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Herpes simplex virus infected cell polypeptide 4 preferentially represses Sp1-activated over basal transcription from its own promoter

(TATA binding protein/*in vitro* transcription/DNA–protein interactions/regulation of transcription)

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ABSTRACT **Herpes simplex virus type 1 infected cell polypeptide 4 (HSV-1 ICP4) is a multifunctional phosphoprotein that is essential for viral infection. It is both a repressor and an activator of viral gene expression depending upon the promoter. ICP4 represses transcription from its own promoter. In the present study, we used general transcription factors from HeLa cell nuclear extracts, recombinant TATA binding protein (TBP) and TFIIB, and the transcriptional activator Sp1 to reconstitute *in vitro* transcription for the ICP4 promoter and to examine the effects of purified ICP4 on transcription. ICP4 was able to effectively repress Sp1-induced transcription from ICP4 promoter templates that contain one or multiple Sp1 binding sites. The observed inhibition required the ICP4 binding site that spans the transcription initiation site. ICP4 did not inhibit basal transcription as inferred by its inability to inhibit transcription when (*i*) Sp1 was not included in transcription reactions, (*ii*) the templates contained no Sp1 binding sites, and (*iii*) TBP was used in place of TFIID in the reactions. The *in vitro* observations were consistent with the behavior of the same constructs expressed in cells from the herpes simplex virus type 1 genome. DNase I footprinting experiments revealed that ICP4 could co-occupy the ICP4 promoter region with TBP-TFIIB, indicating that ICP4 does not necessarily exclude these factors from binding to the TATA region. The data suggest that the repressive effects of ICP4 observed in this study result from ICP4 interfering with the interactions contributing to Sp1-induced transcription.**

The major transcriptional regulatory protein expressed by herpes simplex virus type 1 (HSV-1) is infected cell polypeptide 4 (ICP4). ICP4 is essential for the viral lytic growth cycle, presumably due to its role in the transcriptional activation of most viral genes (1, 2). It is also transcriptionally autoregulatory (3–5). ICP4 exists in cells as a 350-kDa dimer (6, 7) and is a specific DNA binding protein that associates with DNA containing the consensus binding sequence ATCGTCNNNNYCGRC, where N = any nucleotide, Y = pyrimidine, and R = purine (8). Mutational probing studies have confirmed that ICP4, like many transcriptional regulatory proteins, is composed of discrete functional domains that collectively constitute its function (9–11).

The promoter for ICP4 is positively regulated by viral and cellular proteins (12, 13). It contains two upstream cis-acting sites (consensus sequence TAATGARAT) for activation by the virion protein VP16 (12), and at least four (G+C)-rich boxes (consensus sequence GGGCGG) that can serve as binding sites for the cellular transcriptional activator Sp1. Consistent with this, the GC boxes have been shown to be cis activators of transcription in the absence of viral transacting proteins (14). How infection or virus regulatory proteins may affect the function of Sp1 is not known. Despite the relative abundance of sites for upstream activators in the promoter of the ICP4 gene, transcription from this promoter is repressed during the course of infection, in part due to the activity of ICP4 (1–3, 5, 15). A consensus ICP4 binding site spans the transcription initiation site of the ICP4 mRNA (16, 17). From transient transfection studies (18) and studies with mutant viruses (19), it is clear that this site contributes to autoregulation. Several hypotheses may be entertained to explain repression through this binding site. ICP4 bound at the mRNA start site might simply prevent transcription in a manner similar to repressor/operator systems in bacteria. It is also possible that ICP4 precludes the assembly of an active general transcription factor complex by virtue of its size and the general proximity of the ICP4 binding site to the TATA box and the mRNA start site. Lastly, ICP4 may also affect the ability of upstream activators to function. To address these hypotheses, the experiments in the present study examined the transcription of the ICP4 promoter *in vitro* as a function of added ICP4. The effects of the mutation of the ICP4 binding site and presence of Sp1 sites and Sp1 were also examined.

