VIRUS GENES 2:1, 31–48, 1988

Characterization of a Binding Factor that Interacts with the Sequences Upstream of the Vaccinia Virus Thymidine Kinase Gene

ELIZABETH M. WILSON[1], CHERYL EDBAUER[2] AND DENNIS E. HRUBY[1]
[1]*Center for Gene Research, Department of Microbiology, Oregon State University, Corvallis, OR 97331-3804*

Received March 23, 1988
Accepted June 13, 1988

Requests for reprints should be addressed to Dennis E. Hruby, [2]Wadsworth Center, New York State Department of Health, Empire State Plaza, Albany, NY 12201

Key words: VV, thymidine kinase, transcription, gel retention

Abstract

A small 176 base-pair cloned DNA fragment, representing the nucleotide sequences proximal to the 5'-end of the vaccinia virus thymidine kinase (VV TK) gene, was radiolabeled and used in concert with gel retention assays to detect, partially purify, and characterize a promoter binding factor (PBF) extracted from vaccinia virions. The VV TK PBF was purified from solubilized virus particles by a combination of ion-exchange and DNA-affinity chromatographic procedures. The interaction between VV TK PBF and VV TK promoter sequences was relatively specific in that binding to the radiolabeled probe could be effectively inhibited by unlabeled VV TK promoter or VV TK promoter-specific oligonucleotides, but not by similar-sized fragments of control plasmid DNA. The VV TK PBF did, however, bind to other VV early-promoter elements. Glycerol gradient sedimentation provided an estimate of 130–140 kD for the native molecular weight of VV PBF. This correlated well with data from the purification of VV PBF from radiolabeled VV particles that revealed 2 polypeptides, with molecular weights of 70 and 68 kD that co-purified with VV TK PBF activity. Taken together, these results suggest that a heterodimeric promoter-binding factor, which is present within the cytoplasm of VV-infected cells, is capable of specifically interacting with VV early-promoter elements.

Introduction

In many respects, vaccinia virus (VV) represents an ideal self-contained model eukaryotic system with which to investigate a variety of questions related to develop-

ment, virus/host cell interactions, and gene regulation. The latter question, in particular, would appear to be most appropriate to the VV system since this virus apparently encodes more than 200 viral genes whose quantitative and qualitative expression is tightly regulated in a temporal fashion during the viral replicative cycle (1). As an experimental system, VV provides at least two significant advantages for this type of study. First, the 185,000 base-pair DNA genome contains approximately 90% unique sequences (2). This renders it functionally haploid and makes it an effective substrate for both classical and modern molecular genetic approaches. Second, unlike other DNA-containing animal viruses, VV replicates within the cytoplasmic compartment of infected cells (3). Since the virus apparently does not utilize the enzymatic machinery within the host cell nucleus, this necessitates that VV contain or encode many of the enzymes necessary to replicate and express its own genetic information. Thus, in theory, one should have ready access to viral genes encoding essential enzymatic and structural functions as well as the factors that regulate their expression.

When selecting an individual VV gene for detailed molecular genetic analyses, the investigator must consider a large number of practical and theoretical reasons why the locus of choice might be the VV thymidine kinase (TK) gene. a) The sequence of this VV gene and its flanking regions are known. The coding sequences are small and hence easy to manipulate and mutagenize. Furthermore, they have already been cloned and subcloned into plasmid vectors (4,5). b) The transcriptional pattern of this region of the genome has been studied in detail and has been shown to lack many of the complexities associated with VV late gene expression (6,7). c) This gene encodes a protein with a known, easily assayable, and biochemically-selectable activity (8). d) The gene is nonessential in tissue culture, which means that it can be manipulated into and out of the viral genome without killing the recipient virus. e) And most important, the VV TK gene seems to be subject to a variety of regulatory controls (9).

The VV TK gene is an immediate-early viral gene that maps to the central, highly conserved region of the VV genome within the *Hin*dIII J fragment (10). The transcription of this gene is turned on immediately postinfection (p.i.) (9). Enzyme activity and TK mRNA continue to accumulate in the cytoplasm until about 4 hr p.i. when two things happen: transcription of this locus is abruptly attenuated and, the existing TK mRNA is no longer translated into protein (9). Both of these inhibitory events are mediated presumably by immediate-early or early gene products since similar induction and repression kinetics occur in the presence of inhibitors of viral DNA synthesis that prevent the expression of VV late genes. If VV late gene expression is allowed to occur, the otherwise stable VV TK mRNA is rapidly degraded with a function half-life of about 2 hr. Finally, the protein is not active as a monomer, but rather it must be assembled into a tetrameric enzyme complex. This complex catalyzes the conversion of thymidine to dTMP, and its enzymatic activity is subject to feedback inhibition by the final end-products of the biosynthetic pathway, dTDP or dTTP (11). Thus, regulation of this single locus is rather complex, apparently involving a number of viral gene products that include representatives of each of the three major VV kinetic classes.

Viewing the complex expression and regulation scheme employed by the VV TK gene, it becomes obvious that a variety of DNA-protein, RNA-protein, and perhaps protein-protein interactions must be involved. As an initial step in dissecting this complex process, the experiments described in this article have been directed toward identifying the *cis*-acting signals present within the nucleotide sequence of the VV TK gene DNA template and the nature and identity of the *trans*-acting protein factors that interact with them to effect regulated expression.

Materials and Methods

Virus growth and purification

Confluent monolayers of BSC-40 monkey kidney cells in 150 mm dishes were maintained at 37°C in Earle's minimum essential medium (MEM, GIBCO) supplemented with 2 mM L-glutamine, 50 mg/ml gentamycin, and 5% (v/v) heat-inactivated fetal calf serum. Cells were infected at an input multiplicity of infection of 0.1 infectious particles per cell with wild-type vaccinia virus (VV, WR strain). After 48 hr of infection, the infected cells were harvested, broken by osmotic rupture, and the viral particles were purified by two cycles of sucrose gradient velocity sedimentation (3). To metabollically label VV virion proteins, the infections were carried out as described above except that the medium was modified by pre-dialyzing the fetal calf serum against phosphate buffered saline and reducing its final concentration to 3%, by reducing the L-methionine concentration to 10% of normal (1.5 mg/liter), and by adding 2 μCi/ml of [L-^{35}S]methionine (1154 Ci/mmol, New England Nuclear) at 1 hr p.i.

