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The Dual-Specificity Phosphatase Encoded by Vaccinia Virus, VH1, Is Essential for Viral Transcription In Vivo and In Vitro

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The genetic complexity of vaccinia virus is such that as well as encoding its own transcription and replication machinery, it encodes two protein kinases and a protein phosphatase. The latter enzyme, designated VH1, is a prototype for the dual-specificity class of phosphatases. Here we report that the H1 phosphatase is encapsidated within vaccinia virions and describe the construction of a viral recombinant in which expression of the H1 gene is regulated by the presence or absence of isopropylthiogalactopyranoside (IPTG) in the culture medium. When expression of H1 is repressed, the number of viral particles produced is not compromised but the fraction of these particles which is infectious is significantly reduced. The lack of infectivity of the H1-deficient particles is specifically correlated with their inability to direct the transcription of early genes either in vitro or in vivo. A proximal role for the viral phosphatase in regulating the onset of viral gene expression is implied. Prominent among the encapsidated proteins found to be hyperphosphorylated in H1-deficient virions is the 11-kDa product of the F18 gene; this protein is the major DNA-binding component of the viral nucleoprotein complex. The ability of recombinant H1 phosphatase to reverse this hyperphosphorylation in permeabilized virions strengthens the conclusion that the F18 protein is a bona fide substrate for the H1 phosphatase.

The profound roles that phosphorylation and dephosphorylation of proteins play in the regulation of biological phenomena are increasingly apparent. The orderly progression of the eukaryotic cell cycle and the cascade of intracellular signal transduction are two examples of processes driven and modulated by the phosphorylation state of key components. Among the many facets of protein function affected by phosphorylation are subcellular localization, participation in multiprotein complexes, enzymatic activity, and interaction with nucleic acids. The repertoire of protein kinases involved in this regulatory network is extensive, as exemplified by the family of cyclin-dependent kinases (cdks) that coordinate cell cycle progression and the Raf/MEK/ERK kinase cascade that mediates signal transduction. An understanding of the specific phosphatases that serve to counterbalance these kinases is just now emerging (51). A role for phosphatase action in a variety of processes has been inferred from the use of pharmacological inhibitors, but in only a few cases have specific enzymes been linked to specific pathways. Phosphatases with specificity for serine/threonine or tyrosine residues, or for both, have been identified. Among the latter class of dual-specificity enzymes, cdc25 is known to activate cdc2 at the G_2/M transition (6, 10, 30), and the MKP-1 phosphatase is known to reverse the MEK-induced activation of ERK (34, 50, 57). Other members of this class of enzymes have been shown to be induced by mitogens, oxidative/heat stress, or starvation (13, 20, 50), implicating them in the signalling process.

The original and prototypic member of the dual-specificity class of phosphatases is the H1 protein encoded by vaccinia virus (11). The vaccinia virus H1 protein has been expressed in *Escherichia coli*, and its enzymatic activity has been shown to be resistant to okadaic acid, to be sensitive to vanadate, and to involve a catalytic cysteine residue (12). However, neither the authentic substrates nor the facets of the viral life cycle regulated by phosphatase action have been identified. Vaccinia virus infection, which occurs solely within the cytoplasm of infected cells, is characterized by an unusual degree of physical and genetic autonomy from the host (31, 32, 52, 53). The 192-kb viral genome encodes virtually all of the proteins required for transcription of the three temporal classes of genes and replication of the viral DNA, as well as numerous modulators of the host inflammatory and immune responses (48). In addition to the phosphatase, vaccinia virus also encodes two serine/threonine kinases (2, 27, 28, 36, 54, 55), suggesting that its life cycle, much like that of eukaryotic cells, is regulated by a network of phosphorylation and dephosphorylation events. The relative simplicity of the viral system, however, offers some experimental advantages not easily achieved with cellular systems. We therefore felt that a directed genetic approach would allow a precise dissection of the role of the H1 phosphatase and that such an analysis would have implications for the understanding of phosphatases in general.

MATERIALS AND METHODS

Cells and viruses. Stocks of wild-type (*wt*) vaccinia virus (WR strain) were prepared in suspension cultures of mouse L cells; vLacI and v*ind*H1 were amplified in monolayer cultures of monkey BSC40 cells. Virus was purified from cytoplasmic lysates by ultracentrifugation through 36% sucrose. Titrations were performed on BSC40 cells.

