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Vaccinia Virus Gene Encoding a Component of the Viral Early Transcription Factor†

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The gene product of the vaccinia virus open reading frame D6R was synthesized in bacteria and used to raise antiserum against the protein. Using the antiserum as a probe, we demonstrated that the D6R protein is a component of the virion particle, localized to the virus core structure. The D6R protein, purified from virions, has been shown to copurify with the vaccinia virus early transcription factor (VETF). The apparent molecular weight of the D6R polypeptide is identical to that of the smaller of the two VETF-associated polypeptides. Antibodies directed against D6R block both the early promoter-binding and DNA-dependent ATPase activities of VETF, supporting the identity of D6R as a VETF-associated polypeptide. An ATP-binding site was inferred near the amino terminus of the derived D6R amino acid sequence. Thus, the D6R polypeptide could be the source of the ATPase activity associated with VETF. The D6R gene was shown previously to belong to the late class of vaccinia virus genes. Synthesis of the VETF at late times after infection suggests a cascade model for vaccinia virus gene regulation in which class-specific transcription factors are synthesized at the previous phase of the infectious cycle.

Vaccinia virus is the prototypic member of the poxvirus family. The 185,000-base-pair DNA genome of vaccinia virus encodes some 200 gene products that are expressed in a developmentally regulated fashion (reviewed in references 8 and 23). The early class of genes is transcribed immediately upon infection. Following onset of DNA replication, early gene transcription ceases and the intermediate and late classes are transcribed. For each gene class, a distinctive sequence surrounding the transcription start site can be identified. The viral DNA-dependent RNA polymerase presumably is responsible for the transcription of all three classes of genes; however, the mechanisms of promoter selectivity by the RNA polymerase are poorly understood.

Recent progress in the development of systems for the in vitro transcription of vaccinia virus genes has made possible the biochemical analysis of transcriptional regulation (10, 31, 34, 40, 43). Fractionation of vaccinia virion extracts has demonstrated that the early gene transcription machinery is composed of multiple components. Two proteins in addition to the viral RNA polymerase have been identified as being required for accurate, efficient transcription of early genes. One of these factors is required for termination of transcription (36); the other is thought to be required for initiation of transcription (6). The latter factor, the vaccinia virus early transcription factor (VETF), binds specifically to vaccinia virus early promoters and has an associated DNA-dependent ATPase activity (5). The ATPase activity is of possible significance to RNA synthesis as transcription of early genes requires ATP hydrolysis (9, 42).

As a member of the poxvirus family, vaccinia virus spends its entire life cycle in the cytoplasm of the host cell. To the extent of our knowledge, the cell nucleus has minimal influence on viral gene expression. This scenario suggests that vaccinia virus encodes most, if not all, of the components of its transcription machinery. This expectation has been borne out in the case of the multisubunit RNA polymerase (15, 22) and the two-subunit mRNA capping enzyme (21, 24a). In this report, we provide evidence that the VETF is virus encoded as well. Antibody prepared against the product of the D6R open reading frame (ORF) was used to monitor the purification of the protein. Results demonstrated that the D6R protein copurified with VETF, suggesting that the smaller of the two VETF-associated polypeptides is the product of the D6R ORF.

MATERIALS AND METHODS

Cells and virus. Vaccinia virus strain WR was obtained from the laboratory of Bernard Moss, National Institutes of Health, Bethesda, Md., and was propagated on HeLa S3 cells in suspension culture in modified minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with nonessential amino acids and 10% calf serum. HeLa cells were obtained from the laboratory of Michael Rossman, Purdue University. Virus was purified by two rounds of sucrose gradient sedimentation (14).

Bacterial fusion proteins. The vaccinia virus ORF D6R was excised from the vaccinia virus *Hin*dIII D fragment (24) as a 4.3-kilobase *Hpa*II fragment. This fragment was inserted into the *Bam*HI site of the bacteriophage T7 expression plasmid pAR3040 (33) after making the ends of both flush with DNA polymerase I Klenow fragment (New England BioLabs, Inc., Beverly, Mass.) and the four deoxyribonucleoside triphosphates (19). This plasmid, pSB35, encodes all but the amino-terminal 3 codons of the D6R ORF fused to the first 11 codons of the T7 phi10 gene. For protein production, pSB35 was introduced into *Escherichia coli* BL21 (38). The bacteriophage T7 RNA polymerase was provided by infection with bacteriophage lambda strain CE6 to achieve target protein synthesis (38). The infection was allowed to proceed for 6 h before cell harvest.

