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# Herpes simplex virus immediate early infected-cell polypeptide 4 binds to DNA and promotes transcription

(sequence specificity/*in vitro* transcription/regulatory factor/herpes simplex virus glycoprotein gene)

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ABSTRACT **In herpes simplex virus (HSV)-infected cells, there is a sequential expression of viral genes. *In vivo* experiments have implicated the $M_r$ 175,000 immediate early protein ICP4 (infected-cell polypeptide 4) in the regulation of viral RNA synthesis, but the mechanism whereby ICP4 regulates transcription of viral genes is at present unknown. In this report we describe experiments with an *in vitro* transcription system and a purified preparation of ICP4 (estimated 5% of total protein). Using DNA from the HSV glycoprotein D gene (*gD*) as the template, we have observed that (*i*) specific binding occurs between ICP4 and DNA sequences adjacent to the *gD* gene promoter and (*ii*) ICP4 stimulates initiation of transcription from the *gD* gene. The degree of stimulation depends on the amount of ICP4 present in the incubation. The kinetics of RNA synthesis demonstrate that the protein acts at the initiation step of transcription. These results identify ICP4 as a viral transcription factor whose presence on DNA facilitates the formation of transcription complexes.**

Herpes simplex virus (HSV) proteins synthesized in infected cells change in both number and character during productive infection (1, 2). Underlying these changes in viral proteins are changes in the species and abundance of viral mRNAs (3, 4). The sequential appearance of viral mRNAs is believed to be controlled primarily by regulation of transcription, but little is known about the factors or mechanisms that regulate viral RNA synthesis (5). In view of the complex nature of HSV infections and the propensity to establish a latent infection, an understanding of the mechanisms that regulate HSV gene expression might facilitate treatment of this medically important pathogen.

On the basis of their temporal order of expression, three classes of HSV genes have been identified and designated as immediate early or $\alpha$, early or $\beta$, and late or $\gamma$. Temporal control of viral gene expression requires viral proteins: in particular, it has been shown that immediate early proteins are required for transcription from segments of the genome that contain early and late genes (3, 4). Five immediate early genes encode the following infected-cell polypeptides (ICP): ICP4, ICP0, ICP22, ICP27, and ICP47 (1, 6). From experiments with mutant virus and transfection experiments with DNA fragments, a role in regulating transcription has been established for the immediate early protein ICP4 (6–13). This $M_r$ 175,000 protein has been shown to be continuously required for mRNA production from early and late genes (14). It is phosphorylated (15), binds to DNA (15–17) or DNA–protein complex (18), and is found in different forms in the nucleus and cytoplasm (8). Like the EIA protein of adenovirus (19) and the T-antigen of simian virus 40 (SV40) (20), ICP4 appears to modulate transcription.

Studies with DNA viruses have greatly improved our understanding of the DNA sequences involved in regulating mRNA synthesis. Viral genes introduced into cells by transfection can be activated by proteins acting in *trans* (21–24). From these experiments, regulatory elements have been identified, and it appears that these sequences function by interacting with protein factors (25). *In vitro* transcription systems have been developed that are active with viral DNA as template (26, 27) and factors required for correct initiation of RNA synthesis separated from RNA polymerase (28–30). Recently gene-specific transcription factors have been partially purified, and their binding sites have been located on SV40 DNA as well as cellular DNA (30–32). The same cellular factors and nucleotide sequences in HSV DNA are involved in the regulation of HSV gene transcription (33–35).

We have used an *in vitro* transcription system (27) to investigate how a partially purified preparation of the viral protein ICP4 interacts with DNA from the early HSV gene for glycoprotein D (gD). In this paper we present evidence that ICP4 binds specifically to DNA sequences adjacent to the *gD* gene promoter and stimulates accurate transcription from this early gene. The mechanism of stimulation by ICP4 involves an increase in initiation of RNA synthesis. This report identifies a specific step in the transcription process that is regulated by an HSV protein.

