High level expression and purification of herpes simplex virus type 1 immediate early polypeptide Vmw110

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ABSTRACT

Herpes simplex virus type 1 (HSV-1) encodes five immediate early (IE) polypeptides. This paper reports the construction of a baculovirus vector which expresses large amounts of Vmw110, the product of IE gene 1. The expressed protein has been purified to near homogeneity and has a mobility on SDS polyacrylamide gels identical to that of Vmw110 produced during HSV-1 infection. Characterisation of its properties indicated that it forms dimers and perhaps higher order oligomers in solution and that the purified protein binds to both single stranded and double stranded calf thymus DNA cellulose columns. However, filter binding experiments were unable to detect any stable association of Vmw110 with DNA in solution.

INTRODUCTION

The human pathogen herpes simplex virus type 1 (HSV-1) serves as an excellent system to study several aspects of higher eukaryotic molecular biology. Interpretation of the complete sequence of the 150kb double stranded DNA genome has led to the prediction of over 70 unique genes (1). On the basis of the kinetics of synthesis and the effects of metabolic inhibitors, viral gene expression has been divided into three temporal classes termed immediate early (IE), early and late (reviewed in references 2 and 3). At least three of the five IE gene products are involved in the activation of transcription from early and late gene promoters. This report concerns the product of IE gene 1, which is known as Vmw110 or ICP0.

The role of Vmw110 in gene expression was first discovered in transfection assays which demonstrated that it activated expression from a wide variety of promoters in an apparently non-specific fashion (4–7). In certain circumstances the activity of Vmw110 was synergistic with that of another major HSV-1 IE polypeptide, Vmw175 (ICP4). Construction of HSV-1 viruses with deletions in the IE-1 coding region rather surprisingly demonstrated that Vmw110 was not essential for viral growth in tissue culture. However, such mutant viruses exhibited a cell type and multiplicity dependent defect which could be interpreted as arising from inefficient activation of viral gene expression, leading to a lower probability of initiating a productive infection (8–11). Rather interestingly, the outcome of infection with these viruses in the least permissive cell types had many similarities to latency, in that the Vmw110 mutant viral genomes that did

not form plaques were present in the cell in a potentially active form (12). In addition, it has been clearly demonstrated that Vmw110 is both necessary and sufficient for the reactivation of latent HSV in an *in vitro* latency system (13). In contrast, the evidence that Vmw110 plays a role in latency in animal systems is not so clear cut, although it may be required for reactivation from explanted ganglia at normal efficiency (14,15). Thus Vmw110 has been implicated in several key aspects of the HSV-1 life cycle.

The study of the biochemical properties and functions of Vmw110 has been hampered by its rather low levels of expression which presents difficulties for its purification from productively infected cells. It has been shown that it is a phosphorylated nuclear protein which is associated with chromatin, and which binds to calf thymus DNA cellulose columns in crude nuclear extracts (16,17). Analysis of the predicted amino acid sequence of Vmw110 has identified a cysteine-rich region which conforms with a consensus sequence of protein domains which can interact with zinc (18,19). It is significant that this potential zinc finger of Vmw110 is essential for its functions both in transfection assays (20,21) and in the viral genome (10,22). Recently, the potential zinc finger of Vmw110 has been found to be a representative of a class of such elements which is very highly conserved in a number of proteins of diverse evolutionary origin (23). Many of these proteins have been implicated to have roles in important processes, some involving different aspects of DNA metabolism and cell development (24–29). Therefore further investigation of the properties of Vmw110 is pertinent not only to the study of HSV-1, but also to the understanding of the function of its highly conserved crucial region.

This paper reports the use of a baculovirus vector to achieve high level expression of Vmw110 using derivative of the IE-1 gene from which the intron sequences had been removed. The protein has been extensively purified and some of its biochemical characteristics analysed. We found that preparations of Vmw110 contain dimers and perhaps higher order oligomers, and that although the purified protein interacts with both double and single stranded DNA on a solid cellulose support, it does not appear to form a stable complex with DNA in solution.

MATERIALS AND METHODS

Plasmids

Plasmid pAC111 (Figure 1B) was used for the insertion of the Vmw110 coding region under the control of the baculovirus

polyhedrin gene promoter into the baculovirus genome. It was derived from the NcoI-SalI coding region fragment of p110C1, an intron-less derivative of the IE-1 gene of plasmid p111 (30), and the SalI-HpaI region of pJR3 (4), which includes the 3' portion of the IE-1 gene. Initially, a SalI-HindIII fragment of pJR3 (which includes the SalI-HpaI portion) and the NcoI-SalI IE-1 coding region fragment of p110C1 were ligated into the NcoI and HindIII sites of p585.4, a derivative of pET8c (31) to create pT7110. The NcoI-HpaI IE-1 coding region of pT7110 was isolated, blunt ended with Klenow polymerase and ligated into the similarly blunt ended BamHI cloning site of plasmid pAcYM1 (32; Figure 1), thus creating pAC111. All plasmids were maintained in E. coli strain HB101.