Antibodies and immunological methods. The D6R fusion protein was purified by preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (16). Pulverized polyacrylamide gel slices containing approximately 100 μg of ORF D6R protein were used to immunize New Zealand White rabbits. Rabbits were given two boost injections at

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2-week intervals, and serum was collected 2 weeks after the final boost.

Immunoblotting was performed essentially as described previously (39). Proteins were separated electrophoretically on denaturing 10% polyacrylamide gels (16) and then transferred to nitrocellulose membranes (BA 85; Schleicher & Schuell, Inc., Keene, N.H.). The membranes were probed with rabbit antiserum at a dilution of 1:500 to 1:1,000 in 150 M NaCl–10 mM sodium phosphate (pH 7.4)–0.05% Tween 20, followed by ^{125}I-protein A (ICN Pharmaceuticals, Inc., Irvine, Calif.). Protein molecular size standards were carbonic anhydrase (30 kilodaltons [kDa]), ovalbumin (43 kDa), bovine serum albumin (66 kDa), and phosphorylase *b* (92 kDa). For immunoblotting, protein molecular weight standards were ^{14}C-methylated proteins (Amersham Corp., Arlington Heights, Ill.). We determined that the electrophoretic mobility of the methylated proteins differed significantly from that of their unmethylated counterparts. Therefore, the molecular weights of the protein standards used for immunoblotting were corrected for this difference, and molecular weights given in figures are apparent molecular weights. Immunoglobulin G (IgG) fraction was purified from serum as described previously (20).

Protein purification. Purified virus was treated with 0.5% Nonidet P-40–50 mM dithiothreitol (DTT)–50 mM Tris hydrochloride (pH 8.0) to strip away the viral envelope (37). Virus core particles were recovered by centrifugation at 10,000 × *g* for 30 min. Cores were disrupted by Dounce homogenization in a solution of 0.5% sodium deoxycholate, 0.25 M NaCl, 25 mM Tris hydrochloride (pH 8.4), and 50 mM DTT. Insoluble material was removed by centrifugation as above, and the VETF was purified by sequential chromatography on DEAE-cellulose, single-stranded DNA-cellulose, phosphocellulose, and DNA sequence affinity with the vaccinia virus growth factor promoter exactly as described previously (6) except the DNA sequence affinity chromatography step was repeated once.

DNA mobility shift analysis. A radiolabeled 90-nucleotide fragment containing the promoter for the vaccinia virus RNA polymerase (RNAP) 22,000-dalton subunit gene (3) was prepared by phosphorylation at the *Hin*dIII site in the plasmid pSB13 (3) polylinker sequence with T4 polynucleotide kinase and [γ-^{32}P]ATP (19). The radiolabeled promoter fragment was liberated by *Hin*cII cleavage and purified by agarose gel electrophoresis.

For DNA binding, protein fractions (typically 1 to 5 μl) were mixed with ^{32}P-promoter fragment in 20 μl of 10 mM Tris hydrochloride (pH 7.5), 0.1 mM EDTA, 5% glycerol, and 100 μg of poly(dI-dC) · poly(dI-dC) per ml. The poly(dI-dC) · poly(dI-dC) could be omitted from the assay after VETF was purified by DNA sequence affinity chromatography. After 30 min at 20°C, the mixture was loaded directly onto a 4% polyacrylamide gel in 6.7 mM Tris hydrochloride (pH 7.5)–3.3 mM sodium acetate–0.1 mM EDTA. Electrophoresis was at 150 V for 2 h. Gels were dried onto Whatman 3MM paper and exposed to Kodak X-omat film for autoradiography.