## MATERIALS AND METHODS

**Template DNA.** The HSV DNA used in this study was prepared from the plasmid pJB3. This plasmid contains the *Sma* I fragment subcloned from the *Bam*HI fragment J of HSV type 1 (HSV-1) (KOS). The construction pJB3 and a simplified restriction map of the *Sma* I fragment are shown in Fig. 1. More details on the plasmid and its use in mapping the gD mRNA are presented in an earlier publication (36). To obtain the *Ava* I fragment 1 for use in the *in vitro* transcription reactions, plasmid DNA was purified by two cycles of cesium chloride centrifugation, cut with the restriction enzyme *Ava* I (Bethesda Research Laboratories), and extensively extracted with phenol/chloroform, 1:1 (vol/vol). The DNA fragments were precipitated with ethanol, redissolved in buffer, and separated by electrophoresis on 1% agarose gels. The 1.55-kilobase-pair (kbp) *Ava* I fragment 1 was isolated by electrophoresis into a block of low-temperature-gelling agarose, application of heat to 68°C, extraction with phenol, and precipitation with alcohol. The DNA fragment was dissolved in 10 mM Tris chloride, pH 7.5/1 mM EDTA and was used directly as template for *in vitro* transcription.

The *Sst* I (*Sac* I) subclone of pJB3 was constructed by inserting the *Sst* I fragment that contains the *gD* gene into the unique *Sst* I site of pUC18 and was designated pXK3.

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Abbreviations: HSV, herpes simplex virus; ICP, infected-cell polypeptide; gD, glycoprotein D; bp, base pair; SV40, simian virus 40.

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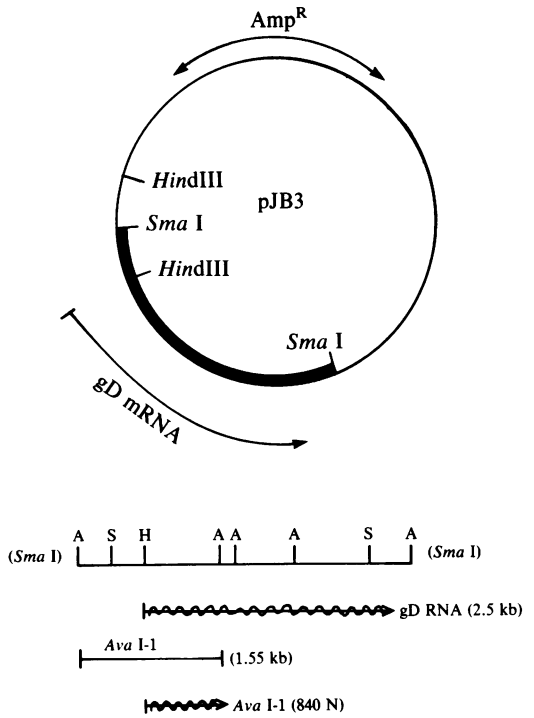


FIG. 1. Structures of recombinant plasmids. The recombinant plasmid pJB3 was constructed by subcloning the 3.6-kbp *Sma* I fragment of the HSV-1 *Bam*HI fragment J into the unique *Sma* I site of plasmid pACYC177. The region of the plasmid that consists of HSV-1 DNA is indicated by a thick line. Below the plasmid is shown a simplified restriction map of the HSV DNA insert in pJB3 in which A is the site for *Ava* I; S, the site for *Sst* I, and H, the site for *Hin*dIII. The position of the 2.5-kilobase (kb) gD mRNA and the 840-nucleotide (N) run-off transcription synthesized *in vitro* from the *Ava* I fragment 1 are shown below the restriction map. $Amp^R$, gene for ampicillin resistance; kb, kilobases.

Restriction enzymes were used under the conditions described by the supplier, and standard procedures were used to grow and transform bacteria. The size markers on the gels were fragments of SV40 DNA digested with *Hin*dIII and labeled with $^{32}P$. DNA quantitation was carried out by comparing the fluorescence of an ethidium bromide-stained sample with a series of DNA standards.

