Genome replication affects transcription factor binding mediating the cascade of herpes simplex virus transcription

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In herpes simplex virus type 1 (HSV-1) infection, the coupling of genome replication and transcription regulation has been known for many years; however, the underlying mechanism has not been elucidated. We performed a comprehensive transcriptomic assessment and factor-binding analysis for Pol II, TBP, TAF1, and Sp1 to assess the effect genome replication has on viral transcription initiation and elongation. The onset of genome replication resulted in the binding of TBP, TAF1, and Pol II to previously silent late promoters. The viral transcription factor, ICP4, was continuously needed in addition to DNA replication for activation of late gene transcription initiation. Furthermore, late promoters contain a motif that closely matches the consensus initiator element (Inr), which robustly bound TAF1 postreplication. Continued DNA replication resulted in reduced binding of Sp1, TBP, and Pol II to early promoters. Therefore, the initiation of early gene transcription is attenuated following DNA replication. Herein, we propose a model for how viral DNA replication results in the differential utilization of cellular factors that function in transcription initiation, leading to the delineation of kinetic class in HSV-productive infection.

DNA viruses | genome replication | transcription | Pol II

The genome of Herpes simplex virus I (HSV-1) is 152 kb of linear double-stranded DNA, containing three origins of replication and over 90 unique ORFs (1–3). Genome replication and transcription occur in the host cell nucleus. HSV-1 encodes its own genome replication machinery (4), and viral gene products augment the RNA Polymerase II (Pol II) machinery of the cell for the transcription of the genome (5). Viral transcription is incredibly robust, allowing a single infecting virus to produce progeny between 4 and 6 h postinfection, culminating in ∼1,000 infectious progeny per cell within 18 h.

The HSV-1 transcriptional cascade was originally defined by adding cyclohexamide (6), canavanine (7), and phosphonoacetic acid (8) to infected cells and assessing peptide and, later, RNA accumulation. These experiments defined immediate early (IE or α), early (E or β), and late (L or γ) genes as those expressed in the absence of de novo viral protein synthesis, in the presence of functional alpha proteins before DNA replication, and after genome replication, respectively. Late genes were further delineated as leaky late (γ_1) and true late (γ_2) based on whether genome replication increases the rate of synthesis or initiates synthesis. α gene promoters possess promoter elements, which bind the viral tegument protein, VP16, activating their transcription (9–12). ICP4 is the product of an α gene that is required for the transcription of viral β and γ genes (13–15). A canonical β gene, thymidine kinase (UL23 or tk), contains upstream promoter elements (UPEs), namely, two GC-boxes and a CAAT-box (16), which bind the cellular transcription factors Sp1 and NF1, respectively (16, 17). A prototypic γ_2 gene, glycoprotein C (UL44 or gC), consists of a TATA box and an initiator (Inr) sequence located near the start site of transcription, the latter of which is important for robust transcription and ICP4 activation (18–21). How this gene architecture is coordinated with genome replication to produce the observed cascade of gene expression is unknown.

We recently demonstrated that the onset of genome replication was sufficient to facilitate γ_2 transcription and the attenuation of early transcription (22). We wanted to determine the transcriptional changes associated with this shift. We report a comprehensive assessment of how genome replication alters the viral transcriptional landscape and facilitates a shift in transcript production. Our results lead us to propose a model of the viral transcriptional cascade which provides insight into a mechanism used by DNA viruses to coordinate efficient virion production.

Results

Classification of Viral DNA Replication-Dependent Transcripts. We first performed a global analysis to obtain a data set of genes in each class as a prelude to determining what molecular features distinguish early and late genes. We infected human fibroblast (MRC5) cells with wild-type HSV-1 (KOS) and a UL30 C-terminal truncation mutant, ΔC1216. UL30 encodes the viral DNA-dependent DNA polymerase (23). The C-terminal tail of UL30 was shown to be essential for interaction with the processivity factor, UL42 (24). In the absence of this interaction, HSV genome replication is inhibited (25). We assessed genome replication and transcriptional activity for this mutant and wild-type virus. As expected, there was no genome replication in ΔC1216-infected cells (Fig. 1*B*). Consistent with previous studies, thymidine kinase and single-stranded DNA-binding protein (UL29) accumulated to higher levels in ΔC1216-infected cells. By the end of the time course almost all other viral transcripts

Significance

Nuclear-replicating DNA viruses employ a common strategy of coupling late gene transcription to genome replication. The mechanism by which genome replication facilitates nascent transcription is unknown. We performed RNA-Seq and ChIP-Seq for Pol II, TBP, TAF1, and Sp1 to quantitatively assess transcription initiation and elongation across the viral genome. The onset of replication permanently altered genomic accessibility and, with the aid of ICP4, facilitated binding of initiation factors to previously silenced promoters. Continued replication precluded binding of transcription factors to promoters active prior to DNA replication. The results provide a model for the mechanism of the transcription switch in HSV infection, which may be applicable to other nuclear-replicating DNA viruses.