ATPase assays. Conditions used to measure ATPase activity varied with the particular application. Before DNA affinity chromatography, ATPase activity was determined in a 50-μl solution of 50 mM Tris (pH 8.0), 1 mM $MnCl_2$, 1 mM DTT, 1 mM [γ-^{32}P]ATP (specific activity of 50,000 to 100,000 cpm/nmol), and 20 μg of single-stranded bacteriophage M13 DNA per ml as the cofactor. The reaction mixture was incubated for 10 min at 37°C. Assays on purified VETF were modified to permit the determination of its relatively weak ATPase activity. The reaction conditions described above were modified by reducing the ATP concentration to 0.1 mM to boost the specific activity by a factor of 10, substituting 100 μg of poly(dI-dC) · poly(dI-dC) per ml for polynucleotide cofactor, and increasing the reaction time to 30 min. ATPase activity was scored by determination of $^{32}P_i$ released from [γ-^{32}P]ATP as described previously (5).

Computer analysis of protein sequences. Protein sequences were compared by the FASTA program of Pearson and Lipman (28). The National Biomedical Research Foundation protein library was searched with the same program utilizing the BIONET system.

RESULTS

Vaccinia virus ORF D6R. The nucleotide sequence of the vaccinia virus *Hin*dIII D fragment predicts the existence of 13 major ORFs (24). One of these, ORF D6R, is a reading frame that could encode a 73-kDa polypeptide of 637 amino acids. It was previously observed that the deduced sequence of the D6R polypeptide shared a region of sequence similarity with the DNA-dependent ATPase nucleoside triphosphate phosphohydrolase I (NPH I), which also is encoded within the *Hin*dIII D fragment (4, 30). While the amino-terminal halves of each protein share 18.6% identity, the noted similarities are strongest in a sequence suggested to comprise the ATP-binding domain of NPH I. These sequences are related to the A-type ATP-binding domains originally described by Walker et al. (41) that are conserved among a plethora of ATPases and ATP-binding proteins. The ATP-binding domain is generally characterized by the consensus sequence GXXGXGKT. ORF D6R encodes the sequence HIMGSGKT in amino acids 45 to 52, suggesting an ATPase activity for its gene product.

Three vaccinia virus enzymes with polypeptide chains in the 60 to 70-kDa range are known to have associated DNA-dependent ATPase activity: NPH I (27), NPH II (27), and VETF (5). NPH I and NPH II are monomeric enzymes of 73 and 67 kDa, respectively (4, 27), and VETF has associated polypeptides previously estimated to be 77 and 83 kDa (5, 6). The gene for NPH I was identified previously; however, the genes for NPH II and VETF have not yet been located.

Expression of D6R protein in *E. coli*. To test the hypothesis that the vaccinia virus ORF D6R encodes a protein with ATPase activity, we sought to express the D6R gene product in *E. coli* so that antibodies to the protein could be produced. For this purpose, we chose the bacteriophage T7 expression system developed by Studier and colleagues (33, 38). The D6R reading frame was fused to the bacteriophage phi10 gene to place expression of the vaccinia virus protein under the control of a T7 promoter. The T7 RNA polymerase was then introduced into cells harboring the D6R expression plasmid by infection with a bacteriophage lambda carrying the T7 RNA polymerase gene (38). Infection resulted in the appearance of a new and abundant polypeptide of about 63 kDa as determined by SDS-polyacrylamide gel electrophoresis (Fig. 1A). The apparent molecular weight of the D6R fusion protein is approximately that expected of the phi10-ORF D6R fusion protein, indicating that expression of the D6R protein had been achieved.

Localization of D6R protein to virion core particle. The D6R protein synthesized in *E. coli* was used to raise antibodies in rabbits. Sera from immunized rabbits were used as probes on immunoblots for the presence the D6R polypeptide in vaccinia virus particles. Immunoblotting of total virus