MATERIALS AND METHODS

General Transcription Factors and Purified Proteins. HeLa cells were used to make nuclear extracts by the method of Dignam *et al.* (20). The method for the fractionation of the general transcription factors from the nuclear extracts (Fig. 1*A*) was as described (21). Human TFIIB was purified from *Escherichia coli* (22). The human TATA binding protein (hTBP) was also purified from *E. coli* (23) as described (24). rTFIIB was used instead of HeLa TFIIB throughout this study, and TBP was used in replace of TFIID as indicated. The purification of ICP4 was conducted as described (7, 25). Sp1 purified from HeLa cells was obtained from Promega.

***In Vitro* Transcription and Primer Extension.** The final concentrations of the components in the *in vitro* transcription reactions were 40 mM Hepes (pH 7.9), 60 mM KCl, 12% glycerol, 8.3 mM $MgCl_2$, 0.6 mM (each) ATP, CTP, UTP, and GTP, 0.3 mM dithiothreitol, and 12 units of RNasin. Template DNA was used at a concentration of 20 μg/ml. General transcription factors, 0.5 μl (1.5 μg) of AB, 5 μl (8 μg) of CB, 3 μl (1.8 μg) of CC, 0.5 μl (50 ng) of rTFIIB, and 5 μl (1 μg) of DB, were mixed before the addition of the above components. When required, 2 μl of Sp1 (40 ng/μl) and the indicated

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Abbreviations: HSV-1, herpes simplex virus type 1; ICP4, infected cell polypeptide 4; tk, thymidine kinase; r, recombinant; h, human.

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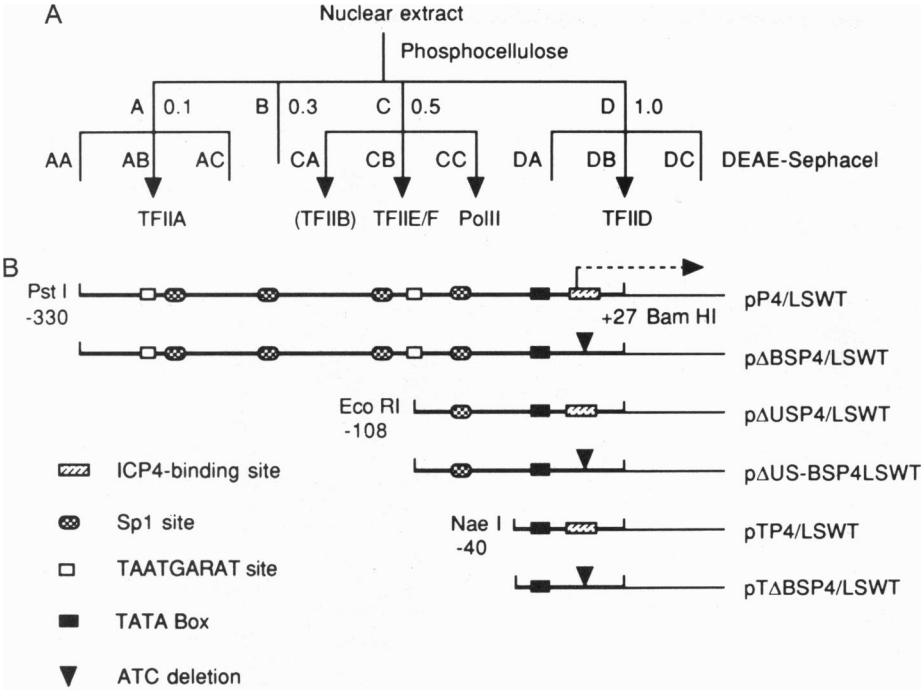