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Data deposition: All data are publicly accessible in the Sequence Read Archive database (accession nos. SRP172751, SRP172780, SRP172782, and SRP172783).

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were produced more robustly in wild-type infection, reaching 50% of total RNA sampled, compared with only ~12% in ΔC1216 (Fig. 1*C*). We clustered viral transcripts based on their accumulation in the presence and absence of genome replication (Fig. 1*A*). Twenty-three genes were excluded from classification, as their reads were present at very low levels (<2,000 MR/MTR/KB) in all samples analyzed. Forty-six viral genes were classified strictly as either β, γ_1, or γ_2. As expected, the β gene class encompassed proteins with a role in genome replication and maintenance. All other transcripts were classified as dependent on genome replication. These more closely defined gene classes (*SI Appendix*, Table S1) were used for all further analyses.

Viral Replication Facilitates Preinitiation Complex Formation on Previously Silent Promoters. We infected MRC5 cells with wild-type HSV-1 in the presence or absence of an inhibitor of viral replication, acyclovir (ACV). Infected cells were harvested at 4 h postinfection (hpi), and ChIP-Seq was performed for Pol II, TATA-binding protein (TBP), and transcription initiation factor TFIID subunit 1 (TAF1) (*SI Appendix*, Fig. S3). Using these data, we were able to assess transcription factor dynamics in conditions optimal for either β or γ gene transcription. ChIP-Seq reads mapped to previously characterized viral promoters demonstrated that each factor was positioned relative to its respective binding element (*SI Appendix*, Fig. S1).

To quantitatively compare inhibited (+ACV) and uninhibited (−ACV) samples we had to account for viral genome replication. Quantification of input samples provided the relative amounts of viral genomes present in each condition (~30-fold higher in −ACV) (Fig. 2*A*). We used this ratio to normalize each immunoprecipitated (IP) sample for the amount of factor per genome. We call this measurement relative factor occupancy and quantified all ChIP-Seq data in the same way. To assess preinitiation complex formation, we quantified relative factor occupancy on viral promoters (*SI Appendix*, Table S3). In the absence of DNA replication (+ACV) there was a complete absence of TBP, TAF1, and Pol II on γ_2 gene promoters. By comparison, in the presence of DNA replication (−ACV) there was a severe reduction in TBP, TAF1, and Pol II on β gene promoters. As expected, γ_1 genes displayed an intermediate phenotype, with initiation factors bound in both conditions but a greater amount in the presence of replication (Fig. 2 *B* and *C*). This trend is quite visible when looking at UL50, UL48, and UL44 as strong examples of β, γ_1, and γ_2 genes, respectively (Fig. 2*D*). These results suggest DNA replication alters promoter accessibility to cellular transcription factors involved in the initiation of transcription.

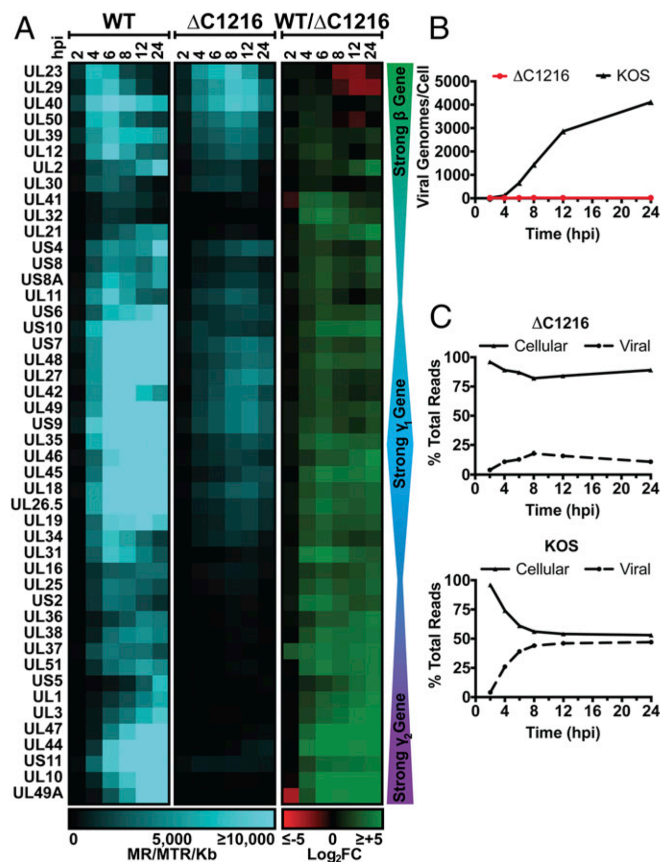

Fig. 1. Replication dependence of viral transcripts. MRC5 cells were infected with WT HSV-1 and ΔC1216. (*A*) RNA-Seq data as mapped reads per million total reads per kilobase (MR/MTR/KB) or $\log_2$ fold-change of WT over ΔC1216. (*B*) Number of viral genomes per cell. (*C*) RNA-Seq reads mapping to the viral or cellular genome as a percentage of total reads.