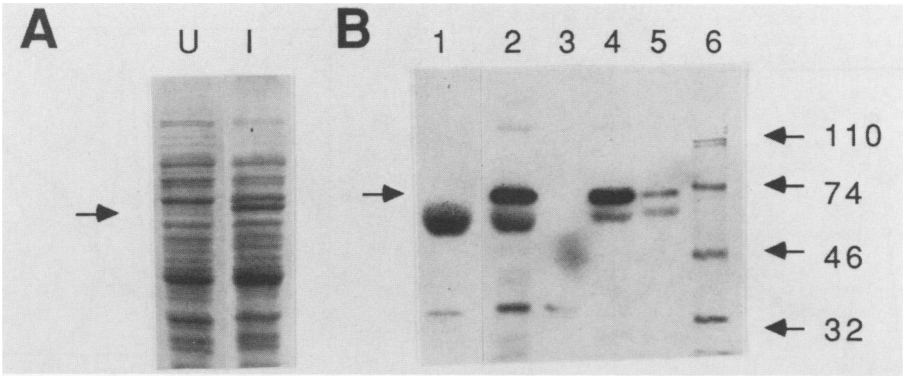

FIG. 1. Localization of the D6R polypeptide to the vaccinia virus core. The vaccinia virus D6R protein was expressed in *E. coli* (A). Total cell proteins from bacteria harboring plasmid pSB35 were resolved by SDS-polyacrylamide gel electrophoresis and visualized with Coomassie blue. Lane U, Uninduced cells; lane I, cells induced to synthesize D6R protein by infection with lambda CE6. The induced polypeptide is indicated by the arrow. (B) Immunoblot of vaccinia virus proteins probed with anti-D6R antiserum. The protein fractions were total virus proteins (lane 2), Nonidet P-40-solubilized envelope proteins (lane 3), core proteins solubilized with 0.5% sodium deoxycholate (lane 4), and sodium deoxycholate-insoluble core proteins (lane 5). Total vaccinia virus proteins probed with preimmune serum are shown in lane 1. The apparent molecular weights ($\times 10^3$) of mobility standards are shown at right.

particle proteins resulted in reaction with a major virus polypeptide of 70 kDa and less prominent polypeptides of about 60 and 34 kDa (Fig. 1B). The bands corresponding to the 60- and 34-kDa proteins were also observed when preimmune serum from the same rabbit was used to probe the immunoblot, indicating that the latter polypeptides are unrelated to the 70-kDa polypeptide and further indicating that immunization with the D6R protein made in bacteria resulted in production of antibodies against a 70-kDa vaccinia virus polypeptide.

The D6R antigen was localized within the virus particle by selective extractions with detergents and DTT. Virus particles were treated with the detergent Nonidet P-40 and DTT to solubilize the viral envelope (Fig. 1B). This treatment did not solubilize the D6R antigen, demonstrating that D6R is a component of the viral core structure. Further treatment of the cores with the detergent sodium deoxycholate and DTT resulted in the solubilization of most of the D6R antigen. This result is similar to that observed for the solubilization of vaccinia virus core-associated enzymes.

Purification of D6R protein. The ability to solubilize the D6R protein from viral cores made it possible to purify the protein from vaccinia virions by using immunoblotting as an assay. The purification scheme was modeled after protocols which have been successful for several other vaccinia virus core-associated enzymes. Because of the putative ATP-binding site described above, we suspected the D6R protein to have ATPase activity. This raised the possibility of D6R encoding the DNA-dependent ATPase activity of either NPH II or VETF. Therefore, during the D6R purification, we also monitored ATPase activity as well as the early promoter DNA-binding activity unique to VETF.

The sodium deoxycholate-soluble fraction of vaccinia virus cores was passed over DEAE-cellulose to remove endogenous DNA and the viral RNA polymerase (6). Immunoblotting of the DEAE-cellulose column fractions demonstrated that the D6R protein did not bind the column (data not shown). The protein was subsequently chromatographed on single-stranded calf thymus DNA (Fig. 2). The D6R protein bound to the column and was eluted at about 0.15 M NaCl (Fig. 2). The position of elution approximated that of VETF, which was assayed by mobility shift analysis with the 22-kDa RNAP promoter. This early vaccinia virus promoter has been demonstrated to require VETF for optimal transcription in vitro (6) and is shown here to form a stable complex with VETF as evidenced by the retarded mobility of the 22-kDa RNAP promoter DNA in a native gel. The D6R elution also approximated that of NPH I, which is responsible for most of the ATPase activity in fractions 20 to 30. The small peak of ATPase activity in fractions 38 to 40 is NPH II as evidenced by the fact that its ability to hydrolyze ATP was enhanced by RNA cofactors such as poly(C) (26; data not shown). Since the D6R polypeptide was resolved completely from NPH II, the two are apparently unrelated.