FIG. 1. Transcription factors and templates used in this study. (*A*) Schematic representation of the fractionation of the different factors required for transcription the ICP4 promoter. The CA fraction containing TFIIB activity was replaced in this study by recombinant TFIIB (rTFIIB). (*B*) Features of ICP4 promoter containing templates used in the study. Bold lines represent the ICP4 promoter and upstream regulatory sequences; thin lines represent thymidine kinase (tk) coding sequences. The ICP4 sequences were cloned into the *Pst* I (−220, relative to the initiation site of the tk gene) and *Bgl* II (+55) sites of pLSWT. The relevant transcription factor binding sites are represented.

amount of ICP4 (0.5 μg/μl) were first included in the general transcription factor mixture. Transcription reaction mixtures (30 μl) were incubated at 30°C for 80 min and reactions were then stopped by the addition of 70 μl of 0.15 M sodium acetate, pH 5.3/15 mM EDTA, pH 8.0. The *in vitro* transcripts were subjected to primer extention analysis using Moloney murine leukemia virus (MMLV) reverse transcriptase, and the ^{32}P-labeled extention products were visualized on denaturing acrylamide gels as described (24).

Promoter/Template Constructs. Six DNA templates that contain ICP4 promoter and different upstream cis elements were used in this work (Fig. 1*B*). Details of construction of these plasmids will be published elsewhere. The ICP4 promoter sequences shown in Fig. 1*B* and extending to +27 were inserted in the tk gene at +55 so the promoter could be introduced into the *tk* locus of the HSV genome. The generation of the mutant viruses used in this work was performed as described (26). An oligonucleotide from +105 to +75 of tk was used for primer extension experiments with RNA from both *in vitro* and *in vivo* sources.

DNase I Footprinting. Dnase I footprinting analysis was conducted as described (24).

RNA Nuclease Protection Assays. [^{32}P]CTP-labeled RNA complementary to the transcripts made from the ICP4-tk hybrid gene was synthesized by using the T7 RNA polymerase Promega riboprobe system as described by the manufacturer. The template used for the synthesis was made by cloning the *Eco*RI at −108 (converted to *Pst* I) to *Sst* I (position +550 in tk coding sequence) fragment from pΔUSP4/LSWT (Fig. 1*B*) into the *Pst* I and *Sst* I sites in pSP72. The conditions for hybridization and RNase digestion were as prescribed by the manufacturer. The protected species were separated on 4% polyacrylamide sequencing gels.

RESULTS

In vitro transcription of the ICP4 promoter has been observed using relatively crude HeLa cell extracts (14) but has not been described using fractionated or recombinant factors. When fractions containing TFIIA, rTFIIB, TFIID, TFIIE/F, Sp1, and RNA polymerase II (polII) were present, reproducible RNA signals from the ICP4 promoter template pP4/LSWT (Fig. 1*B*) were obtained that migrated at the same position as the primer extension product from RNA obtained from cells infected with a virus containing the same promoter (Fig. 2*A*). The observation that Sp1 strongly activates transcription of the ICP4 promoter is consistent with previous results (14). All of the general factor fractions were required to obtain efficient ICP4 transcription, with the possible exception of TFIIA. When TFIID was omitted, transcription was significantly reduced, and the specificity of initiation was also greatly decreased (Fig. 2*A*).