A Single Duplication Alters Genomic Accessibility. Next, we assessed the extent of genome replication required to alter viral transcription dynamics. This was to determine whether the transcriptional shift was due to an immediate cis-effect of DNA replication or an increase in genome number titrating out a repressive factor. MRC5 cells were infected with wild-type HSV-1 for 2, 3, and 4 hpi, and ChIP-Seq for Pol II, TBP, and TAF1 was performed (*SI Appendix*, Fig. S4). HSV-1 genome replication starts between 2.5 and 3 hpi, so each time point represents a different replication state: 2 hpi (prereplication), 3 hpi (1–2 genome duplications), and 4 hpi (3–4 genome duplications) (Fig. 3*A*). Consistent with the results of Fig. 2, there was a complete absence of transcription factors on γ_2 promoters prereplication and a severe reduction of transcription factors on β promoters postreplication (Fig. 3 *B* and *C*). γ_2 promoter occupancy initiated between 2 and 3 hpi. These data suggest genome replication had an immediate cis-effect on the accessibility of γ_2 gene promoters to transcription factors. Conversely, diminished β gene promoter occupancy was not observed until 4 hpi. Thus, genome replication must immediately alter the form or state of the viral genome. This alteration resulted in a global increase in viral promoter accessibility to cellular factors.

Sp1 Preferentially Binds to the Viral Genome Prereplication. Sp1 has been previously shown to bind and activate the viral β gene, thymidine kinase (16, 17). To investigate if Sp1 is globally responsible for activation of β genes we performed ChIP-Seq for Sp1 on MRC5 cells infected with wild-type HSV-1 for 2 and 4 h. Prereplication we observed numerous distinct Sp1 promoter peaks binding to 100% of α genes, 71% of β genes, 22% of γ_1 genes, and 0% of γ_2 genes (Fig. 4 *A* and *B*). We noted a severe reduction in Sp1 binding at 4 hpi, with only the α genes ICP4 and ICP0 retaining strong binding (Fig. 4 *A* and *B*). We noted that promoters bound by Sp1 had decreased Pol II occupancy between 2 and 4 hpi, while promoters always lacking an Sp1 peak did not experience this decrease (Fig. 4*C*). This global decrease in Sp1 binding at 4 hpi may be due to a previously identified phosphorylation event occurring at the same time during infection (26). Alternatively, the amount of Sp1 in the cell may be limiting, or the very act of genome replication precludes Sp1 binding.

Differential TFIID Promoter Context Preferred After Replication. We observed variation in the relative amplitude and shape of TAF1 peaks, with early genes exhibiting weak, broad binding. Genes with a weak TAF1 peak include UL23, UL39, UL50, UL2, and UL12. These promoters still possessed distinct, strong TBP and Pol II binding. To assess whether this binding phenotype was sequence-specific we performed motif discovery to predict TBP- and TAF1-binding motifs. We then determined consensus TATA boxes and Inr elements for each gene class (*SI Appendix*, Table S2). We noted little difference in TATA box motifs between the different gene classes (Fig. 5*A*). On an individual gene basis, TATA boxes matching the consensus, TATAW, were more robustly transcribed.