The D6R-containing fractions from the DNA-cellulose step were pooled and subsequently chromatographed on phosphocellulose. Immunoblotting of column fractions showed that the D6R protein bound to the column and eluted at an NaCl concentration of about 0.15 M, again coincidental with VETF, slightly separated from NPH I (data not shown).

The cochromatography of the D6R protein with the transcription factor VETF suggested that the two are associated or possibly the same protein. Indeed, the D6R polypeptide has an electrophoretic mobility on SDS-polyacrylamide gels that is identical to that of the smaller of the VETF-associated polypeptides. Therefore, the D6R protein fractions from the phosphocellulose chromatography were subjected to DNA sequence affinity chromatography, which has been shown to yield highly purified VETF (6). The column consists of a reiterated DNA sequence encoding the vaccinia virus growth factor promoter coupled to a Sepharose support. Chromatography of the phosphocellulose column fraction containing D6R on the DNA affinity column resulted in the binding of VETF, as determined by DNA mobility shift analysis with the 22-kDa RNAP promoter (Fig. 3). Coincidental with the promoter-binding activity was a DNA-dependent ATPase activity, in agreement with an earlier report (6). When these column fractions were analyzed by immunoblotting with D6R antiserum, it was found that the D6R protein did indeed bind to the affinity column and eluted with the same general profile as that of the VETF. The copurification of the D6R protein with VETF persisted through two rounds of DNA affinity chromatography. The predominant polypeptides in this preparation were 70 and 77 kDa in size, as reported previously (data not shown). VETF was previously reported to contain polypeptides of 83 and 77 kDa (6). In our