Construction of a recombinant baculovirus expressing Vmw110

Plasmid pAC111 was digested with PvuII (sites not shown on Figure 1B), which produces a linear fragment containing the recombinant Vmw110 gene flanked by Autographa californica nuclear polyhedrosis virus (AcNPV) viral DNA, and then transfected by the calcium phosphate method (33) into Spodoptera frugiperda (Sf) cells with infectious AcNPV DNA (kindly provided by Dr. N.D. Stow). The progeny virus stock was harvested, and the required recombinant isolated by enrichment during several rounds of growth of aliquoted dilutions in multiwell dishes followed by plaque purification. At all stages the presence of the recombinant gene containing the Vmw110 coding region was assessed by Southern blot analysis (34), of either XhoI or EcoRI digested total cellular DNA isolated by phenol and chloroform extraction (35). The probe used was pAC111. The required virus contained a characteristic 2.45kb XhoI fragment which includes the 5' portion of the polyhedrin gene linked to the IE-1 coding sequences. The insertion of the 2.8kb IE-1 sequences, which contain no EcoRI sites, into the polyhedrin gene (which has had the internal 0.75kb deleted in vector pAcYM1) increases the size of the AcPNV EcoRI I fragment from 7.4kb to 9.4kb. The virus in the medium was retained as a stock to carry through to the next round of purification. Plaque purification was continued until the stock of recombinant virus (ACV110) contained no trace of contaminating wild type AcNPV genomes.

Purification of Vmw110

(a) Expression and nuclear extract preparation. Sf cells (approximately 2×10^7 per flask) were seeded into 800ml tissue culture flasks with 25ml TC100 medium (Flow Laboratories) supplemented with 5% Foetal calf serum and the antibiotics penicillin and streptomycin. At the same time ACV110 was added at a multiplicity of 2 pfu per cell. The flasks were incubated at 28°C for 3 days. The cells were shaken from the plastic into the medium and harvested by centifugation. The medium was retained as virus stock for use in subsequent experiments. Nuclear extracts were prepared as described (36).

(b) Phosphocellulose chromatography. Nuclear extract from 16 flasks (4ml) was diluted with an equal volume of 50mM Hepes pH 7.0, 10% glycerol, 5mM beta mercaptoethanol and 1mM PMSF (Buffer A), and then applied to a 5ml phosphocellulose column equilibrated with Buffer A plus 0.2M NaCl. After washing with 4 aliquots of 5ml of the equilibration buffer, proteins were eluted with successive steps of Buffer A containing 0.25M, 0.3M, 0.4M and finally 1.0M NaCl. Each ionic strength used 4 aliquots of 5ml of the buffer. Vmw110 eluted principally in

the 0.25M and 0.3M NaCl steps, as judged by SDS polyacrylamide gel electrophoresis (SDS-PAGE).

(c) Q-sepharose chromatography. The appropriate 0.25M and 0.3M NaCl fractions from the phosphocellulose column were mixed and applied directly to a 2ml Q-sepharose column equilibrated with Buffer A plus 0.25M NaCl. After extensive washing with the equilibration buffer, most contaminating proteins were eluted by Buffer A containing 0.3M NaCl. The column was washed with Buffer A containing 0.4M then 1.0M NaCl, using 4 aliquots of 1ml at all stages. Vmw110 eluted in the 0.4M NaCl fractions was judged to be at least 75% pure by examination of SDS polyacrylamide gels stained with Coomassie blue.

SDS polyacrylamide gel electrophoresis

Discontinuous SDS polyacrylamide gels were cast in a BioRad Mini Protean II gel kit using buffers and solutions as recommended by the supplier. Separation gel concentrations were either 7.5% or 10% acrylamide. The molecular weight markers (SDS-6H, Sigma) used were myosin, 205kD; beta galactosidase, 116kD; phosphorylase b, 97kD; bovine serum albumin, 66kD; egg albumin, 45kD and carbonic anhydrase, 29kD. The gels were stained with Coomassie blue, destained and photographed using Kodak ortholith film.

Western blotting to detect Vmw110

SDS-polyacrylamide gels were cast in a Bio-Rad mini gel kit. After electrophoretic separation, the proteins were transferred to nitrocellulose sheets using the compatible Bio-Rad western blotting apparatus. The sheets were blocked at 37°C using 3% gelatin, and then incubated overnight with rabbit antipeptide serum 14711 at a dilution of 1 in 10. Antibody 14711 was raised against a synthetic peptide which corresponds to a sequence near the carboxy terminus of Vmw110 (18). After washing off excess antiserum, the bound antibody was detected using horseradish peroxidase conjugated to protein A.

