insulator activity, several luciferase reporter constructs were developed (see Materials and Methods), modeled after constructs used to characterize the regulation of the chicken β-globin LCR by the enhancer-blocking β-globin insulator (1, 7, 24). The luciferase reporter assays revealed that the LAT enhancer/*rcr* is capable of enhancing transcription from a heterologous promoter (SV40 promoter) by ~3.5-fold, while a CTRL2-containing fragment alone did not influence transcription from the SV40 promoter (Fig. 4A). However, when a CTRL2-containing fragment was positioned between the LAT enhancer and the promoter, it completely blocked the effects of the LAT enhancer (Fig. 4A). This demonstrates that this element possesses classic enhancer-blocking activity and functions as an insulator.

In order to determine whether the repeated CTCF motifs contribute directly to the enhancer-blocking and -silencing activities of this element, a 135-bp fragment containing the nine reiterated CTCF motifs was deleted from the larger insulator fragment used in the above-mentioned experiments. Deletion of the core 135-bp CTRL2 motif cluster resulted in a twofold loss of transcriptional silencing ($P < 0.003$) but no loss of enhancer blocking (Fig. 4B). These data indicate that the silencing function associated with this insulator element is largely dependent on the CTRL2 CTCF cluster, while the enhancer-blocking activity resides within the larger 1.5-kb insulator domain.

Clusters of CTCF motifs are conserved among other alphaherpesviruses. We have demonstrated that the CTCF-containing CTRL2 fragment possesses insulator properties, and we hypothesize that at least some of the other HSV-1 CTCF clusters possess similar functions. If these other elements play a central role in the organization of HSV-1 latent chromatin, we would expect these elements to be conserved among other members of the alphaherpesviruses. In order to investigate this hypothesis, we performed CTCF motif analyses on the genomic sequences of several other alphaherpesviruses. As depicted in Fig. 5, clusters of CTCF motifs were identified in HSV-2 strain HG-52, cercopithecine herpesvirus 1 (herpesvirus simiae, or B virus) (22), varicella-zoster virus (VZV) strain Dumas, and pseudorabies virus (14). Even though several of these viruses contain alternative CTCF motifs (Fig. 5B), the striking feature