Protein purification

All steps of the procedures described below were carried out at 4°C. Purified VV (5.5 mg) was pelleted at 12,000 rpm for 10 min in a TOMY 15A microcentrifuge. The virus pellet was resuspended in 250 ml of buffer A [100 mM Tris-HCl (pH 8.0), 10 mM dithiotreitol (DTT), 250 mM KCl, and 0.1 mM EDTA] and solubilized using 0.2% sodium deoxycholate (12). Following removal of insoluble material by centrifugation, the soluble fraction was applied to a 1 ml DEAE-cellulose (DE-52, Whatman) column that had been pre-equilibrated with buffer A. The column was washed and eluted with 12 × 100 ml aliquots of buffer A that were individually collected. Protein concentrations of each fraction were determined using a BioRad protein assay kit and the peak fractions were pooled (virion protein) and tested for VV TK gene promoter binding factor (PBF) activity.

The virion protein extract was dialyzed overnight against several changes of TGED buffer [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, 0.1% Nonidet P-40, and 20% glycerol] containing 0.1 M KCl. After dialysis, the extract was passed onto a 1 ml phosphocellulose column (BioRad Cellex-P) that had been

preequilibrated with TGED-0.1 M KCl (13). Protein was eluted from the column with sequential washing steps; 1 ml TGED-0.1 M KCl, 1 ml TGED-0.35 M KCl, 1 ml TGED-0.6 M KCl, and 4 ml of TGED-1.0 M KCl. Fractions (500 ml) were collected (PC protein), the protein concentrations determined, and VV TK PBF activity tested.

The PC protein fractions with the highest VV TK PBF activity were pooled and dialyzed overnight against buffer Z [25 mM Hepes (pH 7.8), 12 mM $MgCl_2$, 1 mM DTT, 0.1% Nonidet P-40, and 20% glycerol] containing 10 mM KCl (14). The dialysate was adjusted to a total volume of 2 ml with buffer Z, 220 μg of poly dI-dC was added, and the mixture was placed on ice for 10 min. The mixture was then loaded onto a 0.7 ml DNA affinity column that had been prepared by coupling an annealed synthetic oligonucleotide (containing the sequences from -3 to -36 upstream of the VV TK gene transcriptional start site) to CNBr-activated Sepharose 4B (Pharmacia) (14–16). The effluent was collected and the column washed 4 times with 1 ml aliquots of buffer Z-10 mM KCl. The column was then plugged, 0.8 ml of buffer Z-100 mM KCl added, the beads gently mixed with a siliconized glass rod and allowed to settle for 10 min. The column was unplugged and the effluent and an additional 0.8 ml wash with buffer Z-100 mM KCl collected separately (DAC protein). In order to repass the DAC protein over the affinity column, the effluent was diluted with buffer Z to reduce the [KCl] to 10 mM and the chromatography repeated. The VV TK PBF activity of each fraction was tested. The DAC protein fractions with the highest activity were adjusted to 50% (v/v) glycerol, and frozen in small aliquots at −20°C.

VV TK gene promoter fragment

A 1311 base pair (bp) *Hin*dIII-*Hpa*II VV DNA fragment containing the VV TK gene was cloned into the pUC19 plasmid vector. Setting the 5′-A residue of the *Hin*dIII cleavage site as position 1, the 5′ and 3′ limits of the TK transcriptional unit have been previously mapped to positions 491 and 1070, respectively (4). Using unidirectional *Exo*III deletion mutagenesis (17), the upstream 5′-proximal sequences from positions 0–374 were removed. The truncated fragment containing the VV TK promoter was excised with *Rsa*I, which cut at position 551, and *Eco*RI, which cut upstream of the deletion. The 176 bp fragment was cloned into the *Eco*RI-*Sma*I sites of pUC18 (pUC18:TKp). In order to prepare labeled probe fragment for gel retention assays, pUC18:TKp was linearized with *Eco*RI, treated with alkaline phosphatase, and 5′-labeled using polynucleotide kinase and [g-^{32}P]ATP (18). The 176 bp fragment was then released by *Pst*I digestion and isolated by preparative gel electrophoresis (19). The concentration of the recovered probe fragment was determined by gel electrophoresis of an aliquot together with known concentrations of a control DNA fragment of similar size.

Gel retention assays

In a total volume of 19 μl, protein extracts to be tested for VV TK PBF activity were mixed with 1 μg of poly dI-dC (Pharmacia) in buffer B [10 mM Hepes (pH 8.0), 10% glycerol, 0.5 mM EDTA, 0.5 mM DTT, and 15 mM KCl] and incubated at 0°C for 10 min. One μl of radiolabeled DNA probe fragment (2 ng, ~ 10,000 dpm) was added, the incubation at 0°C continued for 5 min, followed by an additional 30 min incubation at 25°C (4,20). The reaction mixture was then loaded directly onto a low ionic strength 7.5% polyacrylamide slab gel [1.7 mM Tris-HCl (pH 8.0), 0.37 mM sodium acetate, 1 mM EDTA] that had been prewarmed at 15 mA for 2 hr. The buffer was constantly recirculated between the upper and lower chambers during electrophoresis. DNA-protein complexes were subjected to electrophoresis initially at 5 mA for 45 min, after which the power was gradually increased in 5 mM increments to 20 mA. Electrophoresis was continued for an additional 3 hr, at which time the gel was transferred to Whatman 3 MM paper, dried under vacuum, and exposed to Kodak XAR-5 film in a steel cassette with an intensifying screen at −70°C overnight. Competition assays were carried out in an identical manner with the unlabeled DNA fragments added to the reaction mixture at the same time as the labeled probe fragments.

Glycerol gradient sedimentation

Linear 4.8 ml 10–30% (v/v) glycerol gradients in buffer B were prepared in 13 × 51 mm Ultraclear centrifuge tubes (Beckman) and pre-chilled at 4°C for 1 hr. Approximately 25 μg of TK PBF, purified by phosphocellulose chromatography (PC-10), was mixed with 25 μg of a mixture of protein native molecular weight standards (Sigma GF-200), diluted to 250 μl with buffer B (minus glycerol), and applied to the top of the gradient. Gradients were centrifuged at 37,000 rpm at 4°C for 24 hr in a Beckman L8-70M ultracentrifuge. Fractions of 250 μl were collected from the bottom. Ten μl of alternate fractions were tested for VV TK PBF activity. The remainder of each fraction was acetone-precipitated and subjected to polyacrylamide gel electrophoresis (21). Following electrophoresis, the location of the molecular weight markers was determined by staining the gel with Coomassie brilliant blue.