**DNA Binding Immunoassay.** ICP4 was partially purified from the nucleus of HSV-1-infected Vero cells as described by Metzler and Wilcox (37). An additional purification step was carried out by DEAE-cellulose chromatography. The more highly purified preparation of ICP4, which was designated fraction VIII, was used for the experiments reported here. Electrophoresis of fraction VIII on $NaDodSO_4$/PAGE and subsequent staining with Coomassie blue revealed proteins with a wide range of molecular weights; from the relative intensity of the stained bands, it was estimated that ICP4 represents approximately 5% of the total protein mass in fraction VIII (S.F. and K.W.W., unpublished data).

The DNA binding immunoassay was conducted with polyclonal anti-ICP4 IgG and radioactive DNA fragments by a modification of the procedure described by McKay (38). A previous report has shown that this polyclonal anti-ICP4 does not bind to polypeptides from uninfected Vero cells and specifically precipitates ICP4 from fraction VII (the precursor to fraction VIII) (37). Target DNA uniformly labeled with $^{32}P$ was obtained from bacteria grown in medium containing [$^{32}P$]phosphate. Plasmid DNA was purified, cut with restriction enzymes, and repurified. Radioactive DNA fragments were mixed with a 20-fold excess of unlabeled salmon sperm DNA in binding buffer (150 mM NaCl/10 mM Tris chloride, pH 7.5/10 mM $MgCl_2$/2 mM mercaptoethanol/0.5 mM EDTA/50 μg of bovine serum albumin per ml). A sample of fraction VIII was added, and the mixture was incubated for 30 min at 22°C. Antibody was added and allowed to react for 15 min at 22°C. Protein A-bearing *Staphylococcus aureus* was added; after an additional 15 min, the protein A–immunocomplex was recovered and washed by centrifugation. The washed complex was disrupted with buffer containing 2% $NaDodSO_4$, and the DNA fragments in the complex were separated by gel electrophoresis. To locate the [$^{32}P$]DNA fragments that had bound to ICP4, the dried gel was exposed to x-ray film. To determine the molar ratios of DNA fragments within a lane, the x-ray film was scanned with a recording densitometer, and the peak areas were measured with a planimeter. Control experiments (S.F. and K.W.W., unpublished data) have established that DNA fragments are not bound when the IgG fraction from preimmune serum was used or when ICP4 was omitted from the incubation.

***In Vitro* Transcription.** Nuclear extracts from suspension cultures of uninfected HeLa cells were prepared by the procedure of Dignam *et al.* (27). The incubation conditions were those described by Tack and Beard (39) except that a 25-μl reaction volume was used. The $Mg^{2+}$ was 8 mM and the KCl was 70 mM. Unless indicated otherwise, 100 ng of purified *Ava* I DNA fragment 1 was present as template, and the reaction proceeded for 60 min at 30°C. Fraction VIII was dialyzed at 0°C against 20 mM Hepes, pH 7.9/0.1 M KCl/0.2 mM EDTA/0.5 mM dithiothreitol/20% glycerol and stored at 0°C for no more than 2 days. Long-term storage was at −70°C. Antiserum was also dialyzed against this buffer prior to use, and the $K^+$ concentration in the antibody or ICP4 solutions was taken into account when preparing the transcription reaction mixtures.

The [$^{32}P$]RNA was isolated as described (39), and the transcripts were separated after treatment with glyoxal (40) by electrophoresis on a 2% agarose gel. The dried gels were exposed to x-ray film, and the radioactivity in the 840-nucleotide band that corresponds to the *Ava* I fragment 1 run-off transcript (Fig. 1) was measured by excising the band and assaying the $^{32}P$ in a scintillation spectrophotometer. Background values, obtained by assaying gel segments adjacent to the *Ava* I fragment 1 transcripts for radioactivity, were subtracted for each reaction.