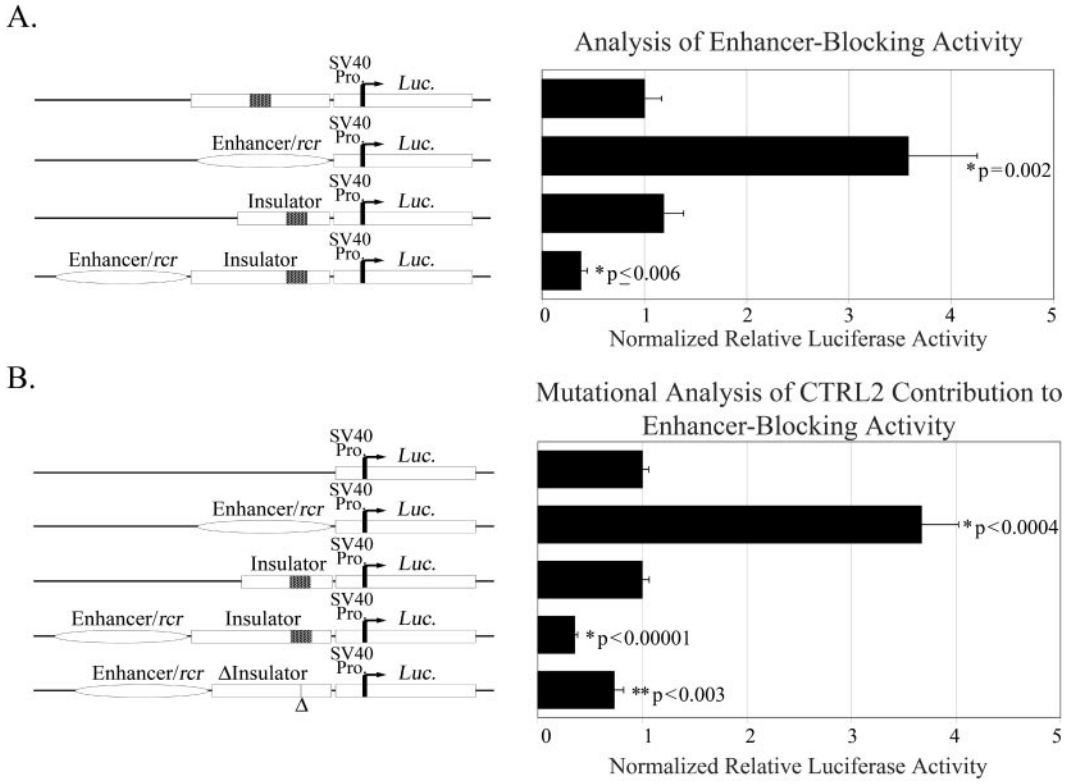

FIG. 4. The 1.5-kb region encompassing CTRL2 functions as an insulator with enhancer-blocking and -silencing activities. (A) Luciferase reporter constructs were generated to test the ability of the LAT insulator to specifically block the LAT enhancer/*rcr*. The data were collected as relative luciferase units, normalized to an internal *Renilla* luciferase control, and expressed relative to the pGL3-SV40 promoter control. The *P* values represent unpaired one-tailed Student's *t* tests in pairwise comparisons to the pGL3-SV40 promoter control. The error bars represent standard deviations. (B) Mutational analysis of a luciferase reporter construct that specifically deletes the 135-bp core CTRL2 cluster. The single-asterisk *P* values represent unpaired one-tailed Student's *t* tests in pairwise comparisons to the pGL3-SV40 promoter control, while the double-asterisk *P* value represents a comparison to the nonmutated enhancer-blocking insulator construct.

is that these motifs also occur in tandem clusters and in a configuration similar to that in HSV-1 (Fig. 5). Specifically, the repeats are often situated so that IE genes are contained within separate domains formed by a pair of clusters, despite the fact that several of the IE genes are located in different relative genomic positions among these viruses. While repeat clusters corresponding to each of those identified in HSV-1 can be identified in these other alphaherpesviruses, there are some differences. As expected, the CT repeats in HSV-2 resemble those of HSV-1, except for a slight shift in their positions, most notably the CTRL2 and CTUS1 homologues (Fig. 5). The most striking difference between the viruses analyzed is that pseudorabies virus and VZV contain a number of additional consensus motifs in the U_L regions of their genomes. Finally, it should be noted for VZV that while there is not a large tandem-repeat cluster of CTCF motifs at the R_L/U_L junction (where the homologue of HSV-1 CTRL1 would be), there are seven CTCF consensus motifs within a 90-bp segment located in this region, a pattern that would predict CTCF binding.

Taken together, these data indicate that the clustering of CTCF-binding motifs is highly conserved across members of the alphaherpesvirus family.

DISCUSSION

We have identified seven clusters of repeated binding motifs for the cellular protein CTCF and demonstrated that CTCF is associated with these clusters on the latent HSV-1 genome. Such a finding is significant, because CTCF is a known transcriptional regulator and a component of all known vertebrate chromosomal insulators (28). Functional analysis of a 1.5-kb fragment containing the cluster of CTCF motifs (CTRL2) located 3′ of the LAT enhancer revealed both enhancer-blocking and silencing activities, properties characteristic of cellular insulators.

The LAT locus is transcriptionally active during latency and is associated with acetylated histones, while ICP0 is transcriptionally repressed and hypoacetylated (16). The 1.5-kb insulator-like element, subsequently referred to as the LAT insulator, is located between these two regions of transcriptionally distinct chromatin, suggesting that the LAT insulator blocks the LAT enhancer from enhancing the ICP0 promoter and other lytic promoters during latency. We postulate that the LAT insulator, as well as the other putative CTCF motif insulators in the HSV genome, plays a dominant role in regulating HSV-1 latent-gene expression at the level of chromatin. For example, during a latent infection, insulators containing the CTRL1 and CTRL2 elements could act as enhancer-blocking insulators to prevent the LAT enhancer from activating transcription of genes outside this chromatin domain. This is consistent with the confined hyperacetylation observed in this region during latency. Reactivation could be initiated by either an alteration or a collapse of the LAT insulator, allowing the LAT enhancer to act on the ICP0 promoter. The production of

