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Vaccinia Virions Lacking Core Protein VP8 Are Deficient in Early Transcription

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When synthesis of the 25-kDa vaccinia virus core protein VP8 is repressed, mature virus particles of normal appearance are produced to approximately 80% of wild-type levels but these particles are over 100-fold less infectious than wild-type particles (D. Wilcock and G. L. Smith, Virology 202:294–304, 1994). Here we show that virions which lack VP8 can bind to and enter cells but the levels of steady-state RNA are greatly reduced in comparison with those for wild-type infections. In vitro assays using permeabilized virions demonstrated that VP8-deficient virions had drastically reduced rates of transcription (RNA synthesis was decreased by 80 to 96%) and that the extrusion of RNA transcripts from these virions was also decreased. Low concentrations of sodium deoxycholate extracted proteins more efficiently from VP8-deficient virions than from wild-type virions. The increased fragility of VP8-deficient virions and their slower RNA extrusion rates suggest that VP8 may be required for the correct formation of the core. Virions which lack VP8 were shown to contain a full complement of transcription enzymes, and soluble extracts from these virions were active in transcription assays using either single-stranded M13 DNA or exogenous plasmid template containing a vaccinia virus early promoter. Thus, the defect in transcription is due not to a lack of specific transcriptional enzymes within virions but rather to the inability of these enzymes to efficiently transcribe the DNA genome packaged within VP8-deficient virions. These results suggest that VP8 is required for the correct packaging of the viral DNA genome and/or for the efficient transcription of packaged virion DNA, which has a higher degree of structural complexity than plasmid templates. Possible roles for VP8 in these processes are discussed.

Vaccinia virus is a large double-stranded (ds) DNA virus which replicates in the cytoplasm of the host cell (21, 39). The 191-kb virus genome has the potential to code for ≥200 proteins (28), around 100 of which are found in the virion (18, 44). The viral DNA genome is packaged as a nucleoprotein complex in the virion core (27, 58). The core itself was originally thought to be dumbbell shaped, but more recent studies using cryoelectron microscopy suggest that it is a dense homogeneous structure which has the same general shape as the virion (13).

There is no evidence for the encapsidation of host DNA-binding proteins in the virion core, but two abundant virus-encoded DNA-binding proteins are present: the 11-kDa phosphoprotein, VP11 (encoded by the F18R gene) (30, 31, 67, 71), and the 25-kDa protein, VP8 (encoded by the L4R gene) (65, 69). VP11 binds strongly to both single-stranded (ss) DNA and dsDNA and shows preferential binding to supercoiled DNA. These binding characteristics are similar to those of histone H1, and, since VP11 also has a tendency to form oligomers, this protein has been suggested to be important for the condensation of vaccinia virus DNA (31). Repression of VP11 synthesis blocks core protein processing and mature particle production, and immature virions with aberrant internal structures are produced (71). VP8 can also bind to both ssDNA and dsDNA. However, although it binds equally well to both forms at low salt concentrations (25 mM NaCl), it shows a binding preference for ssDNA at higher salt concentrations (100 mM NaCl) (69). These DNA binding characteristics, together with stoichiometric considerations, led Yang and Bauer to postulate that VP11 is the major dsDNA-binding protein in the core whereas VP8 may stabilize as much as 20% of the viral genome as ssDNA, perhaps functioning in the initiation of early transcription (69).

In a previous report (66) we described the construction and properties of a recombinant vaccinia virus, vDW4, in which the L4R gene encoding VP8 is inducibly regulated by isopropyl-β-D-thiogalactopyranoside (IPTG). In the presence of IPTG this virus has a wild-type phenotype, but in the absence of IPTG, when VP8 synthesis is repressed, the production of infectious virus progeny is reduced by 97%. In the absence of IPTG, abnormal immature virions are produced which have a gap between the innermost virion membrane and the granular viroplasm contained within it, unlike wild-type immature virions where the viroplasm completely fills the particle. However, core protein processing is not blocked and these aberrant immature virions are able to mature, producing intracellular and extracellular virus particles of normal density, which are indistinguishable from wild-type particles when examined by electron microscopy. Although mature virus particles are produced to approximately 80% of wild-type levels, they are at least 100-fold less infectious. We previously speculated that this reduced infectivity could be caused by either a requirement for VP8 for an essential step in infection or a more indirect requirement whereby essential virion enzymes, or structural components mediating binding to cells, are not packaged in the absence of VP8. Here we show that VP8-deficient (VP8−) virions possess a full set of functional transcription enzymes yet show markedly decreased rates of transcription of virion DNA, indicating that VP8 plays an essential role in the infection

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process by enabling the efficient transcription of early viral genes.

MATERIALS AND METHODS

Cells and viruses. The construction and conditions for propagation of vDW4 have been described previously (66). Virions (intracellular mature virus particles) were purified on sucrose gradients from HeLa S3 suspension cultures (14), except for the virions used for binding assays (see Fig. 1) and for preparing viral cores (see Fig. 4B), which were purified on cesium chloride (CsCl) gradients from RK-13 cells (47).

To prepare viral cores for immunoblotting (see Fig. 4B), virions (harvested 3 days after infection at 0.1 PFU per cell) were incubated in 50 mM Tris-HCl (pH 8.5)–0.5% Nonidet P-40 (NP-40)–50 mM 2-mercaptoethanol for 30 min at 37°C and centrifuged at 6,500 × *g* for 30 min at 4°C and the resulting pellet was resuspended in 50 mM Tris-HCl (pH 8.5)–10 mM 2-mercaptoethanol (32).

Virus binding and entry assays. Radioactively labeled intracellular mature virus was prepared as follows: RK-13 cells were infected at 10 PFU per cell, the infected cells were labeled from 8 h postinfection (hpi) with 125 μCi of [^{35}S]methionine per 175-cm^2 flask, and virus was harvested at 24 hpi and purified on CsCl gradients (47). Peak IMV fractions were pooled, and the radioactivity was measured by liquid scintillation counting before they were used in binding and entry assays on TK$^-$143 cells grown in 24-well plates. Assays were based on previously described methods (3).

The amounts of cell-associated virus were measured as follows. Cells were washed with RPMI containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.4) (RPMI-HEPES), and radioactively labeled IMV (approximately 16,000 trichloroacetic acid [TCA]-precipitable cpm) was added in 200 μl of RPMI–HEPES–2.5% fetal calf serum (FCS). After incubation for various times at 37°C, the inoculum was removed and the cells were washed twice with cold phosphate-buffered saline (PBS). The PBS washes and virus inoculum were pooled and constituted the nonadsorbed virus sample. The cells were then lysed with 200 μl of cold PBS containing 1% NP-40, and this lysate constituted the adsorbed virus sample. The samples were precipitated with 10% TCA and collected on GF/C filters (Whatman, Inc.), and their radioactivity was measured by liquid scintillation counting.