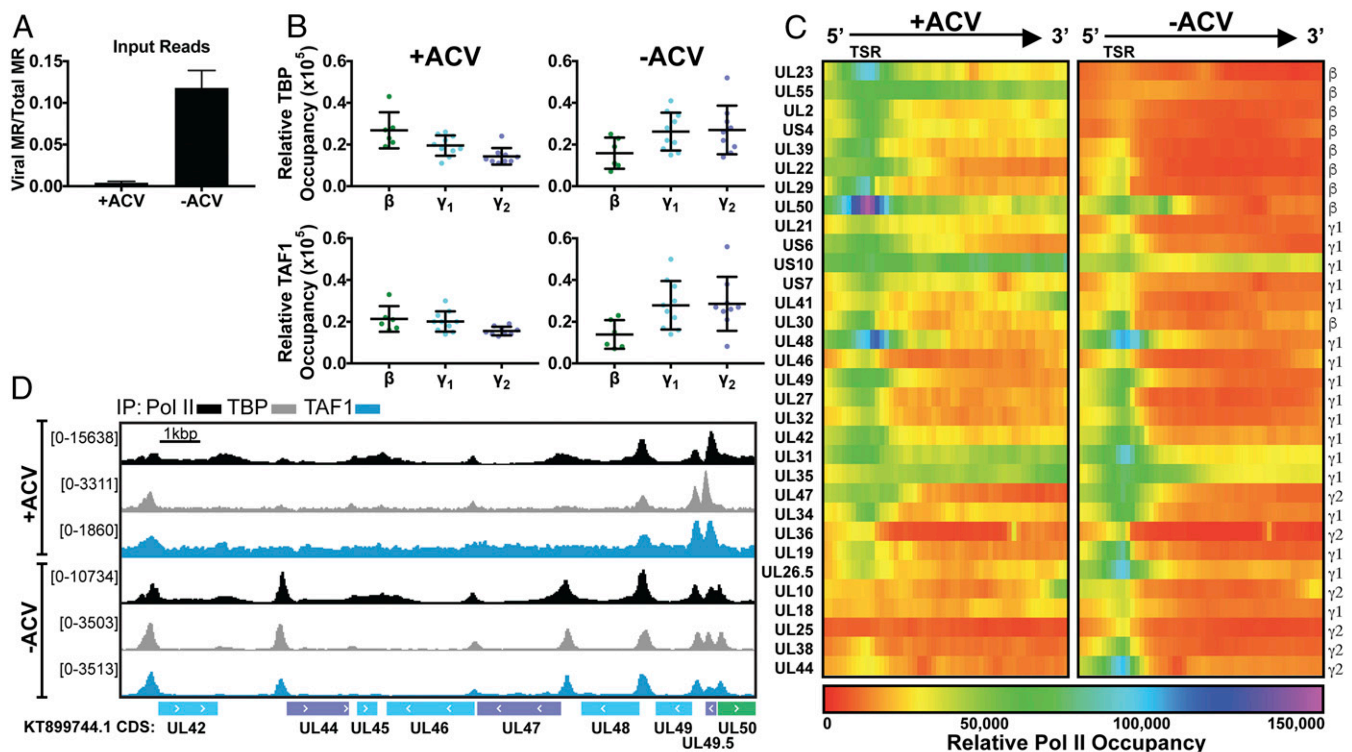

Fig. 2. Initiation complex formation as a function of genome replication. MRC5 cells were infected with HSV-1 in the presence (+ACV) or absence (−ACV) of acyclovir and ChIP-Seq for TBP, TAF1, and Pol II was performed. All data are normalized to the amount of factor per viral genome. (*A*) Viral genomes quantified as input reads mapped to the viral genome per total cellular and viral mapped reads (Viral MR/Total MR). Error bars represent SD. (*B*) TBP and TAF1 promoter occupancy. Each point is a distinct promoter. (*C*) Pol II occupancy heat maps for viral loci. Transcription start region (TSR) indicates the main Pol II promoter peak. (*D*) Overlay of Pol II (black), TBP (gray), and TAF1 (blue) binding mapped to viral UL42-UL50 loci.

There was a distinct deviation in Inr motifs between the gene classes, with γ genes more closely matching the consensus sequence $BBCA_{+1}BW$ (27) (Fig. 5*A*). Unlike the consensus motif, β genes favored adenosine at positions 1 and 2. Promoters containing strong Inr, such as UL44 and UL48, were more robustly transcribed postreplication (Fig. 5*B*). Genes without a canonical