ELISA quantitation of Vmw110

Plastic microtitre dishes were incubated overnight with samples which had been diluted with PBS as appropriate. The wells were then washed, blocked with PBS containing Tween 20 at 0.02%, washed again and then incubated at 37°C for 1 hour with a 1/1000 dilution of rabbit antiserum 14711. After extensive washing, the wells were incubated with horseradish peroxidase linked to protein A and again extensively washed. The bound peroxidase was detected by incubation with the colourigenic substrate ABTS; colour development was quantitated using a microtitre plate reader.

Glycerol gradient centrifugation

Gradients containing 5−20% glycerol in Buffer A plus 0.4M NaCl were made in 5ml centifuge tubes. Samples containing approximately 5μg of purified Vmw110 were layered onto the gradients with 20μg of thyroglobulin (19S), yeast alcohol dehydrogenase (6.7S) and bovine serum albumin (4.3S) as markers. After centifugation at 30,000 rpm for 19 hours, 0.2ml samples were taken sequentially from the top of the gradient with a wide bore pipette. These were analysed by SDS-PAGE and ELISA to detect Vmw110 and the marker proteins.

DNA cellulose chromatography

Double stranded and single stranded DNA cellulose slurries (Sigma) were prepared in Buffer A plus 0.1M NaCl and packed into 0.2ml columns. Purified Vmw110 in the same buffer was applied and the column washed with buffers of increasing salt concentration as described in the text. All operations were performed in the cold. Fractions were collected and assayed for Vmw110 by SDS-PAGE and ELISA.

Nitrocellulose filter DNA binding assays

Nitrocellulose filter circles (0.45μm pore size, Schleicher and Scheull) were boiled in distilled water and then soaked in Buffer A supplemented with 50mM NaCl and 50μg/ml BSA (binding buffer). A probe was prepared by cutting plasmid pgDCAT (37) with HindIII, PvuII and EcoRI and then labelling with Klenow polymerase and alpha ^{32}P dATP. The labelled fragments were purified by phenol and chloroform extraction and ethanol precipitation. Column fractions from the Vmw110 purification procedure were incubated with the probe fragment mixture in binding buffer at 0°C for 20 minutes and then filtered through the nitrocellulose. Filters were washed several times with 1ml aliquots of binding buffer. The amount of radioactivity retained on the filter was determined by scintillation counting. In some instances, the fragments retained on the filters were eluted by incubation with TE buffer containing 0.5% SDS, ethanol precipitated and then analysed on polyacrylamide gels followed by autoradiography.

RESULTS

Construction of a recombinant baculovirus containing the Vmw110 coding region under the control of the polyhedrin gene promoter.

The predicted 775 amino acid residue Vmw110 coding region of HSV-1 IE gene 1 lies in the repeats bounding the long unique segment of the genome and is interrupted by two intervening sequences (Figure 1A). The constuction of an intron-less version of this coding region in plasmid p110C1 has been described previously (10). The NcoI to HpaI IE-1 coding region of p110C1 was transferred to the transfer plasmid pAcYM1 (Figure 1B), which contains a section of the baculovirus genome with a BamHI cloning site 3' of the polyhedrin gene promoter (32). The resultant plasmid was linearised and transfected into Sf cells with infectious baculovirus DNA. The progeny virus stocks were screened for the desired recombinant (as described in detail in the methods section). Virus ACV110, which contains the intronless Vmw110 gene (Figure 1B), was extensively purified and its genome in the region of the expression site thoroughly checked by Southern blotting. Its structure was as predicted (data not shown).

Virus ACV110 expresses large amounts of Vmw110

Wild type AcNPV and ACV110 were used to infect Sf cells at a multiplicity of 2 pfu per cell and parallel plates harvested at various times for analysis by SDS-PAGE. The results showed that ACV110 expressed, at late times of infection, copious amounts of a protein with the mobility expected of Vmw110. The optimal time to harvest the cells was three days after infection (data not shown).

The expression of Vmw110 was investigated in more detail by preparing cytoplasmic and nuclear extracts of the infected cells three days after infection. These extracts were again compared

with parallel extracts made from wild type virus infected cells. The results (Figure 2A) illustrate the expression of Vmw110 by ACV110 (indicated by the upper arrow) and that it is present principally in the nucleus. The most heavily staining band in the wild type virus infected cell extracts is the polyhedrin protein (which is near the bottom of the gel). As expected, this protein is not expressed by ACV110, and, while ACV110 expresses rather less Vmw110 than wild type expresses polyhedrin, the amounts of Vmw110 accumulated are considerable.