In assays for virus entry into cells, the washed cells were chilled on ice for 15 min before addition of virus (approximately 28,000 TCA-precipitable cpm) in 200 μl of RPMI–HEPES–2.5% FCS. After incubation on ice for 3 h, the nonadsorbed virus was removed by two RPMI-HEPES washes, fresh RPMI–HEPES–2.5% FCS was added, and the cells were incubated at 37°C for various times or processed immediately (zero time point). After the 37°C incubation period, the cells were washed twice in PBS and incubated on ice for 2 h in 200 μl of PBS containing proteinase K (Gibco BRL) at 1 mg/ml to remove virus which was bound to the cell surface. The cells were scraped gently off the well, and the suspension was placed in an Eppendorf tube and spun in a microcentrifuge for 1 min. The supernatant was collected, and the pellet was washed twice with 200 μl of PBS. The supernatant and PBS washes were pooled and constituted the adsorbed virus sample. The cell pellet was lysed with PBS containing 1% NP-40 as described above and constituted the internalized virus sample. The samples were TCA precipitated, and their radioactivity was determined as described above.

RNA analysis. RNA was prepared from mock-infected cells or cells which had been infected with purified virions at a multiplicity of 10. (The infectivity of VP8+ virions was determined by a plaque assay, and the same quantity, in A_{260} units, of VP8− virions was used). At 8 hpi, the cells were lysed in a solution containing 4 M guanidinium isothiocyanate, the lysate was sheared with a 20-gauge needle, and the RNA was pelleted through a CsCl step gradient (34). The RNA pellet was dissolved in TES (10 mM Tris-HCl [pH 7.4], 5 mM EDTA, 1% sodium dodecyl sulfate [SDS]) and quantified by spectrophotometry.

Slot blots were prepared by standard methods (53) with Hybond-N nylon membrane (Amersham International). Prehybridizations and hybridizations were performed at 42°C as described previously (60). ^{32}P-labeled probes were synthesized by using the Megaprime DNA labeling system (Amersham International) according to the manufacturer's instructions. Unincorporated label was removed from probe preparations by passage through Sephadex G-50 (Pharmacia) spin columns. Probes used were as follows: vaccinia virus growth factor (VGF), a 534-bp *Dde*I fragment (64) excised from a plasmid, pVGF (made by A. P. Rice, Imperial Cancer Research Fund, London, United Kingdom); TK (thymidine kinase), a 407-bp PCR product; B1R, a 150-bp PCR product; and B5R, a 944-bp PCR product. Following hybridization, filters were washed in the following solutions: 2× SSPE (0.3 M NaCl, 20 mM sodium phosphate, 0.2 mM EDTA [pH 7.4]) containing 0.1% SDS (room temperature, twice for 15 min each), 1× SSPE containing 0.1% SDS (50°C, twice for 15 min each), and 0.2× SSPE containing 0.1% SDS (55°C, twice for 15 min each). Sequential hybridizations were performed on the same filter, which was stripped by boiling in 0.1% SDS after detection of each probe.

For Northern (RNA) blots, RNA was electrophoresed on a 1.2% agarose–formaldehyde gel by standard methods (53). Lanes containing RNA markers plus ethidium bromide were excised from the gel prior to transfer, and the positions of the markers in the gel were recorded under UV light. The remaining RNA lanes were transferred to Hybond-N$^+$ nylon membrane (Amersham International) for 3 h under alkaline conditions (0.05 M NaOH), according to the manufacturer's instructions. Hybridization and washing were performed as described for slot blotting, except that the final 0.2× SSPE–0.1% SDS wash was omitted.

Immunoblotting. Cell extracts were prepared by washing infected cells once in cold PBS, scraping the cells into cold PBS, and centrifuging the mixture at 1,500 rpm for 5 min at 4°C in a Beckmann GPR centrifuge. The cell pellet was resuspended in PBS, and an equal volume of 2× Laemmli sample buffer (35) was added prior to sonication, heat denaturation, and electrophoresis.

Samples for analysis (virus cores or cell extracts) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose (61), and blotted by using the enhanced chemiluminescence detection system (Amersham International) according to the manufacturer's instructions. Antibody to VP8 (P25K/25K) (63) was kindly provided by D. Hruby (Oregon State University). Antibodies to RNA polymerase (29), vaccinia virus early transcription factor (23), small capping subunit (42), poly(A) polymerase (22), RNA polymerase-associated protein 94 (RAP94) (2), and glutaredoxin (1) were kindly provided by B. Moss (National Institutes of Health, Bethesda, Md.). Antibodies to nucleoside triphosphate phosphorohydrolase I (NPH-I) and NPH-II (45) were kindly provided by E. Paoletti (Virogenetics Corporation, Troy, N.Y.). Antibody to vaccinia virus topoisomerase was kindly provided by S. Shuman (Sloan-Kettering Institute, New York, N.Y.). Antibodies to B1R (4) and B5R (15) were produced previously in our laboratory.

In vitro transcription by detergent-permeabilized virions. Transcription reactions were performed in the presence of 0.05% NP-40 as described previously (56) with 0.5 A_{260} units of virus per 200-μl reaction volume. For quantification of total RNA synthesis, aliquots of each transcription reaction mixture were added to 10% TCA after various times of incubation. For extrusion assays, reaction aliquots were separated by centrifugation into released and core RNA fractions before TCA precipitation (56). Precipitated RNA was collected on GF/C filters, and the level of incorporation of [^{32}P]UMP was determined by liquid scintillation counting.

Gel analysis of in vitro transcription products was performed as follows. Aliquots taken after 30 min of incubation were separated into released and core RNA fractions and TCA precipitated to determine the levels of [^{32}P]UMP incorporation. The remainder of each reaction mixture was separated into released and core RNA fractions, and samples were prepared for gel analysis as described previously (56). Approximately 10,000 cpm from each fraction was electrophoresed on a 1% agarose–formaldehyde gel and transferred to Hybond-N overnight in 10× SSC (1.5 M sodium chloride, 0.15 M trisodium citrate) by standard methods (53). The filter was dried, and labeled RNA was visualized by autoradiography.

Preparation of virion extracts. Sodium deoxycholate extracts were prepared from whole virions by a modification of the method described by Rohrmann and Moss (51). Purified virions (4.6 mg) were pelleted by centrifugation at 12,000 × *g* for 10 min at 4°C and resuspended in buffer A (100 mM Tris-HCl [pH 8.0], 10 mM dithiothreitol, 250 mM KCl, 0.2 mM EDTA) at a concentration of 10 mg/ml. The virions were incubated in this buffer for 1 h on ice with gentle vortexing every 10 min. After centrifugation at 12,000 × *g* for 15 min at 4°C, the supernatant (−deoxycholate) was removed and kept on ice. The pellet was resuspended at 10 mg/ml in buffer A, and sodium deoxycholate was added to 0.1% (wt/vol). The incubation (1 h) and centrifugation steps were repeated, and the remaining pellet was resuspended at 10 mg/ml in buffer A and reextracted with 0.2% sodium deoxycholate for 30 min on ice. We found that a reextraction step was necessary for consistent yields of transcription proteins. The supernatants from the two deoxycholate extractions were pooled (+deoxycholate), and each type of supernatant (−deoxycholate or +deoxycholate) was passed through a 23-gauge needle to reduce viscosity before being loaded onto a 0.5-ml DE52-cellulose (Whatman, Inc.) column. The column was washed with buffer A, and the flowthrough material was assayed for protein content by using Coomassie protein assay reagent (Pierce). Peak fractions were pooled and dialyzed at 4°C for 18 h against three changes of 50 mM Tris-HCl (pH 8.0)–0.1 mM EDTA–0.01% NP-40–10% glycerol–2 mM dithiothreitol–50 mM NaCl (70). The protein concentrations of the strain WR, VP8+, and VP8− virion extracts were made equal (42 μg/ml for −deoxycholate and 268 μg/ml for +deoxycholate) by dilution with dialysis buffer, and the extracts were stored in aliquots at −70°C.