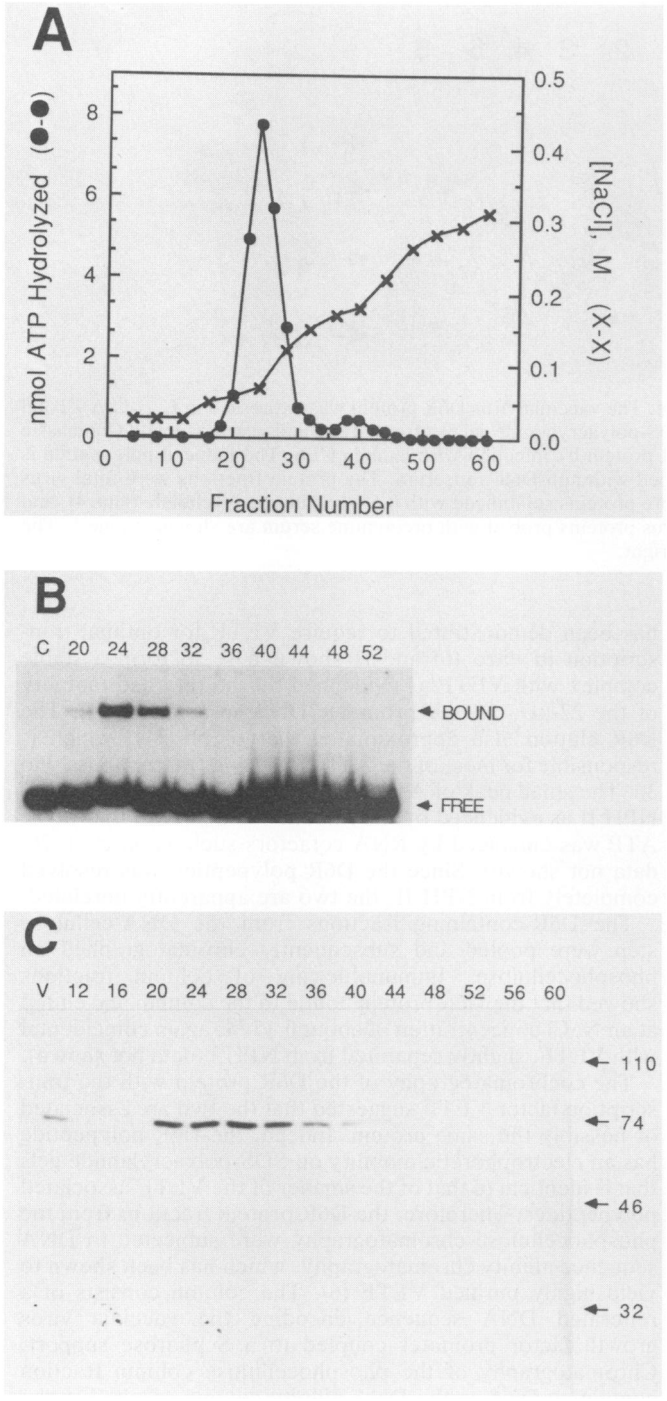

FIG. 2. Chromatography of vaccinia virion extract on DNA-cellulose. Column fractions were assayed for DNA-dependent ATPase activity (A), 22-kDa RNAP promoter-binding activity as determined by DNA electrophoretic mobility shift analysis (B), or D6R polypeptide by immunoblotting (C). Lane C contains free probe alone, and lane V contains total virion proteins. Apparent molecular weights ($\times 10^3$) of protein standards are given on the right of panel C.

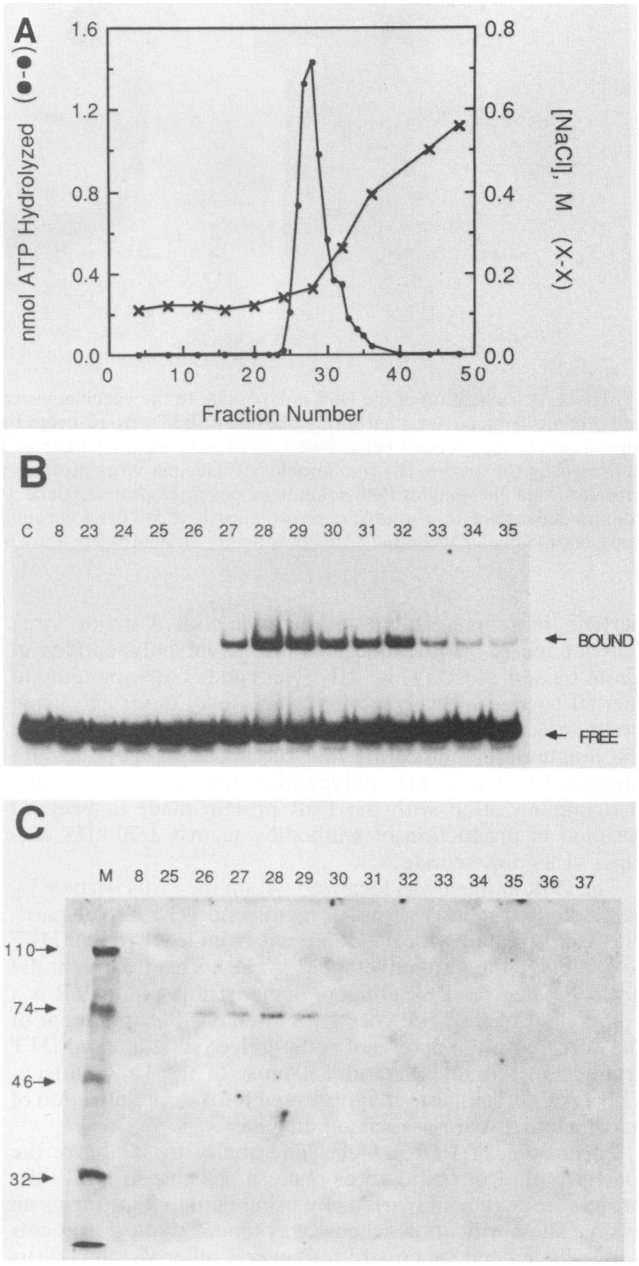

FIG. 3. DNA sequence affinity chromatography of VETF. VETF was purified as described in Materials and Methods and was subjected to a second round of chromatography on an oligonucleotide column consisting of the vaccinia virus growth factor gene promoter linked to a Sepharose support. Column fractions were assayed for ATPase activity (A), 22-kDa RNAP promoter-binding activity by DNA electrophoretic mobility shift analysis (B), and D6R polypeptide by immunoblotting (C). Lane C contains free probe alone. Fraction 8 is among those containing material which did not bind to the column. In panel C, lane M contains protein standards whose apparent molecular weights ($\times 10^3$) are given on the left.