## RESULTS

**ICP4 Binding to the *gD* Gene.** Chromatography of HSV-1-infected cell extract on DNA-cellulose has indicated that ICP4 binds to DNA (15–17). But, the observation that partially purified ICP4 failed to bind to single-stranded DNA-cellulose suggested that the interaction between ICP4 and DNA might be mediated by a cellular protein (18). By use of the DNA binding immunoassay, evidence for specific binding between ICP4 and two regions of pBR322 DNA was obtained (S.F. and K.W.W., unpublished data). The same technique showed that ICP4 in fraction VIII binds to specific fragments of pJB3 DNA, including the *Sac* I fragment that spans the *gD* gene. For further analysis, the *Sac* I fragment was subcloned into pUC18 to generate a plasmid designated pXK3.

Radiolabeled pXK3 was digested with restriction enzymes and then used at the target DNA in the ICP4 binding immunoassay. With fragments from a *Pvu* II/*Hae* II double digest of PXK3, strong binding of ICP4 to a 502 *Pvu* II fragment that spans the 5′ end of the *gD* gene was observed (36), and weaker binding was found to the adjacent 268-bp fragment (Fig. 2). Conducting the assay with a *Pvu* II/*Hae* II/*Hin*dIII triple digest of pXK3 revealed that ICP4 bound strongly to the 274-bp fragment that spans the region from position −263 to +11 relative to the gD mRNA start time. ICP4 bound less strongly to the 228-bp fragment that spans the *gD* gene from +11 to +239. Weak binding of ICP4 to the

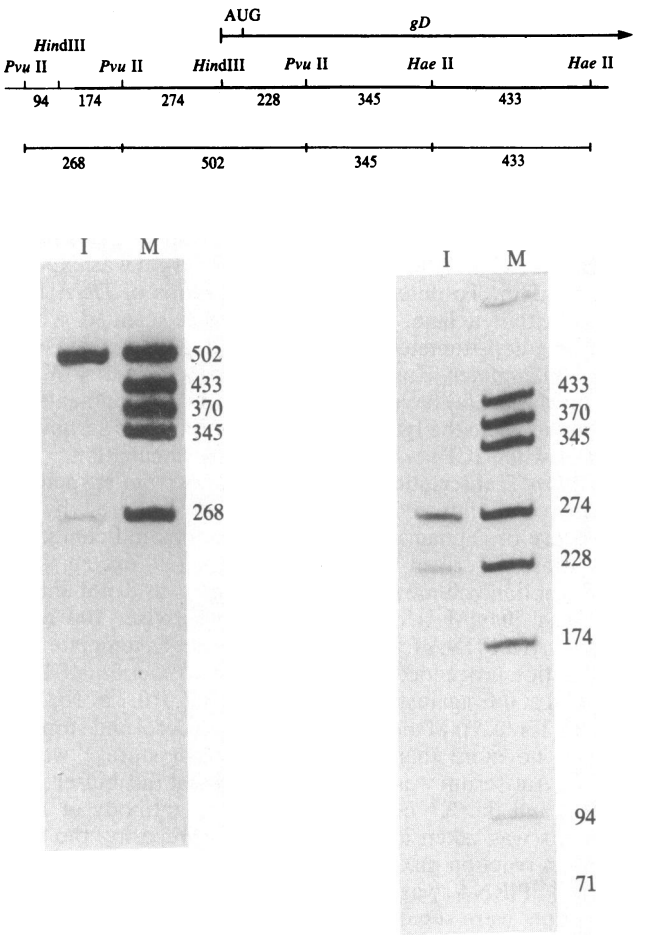


FIG. 2. Binding of ICP4 to specific DNA fragments. (*Upper*) Schematic diagram showing the portion of plasmid pXK3 from a *Pvu* II site in the vector of a *Hae* II site within the *gD* gene. The transcription initiation site (vertical bar), translation initiation site (AUG), and direction of transcription for the *gD* gene are indicated (top line). The sizes of fragments produced from this region by either a *Pvu* II/*Hin*dIII/*Hae*II triple digest (center line) or a *Pvu* II/*Hae*II double digest (lower line) are also shown. (*Lower*) DNA binding immunoassays were performed with radiolabeled pXK3 DNA and fraction VIII under standard conditions. Immunoprecipitated DNA was electrophoresed through a 5% polyacrylamide gel and detected by autoradiography. Two fragments of >1.5-kb size that were derived from the vector and the 3′ end of the *gD* gene were not resolved in these gels and, thus, are not shown. (*Lower Left*) *Pvu* II/*Hae*II-cleaved pXK3 was used as the target DNA. (*Lower Right*) *Pvu* II/*Hin*dIII/*Hae*II-cleaved pXK3 was used as the target DNA. Lanes: M, marker DNA from a corresponding digest of pXK3, with sizes shown in bp; I, immunoprecipitated DNA.