ICP4 was then included in the reaction mixtures along with Sp1 to determine if it could inhibit activated transcription from the ICP4 promoter *in vitro*. Fig. 2*B* shows that ICP4 substantially reduced the amount of transcripts synthesized from the template pP4/LSWT in a concentration-dependent manner. One ICP4 dimer per template was enough to show inhibition of transcription, whereas 20 ICP4 dimers per template dramatically inhibited transcription (Fig. 2*B*). The effect of ICP4 on Sp1-induced transcription was determined using a set of constructs that are derivatives of pP4/LSWT (Fig. 1*B*). Preliminary experiments revealed that Sp1-induced transcription requires the presence of Sp1 sites and exogenously added Sp1 (data not shown). Fig. 3 shows that Sp1 stimulated transcription from the upstream deletion template, pΔUSP4/LSWT (15-fold), as well as from the wild-type ICP4 promoter in pP4/LSWT (20-fold). When ICP4 was included together with Sp1, the level of transcription from pΔUSP4/LSWT, like pP4/LSWT, was greatly decreased. Consistent with the absence of a functional Sp1 binding site, Sp1 did not stimulate transcription from the TATA box only promoter construct, pTP4/LSWT. Interestingly, ICP4 had no effect on the level of transcription from this promoter at concentrations of ICP4 that are inhibitory to Sp1-induced transcription. The ΔBS mutation present in the constructs does not allow ICP4 to bind to the residual sequence (9) and did not allow ICP4 to inhibit Sp1-induced transcription. For the templates shown in Fig. 3, the *in vivo* controls represent primer extension reactions from RNA isolated from cells infected with HSV recombinant viruses containing the P4 and ΔBSP4 promoters.

A series of experiments was conducted to more closely examine the effect of ICP4 on basal transcription of the ICP4 promoter. Fig. 4*A* demonstrates that in the absence of Sp1, with or without Sp1 sites, ICP4 does not inhibit transcription

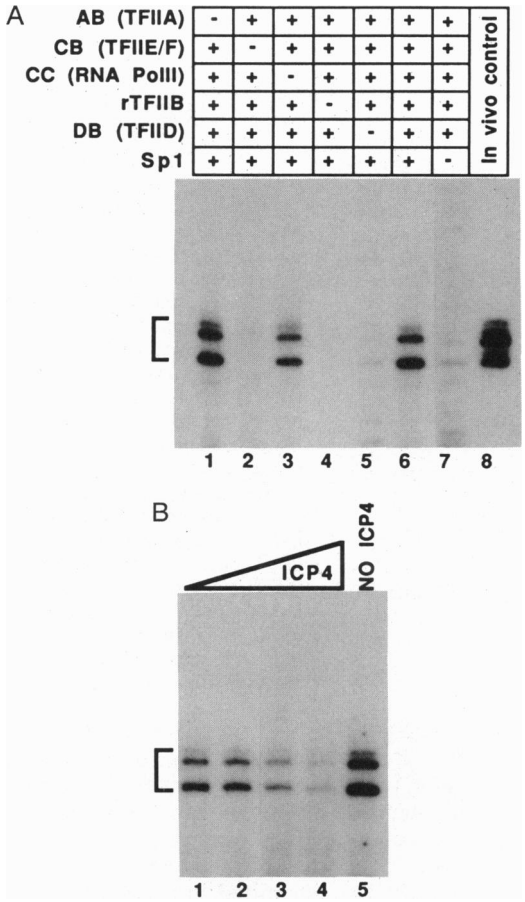

FIG. 2. *In vitro* transcription of ICP4 promoter in pP4/LSWT. (*A*) Transcription reactions were conducted using the intact ICP4 promoter and the indicated fractions and proteins. Shown for comparison are the extension products produced using RNA expressed from the same promoter in the context of viral infection. (*B*) Effect of ICP4 on Sp1-activated transcription. Sp1 (about 5 molecules per template) was included in all the reaction mixtures with no ICP4 (lane 5) and with 1 ICP4 molecule per template (lane 1) to 20 ICP4 molecules per template (lane 4).