Vmw110 was not the only additional band appearing in the ACV110 infected cell nuclear extracts. The other major differences from the wild type virus infected cells are a band at about 35kd (lower arrow in Figure 2A, middle arrow in Figure 2B) and another of low molecular weight (lower arrow in Figure 2B). It is not known if these represent breakdown
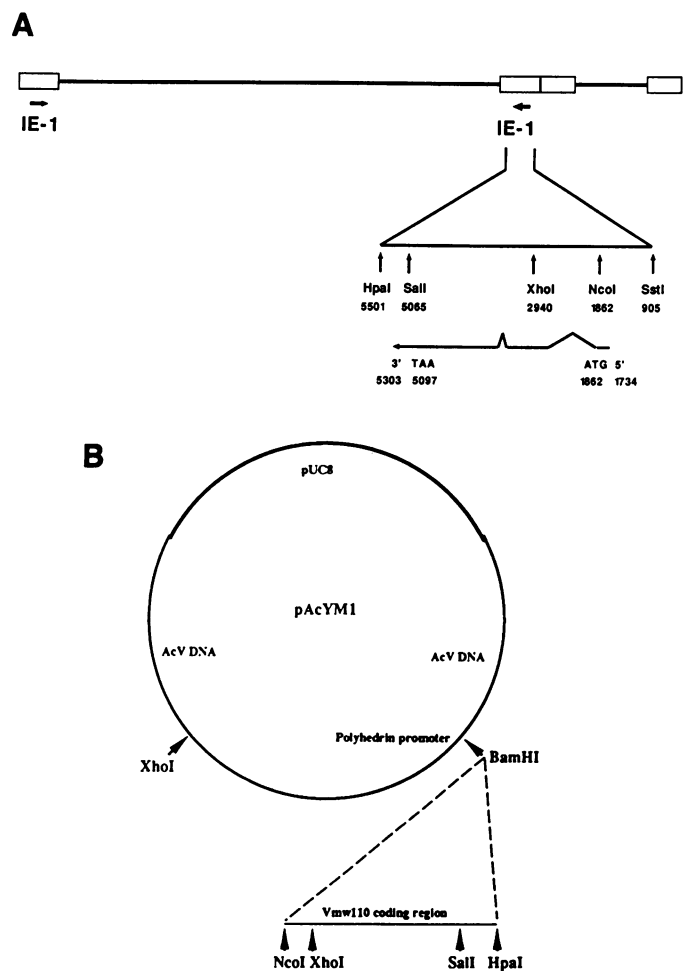

Figure 1. A map of the IE-1 region of HSV-1 and the baculovirus transfer vector pAC111. A. The HSV-1 genome is shown with the repeat sequences marked as boxes. Gene IE-1 lies in the repeat bounding the long unique genome segment. Below is an expansion of the IE-1 region annotated with key restriction sites and the transcribed portion (including the introns) of IE-1. The numbering is of the R_L sequence (18). B. Plasmid pAcYM1 contains a section of ACV EcoRI fragment I cloned into pUC8. An intron-less IE-1 coding region NcoI-HpaI fragment was inserted into the BamHI site 3' of the polyhedrin gene promoter to create pAC111. The transcript derived from the recombinant gene is predicted to express Vmw110 from its natural ATG initiation codon. The XhoI site in the AcNPV vector DNA is marked; there is another 315bp from the NcoI site in IE-1 DNA. The 2.4kb predicted XhoI fragment was used as a screen for recombinant baculovirus genomes which contained the IE-1 coding region.
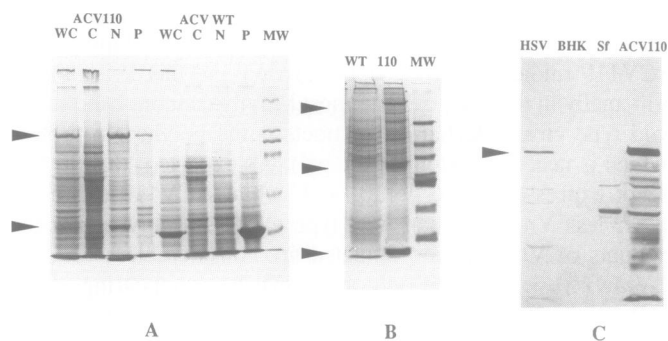
Figure 2. Recombinant virus ACV110 expresses Vmw110. A. Whole cell (WC), cytoplasmic (C), nuclear (N) and insoluble (P) fractions of Sf cells which had been infected for 72 hours with ACV110 and AcNPV wild type virus separated on a Coomassie blue stained 7.5% SDS polyacrylamide gel. The molecular weight markers (MW) are myosin (205kD), beta galactosidase (116kD), phosphorylase b (97kD), bovine serum albumin (66kD), egg albumin (45kD) and carbonic anhydrase (29kD). The upper arrow points out Vmw110 and the lower indicates another prominent band not present in the wild type virus nuclear extract. The major stained band in the whole cell and pellet fractions of AcNPV extracts at about 30kD is the polyhedrin protein. B. Nuclear extracts of AcNPV and A-CV110 separated on a 12.5% SDS polyacrylamide gel. The molecular weight markers are bovine serum albumin (66kD), egg albumin (45kD), glyceraldehyde 3 phosphate dehydrogenase (36kD), carbonic anhydrase (29kD), trypsinogen (24kD) and trypsin inhibitor (20kD). The uppermost of the three arrows indicates Vmw110 while the others indicate proteins not present in ACV WT extracts. C. A western blot of samples of HSV-1 infected (HSV) and uninfected (BHK) cells and ACV110 infected and uninfected (Sf) cells using rabbit antiserum 14711. The arrow points out undegraded Vmw110.
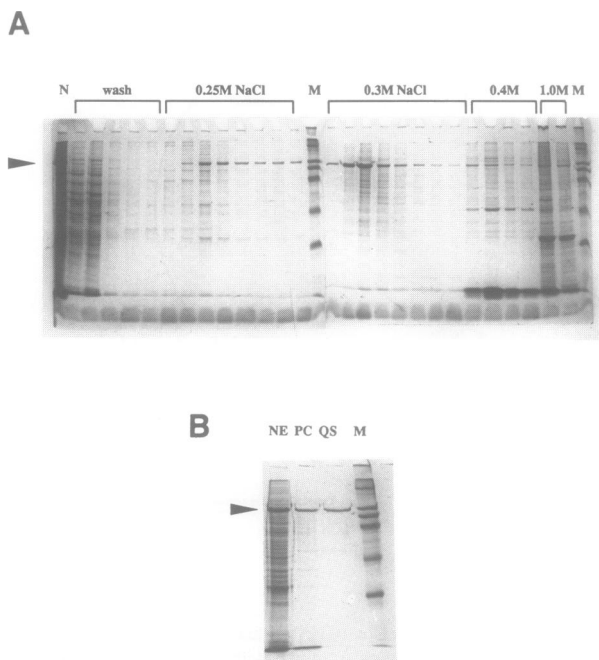