Construction of an H1 null allele by replacement with the Neo^r gene. A plasmid containing 972 bp of sequences derived from the vaccinia *Hin*dIII H fragment (pUC-*Hin*cII-*Sca*I) was cleaved with *Bst*EII (+86) and *Bcl*I (+296), releasing a 211-bp fragment representing 40% of the H1 gene. The Neo^r gene under the regulation of a constitutive viral promoter was excised from pVV:NEO (8) and inserted in place of the deleted H1 segment. Two pH1:NEO constructs, containing the Neo^r cassette in both orientations relative to the flanking sequences, were isolated. These plasmids retained 307 bp of upstream and 454 bp of downstream flanking sequence to facilitate recombination into the vaccinia virus genome. Thirty-five-millimeter-diameter dishes of BSC40 cells were infected with *wt* virus at a multiplicity of infection (MOI) of 0.03 PFU; at 3 h postinfection (p.i.), replicate cultures were transfected with 3.5 μg of a calcium phosphate precipitate of either supercoiled or linearized pH1:NEO DNA. G418

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(2 mg/ml) was added to the medium at 15 h p.i., and cultures were harvested at 3 days p.i. Neo^r isolates from each infection/transfection were purified by two rounds of plaque purification. Isolates were obtained from 18 original infections/transfections, 10 of these having received linearized pH1:NEO (5 in each orientation) and 8 having received supercoiled pH1:NEO (4 in each orientation). Genomic DNA within expanded viral stocks was examined by dot blot hybridization using nick-translated probes representing either the *Hin*dIII D genomic fragment (to normalize the levels of DNA examined), the Neo^r gene, or the 211-bp internal *Bst*EII-*Bcl*I fragment derived from the H1 gene and deleted during the preparation of pH1:NEO.

Preparation and isolation of recombinant vLacI. Plasmid pPR2 (38) contains a copy of the vaccinia virus thymidine kinase (TK) gene that has been disrupted by the insertion of the *lac*I gene under the regulation of the viral p7.5 promoter. This plasmid was used to create the *lac* repressor-expressing virus vLac**I**. Confluent monolayers of TK$^-$143 cells (35-mm-diameter dish) were infected with *wt* vaccinia virus at an MOI of 0.03; at 3 h p.i., linearized pPR2 DNA (3.5 μg) was added in the form of calcium phosphate precipitate. Cells were harvested at 3 days p.i., and TK$^-$ viruses were twice plaque purified on TK$^-$143 cells in the presence of bromodeoxyuridine (50 μg/ml). Insertion of the *lac*I gene into the viral genome was confirmed by DNA dot blot hybridization.

Preparation and isolation of recombinant v*ind*H1. (i) PCR. Recombinant PCR was performed as described previously (61). The primers used to insert the *lac* operator immediately downstream of the endogenous H1 gene promoter were 5′ GggaattCAGTACTCTTAAGAGAGC 3′(primer 1), 5′ C*GAATTGTGAGCGCTCACAATTC*ATGGATAAGAAAAGTTTGT 3′(primer 2), 5′ *TGAGCGCTCACAATTC***GATTTA**TAACGTACAAAT 3′(primer 3), and 5′ GGgatcc TATTCCATATTACTAA 3′(primer 4). Using a plasmid containing the *Hin*dIII H fragment of the vaccinia virus genome as a template, a PCR was performed with primers 1 and 2 to obtain a fragment containing the operator-flanked H1 gene. A PCR performed with the same template and primers 3 and 4 yielded a fragment extending from the *lac* operator through the H1 promoter and the adjacent H2 gene. Portions of the two PCR products, which overlapped by 16 bp, were then used together as templates in a third PCR performed with primers 1 and 4. This reaction yielded a DNA fragment (H1*op*H2) spanning the H1 and H2 genes and within which the 22-bp *lac* operator sequence (shown in italics) had been placed immediately downstream of the H1 promoter (boldface) and upstream of the initiating ATG codon (underlined). This PCR product was cleaved with *Bam*HI (the site is shown in lowercase in primer 4) and cloned into a pUC19 backbone that had been previously cleaved with *Bam*HI and *Sma*I; its DNA sequence was confirmed by using a Sequenase version 2 kit (United States Biochemical). The insert was then recloned into a plasmid that also contained the Neo^r gene (excised from pVV:NEO [8]) under the regulation of a constitutive viral promoter. This construct (pUCneoH1*op*H2) was used for transfection to generate recombinant v*ind*H1 virus. Two additional primers, 5′ CACATTAT TGGTTACCCTTGTCATTATAGT 3′ and 5′ CCTTTTCTCTAACGTATTCT 3′, were used to confirm the insertion of the *lac* operator sequences within the genomes of recombinant viruses (see below).