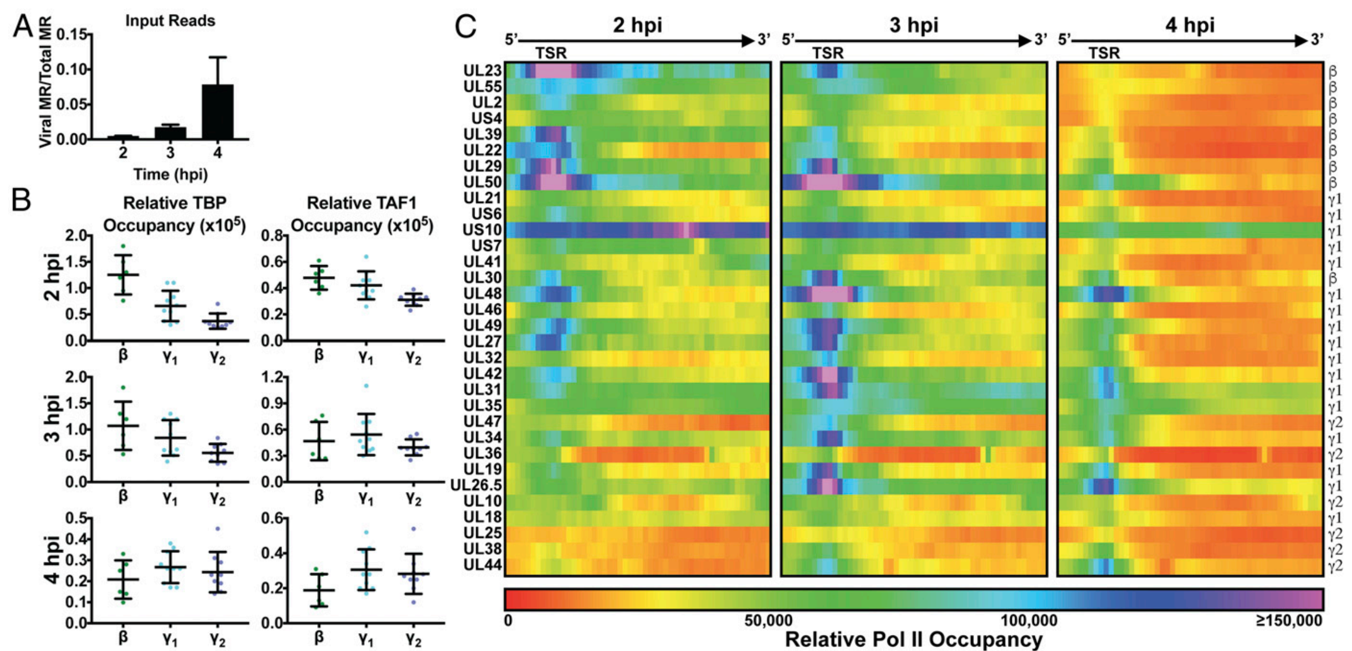

Fig. 3. A single round of genome replication immediately alters cellular initiation factor binding. MRC5 cells were infected with HSV-1 for 2, 3, or 4 h, and ChIP-Seq for TBP, TAF1, and Pol II was performed. All data are normalized as in Fig. 2. (*A*) Viral genomes quantified as in Fig. 2. (*B*) TBP and TAF1 promoter occupancy. Each point is a distinct promoter. (*C*) Pol II occupancy heat maps for viral loci. Transcription start region (TSR) indicates the main Pol II promoter peak.

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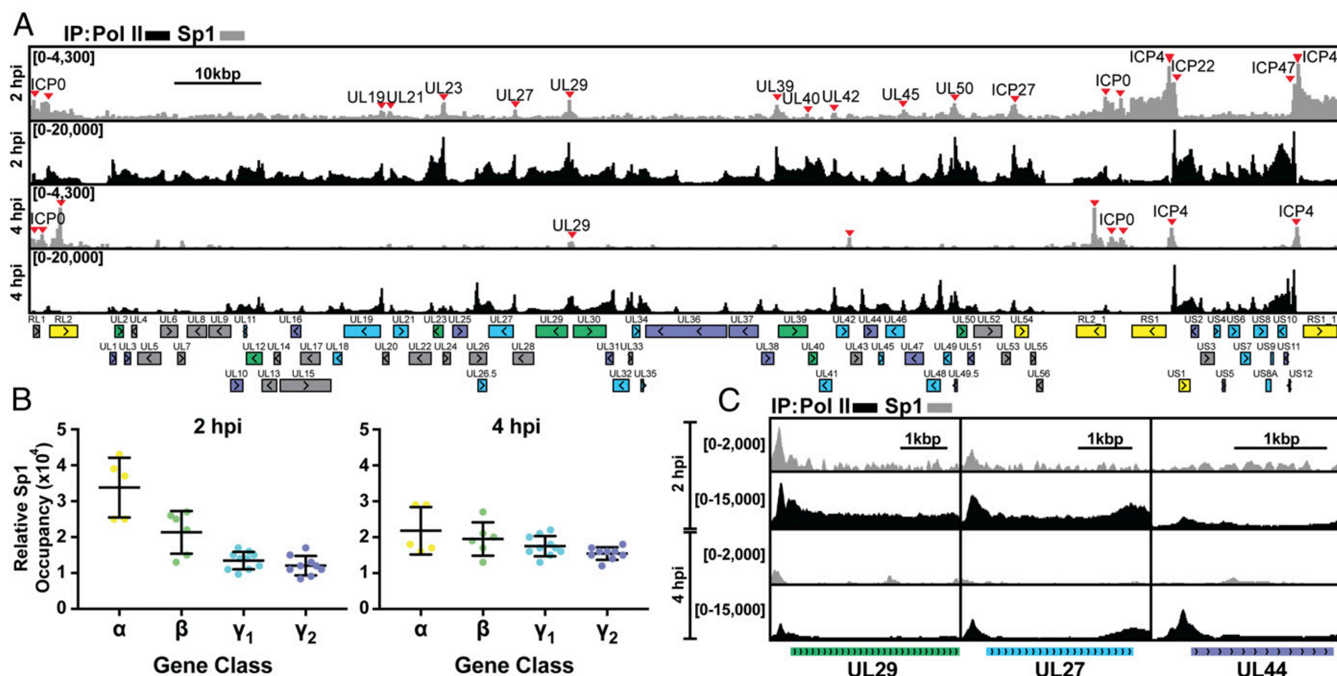

Fig. 4. Genome replication alters the binding of Sp1 to viral promoters. MRC5 cells were infected with HSV-1 for 2 or 4 h, and ChIP-Seq for Pol II and Sp1 was performed. All data are normalized as in Fig. 2. (*A*) Pol II (black) and Sp1 (gray) binding mapped to the viral genome. Distinct Sp1 peaks were highlighted with red arrows; if the peak was located within a viral promoter the gene is annotated above. (*B*) Sp1 promoter occupancy. Each point is a distinct promoter. (*C*) Pol II (black) and Sp1 (gray) binding mapped to UL29 (β gene), UL27 (γ_1 gene), and UL44 (γ_2 gene).