Nonspecific RNA polymerase activity assays. RNA polymerase activity in virion extracts was measured on an ssM13 DNA template in the presence of Mn^{2+} as described previously (70). Reaction mixtures (50 μl) were incubated at 37°C, and aliquots were removed for TCA precipitation at various times.

Specific transcription assays. Specific transcription was assayed by using plasmid pSB24 (made by S. Broyles, Purdue University, and described in reference 37), which contains a G-less cassette under the control of a synthetic early vaccinia virus promoter (12, 54). Assays were performed essentially as described previously (70). Reaction mixtures (50 μl) contained 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 2 mM dithiothreitol, 0.14 mM EDTA, 7.5% glycerol, 6 mM $MgCl_2$, 1 mM ATP, 1 mM CTP, 0.02 mM UTP, 100 μM 3′-*O*-methylguanosine 5′-triphosphate (Pharmacia), 5 μCi of [α-^{32}P]UTP (3,000 Ci/mmol; Amersham International), 200 ng of uncut pSB24, and 250 ng of virion extract. After 30 min at 30°C, the reaction mixtures were adjusted to 100 mM Tris-HCl (pH 7.5)–12.5 mM EDTA–150 mM NaCl–1% SDS–100 μg of tRNA per ml–200 μg of pro-

teinase K per ml and incubated for 30 min at 37°C. The reaction mixtures were extracted once with phenol-chloroform, and the RNA was precipitated three times with ethanol. The pellet was washed with 70% ethanol, resuspended in formamide sequencing loading buffer, and heated to 90°C for 3 min prior to being loaded on a 4% polyacrylamide–urea gel in Tris-borate buffer. RNA markers (0.16 to 1.77 kb; Gibco BRL) were radiolabeled with [γ-^{32}P]ATP and T4 polynucleotide kinase after dephosphorylation. After electrophoresis, the gel was fixed, dried, and autoradiographed. Quantification of bands was performed by using a phosphorimager (Molecular Dynamics).

RESULTS

Virions lacking VP8 can bind and enter cells with normal kinetics. Growth of the recombinant vaccinia virus vDW4 in the absence of IPTG produces mature virus particles which contain greatly decreased levels of VP8 protein. These particles (referred to here as VP8− virions) are similar to vDW4 virions produced in the presence of IPTG (VP8+ virions) on examination by electron microscopy but are at least 100-fold less infectious (66). The stage of infection at which this defect in infectivity is manifested was investigated.

The first stage of vaccinia virus infection involves the binding of a virus particle to the cell surface and its subsequent internalization. We examined these processes using [^{35}S]methionine-labeled VP8+ and VP8− virions. The rates of association of these virions with cultured cells were indistinguishable at 37°C (Fig. 1A). Similar association rates were also observed at 4°C (data not shown). To distinguish virus binding from virus entry, labeled virions were bound to cells at 4°C and then the cells were shifted to 37°C for various times before proteinase K treatment to remove virions still exposed on the cell surface. The rates of accumulation of proteinase K-resistant counts per minute (internalized virus) were similar for both VP8+ and VP8− virions (Fig. 1B). These results suggest that the infection defect observed with VP8− virions occurs after the virus has entered the cell.

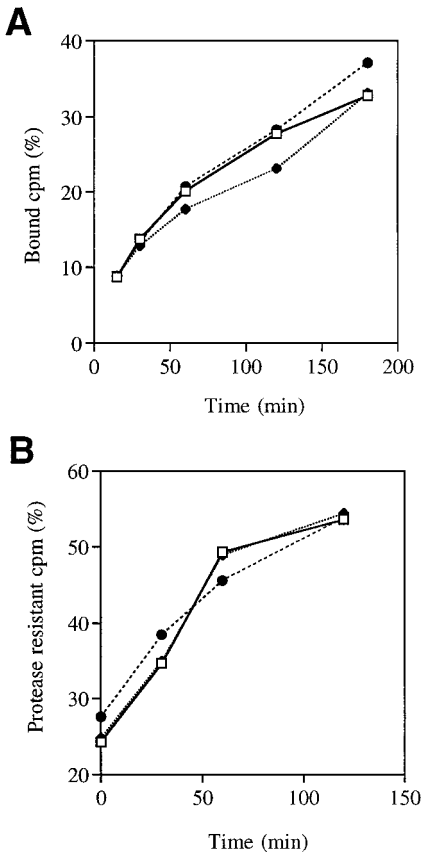

FIG. 1. Virus binding and entry into cells. (A) Virus binding. The amount of virus which had bound to cells at 37°C was measured as described in Materials and Methods. The percentage of virus which had bound to cells [adsorbed/(adsorbed + nonadsorbed), counts per minute × 100] after the indicated times is shown. Each datum point represents the results from duplicate samples. (B) Virus entry. Entry of virus into cells was assayed as described in Materials and Methods. Virus was bound to cells at 4°C, and the cells were shifted to 37°C for various times to assay for entry. The percentage of virus internalized (proteinase K resistant) [internalized/(adsorbed + internalized), counts per minute × 100] is shown after incubation for various times at 37°C. Each datum point represents the results from duplicate samples. Apparent internalization of some virus at zero time is probably due to the incomplete removal of surface virus by proteinase K treatment. □, WR; ◆, VP8+; ●, VP8−.

Levels of RNA synthesis are decreased in cells infected with VP8− virions. Virus cores released into the cytoplasm immediately initiate transcription of early viral genes (39). Early virus transcription was therefore examined by measuring the levels of RNA transcripts produced in cells which had been infected with purified VP8+ or VP8− virions. Figure 2A shows slot blots performed with RNA prepared from cells infected with purified wild-type (strain Western Reserve [WR]), VP8+, or VP8− virions. RNAs transcribed from the early VGF, TK, and B1R promoters, or from the constitutive B5R promoter, were detected with appropriate probes (see Materials and Methods). In each case, the levels of transcripts detected in cells infected with VP8− virions were much lower than those in VP8+ virion-infected cells. Early transcripts in cells infected with VP8− virions were present at 8.9% (VGF), 7.5% (TK), 12.7% (B1R), and 2.5% (B5R) of the levels seen in VP8+ virion-infected cells. At late times (8 hpi), these percentages increased to 24, 38, 23, and 26%, respectively. The presence of early transcripts at late times in VP8− virion-infected cells might be explained by decreased rates of viral transcription in these cells, slowing down the infection, so that 8 hpi is still early in these infections. So, VP8− virions can bind and enter cells normally but are defective in early gene expression, since steady-state levels of viral RNA are reduced by over 85% at early times of infection.