Results

Identification and purification of VV TK gene promoter binding factor (PBF)

Recently, a number of techniques have been developed to study interactions between target DNA sequences and *trans*-acting protein factors (22–24). Each of

these methods relies on the availability of a small cloned DNA fragment containing potential *cis*-regulatory signals as well as a source of biologically active DNA-binding proteins. In order to initiate similar studies with regard to the VV TK gene promoter element, it was necessary to clone an appropriate DNA probe fragment. A schematic representation of the genomic VV TK gene transcriptional unit is shown in Fig. 1A. Previous experiments in which the sequences immediately upstream of the 5'-end of the VV TK transcriptional start site were progressively shortened from either the 5' or 3' direction have demonstrated that only the 30 proximal base pairs (bp) upstream of the transcriptional start site were necessary to obtain full promoter activity when abutted to a reporter gene, bacterial chloramphenicol acetyl transferase, and re-introduced into the viral genome (25, Wilson and Hruby, unpublished data). Therefore, a small 176 bp DNA fragment (TKp), which included the 5'-proximal sequences to the TK gene, was cloned into pUC18 for use as a probe fragment. This fragment contained the VV DNA sequences extending from 124 bp upstream of the VV TK open reading frame (ORF) (including the transcriptional start site) down to 53 nucleotides within the ORF.

As a source of potential *trans*-acting factors, a sodium deoxycholate soluble virion extract was prepared from purified VV and passed over a DEAE-cellulose column to remove the endogenous viral DNA template. The rationale for using this extract as the starting material was that previous studies have shown that such extracts are capable of efficiently and accurately initiating transcription from exogenously added DNA fragments that contain VV early promoter elements. This implies that most, if not all, of the enzymes and factors necessary for early gene transcription are present and functional within the viral particle (12). As the data in Fig. 1B show, there is apparently a VV TK gene promoter binding factor (PBF) present in VV virion extracts. This activity was detected by the use of gel retention assays carried out with radiolabelled VV TKp, in the presence of poly dI-dC to bind up nonspecific DNA binding proteins. Using this procedure, a single DNA-protein complex can be detected with as little as 40 ng of virion protein. By adding increasing amounts of virion protein (up to 1 μg per 2 ng probe) all of the VV TKp fragment becomes complexed into high molecular weight aggregates that are apparently too large to enter the gel. It should be pointed out that the initial virion extract is likely to contain a complex mixture of proteins, many of which will have affinity for DNA. Examples include VV RNA polymerase and VV poly(A) polymerase. Thus, in order to study potential VV *trans*-acting factors it was first necessary to partially purify them.

The soluble virion extract contains at least 30 proteins that are detectable by gel electrophoresis and these probably represent only the major structural species present. Therefore to partially purify the VV TK PBF, the virion extract was fractionated by ion-exchange phosphocellulose chromatography using a step-gradient of increasing concentrations of KCl (Fig. 2). Under these conditions, a major peak of VV TK PBF activity was eluted from the column in fractions 9 and 10 by TGED buffer containing 1.0M KCl. The peak fractions were pooled and designated as PC-10 protein. The binding of PC-10 protein to the VV TKp was titrated

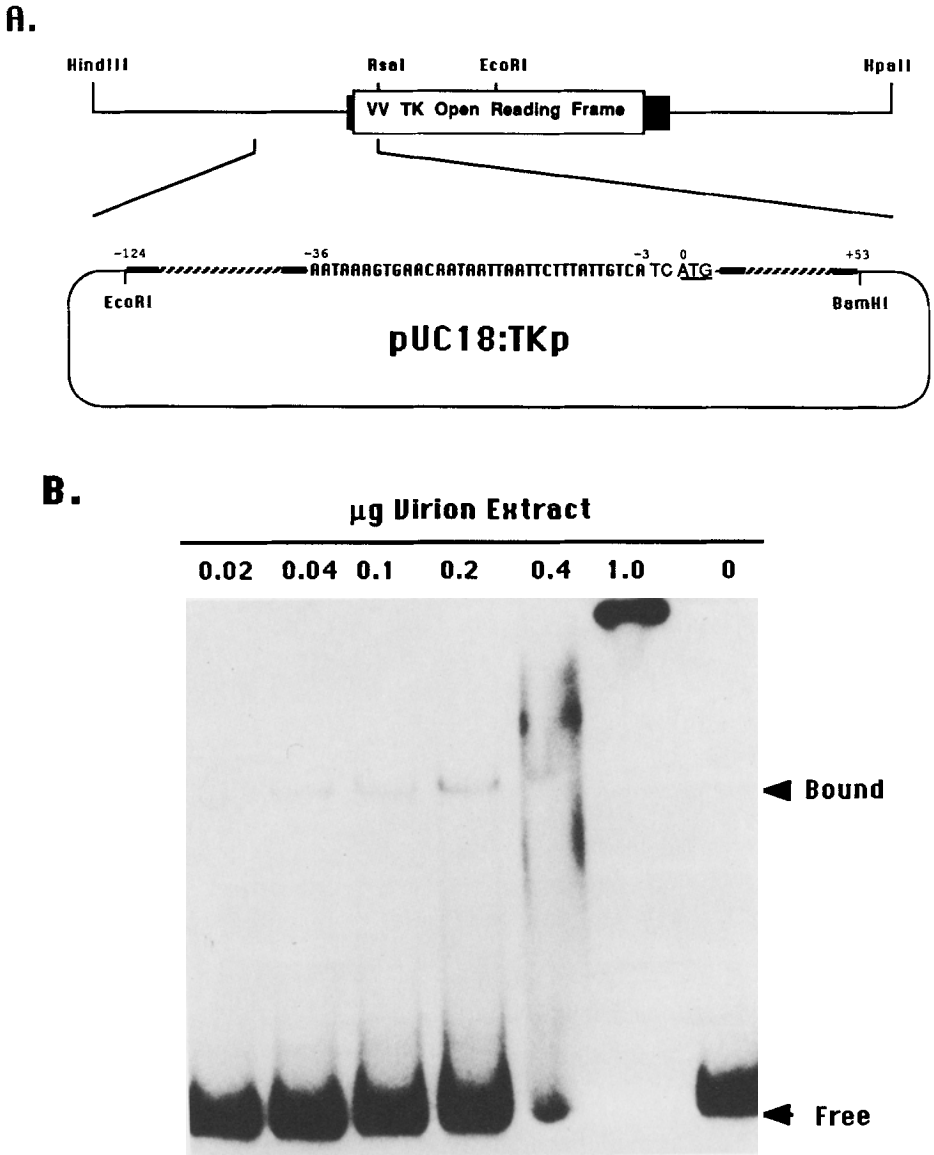

Fig. 1. Interaction of VV TK gene promoter and virion proteins. (A) Schematic representation of the VV TK gene. The open reading frame is indicated by the open box. The 5′- and 3′-untranslated regions are indicated by the filled boxes. The portion that was cloned and used as a TK promoter specific probe is expanded below (pUC18:TKp). The sequence shown is that of the message strand of DNA. The A of the initial ATG of the TK gene open reading frame is designated as position 0. The bold sequence (−3 to −36) is the region that was synthesized and used in subsequent DNA affinity chromatography experiments. (B) Gel retention assay using 2 ng of ^{32}P-labeled 176 bp TKp probe fragment and the indicated amounts (µg) of virion protein extracted from purified VV.