Inr sequence had weak and broad TAF1 peaks. We posit that TAF1 is present in TFIID for all viral promoters; however, the stronger the Inr element, the increased affinity of TAF1 for the Inr, the stronger the cross-linking, and subsequent peak.

The TAF1 ChIP-Seq data in prereplication conditions are indicative of a high level of nonspecific binding (*SI Appendix*, Figs. S3 and S4). As this was consistently present between biological duplicates and only present in 2 hpi or +ACV samples we suspect that this is not an artifact. Because this was not seen with TBP, this signal may represent TAF1 binding independent of TFIID at this time. This remains to be studied.

Continuous Requirement for ICP4 in Viral Transcription. ICP4 is the major viral transcriptional protein and is required for production of β genes (15). We wanted to determine whether ICP4 is always critical for viral transcription, even after γ transcription has been licensed by genome replication. We utilized a temperature-sensitive mutant of ICP4 (tsKos), in which a single point mutation makes the protein unstable and incapable of DNA binding at elevated temperature. MRC5 cells were infected with tsKos and incubated either at the permissive temperature (P), shifted from permissive to nonpermissive temperature at 4 hpi (S), or nonpermissive temperature (N). Immunofluorescence of infected samples at 6 hpi demonstrates an absence of nuclear ICP4 in shifted and nonpermissive conditions (Fig. 6*D*). We measured viral genome replication and mRNA accumulation for two genes robustly transcribed from 4 to 8 hpi (Fig. 6*A*). Infected cells grown entirely at nonpermissive temperature lacked genome replication and transcription. Infected cells grown entirely at permissive temperature underwent genome replication and transcription similar to wild-type kinetics. Infected cells shifted from permissive to nonpermissive temperature at 4 hpi immediately halted transcription after the shift. By comparison, genome replication continued after shift at a similar rate to samples grown at permissive temperature. Thus, even after the onset of DNA replication, ICP4 was essential for continued transcription. To determine which transcriptional stage ICP4 controls we

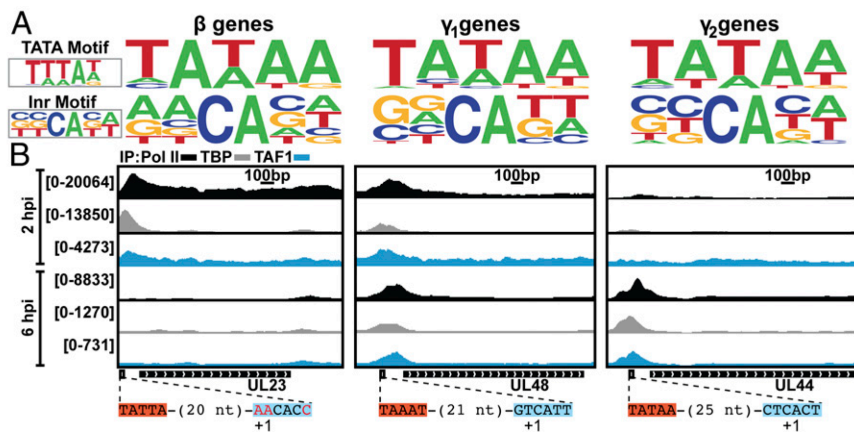

Fig. 5. Analysis of TFIID-binding sites in β and γ promoters. MRC5 cells were infected with HSV-1 for 2 or 6 h, and ChIP-Seq for Pol II, TBP, and TAF1 was performed. All data are normalized to the amount of factor per viral genome. Probability plot of (*A*) TATA box and Initiator elements delineated by gene class. Human consensus is given in gray box. (*B*) Overlay of Pol II (black), TBP (gray), and TAF1 (blue) binding mapped to viral loci. Viral core promoters are outlined, with the TATA box in orange and Inr in blue. Deviations from motif consensus are highlighted in red. Experimentally determined transcription start sites are indicated as "+1" (33–35).

performed Pol II ChIP-Seq on permissive samples, shifted up at 4 hpi, and nonpermissive samples harvested at 6 hpi (Fig. 6 *B* and *C*). In shifted-up cells there was a complete absence of Pol II bound to viral promoters of β and γ genes. Similarly, in nonpermissive conditions, Pol II was absent from viral promoters, with the exception of α genes. Thus, ICP4 is critical for formation of a preinitiation complex and is continuously required for robust transcription of β and γ genes.