VP8− virions synthesize RNA of normal size which can be translated in vivo. RNA prepared from cells infected with purified virions was analyzed by Northern blotting with a probe to the early VGF transcript (Fig. 2B). The level of VGF transcripts detected in preparations from cells infected with VP8− virions was around 9% of that in preparations from VP8+ virion-infected cells, consistent with the results from slot blot analysis (Fig. 2A). Full-length VGF transcripts of approximately 530 nucleotides were produced in cells infected with VP8− virions. The larger band detected in RNA prepared from mock-infected cells might be epidermal growth factor (EGF) RNA, since mouse EGF cDNA is 4.75 kb in length and the EGF and VGF proteins have amino acid similarity (8, 9, 49). To investigate whether early RNA made by VP8− virions could be translated, Western blots (immunoblots) were performed with extracts from cells infected with purified virions (Fig. 3). The 34-kDa early B1R protein kinase was detectable in cells infected with VP8− virions, but its synthesis was delayed relative to that in VP8+ virion infections (Fig. 3A). Levels of B1R protein detectable 2 h after infection with VP8+ virions were only attained 8 h after infection with VP8− virions. Similarly, synthesis of the 42- and approximately 90-kDa forms of the envelope glycoprotein B5R was also delayed in cells infected with VP8− virions (Fig. 3B). B5R was detectable from 4 h in VP8+ infections but only from 8 h in cells infected with VP8− virions. Evidently, RNA synthesized in vivo by VP8− virions is translatable but protein synthesis lags behind

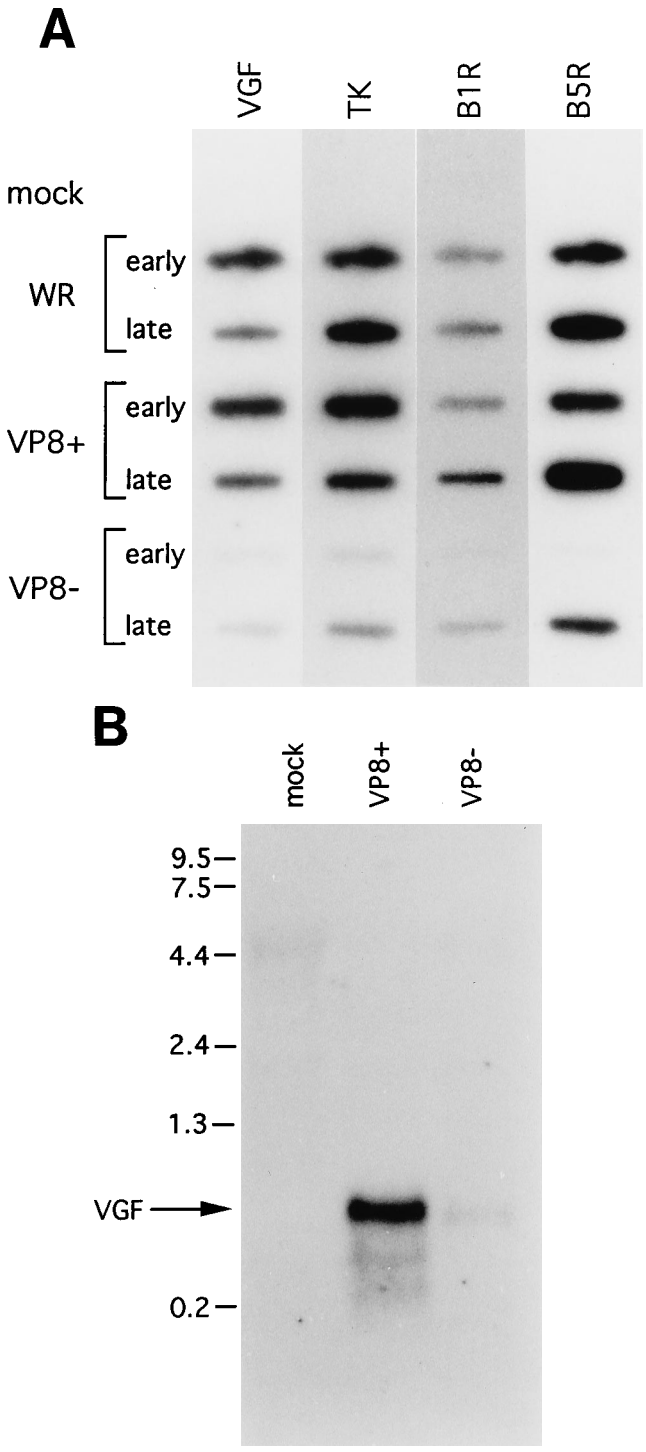

FIG. 2. Analysis of in vivo transcription products. (A) Slot blot analysis. Infections of TK$^-$143 cells were performed with purified WR, VP8+, or VP8− virions (equivalent A_{260} units of VP8+ and VP8− virions were used) in the presence of IPTG and the presence or absence of cycloheximide. Early (with cycloheximide) or late (without cycloheximide) RNA was prepared at 8 hpi as described in Materials and Methods. RNA was adsorbed to a Hybond-N membrane (10 μg per slot) and hybridized with a heat-denatured ^{32}P-labeled probe for either vaccinia virus growth factor (VGF), thymidine kinase (TK), protein kinase (B1R), or glycoprotein of the extracellular virus envelope (B5R). (B) Northern blot analysis. RNA prepared from RK-13 cells by the same method as used for the experiment shown in panel A was electrophoresed on a 1.2% agarose–formaldehyde gel (10 μg per well) and transferred to a Hybond-N$^+$ nylon membrane as described in Materials and Methods. Hybridization was performed with a 534-bp ^{32}P-labeled VGF probe. RNA markers (Gibco BRL) are shown in kilobase pairs. mock, mock-infected cells.

that in wild-type infections because of reduced levels of steady-state RNA.

The packaging of other virion proteins is not affected by a lack of VP8. The defect in early gene expression during infections with VP8− virions may have been caused by their failure to package some other protein(s) into the virion. We therefore examined the protein compositions of VP8+ and VP8− virions, to determine whether VP8− virions possessed a full complement of virion proteins. Gel electrophoresis of whole virions showed no difference in protein profiles (apart from the VP8 protein itself) on Coomassie-stained (Fig. 4A) or on silver-stained (data not shown) gels, indicating that VP8− virions contain normal amounts of the major structural virion proteins. To examine the packaging of core enzymes, Western blots were performed on virion cores with a variety of antibodies (Fig. 4B). VP8− cores had lower levels of VP8 but were not deficient in any of the 12 core enzymes examined. Therefore, the infectivity defect of VP8− virions did not appear to be due to a failure to incorporate other proteins into the virion (although the possibility of defective packaging of an untested component(s) is not ruled out by these analyses).