Figure 4. Preparations of purified Vmw110 contain dimers. A. Glycerol gradient centrifugation of Q-sepharose purified Vmw110. Vmw110 was detected by ELISA using serum 14711 and the marker proteins by SDS-PAGE. The positions of the peaks fractions of the marker proteins, and their sedimentation co-efficients, are indicated. B. Glutaraldehyde cross linking of Vmw110. Q-sepharose purified Vmw110 (110) was treated with .01 and .02% final concentrations of glutaraldehyde and then analysed on a 5% SDS polyacrylamide gel. The lower arrow indicates monomeric Vmw110, migrating slightly faster than the beta galactosidase marker, while the upper points out the dimer band, co-migrating with the myosin marker. Note also the material of very low mobility in the glutaraldehyde treated tracks, which is not present in the untreated control.

products of Vmw110, cellular proteins that become preferentially eluted from the nucleus in the presence of Vmw110, or cellular proteins which are induced by the high level expression of Vmw110.

It was important to compare the Vmw110 proteins expressed by ACV110 and by HSV-1. Accordingly, BHK cells were infected with HSV-1 and harvested 16 hours later. Samples of the HSV-1 and ACV110 infected cells were analysed by SDS-PA-GE with samples of mock infected BHK and Sf cells for comparison. The proteins were transferred to nitrocellulose by Western blotting and Vmw110 detected after incubation with rabbit antiserum 14711, which recognises a carboxy terminal peptide of Vmw110 (10), followed by horseradish peroxidase conjugated protein A. The results (Figure 2C) show that the Vmw110 expressed by ACV110 is of the same mobility as that expressed by HSV-1, and that it possesses the same carboxy terminal epitope. These data suggest that the Vmw110 expressed by ACV110 is very similar to that expressed during HSV-1 infection. The bands of lower molecular weight in the ACV110 track of Figure 2C are probably degradation products of Vmw110 since they are not present in the Sf cell control.

Purification of Vmw110 from ACV110 infected cells

Vmw110 was extensively purified from nuclear extracts of A-CV110 infected cells by two steps of column chromatography. As described in the methods section, nuclear extracts were diluted with Buffer A without sodium chloride in order to reduce the ionic strength to 0.2M. The solution was then loaded onto a phosphocellulose column equilibrated in the same buffer. After extensive washing, bound proteins were eluted with sequential steps of Buffer A containing 0.25, 0.3, 0.4 and 1.0M NaCl.