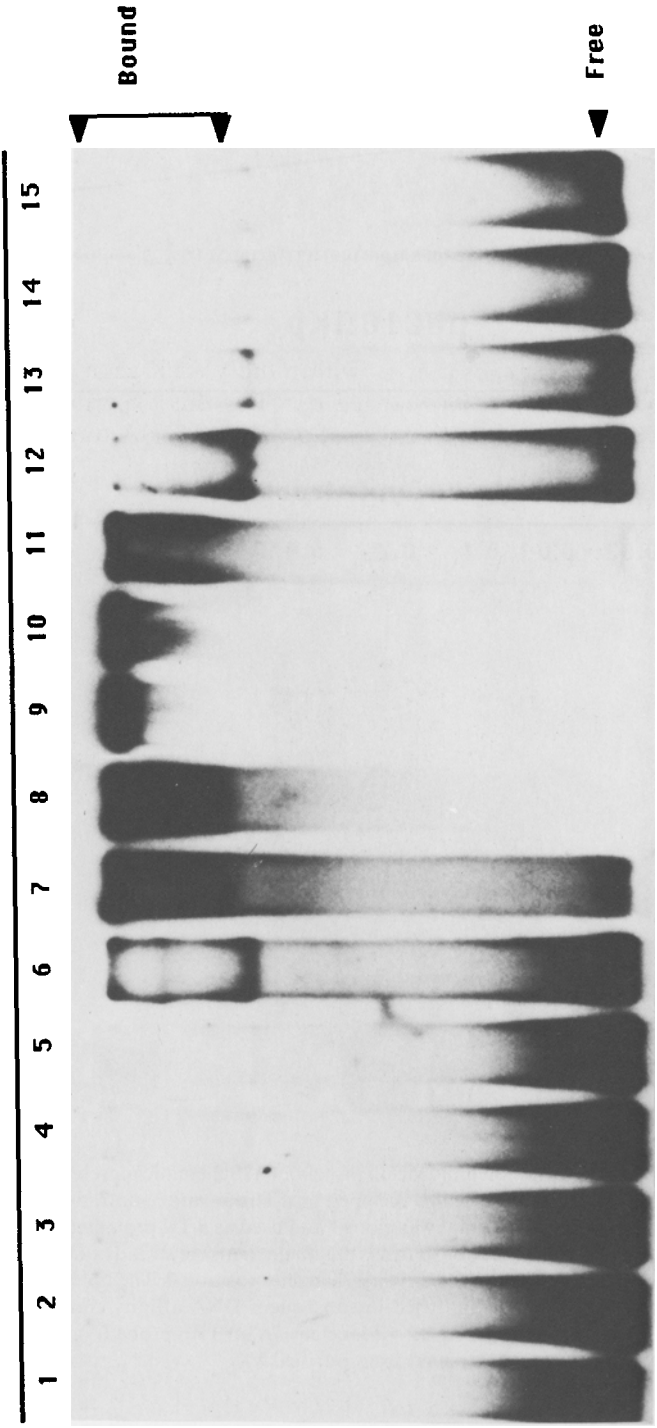

Fig. 2. Ion-exchange chromatography of VV TK PBF present in VV virion extracts. Virion protein (1 mg) was bound to a phosphocellulose column and then eluted using increasing concentrations of KCl (0.1 M to 1.0 M). VV TK PBF activity was measured using 200 ng of protein from each fraction in a gel retention assay with ^{32}P-labeled VV TKp probe fragment.

(Fig. 3) and found to be enriched in VV TK PBF activity relative to the unfractionated virion protein. The addition of 50 ng of PC-10 protein resulted in the formation of a substantial amount of VV TKp-PBF complex. Increasing the amount of PC-10 added to 150 ng resulted in aggregate formation. DNA-binding proteins were also present in other PC fractions, but they did not react specifically with the TKp (data not shown, see below).

Specificity of VV TK PBF binding

To demonstrate that the PBF activity that was being measured was due to a specific interaction with sequences present within the VV TK gene promoter element, a competition experiment was carried out. For this experiment, 2 ng of radiolabeled VV TKp was mixed with 25 ng of unlabeled DNA fragment during both the binding reaction and subsequent gel electrophoresis. The data shown in Fig. 4 indicates that the addition of VV TKp competitor DNA was quite efficient at

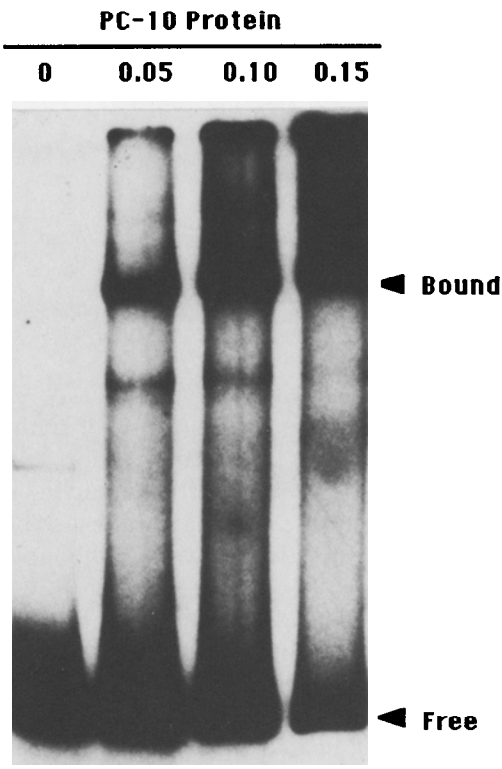

Fig. 3. Titration of phosphocellulose purified VV TK PBF activity. The indicated amounts (μg) of PC-10 protein were mixed with ^{32}P-labeled VV TKp probe fragment and analyzed by gel electrophoresis.