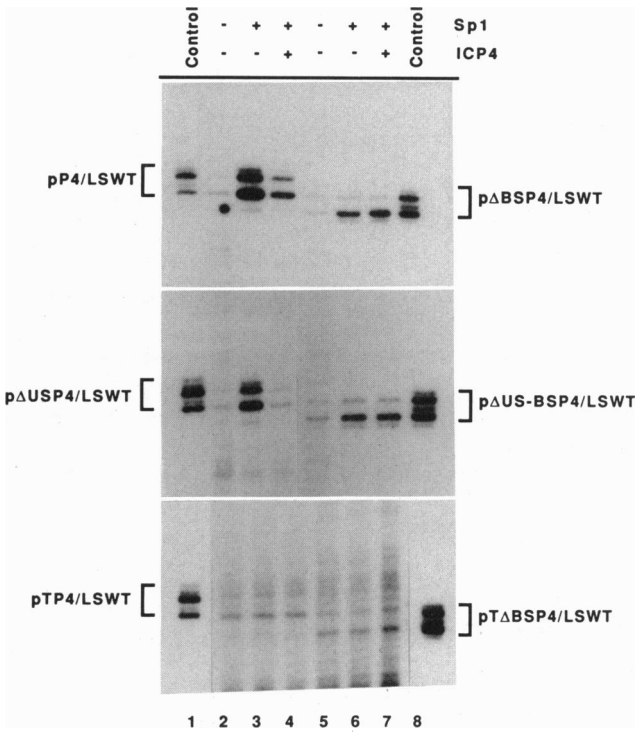

FIG. 3. Effect of ICP4 on Sp1-induced transcription. Transcription conditions for all six templates were the same as described in the legend to Fig. 2. When ICP4 was present in transcription reactions, about 0.5 μg (10–20 ICP4 dimers per template) was added last to the transcription factor mixture before the addition of nucleotides and templates. Lanes 1 and 8, primer extension products for RNA isolated from virus-infected Vero cells. Lanes 2 and 5, transcription from general transcription factors. Sp1 (about 5 molecules per template) was included in the reactions represented by lanes 3 and 6. Lanes 4 and 7, transcription reactions that contained both Sp1 and ICP4.

mediated by the general factors used in these reactions. In addition, deletion of the ICP4 binding site on the templates did not have any quantitative effect on transcription, demonstrating that the apparent lack of inhibition in this experiment for the promoters possessing the binding site was not due to possible compensating positive and negative effects of ICP4.

To further investigate the functional interaction between ICP4 and the general factor transcription complex, recombinant human TBP (rhTBP) was substituted for TFIID in the