174-bp fragment spanning the region from −437 to −264 also was detected. Although it is possible that a single contiguous ICP4 binding site may span a *Hin*dIII or *Pvu* II cleavage site, it is more likely that each of the immunoprecipitated fragments contains a separate ICP4 binding site. The amount of each fragment that was immunoprecipitated was determined by scanning a suitably exposed radioautograph with a densitometer and measuring the areas under the curve. From these measurements, the relative molar amounts of the 274-, 228-, and 174-bp fragments were calculated to be 7.6, 2.8, and 1.0, respectively. These values presumably reflect the relative affinity of ICP4 for sites within these fragments, since equal molar amount of each fragment was observed in the marker lane (Fig. 2). The presence of multiple binding sites in close proximity to the 5′ end of the mRNA start has been reported for the transcription factor Sp1 (31, 33–35), and for the SV40 T antigen (20).

**Effect of ICP4 on *in Vitro* Transcription from the *gD* Promoter of pJB3 DNA.** On the basis of time of appearance, gD is classified as an early protein (41). Measurements of gD mRNA confirm this classification (36), and transfection experiments have demonstrated that ICP4 acts in *trans* to increase gD mRNA levels (10, 22). We have shown that DNA fragments cut from pJB3 plasmid DNA will serve as template for *in vitro* transcription (36). In these experiments, which utilized HeLa whole-cell extract, the sizes of the run-off transcripts were as predicted from the *in vivo* start site for gD mRNA synthesis. These results were confirmed by using the HeLa nuclear extract and S1 nuclease assays (L.I.P. and P.B., unpublished data). With the isolated *Ava* I fragment 1 as template, a single major transcript 840 nucleotides long was synthesized (Fig. 3, lane 1). The radioactive bands in the 1.5-kbp region of the gel correspond in size to the *Ava* I fragment 1 and probably represent end addition of nucleotides to DNA.

Addition of ICP4 (fraction VIII) to a nuclear extract containing the *Ava* I fragment 1 stimulated synthesis of the 840-nucleotide transcript about 4-fold (Fig. 3*A*, lane 2, and Fig. 3*B*). A higher amount of ICP4 somewhat reduced