Figure 3. Purification of Vmw110. A. The elution profile of an ACV110 infected cell nuclear extract (N) separated by successive steps of increasing ionic strength (as indicated) on a phosphocellulose column and analysed on 10% SDS polyacrylamide gels. The molecular weight markers (M) are as in Figure 2A. Vmw110 is indicated by the arrow. B. A 10% SDS polyacrylamide gel stained with Coomassie blue showing ACV110 infected cell nuclear extract (NE), pooled fractions after phosphocellulose column chromatography (PC) and a sample after purification on a Q-sepharose column (QS). Molecular weight markers (M) are as in Figure 2A.
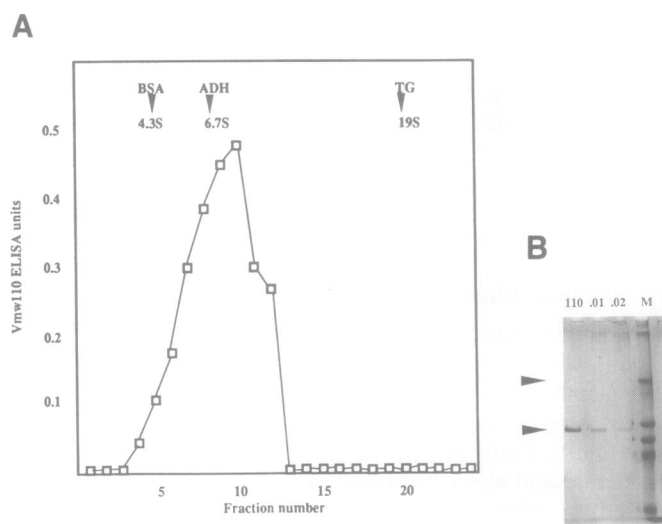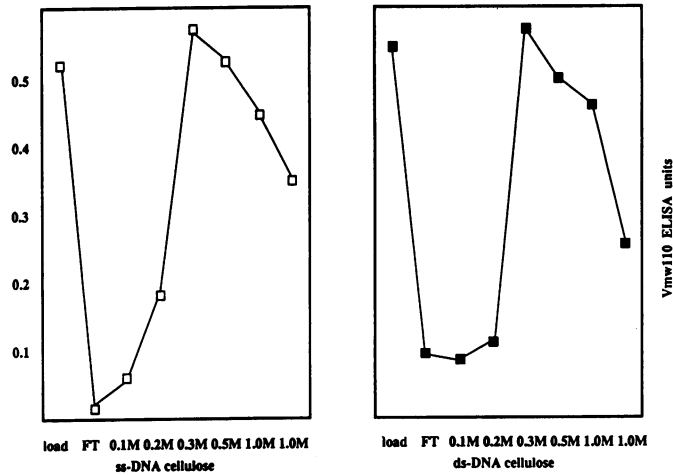
Figure 5. Purified Vmw110 binds to single and double stranded DNA cellulose columns. Vmw110 was loaded onto the indicated columns and eluted by increasing ionic strength as shown. The amount of Vmw110 in each fraction was determined by ELISA.

Figure 6. Analysis of the DNA binding activity in nuclear extracts from ACV110 infected and uninfected Sf cells. A gel retardation experiment using equal amounts of ACV110 and uninfected cell nuclear extracts with the IE-3 EcoRI-BamHI probe and increasing amounts of polydI/polydC competitor.

Vmw110 was detected by SDS-PAGE and was found principally in the 0.3M step elution (Figure 3A). For the second step, fractions from the 0.25M and 0.3M NaCl step elutions were pooled and loaded directly onto a Q-sepharose column equilibrated with Buffer A containing 0.275M NaCl. The column was again washed and bound proteins eluted with steps of 0.3M, 0.4M and 1.0M NaCl. Fractions containing Vmw110 with a high degree of purity were eluted at 0.4M NaCl. A summary of these results, showing the initial extract, the pooled phosphocellulose fractions and the protein eluted from the Q-sepharose column is shown in Figure 3B.

Preparations of purified Vmw110 contain dimeric molecules

The purified Vmw110 preparation illustrated in Figure 3B was analysed by glycerol gradient centrifugation. Gradients were loaded with a sample that contained Vmw110 and control proteins bovine serum albumin, yeast alcohol dehydrogenase and bovine thyroglobulin. After centrifugation fractions were collected from the top of the tubes, and those containing the marker proteins were identified by SDS-PAGE (data not shown). Suitable amounts of the fractions were used to coat wells for an ELISA estimation of the amount of Vmw110 using rabbit antiserum 14711. The results are shown in Figure 4A. Yeast ADH has a native molecular weight of 150kD and a sedimentation coefficient of 6.7S. Vmw110 is clearly sedimenting with a peak position of slightly higher S value (8.2S) than ADH. This is consistent with the presence of dimeric Vmw110 molecules. To investigate this further, the purified Vmw110 preparation was incubated in the presence of 0.01% glutaraldehyde at room temperature for 1 hour. Subsequent SDS-PAGE showed that glutaraldehyde treatment resulted in the appearance small amounts of a protein of exactly twice the apparent molecular weight of Vmw110 (Figure 4B). Thus it is clear that the preparation of Vmw110 contains dimeric molecules. However, treatment with glutaraldehyde also converted some of the input protein into material with very low gel mobility, seen as faint bands at the top of the treated tracks, which is not present in the control track. Thus it is possible that higher order multimers of Vmw110 can also be formed. Glutaraldehyde treatment of control proteins
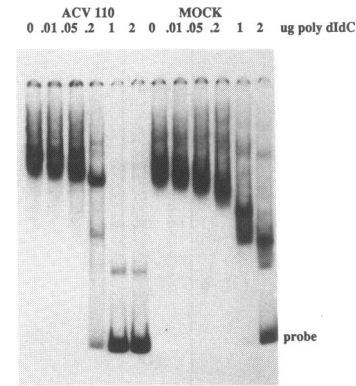
known to be monomeric in solution did not produce novel bands at the position expected of dimers (data not shown).