(ii) Generation of v*ind*H1. v*ind*H1 was isolated by transient dominant selection (7, 61). Confluent monolayers of BSC40 cells (60-mm-diameter dish) were infected with vLac**I** at an MOI of 0.03; at 3 h p.i., supercoiled pUCneoH1*op*H2 DNA (10 μg) was applied in the form of calcium phosphate precipitate, and isopropylthiogalactopyranoside (IPTG) was added to 5 mM. G418 was added to a concentration of 2 mg/ml at 15 h p.i. Cells were harvested at 3 days p.i., and Neo^r viruses were twice plaque purified in the presence of G418 and IPTG (2 mg/ml and 5 mM, respectively). A third round of plaque purification was then performed in the absence of G418; DNA dot blot hybridization was performed to screen the plaques which had lost Neo^r by homologous recombination between the tandemly repeated H1 and H2 genes. Resolved plaques were subjected to a second round of plaque purification and then examined phenotypically (titration with or without IPTG) and by PCR screening to distinguish those that contained a *wt* H1 locus from those containing the H1*op*H2 locus. v*ind*H1 isolates formed smaller plaques than *wt* virus and gave PCR products of 198 bp (rather than 173 bp) when assayed with primers flanking the H1 promoter. The presence of *lac* operator sequences within these PCR products was confirmed by Southern blot analysis.

Preparation of H1$^+$ and H1$^-$ viral stocks. Forty 15-cm-diameter dishes of BSC40 cells were infected with v*ind*H1^{131} at an MOI of 5. The inoculum was removed at 1 h p.i. and replaced with Dulbecco modified Eagle medium–5% fetal calf serum with and without 5 mM IPTG for H1$^+$ and H1$^-$ stocks, respectively. Cultures were harvested at 24 h p.i., and virus was purified from cytoplasmic extracts by ultracentrifugation through a 36% sucrose cushion and banding on a 25 to 40% sucrose gradient. Purified virus was resuspended in 1 mM Tris (pH 9.0). Virions were quantitated by measuring the optical density at 260 nm (OD_{260}), assuming that 1 OD unit corresponds to 1.2×10^{10} particles per ml. Infectious virus was quantitated by titration in the presence of 5 mM IPTG.

Preparation of ^{32}P-labeled *wt* and H1$^-$ virions. Confluent monolayers of BSC40 cells were infected with either *wt* virus or v*ind*H1 at an MOI of 1. After adsorption of the inoculum for 1 h, cultures were maintained in the absence of IPTG in phosphate-free Dulbecco modified Eagle medium supplemented with 5% dialyzed fetal calf serum (dialyzed against Tris-buffered saline) and $^{32}P_i$ (50 μCi/ml). Cells were harvested at 24 h p.i., and ^{32}P-labeled virions were purified by ultracentrifugation as described above.

Analysis of viral adsorption and entry. Confluent monolayers of BSC40 cells (35-mm-diameter dishes) were inoculated with 840 particles of *wt*, H1$^+$, or H1$^-$ virions. After 40 min of adsorption (or immediately, as indicated), monolayers were washed five times with phosphate-buffered saline and treated with 0.05% trypsin–0.53 mM EDTA. Cells were then recovered by centrifugation; cellular lysates were fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the levels of cell-associated F18 protein were determined by immunoblotting, using an alkaline phosphatase-conjugated second antibody and colorimetric development (Bio-Rad).

NP-40 and α-toxin permeabilization of purified virions and in vitro transcription. Nonidet P-40 (NP-40) permeabilization was accomplished by incubation of virions in 50 mM Tris (pH 8.0)–50 mM dithiothreitol (DTT)–0.05% NP-40 for 30 min at 37°C. When appropriate, subviral cores were isolated from permeabilized virions by sedimentation. For permeabilization with *Staphylococcus aureus* α-toxin, virions were incubated in 50 mM Tris-HCl (pH 8.0) for 30 min at 37°C in the presence of 30 U of α-toxin (Calbiochem) per ml. In vitro transcription was then performed as previously described (46), with slight modifications. Briefly, purified vaccinia virions ($0.023\ A_{260}$ unit, 2.8×10^8 virions) were incubated at 37°C in a 100-μl reaction mixture containing 60 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM DTT, 5 mM ATP, 1 mM CTP, 1 mM GTP, 0.2 mM [α-^{32}P]UTP (1,500 mCi/mmol), and 10 μM adenosylmethionine. At various times, aliquots were removed from the reaction mixture and quenched on ice for >30 min with an equal volume of 20% trichloroacetic acid–0.2 M Na_2PPi. Trichloroacetic acid-insoluble precipitates were recovered on glass fiber filters, and [α-^{32}P]UTP incorporation was determined by β-scintillation counting.