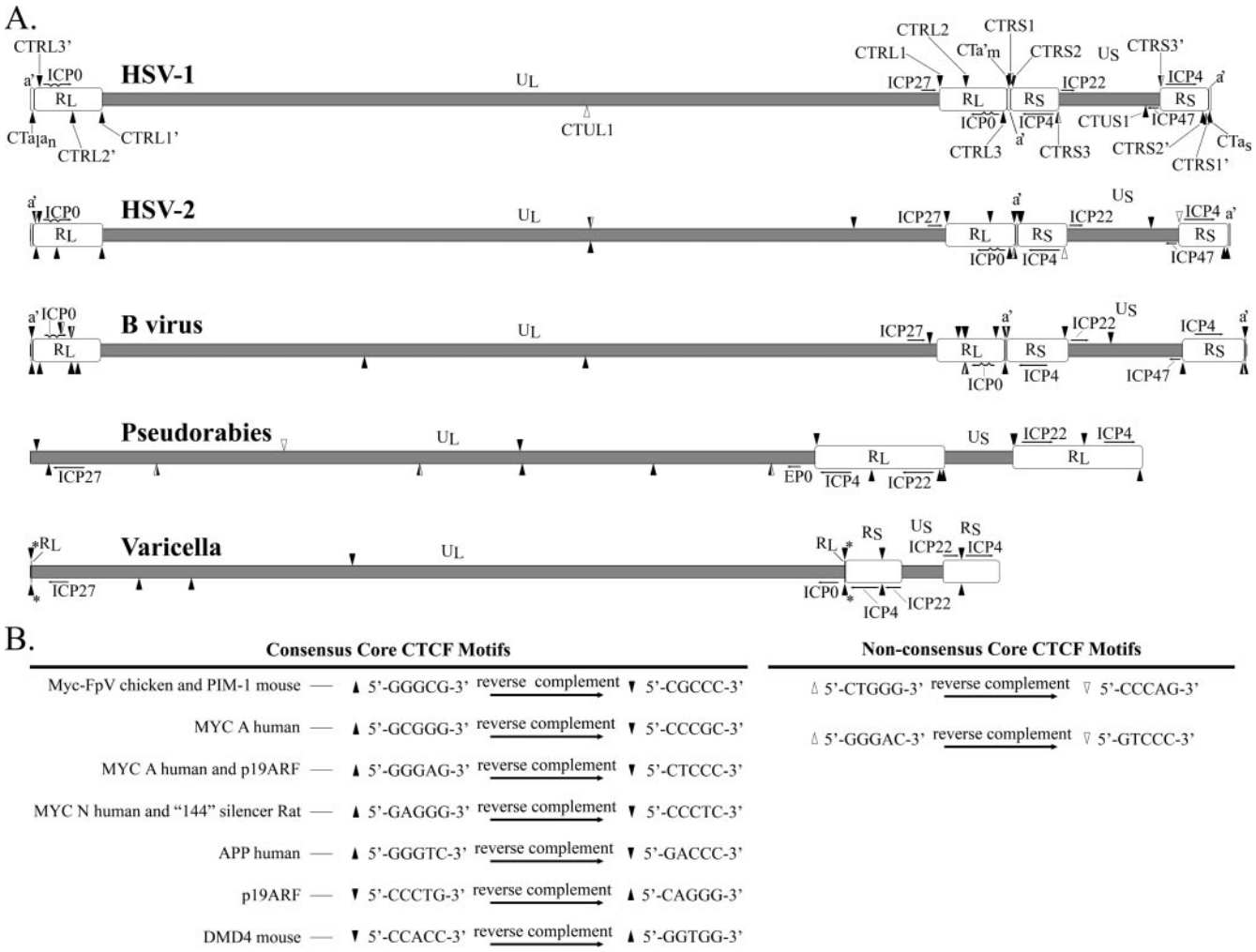

FIG. 5. Clustered CTCF-binding sites are conserved across the *Alphaherpesvirus* family and flank the immediate-early genes. (A) The algorithm used to analyze the HSV-1 strain 17*syn*+ genome was applied in the same manner to analyze 1,000-bp segments of each alphaherpesvirus genome to determine the frequency of CTCF-binding sites on the positive (direct) or negative (complement) DNA strand. Additionally, tandem-repeat analysis was performed to characterize the CTCF motif clustering (2). Analyses were performed using published NCBI GenBank sequences for HSV-2 strain HG52 (NC_001798; D. J. McGeoch), suid herpesvirus 1 (pseudorabies virus) (BK001744; L. W. Enquist), human herpesvirus 3 strain Dumas (varicella-zoster virus) (X04370; J. E. Scott), and cercopithecine herpesvirus 1 (monkey B virus) (NC_004812; J. K. Hilliard). (B) Representative CTCF pentanucleotide motifs found clustered within the *Alphaherpesvirus* family members. The solid triangles represent consensus CTCF motifs previously reported to bind CTCF (17, 21). The open triangles represent nonconsensus CTCF pentanucleotide motifs. Partially solid/open triangles represent clusters composed of interleaved consensus and nonconsensus motifs. The pointed end of each triangle indicates the DNA strand direction (direct or complement).

ICP0, which has chromatin-remodeling activities (17), would potentially promote a shift from transcriptionally nonpermissive hypoacetylated chromatin to a more permissive state and facilitate the activation of other lytic genes. Work to determine whether these changes in chromatin occur following stress is under way.

A significant finding in this study is that the transcriptional silencing activity within the LAT insulator is dependent on the CTCF motifs, whereas the enhancer-blocking activity is not. Since CTCF is known to be a core component of insulators, one might initially assume that deleting the binding motifs would abolish all insulator functions. On the contrary, characterization of cellular insulators has shown this not to be the case. For example, Prawitt et al. have recently shown that deletion of a complete cluster of seven CTCF motifs in the *IGF2*-regulating center alone is incapable of disrupting insulator activity and that additional deletions or rearrangements of surrounding *cis* components are necessary to abolish the insulator function (23). Even more analogous to the LAT insulator, the chicken lysozyme insulator possesses enhancer-blocking and silencing activities. In this case, enhancer blocking is not dependent on CTCF but instead depends on a nearby thyroid hormone response element (19). In contrast, the silencing activity shows a direct dependence on CTCF binding (3). Therefore, the observation that enhancer-blocking and silencing functions of the LAT enhancer seem to be differentially dependent on CTCF is consistent with those of other characterized insulators and suggests that there are other elements within the 1.5-kb insulator fragment that provide accessory functions.