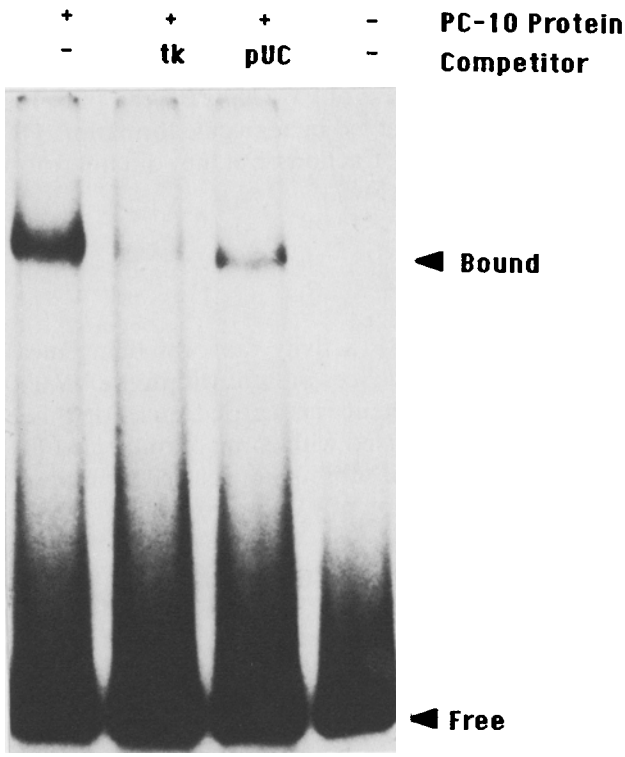

Fig. 4. Competition for TK PBF binding activity present in PC-10 protein. DNA-protein binding reactions were set up and analyzed using 50 ng of PC-10 protein and 2 ng of ^{32}P-labeled VV TKp 176 bp probe fragment. In the reactions indicated, 25 ng of either unlabeled VV TKp DNA (tk) or a 220 bp pUC18:*Ava*II DNA fragment (pUC) were included in the mixtures.

inhibiting PBF activity. An equivalent concentration of an unrelated pUC18 plasmid DNA fragment was less effective in this competition assay. It is interesting to note that, if competition experiments were carried out using the initial virion protein extract prior to phosphocellulose chromatography, the VV TKp and pUC18 competitor fragments were equally effective with no demonstratable VV TK promoter specificity (data not shown).

The apparent specificity of the binding interaction between VV TKp and PBF was then taken advantage of by utilizing DNA-affinity chromatography to further purify the PBF activity. Oligonucleotides representing the positive and negative strand sequences found from −3 to −36 bp upstream of the VV TK ORF (see Fig. 1A) were synthesized, annealed, ligated, and coupled to Sepharose beads. PC-10 protein was passed onto this matrix in the presence of 10 mM KCl. After washing the column with additional buffer containing 10 mM KCl, the PBF activity was eluted from the column using 100 mM KCl (DAC protein). Under these con-

ditions, 98%–99% of the PC-10 protein flowed through the column in 10 mM KCl with 1%–2% being bound until the salt concentration was elevated to 100 mM (data not shown). The PBF that was recovered in the 100 mM salt wash was highly active and retained its specificity for VV TKp binding. The data in Fig. 5 demonstrates that only a small amount (5–10 pg) of DAC protein was required to produce an easily detectable TKp-PBF complex. Furthermore, the DAC PBF apparently retained its binding specificity, in that PBF association with the probe fragment could be inhibited almost completely by the addition of unlabeled TKp or the double-stranded synthetic oligonucleotide that was used in the purification. In contrast, there was no reduction in PBF activity in the presence of a competitor fragment isolated from pUC18 plasmid DNA.

Having demonstrated that the DAC-purified VV TK PBF has an affinity for the DNA sequences that reside upstream of the VV TK gene transcriptional start site, it was of interest to determine if this interaction was limited to the TK gene or if the

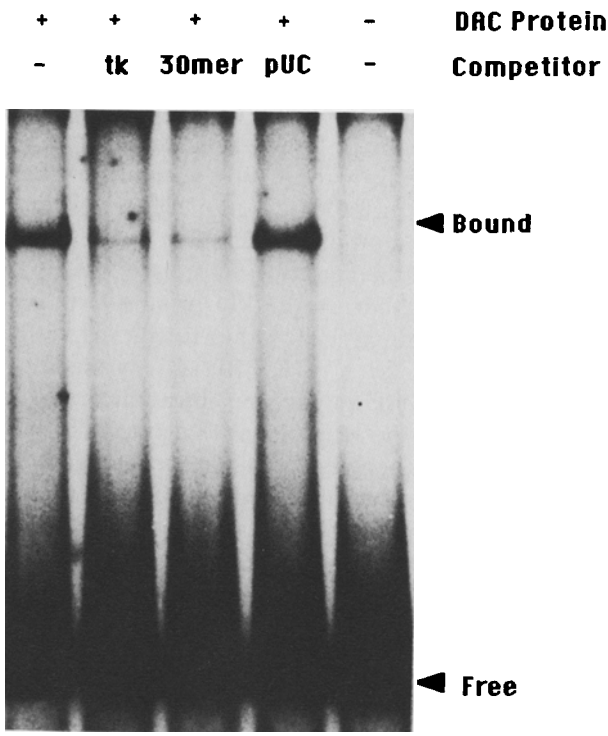

Fig. 5. Activity and specificity of VV TK PBF purified by DNA affinity chromatography. PC-10 protein (450 μg) was further purified by three cycles of DNA affinity chromatography (DAC). Binding reactions were assembled using 4μl of DAC protein and 2 ng of ^{32}P-labeled VV TKp fragment in the presence of no competing unlabeled DNA (−), 20 ng of VV TKp (tk), 10 ng of annealed-ligated oligonucleotide corresponding to the region immediately upstream of VV TK gene start site (30 mer), or 20 ng of pUC18:*Ava*II DNA fragment DNA-protein complexes were analyzed by gel electrophoresis.

PBF would bind also to other VV early-gene promoter elements. To address this question, the ability of the DAC-purified VV TK PBF to bind to two other VV early promoter elements was tested. As can be seen in Fig. 6, the VV TK PBF binds equally well to the VV TK, 7.5 kD (26), and *Hin*dIII F (27) early promoter elements. Furthermore, each of these promoter elements is able to cross compete for VV TK PBF binding (data not shown).

Physical characterization of VV TK PBF

Recognizing that the procedures employed thus far were not sufficient to completely purify the VV TK PBF, it was nevertheless of interest to attempt to learn something about the molecular properties of this activity. In order to determine the nature of the proteins that co-purify with VV TK PBF, a stock of [L-^{35}S]methionine-labeled vaccinia virus was grown and subjected to the same series of purification procedures as outlined above. At each step, an aliquot was reserved and tested for VV TK PBF to insure that biological activity was retained. The protein composition of each fraction was analyzed by gel electrophoresis and autoradiography (Fig. 7). The complexity of the protein mixture present in the virion extract after passage over DEAE-cellulose is easily appreciated. The phosphocellulose chromatography results in the apparent purification of a 23 kD protein which is evident in the PC-10 fraction. However, analysis of the eluate from the DAC column indicates that there is still a large number of other proteins in the PC-10 fraction but that their concentration is low compared to that of the 23 kD protein. It is of interest that the 23 kD protein apparently interacts quite strongly with the DAC column and could be eluted only with very high salt concentrations, in excess of 1 M. No VV TK PBF activity was found in that fraction. The majority of the VV TK PBF activity was found in the initial 0.1 M KCl wash fraction from the DAC column. Only two labeled proteins, of estimated molecular weights of 70,000 and 68,000 (denoted A and B), were evident in this fraction. This result was confirmed by silver staining similar unlabeled DAC-purified PBF fractions that had been subjected to gel electrophoresis (data not shown).