Expression and purification of recombinant H1. The H1 open reading frame was inserted into pET14B, using a combination of genomic sequences and sequences generated by PCR. PCR was used to synthesize an N-terminal fragment extending from the initiating ATG codon to a *Bst*EII site located 87 nucleotides (nt) downstream. The upstream primer (5′ GGG*GTCAAC***CATATG** GATAAGAAAAGTTTG 3′) introduced *Hin*cII (italicized) and *Nde*I (boldface) sites upstream of and overlapping, respectively, the initiating ATG (underlined); the downstream primer (5′ CACATTATT**GGTTACC**CTTGTCATTATAGT 3′) extended through the *Bst*EII site (boldface). The PCR product was digested with *Nde*I and *Bst*EII. The remainder of the H1 open reading frame was obtained from a cloned genomic fragment containing sequences extending from this *Bst*EII site to an *Eco*RV site located 42 nt downstream of the TAA termination codon. The reconstructed gene was inserted into a pET14B vector that had been previously digested to have *Nde*I and *Bam*H1 (blunt) termini. This vector was predicted to encode a modified H1 protein containing a 20-amino-acid 5′ extension including a hexahistidine tag.

HMS174 transformants carrying pET14B/H1 were induced to express H1 by infection with bacteriophage lambda CE6, which encodes the T7 RNA polymerase (21, 22, 36, 49). After adsorption, infected cultures were maintained at 37°C with vigorous agitation for 1.5 h. Cells were harvested by sedimentation and resuspended at 1/25 the original culture volume in 20 mM Tris (pH 7.9)–500 mM NaCl–5 mM imidazole. After three cycles of freeze-thawing, Triton X-100 was added to 0.1%, and lysates were vortexed for 15 s and sonicated for 30 s. Insoluble material was removed by sedimentation at $12{,}000 \times g$ for 30 min at 4°C. To purify His-tagged H1, soluble lysates were applied to nickel-Sepharose columns (Novagen, Madison Wis.). Columns were washed sequentially with binding buffer and then with binding buffer containing increasing concentrations of imidazole; H1 carrying the hexahistidine tag eluted between 150 and 250 mM imidazole. Pooled samples were dialyzed against 50 mM Tris (pH 7.4)–50 mM NaCl–1 mM DTT, and samples were concentrated by the direct addition of polyethylene glycol to the dialysis bag. Protein concentration was determined using the Bio-Rad colorimetric assay with lysozyme as a standard.

Phosphatase activity was measured by monitoring the OD_{410} after incubation with the colorimetric substrate *p*-nitrophenyl phosphate (pNPP). Reaction mixtures contained 50 mM Tris (pH 7.4), 50 mM DTT, and 15 mM pNPP.

Expression and purification of recombinant H1^{c110s}. The original plasmid (pGEX-KG) containing the *cys*110*ser* allele of the H1 phosphatase was kindly supplied by J. Dixon (University of Michigan) (11). The phosphatase sequences were released as a fragment containing *Eco*RI (blunt)-*Bam*HI termini and ligated into a pET14B vector previously treated to have *Xho*I (blunt)-*Bam*HI termini. The resulting plasmid was predicted to encode a derivative of H1^{c110s} containing a 25-amino-acid N-terminal extension including a hexahistidine tag. Expression and purification of the mutant phosphatase were performed as described above for the *wt* allele.

Phosphatase treatment of ^{32}P-labeled virions. ^{32}P-labeled virions (4×10^8 particles) were incubated with 1 μg of H1 phosphatase or H1^{c110s} phosphatase for 90 min at 37°C in 40-μl reaction mixtures containing 50 mM Tris (pH 8.0) with or without 50 mM DTT as indicated and with or without 0.05% NP-40 as indicated. Reaction mixtures were heated to 100°C prior to the addition of Laemmli sample buffer and then fractionated on SDS–17% polyacrylamide gels. Gels were transferred to a polyvinylidene difluoride membrane, which was then examined by autoradiography. The same membrane was subsequently incubated simultaneously with three antisera directed against the 11-kDa F18 protein, the H1 phosphatase, and the L4 protein and developed colorimetrically after incubation with a second antibody as instructed by the manufacturer (Bio-Rad).