Analysis of Vmw110 by SDS-PAGE in the absence of reducing agent indicated that the dimers or multimers were not covalently linked by disulphide bridges (data not shown). Attempts to characterise further the nature of purified Vmw110 using native polyacrylamide gel electrophoresis were not successful; the protein migrated as a high molecular weight smear (data not shown).

Purified Vmw110 binds to both single stranded and double stranded DNA cellulose matrices

One of the crucial questions about the properties of Vmw110 is whether it interacts directly with DNA, and, if so, whether this interaction is sequence specific. Earlier experiments had shown that Vmw110 in crude nuclear extracts is retained on calf thymus DNA cellulose columns (17). However, this could be explained by an interaction between Vmw110 and one or more proteins which were binding to the column. The binding of Vmw110 to both single stranded and double stranded DNA cellulose was investigated using the purified preparation. The results (Figure 5) showed that the protein did indeed bind to DNA cellulose, eluting mainly at salt concentrations higher than 0.2M, and that its binding to double stranded DNA was essentially indistinguishable from its binding to single stranded DNA. These results show that Vmw110 can interact directly with DNA. (Vmw110 prepared from HSV-1 infected BHK cells does not bind to cellulose slurries; unpublished data).

Purified Vmw110 does not appear to form a stable complex with DNA in solution

That Vmw110 binds to DNA cellulose columns does not necessarily imply that it is a DNA binding protein, because its retention by these columns could be explained by a non-specific electrostatic interaction, in the same way that it binds to phosphocellulose. Therefore several attempts were made to investigate whether purified Vmw110 interacts with DNA in solution. Initial experiments adopted gel retardation technology to compare the DNA binding activities of nuclear extracts of A-CV110 infected and uninfected cells. The probe contained the IE-3 promoter region from coordinates −108 to +27, and the methods were exactly as described (38). The results (Figure 6)
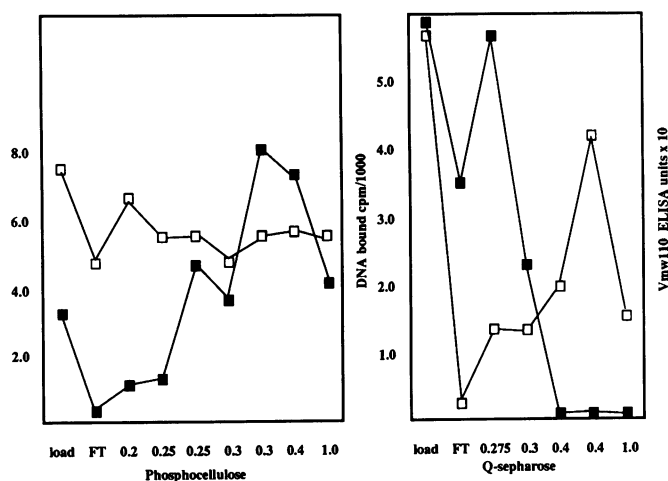
Figure 7. The DNA binding activity in ACV110 infected cell nuclear extracts does not co-purify with Vmw110. A nuclear extract containing Vmw110 was fractionated on phosphocellulose and Q-sepharose columns as indicated. The load, flowthrough, 0.2M, 0.25M (2 aliquots), 0.3M (2 aliquots), 0.4M and 1.0M NaCl samples were used to detect the amount of Vmw110 by ELISA (open boxes) and the amount of DNA binding activity in filter binding experiments (filled boxes). The vertical scale on the display on the left refers to the DNA binding activity (cpm×10⁻³), normalised for the amount of protein in each sample. That to the left of the righthand display gives the amount of Vmw110 ELISA units.

showed that nuclear extracts containing Vmw110 displayed a similar general or non-specific DNA binding potential to uninfected nuclear extracts. Both contained sufficient DNA binding activity to form complexes of low mobility (which probably contain many different proteins) in the absence of competitor polydI/polydC. Increasing the amount of competitor resolved bands of higher mobility, but there was no indication of any major differences between the two types of extract.

A gel retardation experiment of necessity can investigate binding to only a limited set of DNA sequences. Therefore a filter binding assay, using of a mixture of probe fragments derived from plasmid pgDCAT, was also used. Plasmid pgDCAT responds to activation by Vmw110 in transfection experiments (37). The probe fragments were incubated with the ACV110 nuclear extract and the mixture filtered through nitrocellulose. The DNA fragments bound to the filter by proteins in the extract were eluted with SDS and analysed on polyacrylamide gels. There was no preferential retention of any of the fragments; all were equally sensitive to competition by the addition of increasing amounts of polydI/polydC (results not shown). These results suggests that Vmw110 does not bind with high affinity to any specific DNA sequences in pgDCAT, but (taken with the results of the gel retardation experiment) they do not exclude the possibility that Vmw110 has a non-specific DNA binding ability which was masked by the total DNA binding capacity of the crude nuclear extract (Figure 6).