The rate of in vitro transcription by permeabilized VP8− virions is reduced. We performed a number of in vitro assays in order to define the infectivity defect of VP8− virions more precisely. Once a virus particle has entered the cell, early viral genes are transcribed by enzymes which enter the cell packaged in the virion. This process can be mimicked in vitro by permeabilizing virions with detergent and incubating them with nucleoside triphosphates and magnesium ions (40). The inclusion of a radioactively labeled nucleoside triphosphate in the reaction mixture allows the levels of newly synthesized RNA to be measured. Figure 5 shows the results of such an assay performed on WR, VP8+, and VP8− virions purified from cells at 24 hpi. The transcription rates of WR and VP8+ virions were very similar, whilst those of VP8− virions were much lower. After 30 min, VP8− virions had produced only 4% of the level of TCA-precipitable RNA made by VP8+ virions. This percentage varied among different virion preparations and appeared to be dependent on the multiplicity of infection and the time at which the cells were harvested for virion purification. Levels of up to 20% were observed with some preparations which were harvested at 3 days postinfection. This variability is probably explained by increased levels of VP8 in virion preparations harvested at later times of infection relative to VP8 levels in preparations harvested earlier, since repression of VP8 gene expression in the absence of IPTG is incomplete (50, 66). Also, the percentage of virus mutants or recombinants which had escaped IPTG-dependent regulation of VP8 gene expression was probably higher in cultures which were infected for longer times before harvesting.

The extrusion of RNA from permeabilized VP8− virions is defective. RNA synthesized in vitro by virus cores or by permeabilized virions accumulates transiently in the virus particle before its release via an ATP-dependent process which is independent of the rate of RNA synthesis (32, 56). The rate of release of newly synthesized RNA from permeabilized virions was measured by performing transcription reactions as described for the experiment shown in Fig. 5 and centrifuging aliquots at various times to separate released RNA from core-associated RNA. After a 30-min incubation, WR and VP8+ virions had each released 73% of the newly synthesized RNA (Fig. 6A). The levels of RNA synthesized by VP8− virions were much lower than those for VP8+ virions (compare *y* axes in Fig. 6A), as shown in Fig. 5. Notably, the rate of extrusion of RNA from VP8− virions was significantly lower than it was

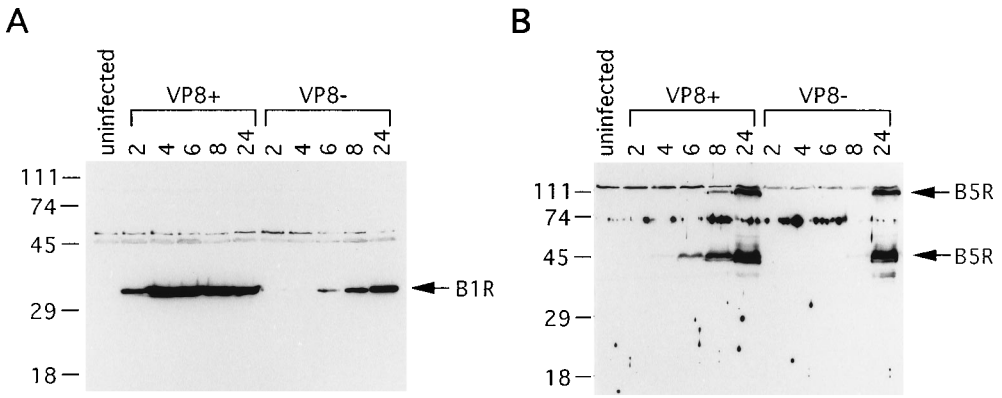

FIG. 3. Time course of early viral protein synthesis. Purified VP8+ or VP8− virions were used to infect TK$^-$143 cells at a multiplicity of infection of 10 in the absence of IPTG. Cells were harvested at 2, 4, 6, 8, and 24 hpi as described in Materials and Methods. Aliquots of each extract were electrophoresed on an SDS–12% polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with antibodies to the early virus proteins B1R (A) and B5R (B) as described in Materials and Methods. Numbers above the lanes indicate time in hours, and numbers on the left indicate molecular mass in kilodaltons.

from VP8+ virions, with only 34% being released after 30 min. When *S*-adenosyl-L-[methyl-^{3}H]methionine (Amersham International) was included in extrusion assay mixtures, the rates of methyl-^{3}H incorporation mirrored those of [^{32}P]UMP incorporation, indicating that the RNA produced by VP8− virions is capped normally (data not shown).

The size distribution of the RNAs synthesized by permeabilized virions was determined by electrophoresing the transcription products present in the core and those released from the core on a formaldehyde-agarose gel and detecting the labeled RNA by autoradiography (Fig. 6B). For all three types of virion, the size distributions of both the released and the core-associated RNAs were the same, with a peak at around 1.2 kb, as was observed previously for wild-type virions (56). Apparent differences in the range of RNA sizes among lanes were probably due to loading differences, since the big difference in levels of RNA production made it difficult to accurately quantify samples for gel loading. These data show that the RNA produced by VP8− virions in vitro is capped and of normal size but is not extruded as efficiently as it is from VP8+ virions.

Soluble extracts from VP8− virions can efficiently transcribe exogenous DNA from an early vaccinia virus promoter. The greatly diminished transcriptional activity of VP8− virions observed both in vitro and in infected cells contrasted with the normal profile of virion proteins (except VP8) and enzymes in VP8− virions. This suggested that either some other untested key transcriptional component was absent or the transcriptional enzymes were all present but were unable to efficiently utilize the VP8− DNA template. To address the latter possibility, we performed transcription assays using exogenous DNA templates. Disruption of virions with sodium deoxycholate allows the preparation of a soluble virion extract which is capable of in vitro transcription of plasmid templates containing early vaccinia virus promoters (25, 51). Extracts were prepared from WR, VP8+, and VP8− virions by sequential treatment with 0.1 and 0.2% sodium deoxycholate (+deoxycholate extracts; see Materials and Methods). The protein profiles of these extracts were indistinguishable, except for the reduced amount of VP8 in VP8− virion extracts (Fig. 7A). Unlike the situation with the major core proteins 4a and 4b, a substantial fraction of the VP8 protein was extracted by these concentrations of deoxycholate (reference 69 and data not shown). WR, VP8+, and VP8− virion extracts contained similar levels of nonspecific RNA polymerase activity when tested on an ssM13 template (Fig. 7C). Although the VP8− extracts