As an additional approach to determining the estimated size of the VV TK PBF, the sedimentation rate of the native protein(s) responsible for this activity was determined. PC-10 protein was separated according to size by centrifugation through a 10%–30% glycerol gradient. Fractions were collected and tested for PBF activity (Fig. 8). A single peak of VV TK PBF activity was detected in fractions 10–12, giving rise to a DNA-protein complex with the same mobility as the positive control unfractionated PC-10. Other binding activities were detected also in fractions 16 and 18, but the protein-DNA complexes migrated at a different position from VV TK PBF-TKp and were not competable by unlabeled TKp (data not shown). By comparison to the sedimentation rates of marker proteins that were included in the gradient, the size of the VV TK PBF was estimated to be between 130 kD and 140 kD.

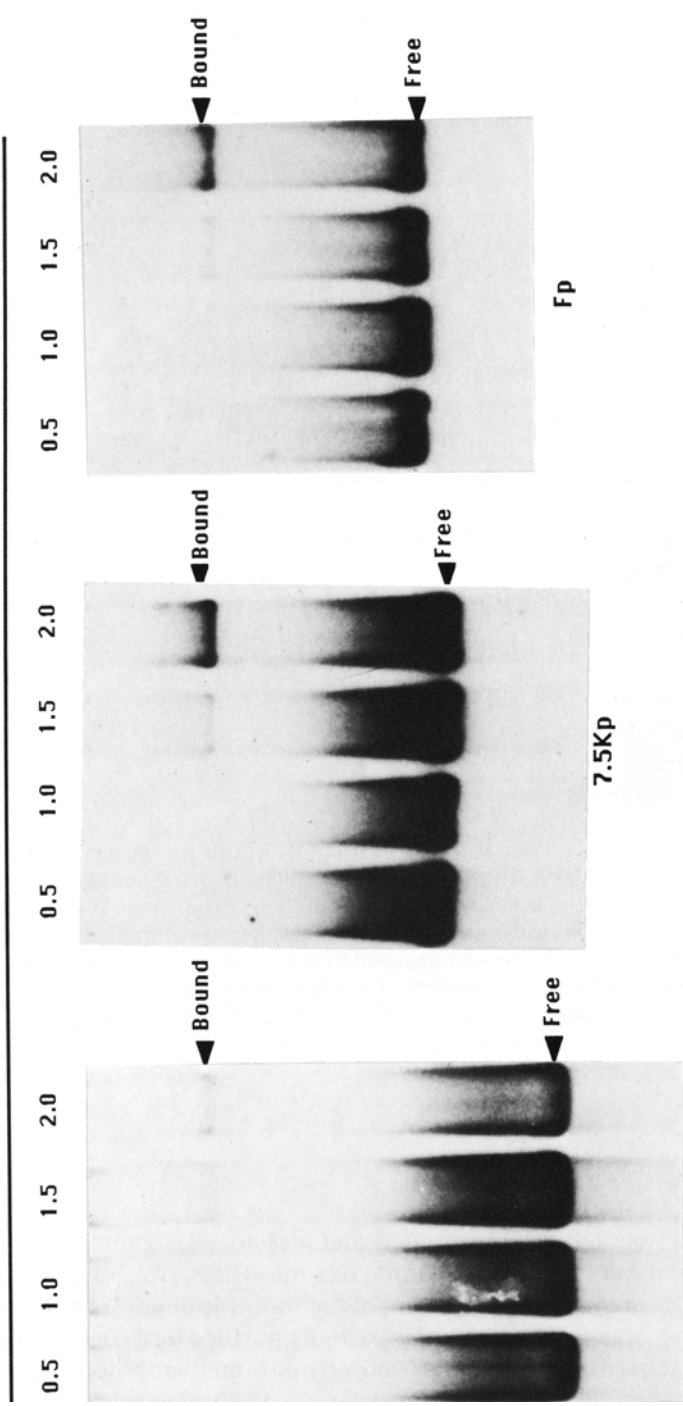

Fig. 6. Specificity of VV TK PBF activity. The indicated amounts of DAC protein were mixed with 2 ng of radiolabeled VV promoter fragments and DNA-protein complexes resolved by gel electrophoresis. The probe fragments used were the VV TK gene promoter (VV TKp, 176 bp), the VV 7.5 kD gene promoter (7.5Kp, 200 bp *Nde*I-*Sst*I fragment), and the VV early promoter adjacent to the *Bam*HI site within the VV *Hin*dIII F DNA fragment (Fp, *Nru*I-*Bam*HI 330 bp fragment).