Discussion

Our findings provide a global mechanism by which HSV-1 genome replication controls transcription. Due to the sensitivity and specificity of the approaches used, we were able to make conclusions regarding the transcriptional activity or priming of individual viral promoters. Specifically, it should be noted that the majority of transcriptional events take less time and occur earlier in infection than previously assumed. Transcription for most viral genes has at least been initiated by 3 hpi and decreases by 4 hpi. This trend continues and is ever more drastic at 6 hpi. Since data were quantified as transcription factor occupancy per genome, this suggests a segregation in genome function. Recent work from the N.A.D. laboratory following prelabeled viral genomes found the genome to be associated with transcription factors at 3 hpi and that by 6 hpi the genome was predominantly associated with packaging and assembly factors (28). Furthermore, replication forks were more enriched for transcription factors than previously replicated DNA (22). These data lead us to conclude that before genome replication there is little segregation of function, most genomes are actively transcribed. We propose that after two rounds of genome replication there is a functional coupling, in which newly synthesized genomes are actively transcribed, whereas "older" genomes begin the assembly and packaging process. This functional coupling results in efficient virion production from 5 to 18 hpi.

Our data have allowed us to propose how promoter architecture and genome replication determine transcriptional kinetics. Before replication the genome exists in a state that is not accessible to general transcription factors (GTF) on γ_2 promoters. What is not clear at present is why the viral chromatin at this time does not allow for TFIID and, hence, Pol II binding on promoters just containing TBP/TAF1-binding sites. Perhaps the restrictive chromatin is due to histone presence, or a specific distribution of viral and cellular genome-binding proteins. Before replication, initiation complexes form only on promoters containing UPEs, i.e., TAATGARAT sites, GC-boxes, and CAAT-boxes, and thus they are robustly transcribed. As expected, we observed Sp1 binding to the promoters of most α and β genes before replication. Select γ_1 genes were also transcribed at this time, likely due to upstream promoter elements. In these conditions, robust transcription does not require an Inr element. We posit that early during infection, the relatively high density of ICP4 on the viral genome results in the recruitment of TFIID to viral promoters lacking a strong initiator element (29–31), which have been rendered accessible by the function of upstream activators. We believe this allows for stable TFIID binding to promoters with weak or nonexistent Inr elements, facilitating robust β gene transcription and some leaky γ_1 gene transcription.