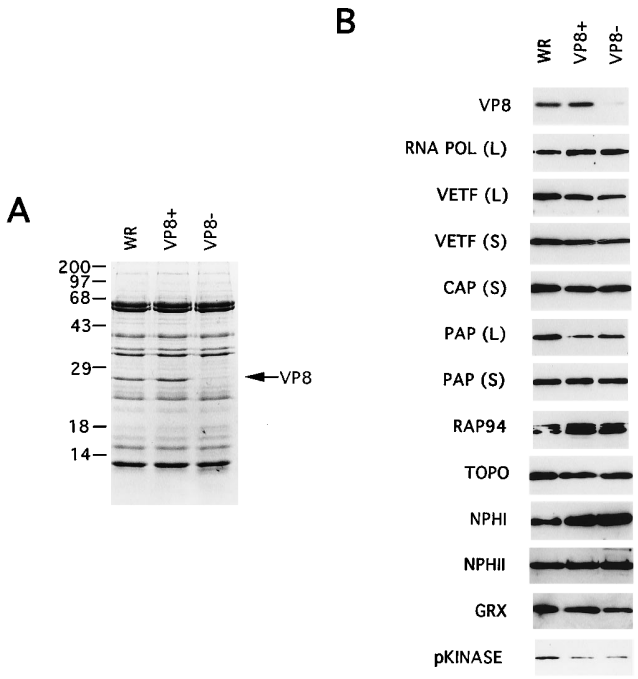

FIG. 4. Protein composition of wild-type and mutant virions. (A) Protein profiles of purified virions. Virions were purified from HeLa cells infected with wild-type virus (WR) or with vDW4 in the presence (VP8+) or absence (VP8−) of IPTG and analyzed by SDS-PAGE on a 15% gel (0.2 A_{260} units of purified virion preparation per well). Proteins were detected by Coomassie staining. The arrow indicates the position of VP8. Marker sizes (Gibco BRL) are shown in kilodaltons. (B) Immunoblots of virion cores. Virus cores prepared from purified virions (see Materials and Methods) were electrophoresed on an SDS-polyacrylamide gel (0.04 A_{260} units [top panel] or 0.125 A_{260} units [other panels] of core preparation per lane). The electrophoresed proteins were transferred to nitrocellulose and probed with one of 13 antibodies to vaccinia virus core proteins. A composite of the bands obtained is shown. Antibody targets (in descending order) were as follows: VP8 (P25K/25K), RNA polymerase (POL) (only the 147- and 132-kDa large [L] subunits are shown, though these appear as a single band at this resolution; other Western blots confirmed that all the RNA polymerase subunits were present in VP8− virions [data not shown]); vaccinia virus early transcription factor (VETF) large (L) and small (S) subunits, capping enzyme (CAP) small subunit (S), poly(A) polymerase (PAP) large (L) and small (S) subunits, 94-kDa RNA polymerase-associated protein (RAP94), vaccinia virus type I topoisomerase (TOPO), nucleoside triphosphate phosphohydrolases (NPH) I and II, glutaredoxin (GRX), and B1R protein kinase (pKINASE).

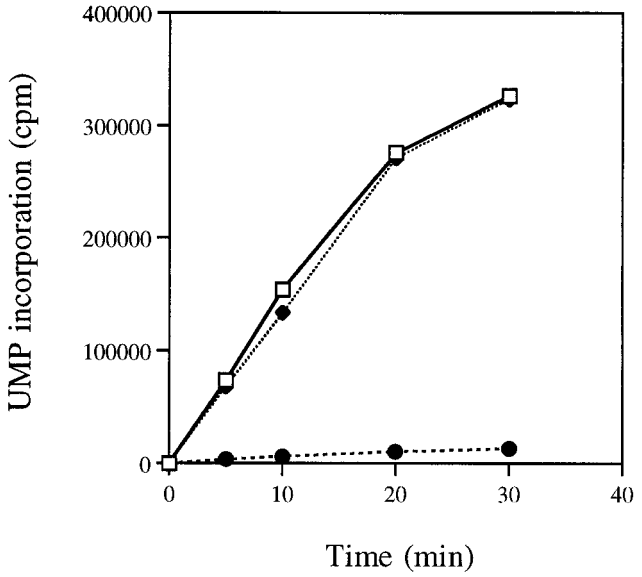

FIG. 5. In vitro transcription by permeabilized virions. Purified WR, VP8+, or VP8− virions were used in transcription assays performed in the presence of 0.05% NP-40 as described in Materials and Methods. The graph shows the incorporation of TCA-precipitable counts per minute with time. The mean number of counts per minute from duplicate experiments is shown. □, WR; ◆, VP8+; ●, VP8−.

contained wild-type levels of active RNA polymerase, it remained possible that factors conferring transcriptional specificity for early vaccinia virus promoters might be altered in these extracts. This was examined by using pSB24, a plasmid which contains a strong, synthetic early vaccinia virus promoter (12) upstream of a 382-nucleotide cassette which lacks guanylate residues in the nontemplate strand (54). Thus, specific in vitro transcription from the vaccinia virus promoter produces a transcript of approximately 390 nucleotides when the reaction is performed in the absence of GTP (37). Figure 7E demonstrates that soluble extracts prepared from all three types of virions were able to specifically transcribe a pSB24 template. Quantitative analysis with a phosphorimager showed that the level of transcription by VP8− virions was equal to that by VP8+ virions. Reactions performed for shorter time periods with titrations of virion extract also failed to detect a defect in specific transcription by VP8− virions (data not shown). Consequently, although VP8− virions showed a greatly reduced rate of early transcription of endogenous virion DNA (Fig. 5), extracts from these virions were able to efficiently transcribe from an early promoter on an exogenous plasmid DNA template (Fig. 7E).

Transcription proteins are preferentially released from VP8− virions. During initial experiments to optimize extraction conditions for the preparation of soluble virion extracts, we noticed that proteins were more easily extracted from VP8− virions than from VP8+ virions with low concentrations of sodium deoxycholate. In fact, significantly more protein was released from VP8− virions than from VP8+ virions after incubation in extraction buffer which contained no deoxycholate at all (Fig. 7B; this gel shows undiluted extracts obtained prior to passage down a DE52 column). The levels of a number of proteins released from VP8− virions were markedly reduced in comparable extracts from VP8+ virions. Variability in the efficiency of deoxycholate extraction of a number of core enzymes from different wild-type virion preparations has been noted previously (57). However, we believe that proteins from VP8− virions are inherently more easily extracted since this effect was repeatedly observed with at least three independent preparations of both VP8+ and VP8− virions.

The increased level of protein extraction from VP8− virions in the absence of deoxycholate was reflected in the elevated levels of nonspecific RNA polymerase activity detected in these extracts (Fig. 7D). The detergent-free extracts from VP8− virions were specifically enriched in transcription proteins, since 250 ng of VP8− extract synthesized 10-fold more RNA from a pSB24 template than was produced by the same quantity of VP8+ extract (Fig. 7F). These results show that the viral transcription machinery is preferentially released from VP8− virions under these conditions.

DISCUSSION

Previously, a recombinant virus, vDW4, in which VP8 gene expression was conditionally blocked by the *Escherichia coli lac* repressor protein was described (66). Despite the greatly diminished expression of this major core protein, morphologically normal intracellular and extracellular virions were formed at 80% of wild-type levels. These virions were, however, 100-fold less infectious. This paper addresses the basis for this greatly diminished infectivity and shows that VP8− virions are defective in early transcription. This defect is due not to a lack of specific transcriptional enzymes within virions but rather to the inability of these enzymes to efficiently transcribe the DNA genome packaged within VP8− virions.

VP8− virions can bind to and penetrate cells normally, but the levels of early RNA and protein synthesis are greatly reduced, though not abolished. This residual activity suggests that either some transcription can occur inefficiently in the absence of VP8 and/or the small quantity of VP8 which is present as a result of leakiness of the repression system can support a low level of transcription. The variability in the level of in vitro transcription observed, depending on the time of virion harvesting, favors the latter possibility. In this regard, it would be interesting to compare the levels of transcription by VP8− virions with those by the temperature-sensitive (*ts*) VP8 mutant *ts*85, which does not even form small plaques at the nonpermissive temperature and may therefore be less leaky than the vDW4 conditional mutant (16, 17).