experiment, these polypeptides migrated slightly faster through SDS-polyacrylamide gels, with mobilities corresponding to 77 and 70 kDa, respectively. This preparation of VETF was judged to be about 80 to 90% homogeneous with respect to the 77- and 70-kDa polypeptides by SDS-polyacrylamide gel electrophoresis and silver staining. The fact that the D6R polypeptide copurifies with VETF and comigrates on SDS-polyacrylamide gels with the smaller VETF-associated polypeptide strongly argues that the D6R protein constitutes the smaller of the VETF polypeptides.

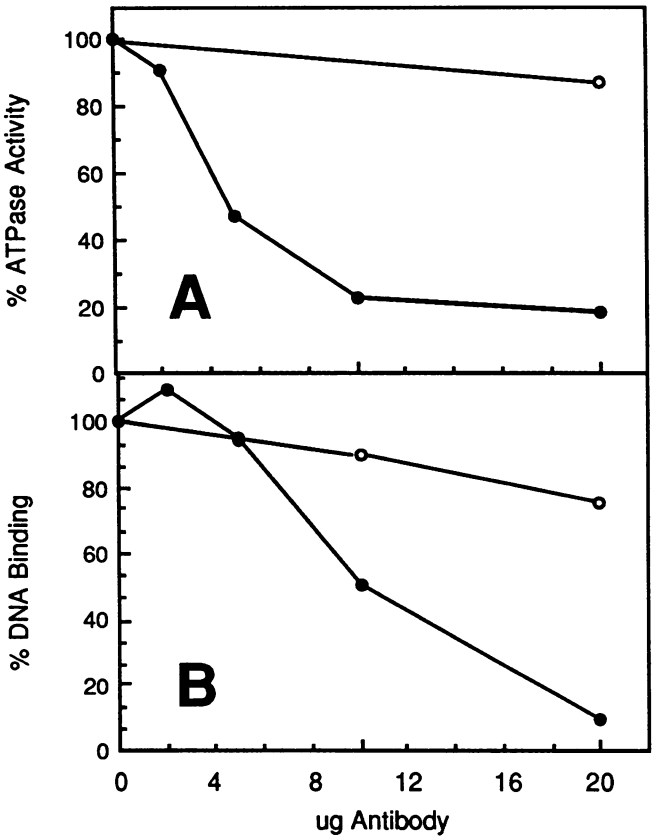

FIG. 4. Effect of anti-D6R antibodies on the VETF-associated early promoter-binding and DNA-dependent ATPase activities. The indicated quantities of purified IgG from immune (●) or preimmune (○) rabbits were incubated with units of purified VETF at 4°C for 1 h. At that time, VETF was assayed for DNA-dependent ATPase activity (A) or promoter binding by DNA electrophoretic mobility shift of the 22-kDa RNAP promoter (B). DNA binding was quantitated by densitometry of the autoradiography film.

Effect of anti-D6R antibodies on VETF-associated activities. The effect of anti-D6R antibodies on the described early promoter-binding and DNA-dependent ATPase activities of VETF was examined. Purified VETF was preincubated with purified IgG fractions and assayed for these activities. The ability of VETF to form a stable complex with the 22-kDa RNAP promoter, as assayed by DNA mobility shift analysis, was severely inhibited by the anti-D6R IgG; however, IgG from the preimmune rabbit had little effect on promoter binding (Fig. 4). Similarly, the immune IgG inhibited the DNA-dependent ATPase activity associated with VETF, while the preimmune IgG had correspondingly less effect. Previous studies showed that antibodies directed against vaccinia virus NPH I did not affect the ATPase activity of VETF (5). We were unable to examine the effect of antibody on the VETF activity because of the presence of RNase activity in our antibody preparations.