As mentioned in the introduction, cellular insulators often have cell type specificity; therefore, the establishment of HSV-1 CTCF insulator complexes may be cell type specific. If this is the case, these insulators could play a role in regulating the tropism of HSV-1 latency in sensory neurons. Since it has been suggested that only a subset of sensory neurons are permissive for latent infection (29), it is possible that only this subset of sensory neurons can activate the enhancer-blocking activity of the CTRL2-containing LAT insulator. It should be noted that the LAT promoter and LAT enhancer also have cell type specificities (10), and it is conceivable that they, in combination with the insulator, may act as a multicomponent regulator. It is intriguing to speculate that the presence of such a multicomponent switch could explain why stress induces only a portion of the latently infected pool of neurons to reactivate at any given time. The regulation of the LAT insulator, the LAT enhancer, and the ICP0 promoter would provide several upstream levels of regulatory control that would allow the virus to balance between latency and reactivation, even in the context of relatively global stress stimuli.

While we have discussed the potential role of the LAT insulator as a chromatin level regulator of LAT and ICP0 transcription, this does not include a role for the other CTCF motif-containing elements that we have identified. The fact that the CTCF elements exist in a genomic arrangement so that each of the IE genes is bounded by separate insulators suggests the possibility that each could be regulated as a separate chromatin domain. While our analyses by ChIP have failed to detect any significant differences in the acetylation statuses of ICP4, ICP27, and ICP0, this does not rule out the possibility that the promoters for these genes could each be associated with other specifically modified histones that would confer different transcription activation potentials. The conservation of these elements among other alphaherpesviruses implies they may play significant regulatory roles. It is interesting to speculate that some of the additional CTCF elements present in VZV could play a role in the transcriptional activity observed for some lytic VZV genes during latency (8), specifically, by generating more permissive transcriptional domains surrounding some lytic genes than others. An alternative hypothesis for the existence of separate chromatin domains surrounding lytic gene compartments in HSV-1 is that the separate insulation of each IE gene is redundant and helps prevent any LAT enhancer activity that might leak beyond the LAT insulators from altering the regulation of the IE genes, as long as the separate insulators are in place. Such "back-up" insulation has been observed in the regulation of several cellular chromatin domains (11, 28). The specific enhancer-blocking and boundary properties possessed by each of the CT elements both individually and in combination remain to be tested experimentally.