The transcriptional deficiency of VP8− virions was examined in more detail in vitro by using purified VP8+ or VP8− virions. Consistent with the data obtained from infected cells, the level of transcription by VP8− permeabilized virions was drastically reduced in comparison with levels for wild-type or VP8+ virions (Fig. 5). This defect was not attributable to a lack of other virion polypeptides (Fig. 4A) or specific virion enzymes (Fig. 4B). In fact, extracts from VP8− virions showed normal, or greater, transcriptional activity on nonspecific or specific exogenous templates (Fig. 7). Two explanations, which are not mutually exclusive, seem plausible: either VP8− virions have the DNA genome packaged incorrectly so that the transcription enzymes cannot efficiently access or transcribe the template or VP8 is directly required for transcription of packaged virion DNA but not of exogenous plasmids containing vaccinia virus early promoters.

Structural virion proteins which also function in transcription have been reported for other viruses. The herpes simplex virus type 1 α-*trans*-inducing factor (αTIF, Vmw65, or VP16) is an abundant virion tegument protein which complexes with cellular proteins and activates immediate-early virus gene transcription (5, 48). The varicella-zoster virus proteins encoded by open reading frames 4 and 63 have been suggested to have dual structural and transcriptional regulatory roles (33). Also,

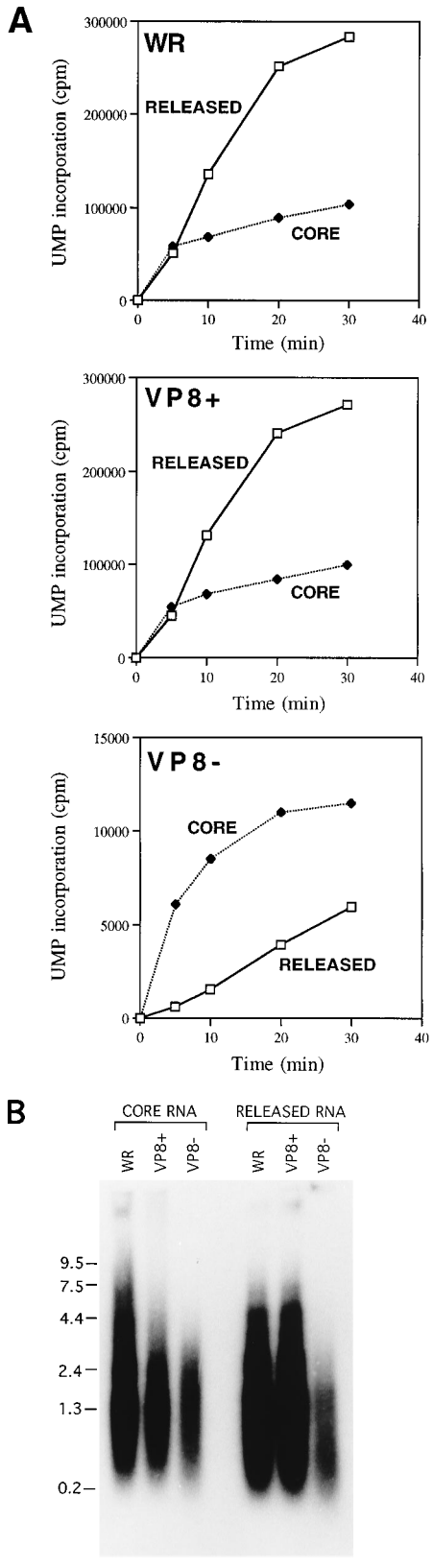

FIG. 6. RNA extrusion by permeabilized virions. (A) Rates of extrusion. Transcription reactions were performed with WR, VP8+, or VP8− virions as in the experiment shown in Fig. 5, and the products were separated into core and released fractions as described in Materials and Methods. Graphs were plotted as for Fig. 5. (B) Gel analysis of transcription products. Transcription reactions were performed as described for panel A, and the labeled products in the core and released RNA fractions were analyzed on a 1% agarose–formaldehyde gel (see Materials and Methods). RNA markers were as described for Fig. 2B.

there is circumstantial evidence suggesting that African swine fever virus DNA-binding protein, p10, may function in early transcription (41, 52).

In addition to reduced rates of transcription, we observed reduced rates of RNA extrusion from VP8− virions (Fig. 6A). Others have observed that when in vitro transcription reaction conditions are changed to alter the rate of RNA synthesis by vaccinia virus core particles, for each doubling of the rate of RNA synthesis there is an approximate doubling of the steady-state level of RNA inside the cores. Thus, even though RNA synthesis is increased, the rate of RNA release from cores remains constant, that is, the rate of RNA extrusion is independent of the rate of RNA synthesis (32). This suggests that the extrusion defect we observed was not simply a consequence of reduced transcription rates but was due to a requirement for VP8 either in the extrusion process or in the formation of a structure needed for extrusion. VP8 does not bind to RNA and is therefore unlikely to be directly involved in the extrusion process (27). The requirement for VP8 is more likely to be at the level of virion structure. In this regard, the gap observed in VP8− immature virions (66) and the increased sensitivity of VP8− virions to DOC extraction (Fig. 7) provide support for the idea that VP8 may be important for correct formation of the core periphery. Thus, the number of functional extrusion sites may be reduced in VP8− virions. We cannot rule out the possibility that reduced RNA extrusion is the cause of the decreased rate of early transcription in VP8− virions but think this is unlikely, since the quantity of RNA accumulated in VP8− virions after 30 min is only 11% of that found in VP8+ virions which are transcribing normally, suggesting that these levels of core transcripts are not inhibitory to transcription (Fig. 6A).

Studies of mammalian transcription have suggested an important role for chromatin structure in the regulation of transcription (reviewed in references 46 and 68). It has been proposed that transcriptionally repressed chromatin must be decondensed and unfolded to a derepressed state before true activation of transcription by sequence-specific transcription factors can occur (46). Sequence-independent DNA-binding proteins have been shown to regulate transcription in mammalian systems. The addition of nucleosome cores and histone H1 to naked DNA templates results in a marked decrease in RNA polymerase II transcription in vitro (36). The high-mobility-group proteins 1 and 2 (HMG 1/2) are also implicated in transcriptional regulation: HMG 2 represses transcription in a highly purified RNA polymerase II transcription system, whereas other studies report stimulation of transcription by HMG 1/2, with HMG 1 apparently enhancing gene expression by altering chromatin structure (43, 59, 62). It is interesting to compare these mammalian proteins with the two major sequence-independent DNA-binding proteins of vaccinia virus. Comparisons between VP11 and histone H1 have already been made (31), and it might be predicted that, like H1 binding, VP11 binding to DNA inhibits transcription by altering the structure of the DNA template. Parallels can also be drawn between VP8 and HMG 1/2, since both are relatively abundant, bind preferentially to ssDNA, and appear to be required for normal levels of transcription from complex templates.