Therefore, the DNA binding activity in fractions throughout the Vmw110 purification procedure was measured by filter binding experiments using the pgDCAT probe mixture. The amount of Vmw110 in each fraction was quantitated by ELISA. The results (Figure 7) showed that fractions from the phosphocellulose column contained DNA binding activity, but binding was not proportional to the amount of Vmw110 in the fractions. The 0.25M and 0.3M NaCl step fractions from the phosphocellulose column were pooled and passed down a Q-

seharose column. The binding activity clearly eluted with a peak at a much lower ionic strength than the Vmw110 elution peak. Indeed, DNA binding activity in the peak Vmw110 fractions was essentially undetectable. These data can not be explained by inactivation of the DNA binding activity of Vmw10 during Q-sepharose chromatography since the recoveries of both Vmw110 and DNA binding activity were about 70%. The assays using the peak Vmw110 fractions contained a final salt concentration (90mM) which did not inhibit binding of Vmw110 to DNA cellulose columns (Figure 5). The fractions containing purified Vmw110 were also completely inactive in a gel retardation experiment in the absence of polydI/polydC competitor (results not shown).

These results strongly suggest that purified Vmw110 is unable to form a stable complex with DNA in solution. It did not display non-specific DNA binding activity under a variety of conditions. If it were a sequence specific DNA binding protein, even if its recognition site is not present in a plasmid which responds to its activity, it would be expected to bind to other DNA sequences in a plasmid the size of pgDCAT in the absence of competitor.

DISCUSSION

A fundamental understanding of the mechanism of action of Vmw110 has been impeded by its low level of expression during a normal virus infection; it has been difficult to obtain the protein in any great quantity or purity. The construction of a baculovirus recombinant which expresses large quantities of Vmw110, and the subsequent isolation of highly purified preparations of the protein, is a first step towards a detailed understanding of its biochemistry and its interactions with other macromolecules. This paper describes the initial steps towards this goal: we have found that Vmw110 forms multimers, including dimers, in solution and that it is apparently unable to form a stable interaction with DNA in solution.

The interaction of Vmw110 with DNA is of considerable interest given its role in transcriptional activation. This study confirms a previous finding that Vmw110 binds to DNA cellulose columns (17) and extends it to show that highly purified protein is still able to bind, both to double and single stranded DNA. However, the key question is whether this binding represents a simple ionic interaction with the phosphate backbone of DNA, a non-specific interaction with the nucleotide bases, or a specific interaction with a defined sequence of bases. There is no evidence of any sequence specific interaction, since the DNA binding activity of extracts containing Vmw110 could not be shown to select any specific sequence from fragments comprising a plasmid which responds to activation by Vmw110. Perhaps this is not surprising as no specific sequences have been defined which are required for activation by Vmw110. If Vmw110 interacts non-specifically with the nucleotide bases, this interaction must be sufficiently weak to be undetectable by a non-specific filter binding assay conducted under conditions which would allow binding of a wide range of DNA binding proteins (Figure 7). Therefore, since Vmw110 binds to negatively charged matrices during purification, it seems likely that its retention on DNA cellulose columns is simply a reflection of this ionic interaction. It follows that its ability to activate gene expression is likely to result from an interaction with other proteins or macromolecules.

As illustrated by a number of recent observations, activation of gene expression can be achieved without direct interaction with DNA by a variety of different mechanisms. These include:

activation of a bound cellular transcription factor (39,40); removal of repressors complexed to transcription factors (41−44); modification of the DNA binding specificity of a transcription factor (45), and inhibition of the formation of a restrictive chromatin structure (46). The availability of large amounts of purified Vmw110 should enable studies to determine which (if any) of these stategies it employs.

A further area of interest with respect to Vmw110 is the recent discovery that a potential zinc finger domain within its second exon is very highly conserved among a number of proteins of diverse evolutionary origin (23−29). Some of these proteins seem to be involved in a number of different aspects of DNA metabolism and gene expression. The highly conserved nature of the potential zinc finger (which is distinguishable from the two other well described classes of zinc finger) suggests that it is involved in a process of general importance. It has been suggested, as cited above, that the conserved motif might be involved in DNA sequence recognition since it is similar to other zinc finger motifs which perform this function. However, the results presented here imply that DNA binding is not the role of at least this member of this particular class of zinc finger. The availability of purified preparations of one member of this family of proteins will enable investigations relevent not only to herpes viruses and gene regulation, but also to a wide range of other aspects of DNA function.

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