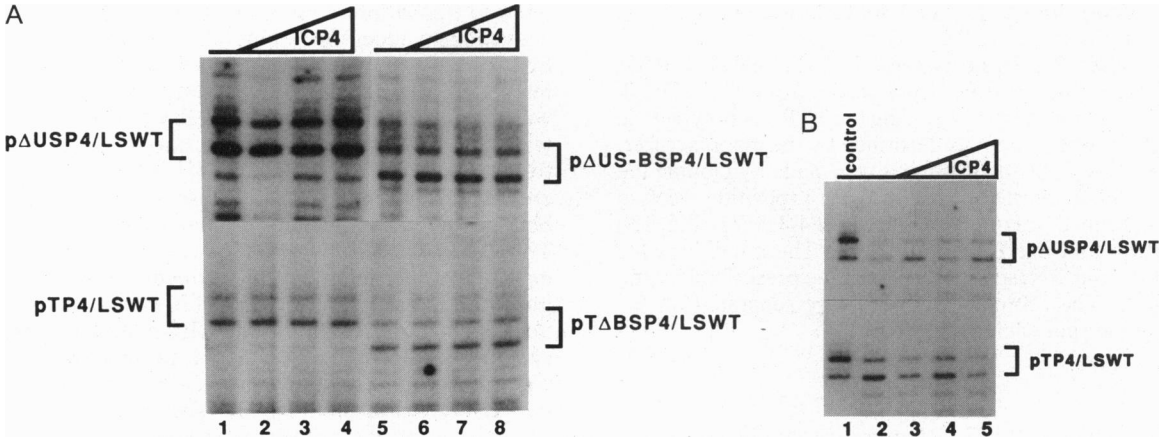

FIG. 4. Effect of ICP4 on basal transcription. (*A*) Effect of ICP4 on transcription in the absence of Sp1 activation. Transcription conditions were the same as described in the legend to Fig. 2 except that Sp1 was not added to the transcription reactions. The proteins included in the reactions were as follows: no ICP4 (lanes 1 and 5), 0.2 μg of ICP4 (lanes 2 and 6), 0.4 μg of ICP4 (lanes 3 and 7), and 0.6 μg of ICP4 (lanes 4 and 8). (*B*) Effect of ICP4 on TBP-mediated transcription. Transcription conditions were the same as in *A* except that 1.5 μl of TBP was used in place of TFIID. The proteins included in the reactions were as follows: *in vivo* control (lane 1), no ICP4 (lane 2), 0.2 μg of ICP4 (lane 3), 0.4 μg of ICP4 (lane 4), and 0.6 μg of ICP4 (lane 5).

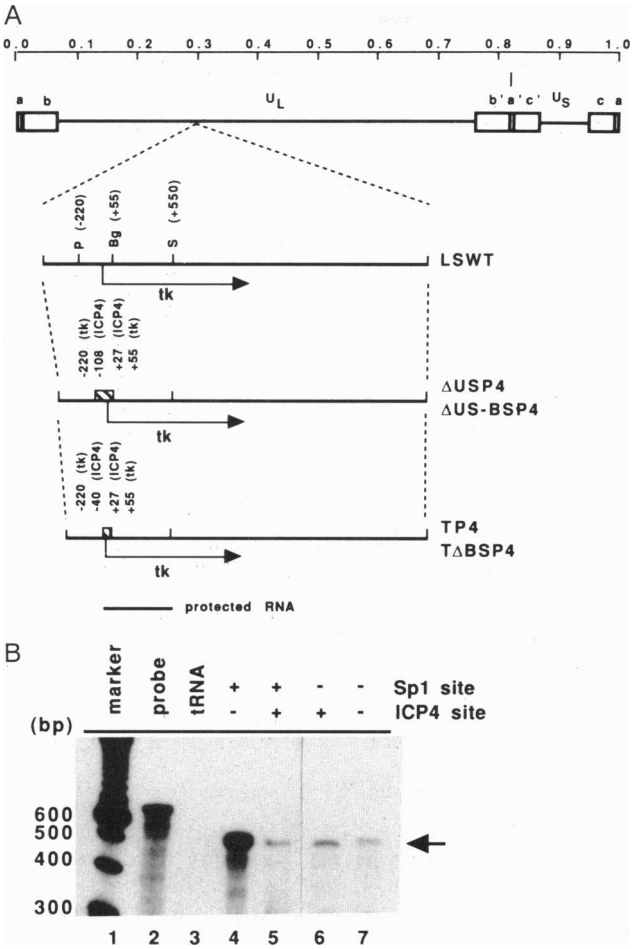

FIG. 5. Effect of ICP4 on Sp1-activated transcription in the context of viral infection. (*A*) Diagram of the HSV genome, the *tk* locus, and the location and identity (hatched boxes) of the ICP4 sequences inserted in place of the tk promoter in the viral genome. (*B*) Nuclease protection assay for RNA isolated from virus-infected cells in the presence of ICP4. Twenty micrograms of RNA from 8-hr postinfection cells was used for nuclease protection. The lanes contained 100-bp molecular weight ladder (lane 1), untreated probe used in the assay (lane 2), tRNA (lane 3), RNA from pΔUS-BSP4/LSWT (lane 4), RNA from pΔUSP4/LSWT (lane 5), RNA from pTP4/LSWT (lane 6), and RNA from pTΔBSP4/LSWT (lane 7).