Documented examples of transcription stimulation by other sequence-independent ssDNA-binding proteins (SSBs) are

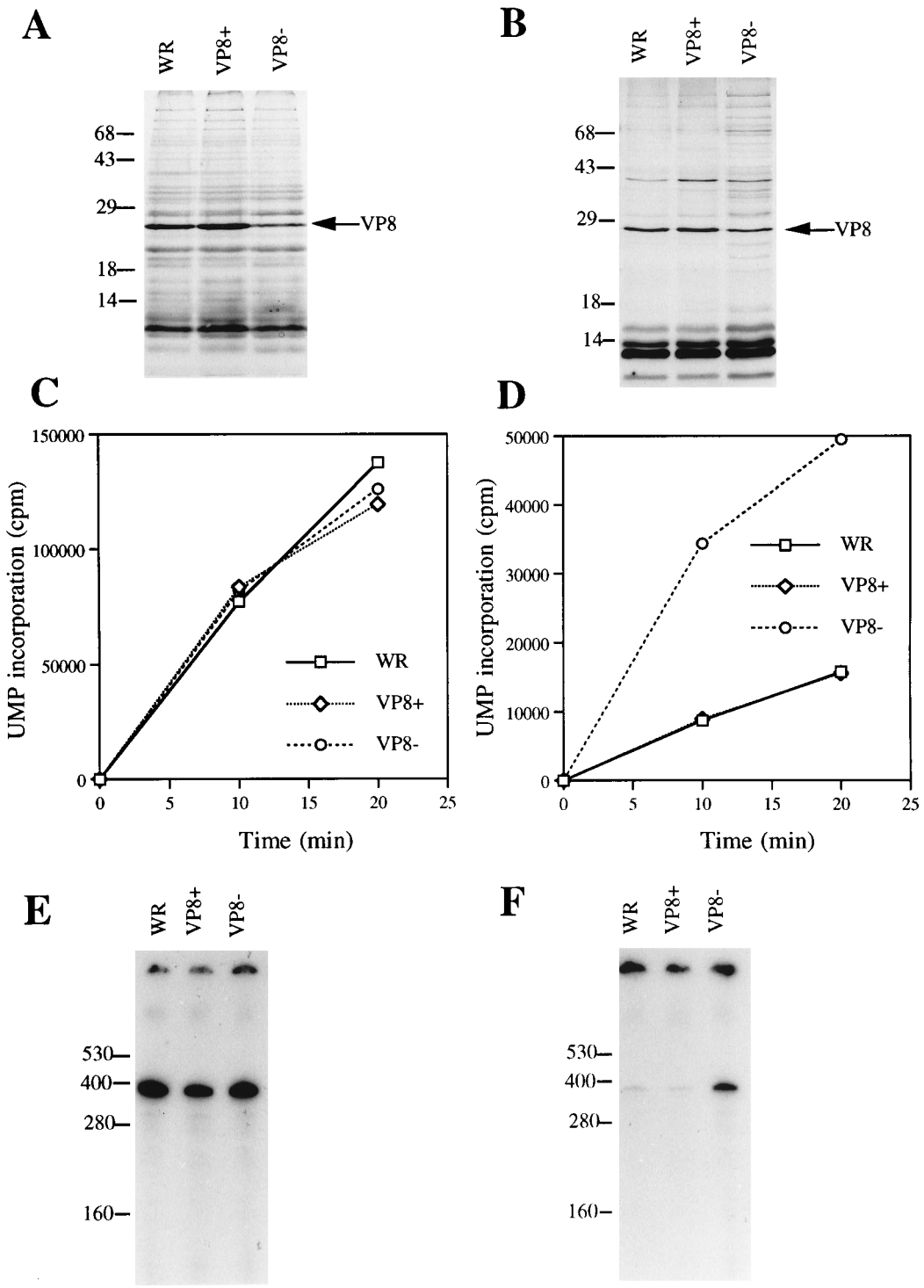

FIG. 7. Protein composition and transcription activity of soluble virion extracts. Soluble virion extracts were prepared in the presence (A, C, and E) or absence (B, D, and F) of sodium deoxycholate as described in Materials and Methods. The virions used were harvested 3 days postinfection, and the VP8− virions produced 20% of VP8+ RNA levels in permeabilized virion transcription reactions. (A and B) Protein profiles of virion extracts. Five-microliter aliquots of extracted proteins (undiluted supernatant material obtained prior to passage down a DE52-cellulose column) were run on SDS–15% polyacrylamide gels and detected by silver staining. The position of VP8 is indicated by an arrow. Sizes of protein markers are shown in kilodaltons. (C and D) Nonspecific RNA polymerase activity of virion extracts. Ten microliters of the extract material shown in panels A and B (again undiluted supernatant material obtained prior to passage down a DE52-cellulose column) was assayed for nonspecific RNA polymerase activity on an M13 template as described in Materials and Methods. (E and F) Specific transcription activity of virion extracts. Extracts which had been passed down a DE52-cellulose column and dialyzed were assayed as described in Materials and Methods by using 250 ng of extract and a plasmid template, pSB24, containing an early vaccinia virus promoter followed by a G-less cassette. Sizes of ^{32}P-labeled RNA markers (Gibco BRL) are shown in nucleotides.

rare. The adenovirus DNA-binding protein (DBP) has ssDNA binding activity and activates transcription from the adenovirus major late promoter by inducing a change in DNA structure which enhances binding of upstream stimulatory factor, a sequence-specific transcription factor (10, 72). The bacteriophage N4 makes use of two SSBs to stimulate transcription of its genes: early gene transcription by the N4 virion RNA polymerase is stimulated by the host (*E. coli*) SSB, and late transcription of N4 genes by the *E. coli* σ^{70} RNA polymerase is activated by the N4 SSB. In the first case, *E. coli* SSB is proposed to stabilize ss regions in the promoter, facilitating polymerase binding. In the second case, activation is thought to occur through direct protein-protein interactions between N4 SSB and *E. coli* σ^{70} RNA polymerase (11, 24, 38).

So, how does VP8 activate early transcription? Metabolic labeling experiments have shown that VP8− virions have a normal DNA content (66), but VP8 may be required for correct packaging of this DNA into a form which can be transcribed, as discussed previously. Alternatively, VP8 may have a more direct role in transcription. It has been shown that the vaccinia virion enzyme NPH-II is an RNA helicase (55) and also has DNA helicase activity which can unwind short DNA duplexes and can be stimulated to unwind longer DNA duplexes by *E. coli* SSB (6). Recent data have shown that VP8 also stimulates NPH-II to unwind a longer (45-bp) DNA duplex (7). *ts* mutants with mutations in the I8R gene encoding NPH-II have a phenotype similar to that of the VP8 mutant (19, 20, 66), producing morphologically normal, noninfectious virions, which contain a full complement of transcription enzymes, but are defective in early transcription (26). We therefore suggest a model whereby NPH-II and VP8 cooperate to unwind early promoter regions within the vaccinia virus DNA genome, producing a derepressed conformation to which the transcription complex can bind efficiently. Reconstituted transcription assays using DNA templates which have a higher degree of structural complexity than naked DNA could be used to test this model.

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