DISCUSSION

The deduced sequence of the vaccinia virus *Hin*dIII D6R ORF gene product suggests the presence of an ATP-binding site. Based on this prediction, it was proposed that D6R might encode the DNA-dependent ATPase NPH II (4). The D6R sequence predicts a polypeptide of 73 kDa with a potential ATP-binding site that was expressed as a late protein, all of which are consistent with NPH II (25, 27). However purification of the D6R polypeptide from vaccinia virions showed that it was readily separable from NPH II, suggesting that NPH II was unrelated to D6R. Indeed, when the D6R polypeptide was purified, it was found to cochromatograph on four different columns with VETF. The electrophoretic mobility of the 70-kDa D6R protein determined on immunoblots is identical to that of the smaller VETF-associated polypeptide. It therefore is reasonable to assume that D6R is that polypeptide. The putative ATP-binding site in the D6R polypeptide further suggests that it is the origin of the ATPase activity associated with VETF. Because of the insolubility of the protein synthesized in bacteria, we were unable to demonstrate an ATPase activity directly.

Aside from the possible ATP-binding site, no other structural motifs commonly found in transcription factors could be deduced from the primary sequence of D6R. There are no groupings of cysteines or histidines indicative of zinc finger DNA-binding domains (2); nor are there any obvious leucine zipper domains as found in several dimeric transcription factors (17). In addition, a search of the National Biomedical Research Foundation protein library with the FASTA program revealed no significantly similar proteins.

Several eukaryotic transcription factors have been observed to have nonidentical polypeptides on SDS-polyacrylamide gels, as does VETF. In the cases of the transcription factors SP1 and AP-1, the polypeptides of differing mobilities appear to be the result of variable glycosylation (13). The fact that the D6R antibody recognized only the smaller of the VETF-associated polypeptides suggests that the two polypeptides are unrelated and are not modified forms on one another. Therefore, we would anticipate that the large VETF-associated polypeptide would be encoded by a separate gene in the vaccinia virus genome.

Previous studies by Lee-Chen and Niles (18) have demonstrated that the D6R gene belongs to the late class of vaccinia virus genes. Transcription of D6R occurs only at late times after infection and is completely blocked by DNA synthesis inhibitors. Transcription signal motifs in the D6R gene are consistent with late gene expression. The consensus sequence TAAATG is found at the site of transcriptional-translational initiation (11, 32). In addition, five early gene transcriptional termination signals of the sequence TTTT TNT (44) are found within the D6R ORF, making it highly unlikely that D6R is transcribed early in the infectious cycle. Our immunoblot analysis of infected-cell extracts with anti-D6R antiserum are consistent with D6R being a late-class protein (data not shown).

The timing of expression of VETF suggests a model for the regulation of vaccinia virus gene expression throughout the infectious cycle. The fact that VETF is synthesized late in infection for use in the early stage of a subsequent round of infection suggests that VETF is part of a cascading progression of transcription factors that regulate the temporal expression of vaccinia virus proteins. The factors required for transcription of a given gene class would be expected to be synthesized in the preceding phase of the infectious cycle. Just as VETF is synthesized as a late protein, one might expect that intermediate and late gene transcription factors would be synthesized as early and intermediate proteins, respectively. This scenario is analogous to the pattern of gene expression in herpes simplex virus type 1 (1, 7, 29). The herpesvirus equivalent to VETF is the protein αTIF that is required for immediate-early protein synthesis. It is synthesized as a late-class protein and

is packaged into virions for use in the subsequent infectious cycle.

The mapping of conditional lethal mutations to the D6R ORF demonstrates that the D6R gene product is essential for vaccinia virus growth (35). The phenotype of D6R temperature-sensitive mutants is that of faulty virus assembly with no apparent defect in early gene expression. This is similar to the phenotype of some mutations mapping in the genes for obvious components of the early transcription machinery such as RNAP subunits (12). Indeed, no vaccinia virus mutants have been reported to be defective in early gene expression. Therefore, it is not surprising that available D6R mutants are not defective for early transcription. The identification of ORF D6R as one of the genes encoding VETF will permit a systematic approach to mutation of the gene.

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