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REFERENCES

1. **Bell, A. C., A. G. West, and G. Felsenfeld.** 1999. The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. Cell **98:**387–396.
2. **Benson, G.** 1999. Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Res. **27:**573–580.
3. **Burcin, M., R. Arnold, M. Lutz, B. Kaiser, D. Runge, F. Lottspeich, G. N. Filippova, V. V. Lobanenkov, and R. Renkawitz.** 1997. Negative protein 1, which is required for function of the chicken lysozyme gene silencer in conjunction with hormone receptors, is identical to the multivalent zinc finger repressor CTCF. Mol. Cell. Biol. **17:**1281–1288.
4. **Cai, H. N., and M. Levine.** 1997. The gypsy insulator can function as a promoter-specific silencer in the *Drosophila* embryo. EMBO J. **16:**1732–1741.
5. **Capelson, M., and V. G. Corces.** 2004. Boundary elements and nuclear organization. Biol. Cell **96:**617–629.
6. **Chao, W., K. D. Huynh, R. J. Spencer, L. S. Davidow, and J. T. Lee.** 2002. CTCF, a candidate *trans*-acting factor for X-inactivation choice. Science **295:**345–347.
7. **Chung, J. H., A. C. Bell, and G. Felsenfeld.** 1997. Characterization of the chicken beta-globin insulator. Proc. Natl. Acad. Sci. USA **94:**575–580.
8. **Cohrs, R. J., J. Randall, J. Smith, D. H. Gilden, C. Dabrowski, H. van Der Keyl, and R. Tal-Singer.** 2000. Analysis of individual human trigeminal ganglia for latent herpes simplex virus type 1 and varicella-zoster virus nucleic acids using real-time PCR. J. Virol. **74:**11464–11471.
9. **Deshmane, S. L., and N. W. Fraser.** 1989. During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. J. Virol. **63:**943–947.
10. **Dobson, A. T., T. P. Margolis, W. A. Gomes, and L. T. Feldman.** 1995. In vivo deletion analysis of the herpes simplex virus type 1 latency-associated transcript promoter. J. Virol. **69:**2264–2270.
11. **Donze, D., C. R. Adams, J. Rine, and R. T. Kamakaka.** 1999. The boundaries of the silenced HMR domain in *Saccharomyces cerevisiae*. Genes Dev. **13:**698–708.
12. **Du, M., L. G. Beatty, W. Zhou, J. Lew, C. Schoenherr, R. Weksberg, and P. D. Sadowski.** 2003. Insulator and silencer sequences in the imprinted region of human chromosome 11p15.5. Hum. Mol. Genet. **12:**1927–1939.
13. **Felsenfeld, G., B. Burgess-Beusse, C. Farrell, M. Gaszner, R. Ghirlando, S. Huang, C. Jin, M. Litt, F. Magdinier, V. Mutskov, Y. Nakatani, H. Tagami, A. West, and T. Yusufzai.** 2004. Chromatin boundaries and chromatin domains. Cold Spring Harbor Symp. Quant. Biol. **69:**245–250.
14. **Klupp, B. G., C. J. Hengartner, T. C. Mettenleiter, and L. W. Enquist.** 2004. Complete, annotated sequence of the pseudorabies virus genome. J. Virol. **78:**424–440.
15. **Kubat, N. J., A. L. Amelio, N. V. Giordani, and D. C. Bloom.** 2004. The herpes simplex virus type 1 latency-associated transcript (LAT) enhancer/*rcr* is hyperacetylated during latency independently of LAT transcription. J. Virol. **78:**12508–12518.
16. **Kubat, N. J., R. K. Tran, P. McAnany, and D. C. Bloom.** 2004. Specific histone tail modification and not DNA methylation is a determinant of herpes simplex virus type 1 latent gene expression. J. Virol. **78:**1139–1149.
17. **Lomonte, P., K. F. Sullivan, and R. D. Everett.** 2001. Degradation of nucleosome-associated centromeric histone H3-like protein CENP-A induced by herpes simplex virus type protein ICP0. J. Biol. Chem. **276:**5829–5835.
18. **Lutz, M., L. J. Burke, G. Barreto, F. Goeman, H. Greb, R. Arnold, H. Schultheiss, A. Brehm, T. Kouzarides, V. Lobanenkov, and R. Renkawitz.** 2000. Transcriptional repression by the insulator protein CTCF involves histone deacetylases. Nucleic Acids Res. **28:**1707–1713.
19. **Lutz, M., L. J. Burke, P. LeFevre, F. A. Myers, A. W. Thorne, C. Crane-Robinson, C. Bonifer, G. N. Filippova, V. Lobanenkov, and R. Renkawitz.** 2003. Thyroid hormone-regulated enhancer blocking: cooperation of CTCF and thyroid hormone receptor. EMBO J. **22:**1579–1587.
20. **Margolis, T. P., F. Sedarati, A. T. Dobson, L. T. Feldman, and J. G. Stevens.** 1992. Pathways of viral gene expression during acute neuronal infection with HSV-1. Virology **189:**150–160.
21. **Ohlsson, R., R. Renkawitz, and V. Lobanenkov.** 2001. CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. Trends Genet. **17:**520–527.
22. **Perelygina, L., L. Zhu, H. Zurkuhlen, R. Mills, M. Borodovsky, and J. K. Hilliard.** 2003. Complete sequence and comparative analysis of the genome of herpes B virus (Cercopithecine herpesvirus 1) from a rhesus monkey. J. Virol. **77:**6167–6177.
23. **Prawitt, D., T. Enklaar, B. Gartner-Rupprecht, C. Spangenberg, M. Oswald, E. Lausch, P. Schmidtke, D. Reutzel, S. Fees, R. Lucito, M. Korzon, I. Brozek, J. Limon, D. E. Housman, J. Pelletier, and B. Zabel.** 2005. Microdeletion of target sites for insulator protein CTCF in a chromosome 11p15 imprinting center in Beckwith-Wiedemann syndrome and Wilms' tumor. Proc. Natl. Acad. Sci. USA **102:**4085–4090.
24. **Recillas-Targa, F., A. C. Bell, and G. Felsenfeld.** 1999. Positional enhancer-blocking activity of the chicken beta-globin insulator in transiently transfected cells. Proc. Natl. Acad. Sci. USA **96:**14354–14359.
25. **Rock, D. L., and N. W. Fraser.** 1985. Latent herpes simplex virus type 1 DNA contains two copies of the virion DNA joint region. J. Virol. **55:**849–852.
26. **Stevens, J. G., E. K. Wagner, R. G. B. Devi, M. L. Cook, and L. T. Feldman.** 1987. RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science **235:**1056–1059.
27. **Wang, Q. Y., C. Zhou, K. E. Johnson, R. C. Colgrove, D. M. Coen, and D. M.**