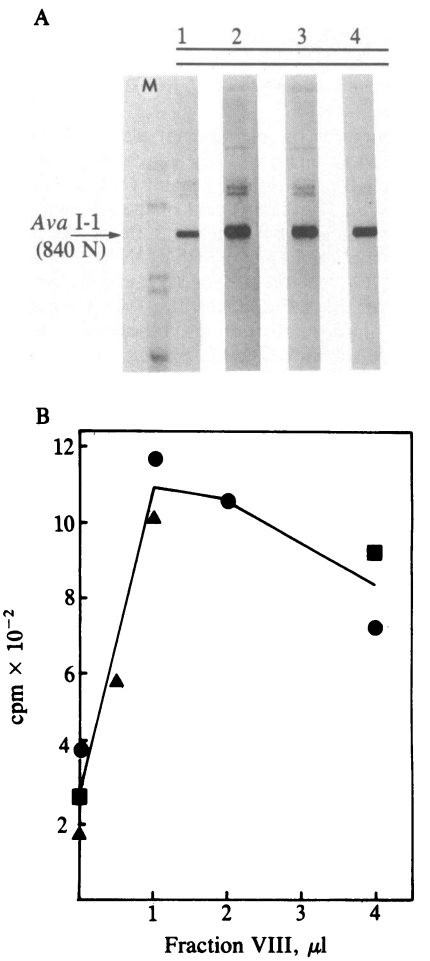


FIG. 3. Effect of ICP4 on transcription. (*A*) Transcription from 100 ng of the 840-nucleotide (N) *Ava* I fragment 1 in the absence (lane 1) or presence (lanes 2–4) of increasing amounts of ICP4: 1 μl (lane 2), 2 μl (lane 3), and 4 μl (lane 4) of fraction VIII. The DNA and fraction VIII were preincubated in reaction buffer for 5 min at 26°C; then nuclear extract was added, and transcription was allowed to proceed for 60 min at 30°C. Lane M shows *Hin*dIII-cut SV40 DNA used as size markers. (*B*) The radioactivity in the 840-nucleotide run-off bands was measured in a scintillation counter, and the data are plotted for the experiment shown in *A* (●). The data from two additional experiments with different nuclear extracts are also shown (■ and ▲) on this figure.

stimulation (Fig. 3*A*, lane 4, and Fig. 3*B*), suggesting that other proteins in fraction VIII may interfere with the assay.

To obtain information on how ICP4 affects transcription, we followed the kinetics of gD mRNA synthesis in the presence or absence of fraction VIII. The autoradiogram in Fig. 4 *Upper* demonstrates that ICP4 increased the rate of transcription. By measuring the radioactivity in the 840-nucleotide band after different times of synthesis, the data shown in Fig. 4 *Lower* was obtained. The presence of ICP4 in the reaction was found to have two effects: it reduced the lag before RNA synthesis started and extended the duration of RNA synthesis. In the absence of ICP4, RNA synthesis stops after about 35 min. When initiation of transcription was blocked after 15 min and the reaction was allowed to proceed for an additional 45 min, the results shown in Fig. 4, lanes 9 and 10, were obtained. In the absence of ICP4, [$^{32}$P]UTP incorporation was the same as that found with standard incubation conditions (Fig. 4, lanes 5 and 7). When ICP4 was present, incorporation was reduced (Fig. 4, lane 10) in comparison with the 60-min point (lane 8). These results show that, in the absence of ICP4, initiation complexes are formed within 15 min, and a single round of transcription takes place. When ICP4 is present, the initiation complex forms more rapidly and reinitiation can occur. Taken together, these data indicate that ICP4 facilitates initiation of RNA synthesis at the *gD* promoter.