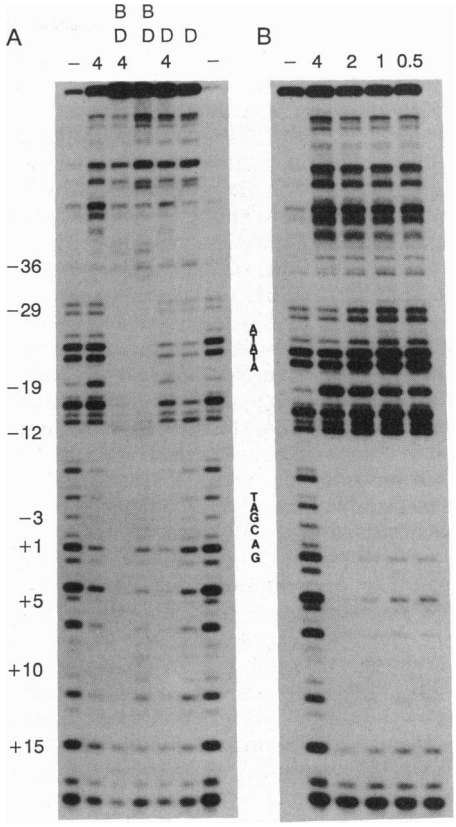

FIG. 6. Simultaneous binding of ICP4 and TFIIB-TBP on ICP4 promoter. (*A*) DNase I footprints generated after the inclusion of the indicated proteins. The labels above the lanes correspond to no added protein (−), ICP4 (4), TFIIB (B), and TBP (D). The ICP4 binding site, GACGAT, and the TATA box sequence, ATATA, of the ICP4 promoter are shown alongside the footprint. (*B*) DNase I footprints of the ICP4 binding site by different concentrations of ICP4. The numbers above the lanes indicate the volume of ICP4 in microliters added to the reaction.

reactions. TBP will substitute for HeLa TFIID for transcription *in vitro* (23, 27); however, it lacks the many TBP associated factors (TAFs) that serve as coactivators. As a consequence, TBP does not support activation by many transcription factors, including Sp1. In the present study, ICP4 failed to inhibit transcription mediated by TBP (Fig. 4*B*), further indicating that the binding of ICP4 to its binding site does not inhibit basal or general factor-mediated transcription and most likely requires TAFs. The addition of Sp1 to the TBP reactions had no effect on the levels of RNA seen in any of the reactions of Fig. 4*B* (data not shown).

To address the biological significance of the effects described above the appropriate promoter constructs were inserted into the genome of HSV and expression from these promoters was measured in the presence of ICP4 expressed during productive HSV infection. The four constructs in Fig. 1*B* that lack the upstream TAATGARAT sites were recombined into the *tk* locus of HSV such that expression from these promoters will result in the transcription of HSV tk mRNA (Fig. 5*A*). The constructs used address the involvement of Sp1 and ICP4 by analysis of message levels resulting from the initiation at promoters that possess or lack the binding sites for these proteins. As seen in Fig. 5*B*, the presence of an Sp1 binding site resulted in greatly enhanced transcription only in the absence of an ICP4 binding site. Without an Sp1 binding site, the level of RNA was not greatly affected by the presence of an ICP4 binding site. Therefore, in the context of viral infection, ICP4 did not efficiently inhibit transcription mediated solely from the TATA box but, again, had a dominant repressive effect on Sp1-induced transcription.

The hypothesis that the binding of ICP4 does not inhibit TBP-mediated transcription in cis requires that TBP and ICP4 can occupy their respective sites on the same promoter. The DNase I footprinting experiment shown in Fig. 6 addresses this question. ICP4 footprints a large region of the promoter spanning from −12 to +15 (Fig. 6*B*). The simultaneous addition of TBP (labeled D) and TFIIB (labeled B) results in the strong protection of the TATA box region from −35 to −15 and a weaker protection toward the initiation site. TBP alone only weakly protected the TATA box at this concentration. The footprint of TBP (and TFIID) over the TATA box is greatly enhanced by the addition of TFIIB (28). When ICP4 was included with TBP and TFIIB, a strong protection resulted, extending from −35 to +15 with a region of DNase sensitivity from −10 to −14. In fact, the presence of TBP and TFIIB resulted in a stronger ICP4 footprint at this concentration. These results are consistent with the ability of TBP (and probably TFIID) and ICP4 to simultaneously bind to the same promoter element. We have also observed that ICP4, TBP, and TFIIB can form a tripartite complex as determined on these sites in the ICP4 promoter (28).