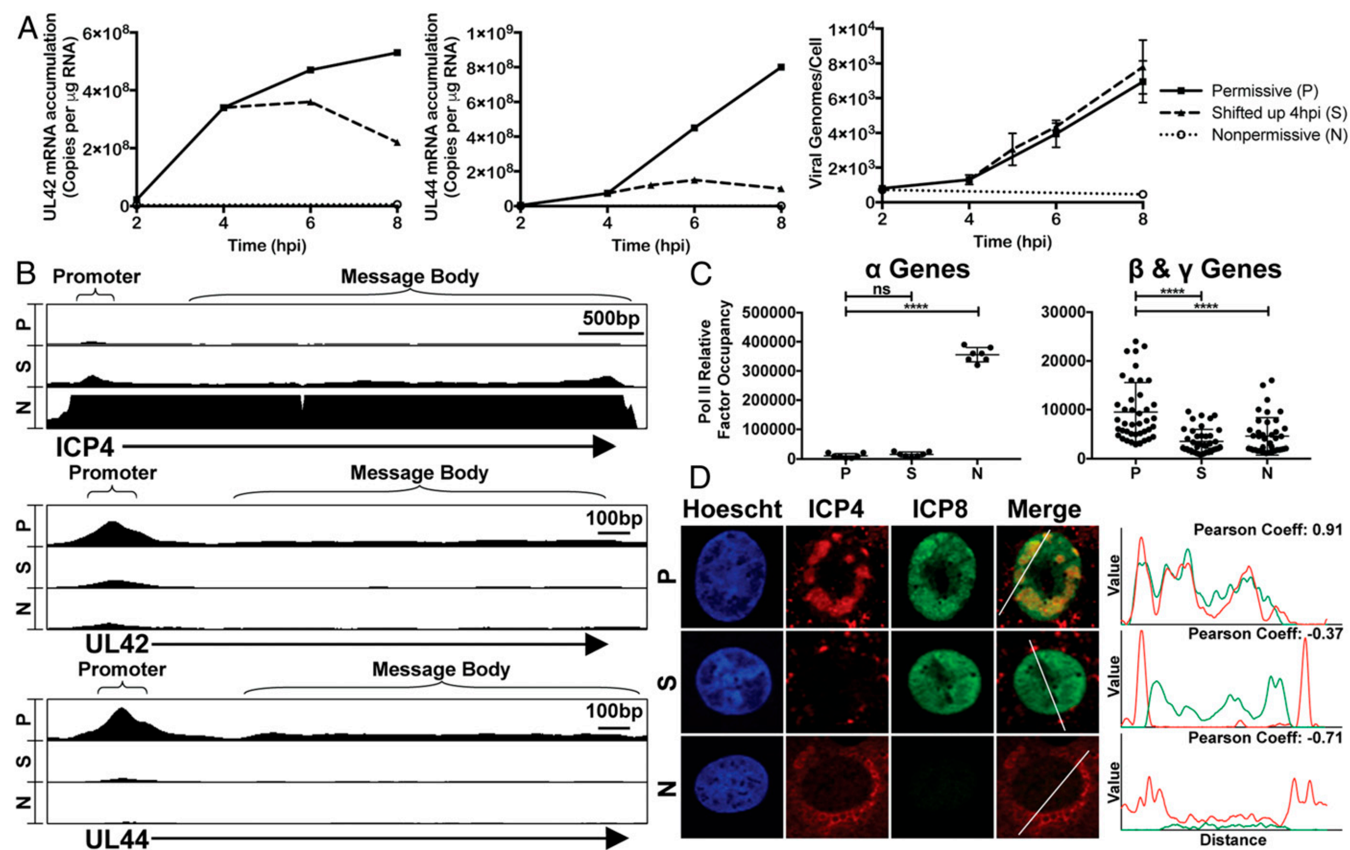

Fig. 6. ICP4 is continuously required for transcription after the onset of genome replication. MRC5 cells were infected with tsKos and grown at permissive conditions (P), shifted up from permissive to nonpermissive conditions at 4 hpi (S), or nonpermissive conditions (N). (*A*) Number of viral genomes per cell. UL42 (γ_1 gene) and UL44 (γ_2 gene) mRNA copies per microgram total cellular RNA. Error bars represent the SD of biological duplicates. (*B* and *C*) ChIP-Seq for Pol II was performed on P, S, and N samples at 6 hpi. All data are normalized to the amount of factor per viral genome. (*B*) Pol II traces mapped to ICP4 (α gene), UL42 (γ_1 gene), and UL44 (γ_2 gene) loci. *y* axes are 0–7500. (*C*) Pol II promoter occupancy. Each point is a distinct promoter. (*D*) Images of infected Vero cultures grown for 6 hpi. Pearson correlation test was performed on the red and green intensity profiles.

At the onset of genome replication there is an immediate alteration to the structure of the viral genome, such that promoters from all genes classes had an increase in Pol II promoter occupancy. This alteration and the presence of ICP4 was critical for the shift to robust viral transcription. As the number of viral genomes increases, promoters possessing initiator elements that make strong TAF1 contacts are favored. Most γ genes robustly recruited TAF1 and possessed strong Inr elements matching the consensus motif, BBCABW. We propose that the increase in viral genomes reduces the relative concentrations of GTFs, such as Sp1, TFIIA, TFIID, and ICP4. Ultimately, the relative decrease in host GTFs and absence of Inr elements resulted in attenuation of β gene transcription. Viral genome numbers continued to increase, resulting in prolonged robust γ gene transcription, despite the average transcriptional activity per genome being decreased.

We believe our findings elucidate the major mechanisms by which HSV-1 controls transcription. Our study found that a single round of genome replication permanently altered the transcriptional landscape of HSV-1. The alteration facilitated an increase in genome accessibility to RNA Pol II, TBP, and TAF1. Our results suggest that genome replication was itself responsible for promoting this shift, rather than titration of a factor. This mechanism acted as the switch necessary to promote a global increase in viral transcription and initiate synthesis of previously silent promoters. In this way, synthesis of genes required for later stages of the life cycle, i.e., capsid assembly and egress, is not initiated until sufficient production of earlier viral gene products and recruitment of essential cellular factors. This general mechanism could explain the coupling of genome replication and nascent transcription for other viruses which undergo nuclear DNA replication. This allows for an initial "colonization" stage of infection before a massive wave of proliferation, also rendering the infection less detectable to host defense mechanisms before virion production.

Methods

Cells and Viruses. MRC5 (human fetal lung) or Vero (African green monkey kidney) cells were obtained from and propagated as recommended by ATCC. HSV-1 strains used in this study include mutants ΔC1216 and tsKos and wild-type KOS.

Infection. Cells were infected with 10 pfu per cell. Virus was adsorbed in TBS for 1 h at room temperature. Viral inoculum was removed, and cells were washed quickly with tricine buffered saline (TBS) before adding 2% FBS media.

RNA-Seq. RNA was harvested using the Ambion RNAqueous-4PCR kit and quantified using the Agilent RNA 6000 Nano kit. Libraries were generated from 2 μg RNA using NEBNext kits, #E7490 and #E7420.

ChIP-Seq. ChIP-Seq was performed as described previously (32). Samples were immunoprecipitated with 25 μg of the following antibodies: Pol II 4H8 (AbCam #ab5408), Pol II 8WG16 (AbCam #ab817), TBP (AbCam #ab51841), TAF1 (SantaCruz #sc-735), or Sp1 (SantaCruz #sc-17824). Libraries were generated from 2 to 20 ng of using NEBNext kit, #E7103S.

Data Availability. All data are publicly accessible in the SRA database (SRP172751, SRP172780, SRP172782, and SRP172783).

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