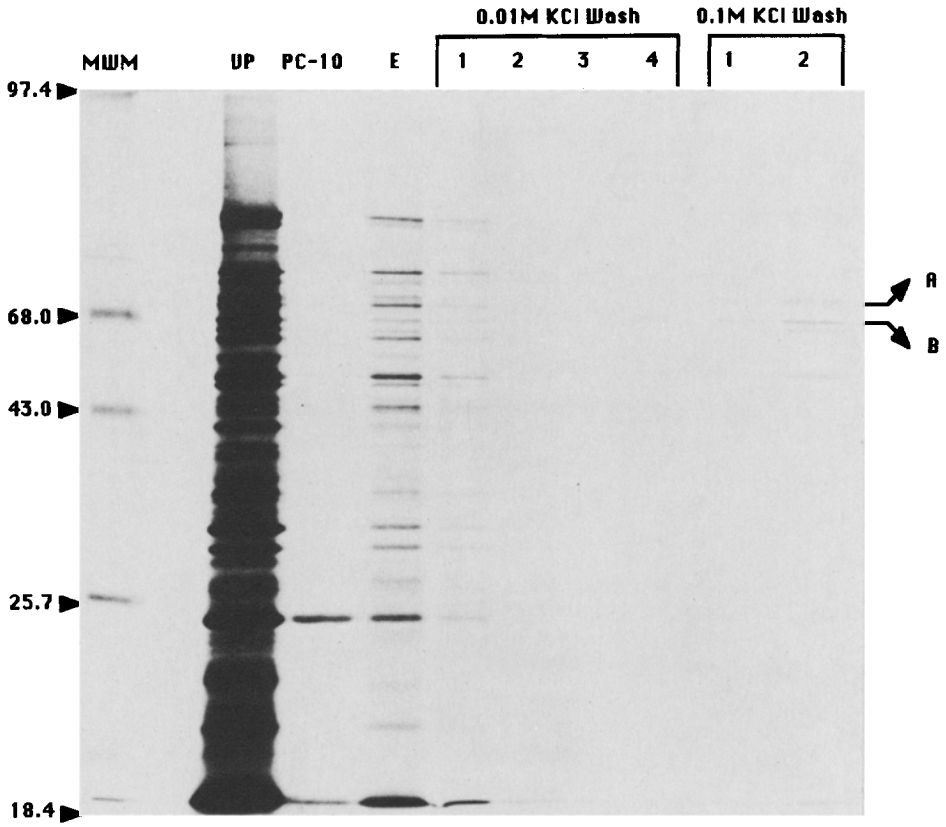

Fig. 7. Analysis of the viral proteins associated with VV TK PBF activity. [L-^{35}S]methionine-labeled VV was grown and purified. The labeled virion proteins were solubilized and purified by phosphocellulose (PC) and DNA affinity chromatography (DAC). A portion (250 µl) of the DAC effluent (E) and each of the DAC fractions, along with a small aliquot (10 µl) of the original virion extract (VP) and phosphocellulose-purified protein (PC-10), were analyzed by sodium dodecyl sulfate:polyacrylamide gel electrophoresis and fluorography. The numbers at the right indicate the positions and sizes of radioactive protein molecular weight size markers (MWM). The letters at the right (A & B) point out the two major proteins that are present in the initial 0.1 M KCl DAC wash fraction that contains high VV TK PBF activity.

Discussion

The data presented here have demonstrated that a factor (designated as VV TK PBF), extracted from vaccinia virus virions, has an affinity for binding to the nucleotide sequences immediately upstream of the transcriptional start site of the VV thymidine kinase gene. This protein was partially purified by a combination of ion-exchange and sequence-specific DNA affinity chromatographic procedures. Following purification, the VV TK PBF retained its biological activity and

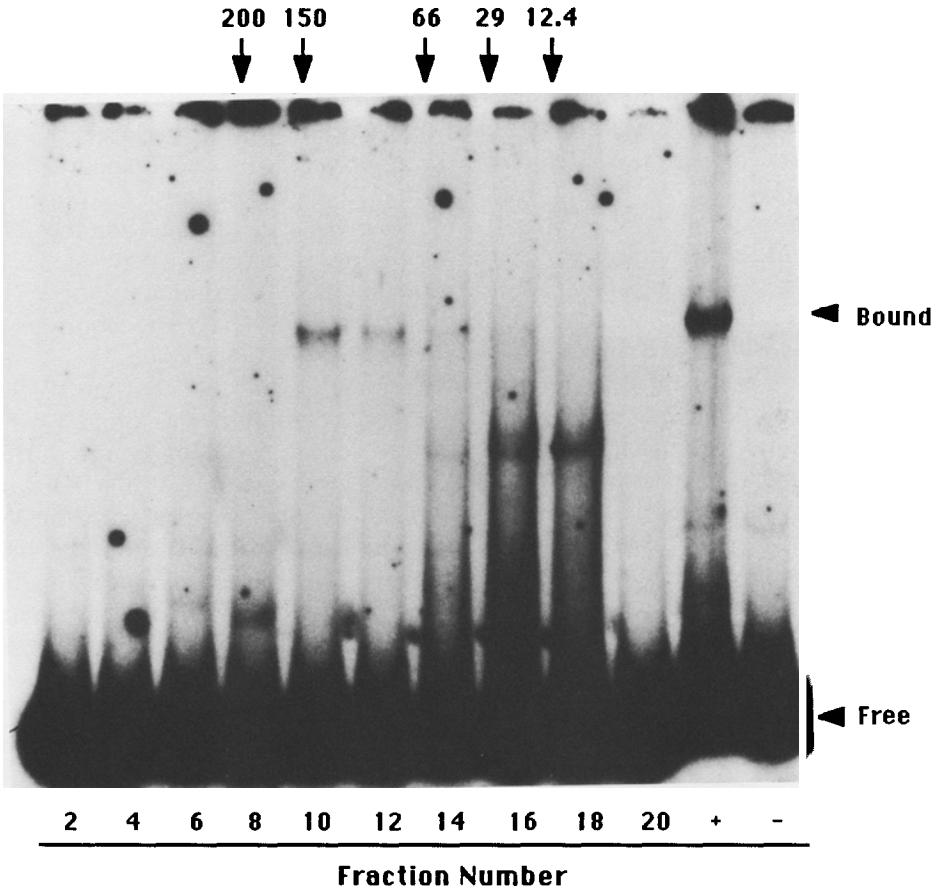

Fig. 8. Velocity sedimentation of VV TK PBF activity. An aliquot of PC-10 (25 μg) protein was separated according to native molecular weight by centrifugation through a glycerol gradient. Gradient fractions were assayed for their VV TK PBF activity using VV TKp and the gel retention assay. Control reactions with (+) and without (−) PC-10 protein were included. The numbers indicate the positions and sizes (in kD) at which protein molecular weight standards sedimented under the centrifiguation conditions used: 12.4, cytochrome C; 29, carbonic anhydrase; 66, bovine serum albumin; 150, alcohol dehydrogenase; 200, β-amylase.

specificity, binding to DNA fragments containing VV early promoter elements, but not reacting with promoterless probes. The PBF activity responsible for binding to the VV TK gene promoter had an apparent native molecular weight of 130–140 kD when analyzed by glycerol gradient sedimentation. When taken together with the observation that two viral proteins, with estimated molecular weights of 70 and 68 kD, co-purify with the binding activity, this suggests that the VV TK PBF is a heterodimeric complex, although the possibility that additional components may be required for activity cannot be excluded at this time. Verification of this in-

itial identification will await further purification procedures and genetic mapping of the gene(s) that encode VV PBF. It is possible, for example, that only one of the two peptides present in the DAC protein fraction is involved with VV TKp binding, with the other being a contaminant that co-purifies. Furthermore, the experiments carried out here, while demonstrating a specific interaction between VV PBF and VV TKp, do not formally establish that VV PBF functions as a transcriptional activator, or repressor, in vivo. In order to investigate this question it will be necessary to use either virion-derived transcription systems or directed genetics of the VV PBF gene, which is obviously yet to be mapped. Unfortunately, the VV tk promoter is a relatively poor template in vitro so it is likely that more laborious in vivo methods will have to be used for these studies.