DISCUSSION

ICP4 repressed Sp1-induced transcription from the ICP4 promoter in the presence of the ICP4 binding site located at the start site of ICP4 transcription. Repression of Sp1-induced transcription by ICP4 occurred whether the promoter contained one or multiple Sp1 binding sites. Several observations indicate that the concentrations of factors and ICP4 that were sufficient to inhibit Sp1-induced transcription were not sufficient to inhibit transcription mediated by the TATA box promoter alone. (*i*) ICP4 did not inhibit transcription in the absence of Sp1. (*ii*) ICP4 did not repress transcription in the absence of Sp1 sites. (*iii*) ICP4 did not inhibit transcription when TBP was substituted for HeLa TFIID. The data in this study demonstrate that ICP4 can preferentially repress transcription by a mechanism other than simply blocking the transcriptional start site by binding there. However, the data do not rule out that ICP4 can inhibit transcription by such mechanisms. Overexpression of ICP4 in cells may repress transcription by this mode. Expression of the DNA binding domain of ICP4 in transient assays and from high-copy-number transformed cells results in a modest repression of ICP4 transcription (7, 11). In such cases it might be expected that basal transcription would also be inhibited.

Other examples of repressors include the *Drosophila* Kruppel protein (29) and the human YY-1 protein (30), both of which, like ICP4, can repress and activate transcription under certain conditions. It has also been shown that the intermediate early 2 (IE2) protein of human cytomegalovirus represses its own promoter *in vitro* (31). The repression modes of all these proteins require DNA binding. However, in some cases additional regions of the proteins are also required for repression, suggesting that the mechanism of repression can be more sophisticated than stearic interference caused solely by the presence of the molecule on DNA. Therefore, repression may result from additional interactions with proteins assembled at the promoter to initiate transcription. These interactions may interfere with the activity of specific factors. Alternatively, interactions between the repressor and specific factors may interfere with the further assembly of an active transcription complex.

The co-occupancy of the ICP4 promoter/start site region with the general factors and ICP4 may preclude or alter the interaction of Sp1 or its coactivator, or TAF (32). A plausible hypothesis is that ICP4 and the Sp1 coactivator compete for sites of action in the transcription complex. Whether or not ICP4 can inhibit or affect Sp1-induced transcription under conditions where ICP4 is presented differently to the general transcription complex than in the case of the ICP4 promoter remains to be determined and may possibly provide a basis for the involvement of the repressor activity of ICP4 in temporal regulation. It is clear that Sp1 is utilized in the expression of viral genes more at early times after infection than at late times. IE gene promoters have many Sp1 sites, E gene promoters have one or two, and late gene promoters have none. This would be consistent with previous results (26, 33) showing that the presence of ICP4 lessens the requirement for Sp1 for full expression of the tk gene.

While the present study examines the functional interaction between Sp1 and ICP4 in the repression of the ICP4 gene, it is also possible that the intimate involvement of ICP4 at the transcription initiation complex may affect the activity of other upstream activating proteins. This would occur by impeding with the mechanisms by which upstream activators communicate with the general transcription complex. In the case of the ICP4 promoter, TAATGARAT sequences in the upstream region of the promoter mediate activation by VP16 (13, 14). In transient assays, the repressive effects of ICP4 on its own promoter are dominant over activation by VP16 (4). However, in the context of viral infection, repression of VP16-induced transcription by ICP4 binding at the start site is not as pronounced as it is for Sp1 (unpublished observations). Reflecting its potency as an activator, it may be that VP16 competes with ICP4 better than Sp1 does for sites of interaction with the transcription complex.

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