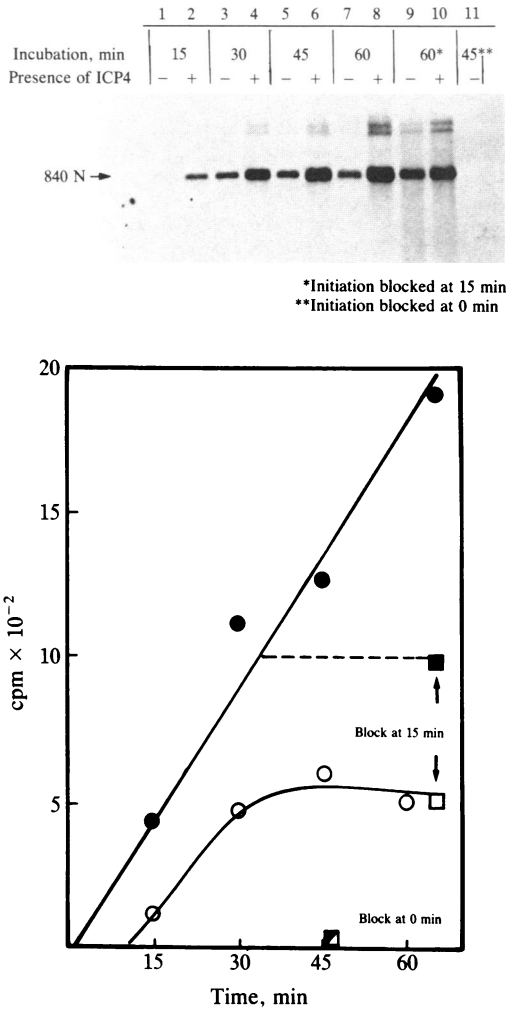


FIG. 4. Effects of ICP4 on the kinetics of *Ava* I fragment 1 transcription. (*Upper*) Standard reactions with *Ava* I fragment 1 DNA were set up with (lanes 2, 4, 6, 8, and 10) or without (lanes 1, 3, 5, 7, and 9) ICP4. When 1 μl of fraction VIII was present (lanes 2, 4, 6, 8, and 10), it was preincubated with the DNA prior to the addition of nuclear extracts. The incubations were stopped after 15 min (lanes 1 and 2), 30 min (lanes 3 and 4), 45 min (lanes 5 and 6) and 60 min (lanes 7 and 8) by the addition of $NaDodSO_4$ and EDTA. RNA polymerase initiation was blocked after 15 min of incubation (lanes 9 and 10) by making the reaction 100 mM in $(NH_4)_2SO_4$ and 1.7 mM in $MnCl_2$ (42). The incubation was continued for another 45 min. Lane 11 shows the control in which initiation was blocked at 0 time and 45 additional min was allowed for chain elongation. The radioautogram of the separation gel is shown. (*Lower*) Data obtained by cutting the 840-nucleotide (N) bands and measuring the radioactivity. ○, Data from reactions without ICP4; ●, data obtained from reactions with ICP4; ■ and □, data from reactions with (■) and without (□) ICP4 where initiation was blocked at 15 min; ◧, data from the control in which initiation was blocked at 0 min.

To determine whether ICP4 can reactivate transcription, a second experiment was carried out in which ICP4 (fraction VIII) was added to the incubation after 30 min, and an additional 30 min was allowed to complete the reaction. The radioactivity in the 840-nucleotide band was determined and compared with that in bands from incubations that lacked ICP4 or had ICP4 added at zero time. When ICP4 was absent, the 840-nucleotide band contained 310 cpm; when ICP4 was present from the start of the reaction, the band contained 1550 cpm; when ICP4 was added at 30 min, the band contained 375 cpm. These data show that ICP4 had no effect when added after transcription had started. It appears that in the absence of ICP4 a component of the transcription system, possibly an initiation factor, is inactivated during the incubation. ICP4 cannot substitute for this component or reverse its inactivation. This result is also consistent with ICP4 acting at initiation of transcription.

**Effect of Anti-ICP4 Antibody on Stimulation of Transcription by ICP4.** Because fraction VIII contains several proteins, we wished to obtain additional evidence that the stimulation of transcription was due to ICP4. We took advantage of the specificity shown by the anti-ICP4 antibody (37) and tested the effect of the antibody on transcription. When the ICP4 preparation was incubated with antibody for 15 min prior to the addition of *Ava* I fragment 1 template DNA, the amount of RNA synthesized was markedly reduced but not to the level seen in the absence of ICP4 (Fig. 5, lane 3). Antibody in the absence of ICP4 lowered transcription by 15% (Fig. 5, lane 4); increasing the amount of antibody in control experiments reduced transcription even further, so it was not feasible to test the effect of more antibody on stimulation by ICP4. Preimmune IgG also reduced transcription about 15% (data not shown). Because ICP4 can bind to DNA in the presence of antibody (S.F. and K.W.W., unpublished data), some stimulation of transcription by ICP4 could occur in the presence of antibody. The results with the anti-ICP4 antibody, while not unambiguous, support the view that ICP4 stimulates transcription *in vitro*.

## DISCUSSION

The *in vitro* experiments described in this paper extend the *in vivo* experiments that identify ICP4 as a HSV regulatory protein. The demonstration that ICP4 binds specifically to segments of the HSV *gD* gene and stimulates transcription from this gene provides evidence for ICP4's mechanism of action and raises interesting questions about the DNA sequences and proteins involved in viral gene regulation. The binding of ICP4 is not restricted to HSV DNA; two sites have been identified on the bacterial plasmid pBR322.[¶] A comparison of the nucleotide sequences in pBR322 and the *gD* gene has indicated regions of sequence homology in these DNAs. Based on the requirement for a functional ICP4 to obtain early and late HSV gene expression, we would expect

[¶]Farber, S. & Wilcox, K. W., Xth Herpesvirus Workshop, Univ. of Michigan, Ann Arbor, August 11–16, 1985, p. 64.