From the evidence thus far obtained, there is no direct proof that the VV TK PBF is a virally encoded function. The alternative possibility exists that VV TK PBF is a cellular factor that becomes associated with the viral chromatin during infection and packaged into the VV virion during viral assembly. While there are certainly precedents for both the association of cellular proteins with the VV transcriptional apparatus (28) and packaging of non-viral proteins into the viral particle (29), this explanation for the origin of VV TK PBF seems improbable for several reasons. First, the nucleotide sequences upstream of most VV early genes are quite unique when compared to typical eukaryotic promoter elements (26), thus making it unlikely that a pre-existing cellular factor would interact with these elements in a specific manner. Second, the two proteins that co-purify with the VV TK PBF were radiolabeled in viral-infected cells under conditions when the translation of most cellular proteins is shut down. Finally, we have recently been able to demonstrate that VV TK PBF can be detected in vivo in extracts from VV-infected, but not uninfected cells (data not shown). Final proof of the viral origin of the VV TK PBF will require the mapping of its genomic location and genetic studies to verify that this function is indeed virally encoded.

It was of interest to note that VV TK PBF bound not only to the sequences upstream of the VV TK gene, which were used to affinity purify it, but also to other VV early promoter elements, despite the absence of any striking inter-promoter homologies (4). This observation is in agreement with that of Yuen et al. (30) who have recently reported on a early promoter-binding factor isolated from vaccinia virions that binds to the promoter of the VV growth factor gene as well as the 7.5 kD promoter element. It is unclear if the VGF-binding factor is the same as the one here designated as VV TK PBF. They share the properties of being isolated from VV virions, having similar native molecular weights, and reacting with several different VV early promoters. They differ in that, although lesser amounts of virion extract were required to detect VV TK PBF as opposed to VGF PBF, the latter activity was competable in crude extracts whereas the TK PBF had to be partially purified before exhibiting specificity. It is possible that these activities represent similar but distinct factors. Alternatively, the differences observed may stem from the probe fragments used for these experiments with the VGF fragment having a high affinity for PBF and the TK fragment having a relatively low affinity for

this factor. This latter possibility is supported by the previous observation that, when compared to either the 7.5 kD or VGF promoter elements, the VV TK gene is transcribed rather poorly in virion extracts (12), and by the fact that, in order to affinity purify VV TK PBF, it was necessary to bind and elute with substantially lower salt concentrations than have previously been described for transcriptional factor purification (14). This observation is of importance as the relatively moderate affinity between VV PBF and VV TKp has made footprinting of the DNA-factor contact points a difficult proposition (data not shown).

Thus, it remains an open question as to whether the in vivo expression and regulation of the multiple kinetic subclasses of VV early genes (31) will be modulated by the same transcriptional factor, multiple transcriptional factors, the binding affinities between promoter elements and factors, or a combination of these mechanisms. In any case, the identification of VV TK PBF activity represents only the initial step in elucidating the elements responsible for VV TK gene expression. It will now be of interest to use purified VV TK PBF together with footprinting and mutagenesis procedures to identify the *cis*-regulatory signal with which the VV TK PBF is interacting. Also, it should be noted that, within the infected cell, transcription of the VV TK gene is abruptly switched off at 4 hr p.i. coincident with the onset of viral late gene expression. It remains to be established whether this phenomenon is due to an alteration in the viral DNA-dependent RNA polymerase, turnover or modification of VV TK PBF, or synthesis of a repressor with higher affinity for the VV TK promoter. Future studies of the interaction between the VV TK gene and VV TK PBF should address these important biological questions.

Acknowledgments

We thank Walter Hodges for his efforts in growing and purifying the vaccinia virus for these studies. The oligonucleotides were synthesized by the Oregon State University Center for Gene Research Central Services Facility. This work was supported by a Research Career Development Award from the National Institutes of Health (AI-00666) and a research grant (MV-194) from the American Cancer Society. This is Oregon Agricultural Experiment Station Technical Paper No. 8536.

References

1. Moss B., Comprehensive Virology *4*, 405–474, 1974.
2. Baroudy B.M. and Moss B., J Biol Chem *255*, 4372–4380, 1980.
3. Hruby D.E., Guarino L.A. and Kates J.R., J Virol *29*, 705–715, 1979.
4. Hruby D.E., Maki R.A., Miller D.B. and Ball L.A., Proc Natl Acad Sci USA *80*, 3411–3415, 1983.
5. Weir J.P. and Moss B., J Virol *46*, 530–537, 1983.

6. Bajszar G., Wittek R., Weir J.P. and Moss B., J Virol *45,* 62-72, 1983.
7. Patel D.D. and Pickup D.J., EMBO Journal *6,* 3787-3794, 1987.
8. McAuslan B.R., Virology *20,* 162-168, 1963.
9. Hruby D.E. and Ball L.A., J Virol *40,* 456-464, 1981.
10. Hruby, D.E. and Ball L.A., J Virol *43,* 403-409, 1982.
11. Hruby D.E., Virus Res *2,* 151-156, 1984.
12. Rohrman G., Yuen L. and Moss B., Cell *46,* 1029-1035, 1986.
13. Dynan W.S. and Tjian R., Cell *32,* 669-680, 1983.
14. Kadonaga J.T. and Tjian R., Proc Natl Acad Sci USA *83,* 5889-5893.
15. Briggs, M.R., Kadonaga J.T., Bell S.P. and Tjian R., Science *235,* 47-52, 1986.
16. Lee W., Mitchell P. and Tjian R., Cell *49,* 741-752, 1987.
17. Henikoff S., Gene *28,* 351-359, 1987.
18. Maniatis T., Fritsch E.F., Sambrook J., *Molecular Cloning, A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.
19. Benson S.A., BioTechniques *2,* 66-68, 1984.
20. Samuels M., Fire A. and Sharp P.A., J Biol Chem *257,* 14419-14427, 1982.
21. Studier F.W., J Mol Biol *79,* 237-248, 1979.
22. Carthew R.W., Chodosh L.A. and Sharp P.A., Cell *43,* 439-448, 1985.
23. Dunaway M. and Reeder R.H., Mol Cell Biol *5,* 313-319, 1985.
24. Wu C., Nature *317,* 84-87, 1985.
25. Weir J.P. and Moss B., Virology *158,* 206-210, 1987.
26. Cochran M.A., Puckett C. and Moss B., J Virol *54,* 30-37, 1985.
27. Panicali D. and Paoletti E., Proc Natl Acad Sci USA *79,* 4927-4931, 1982.
28. Morrison D.K. and Moyer R.W., Cell *44,* 587-596, 1986.
29. Franke C.A. and Hruby D.E., Arch Virol *94,* 347-351, 1987.
30. Yuen L., Davison A.J. and Moss B., Proc Natl Acad Sci. USA *84,* 6069-6073, 1987.
31. Pennington T.H., J Gen Virol *25,* 433-445, 1974.