Knipe. 2005. Herpesviral latency-associated transcript gene promotes assembly of heterochromatin on viral lytic-gene promoters in latent infection. Proc. Natl. Acad. Sci. USA **102:**16055–16059.
28. **West, A. G., M. Gaszner, and G. Felsenfeld.** 2002. Insulators: many functions, many mechanisms. Genes Dev. **16:**271–288.
29. **Yang, L., C. C. Voytek, and T. P. Margolis.** 2000. Immunohistochemical analysis of primary sensory neurons latently infected with herpes simplex virus type 1. J. Virol. **74:**209–217.
30. **Yao, S., C. S. Osborne, R. R. Bharadwaj, P. Pasceri, T. Sukonnik, D. Pannell, F. Recillas-Targa, A. G. West, and J. Ellis.** 2003. Retrovirus silencer blocking by the cHS4 insulator is CTCF independent. Nucleic Acids Res. **31:**5317–5323.
31. **Yusufzai, T. M., H. Tagami, Y. Nakatani, and G. Felsenfeld.** 2004. CTCF tethers an insulator to subnuclear sites, suggesting shared insulator mechanisms across species. Mol. Cell **13:**291–298.
32. **Zhao, H., and A. Dean.** 2004. An insulator blocks spreading of histone acetylation and interferes with RNA polymerase II transfer between an enhancer and gene. Nucleic Acids Res. **32:**4903–4919.