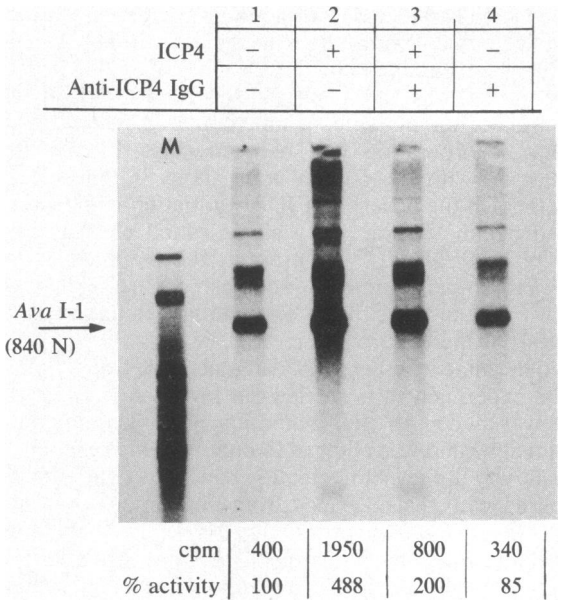


FIG. 5. Effects of anti-ICP4 antibody. ICP4 (1 μl of fraction VIII) (lanes 2 and 3) was preincubated with 40 μg of anti-ICP4 IgG (lanes 3 and 4) or buffer (lanes 1 and 2) for 15 min at 26°C prior to the addition of *Ava* I fragment 1 DNA and the components of the transcription assay. After an additional 5 min, the nuclear extract was added, and the incubation was allowed to proceed for 60 min. The radioautogram was developed for 24 hr, and the radioactivity in the *Ava* I fragment 1 run-off band was measured in a scintillation counter. Lane M, molecular weight markers; % activity is relative to the control activity as 100%.

ICP4 to bind specifically to regulatory regions of many HSV genes. We have shown that purified ICP4 stimulates *in vitro* transcription from the HSV *gC* gene and inhibits *in vitro* transcription from the immediate early gene for ICP22 (L.I.P. and P.B., unpublished data).

An interesting feature of ICP4 binding to the *gD* gene is that there are three sites that, on the basis of the DNA fragment binding assay, have different affinities. Multiple binding sites for regulatory proteins may be a common feature of transcriptional control. The cell factor Sp1 binds to two or more sites on SV40 and to HSV genes (25, 34, 35). A potential advantage to using several factors and multiple sites to control transcription is that small changes in the concentration of transcription factors can be amplified, leading to large differential effects on RNA synthesis. The regulatory effects could be positive or negative in nature, and interactions between the factors or the DNA binding sites might magnify the effects of factor binding. An interactive system of this type could explain the observed changes in HSV mRNA levels that occur during infection. Fluctuations in the concentrations of ICP4 and other immediate early proteins such as ICP0 (22, 43, 44) could have major effects on the binding of RNA polymerase to viral genes and the effectiveness of the DNAs as templates for RNA synthesis. From *in vivo* experiments, we expect that ICP4 would stimulate RNA synthesis from early and late HSV genes and possibly reduce synthesis from immediate early genes. The data obtained from *in vitro* transcription experiments are, therefore, consistent with *in vivo* observations. To obtain information on the interaction between viral and cellular factors, additional experiments with purified components will be required.

It appears that the combination of viral proteins, cellular factors, and regulatory sequences in the DNA controls the sequential activation of HSV genes. Tissue trophisms and abortive infections are also probably influenced by these factors. The fact that ICP4 is the one viral protein expressed in the ganglia of latently infected animals (45) may imply that this protein is important in establishing or maintaining the latent state. It is attractive to hypothesize that fluctuations in the amount of ICP4 in cells could increase and/or reduce the transcription from viral genes and control the transition from a latent to an active infection.

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