Preparation of anti-H1, anti-F18, and anti-L4 antisera. The majority of the H1 open reading frame was cloned into a pATH vector to allow production of a

TrpE-H1 fusion protein (23). A *Bst*EII (+86)-*Eco*RV (+556) fragment was excised from pUC-*Hin*cII-*Sca*I, made blunt ended with the Klenow fragment of DNA polymerase I, and inserted into *Sma*I-digested pATH11 DNA. *E. coli* HB101 transformants containing this plasmid directed the inducible synthesis of a 54-kDa polypeptide containing the amino-terminal 336 residues (37 kDa) of *E. coli* TrpE protein and amino acids 30 to 171 of the H1 protein (17 kDa). The F18 gene was amplified by PCR using appropriate primers, and an *Eco*RI-*Bam*HI fragment extending from nt 3 through the rest of the gene was cloned into a pATH3 vector. *E. coli* transformants containing this plasmid directed the inducible synthesis of a 48-kDa fusion protein containing 37 kDa of *E. coli* TrpE and 11 kDa of F18. The L4 gene was amplified by PCR using appropriate primers and inserted into a pUC vector; a *Bgl*II-*Hin*dIII fragment extending from nt 115 of the L4 gene through the stop codon into adjacent vector sequences was inserted into a pATH11 vector previously digested with *Bam*HI and *Hin*dIII. This vector directed the inducible synthesis of a fusion protein containing 37 kDa of the *E. coli* TrpE protein and 25 kDa of the L4 protein (containing all but the N-terminal 38 amino acids of the initial translation product and all but 6 amino acids of the mature, proteolytically cleaved protein). The fusion proteins were excised from SDS-polyacrylamide gels and used to inoculate rabbits. The specificity of the resultant polyclonal antisera was confirmed by immunoblotting and immunoprecipitation.

RESULTS

The vaccinia virus H1 gene is known to be expressed at late times after infection and to encode a 20-kDa, dual-specificity protein phosphatase (11, 40). We have taken a directed genetic approach to determine what role(s) the phosphatase plays in the viral life cycle. To determine whether the H1 gene is essential, we initially attempted to construct viral isolates containing only an interrupted, null allele of the H1 gene.

Viruses lacking a functional H1 allele are not viable. Targeted insertion of a dominant selectable marker has been used effectively to inactivate nonessential vaccinia virus genes; conversely, the failure to recover viruses with interrupted alleles has been considered compelling evidence of a gene's essentiality. Our targeting vector contained the H1 gene and flanking sequences. A central 211-bp sequence from within the H1 gene was removed and replaced with a Neo^r gene under the control of a constitutive viral promoter (8, 36). Infected cells were transfected with circular or linearized plasmid DNA, and Neo^r isolates were twice plaque purified. Genomic DNA from 18 isolates, each representing an independent infection/transfection, was analyzed by Southern dot blot hybridization using an internal H1 fragment as a probe. All 18 Neo^r viruses had retained an intact H1 allele (data not shown). Were the H1 gene nonessential, the tandemly repeated H1 alleles resulting from integration of the circular plasmid would be predicted to recombine, leaving a genome containing only an interrupted H1 allele. Likewise, transfection with linearized DNA would be predicted to direct a replacement of the H1 locus with the H1/Neo^r cassette via a double-crossover event. That none of the plaques recovered contained this genomic structure strongly suggested that the H1 gene was essential for virus viability.

Construction of a viral recombinant in which the H1 gene is under the control of the *lac* repressor/operator system. Given the essential nature of the H1 gene, an investigation of its role in the viral life cycle was clearly of interest. In the absence of any temperature-sensitive mutants with lesions in the H1 gene, we chose to construct a viral recombinant in which H1 expression could be controlled experimentally. The strategy chosen was to appropriate the regulatory components of the *E. coli lac* operon and manipulate H1 expression by performing infections in the presence or absence of IPTG. The *lac* operator was inserted immediately downstream of the H1 promoter by recombinant PCR technology; this allele was inserted into the viral genome in place of the endogenous H1 gene by transient dominant selection (7, 59, 60). Briefly, circular plasmid DNA containing both a Neo^r gene and the H1-operator-H2 sequence was introduced into cells infected with a virus containing the *lac* repressor gene within the nonessential TK locus (vLacI). Neo^r viruses were twice plaque purified, and then the tandemly repeated H1-H2 sequences were allowed to resolve by passage in the absence of G418. Two further rounds of plaque purification were performed in the absence of G418, and plaques that had successfully resolved (and hence lost the integrated Neo^r gene) were identified by Southern dot blot hybridization. To distinguish viral isolates that had retained only an operator-flanked H1 allele from those that had reverted to the *wt* genomic structure, a combination of phenotypic screening and PCR analysis was used. The results of PCR analysis of two such plaque-purified isolates (designated v*ind*H1) are shown in Fig. 1. Using primers flanking the H1 promoter, we observed a 173-nt product with a *wt* genomic template, whereas the recombinant genomic DNA directed the amplification of a 198-nt product. Southern hybridization analysis of the PCR products confirmed that this increase in product length reflected the insertion of the *lac* operator sequences (not shown).

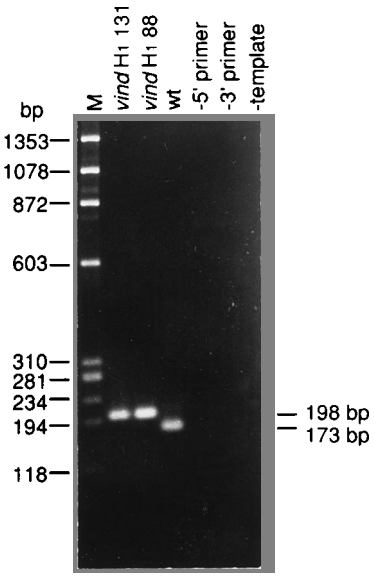

FIG. 1. Confirmation of the insertion of *lac* operator sequences in v*ind*H1 isolates by PCR. Viral DNA was prepared from BSC40 cells infected with either *wt* virus or two isolates of v*ind*H1. Five nanograms of each genomic DNA was used as a template in PCRs containing primers flanking the H1 promoter. Control reactions lacking either the 5′ primer, 3′ primer, or template are also shown. Lane M contains DNA standards whose sizes are shown at the left. The 173- and 198-bp products directed by *wt* and v*ind*H1 genomic DNAs, respectively, are indicated at the right.

S1 nuclease analysis was used to compare the levels of H1 transcription in the presence and absence of IPTG. As shown in Fig. 2, IPTG had no effect on H1 transcription during infections with the parental vLacI virus (lanes 3 and 4) but was clearly required for significant expression from the v*ind*H1 isolate (lanes 5 and 6). We estimate that the residual expression observed under repressed conditions was <15% of that seen in the presence of IPTG induction.

Repression of H1 expression causes a reduction in plaque size and a decrease in the yield of infectious virus. As a first examination of the impact of reduced H1 expression on the viral life cycle, vLacI and two isolates of v*ind*H1 (v*ind*H1^{88} and v*ind*H1^{131}) were titrated in the presence and absence of IPTG (Fig. 3). As expected, no effect of IPTG on plaque size or morphology was seen with vLacI. However, a clear reduction

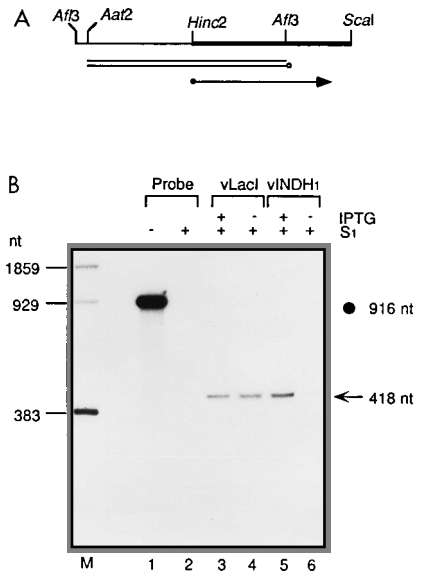

FIG. 2. S1 analysis of the steady-state level of the H1 transcripts. BSC40 monolayers were infected with vLacI or v*ind*H1 at an MOI of 15, and cultures were maintained in the presence or absence of IPTG as indicated. At 8 h p.i., cells were harvested and total RNA was isolated. Twenty-four micrograms of each RNA preparation was hybridized with an H1-specific radiolabeled probe at 42°C and subjected to S1 nuclease treatment and denaturing electrophoresis (37). (A) An appropriate DNA plasmid was radiolabeled at a 5′ terminus (filled circle) generated by *Aff*III digestion and then subdigested with *Aat*II to yield a singly radiolabeled 916-bp probe. The mRNA is shown schematically beneath the probe by a solid line with an arrowhead. (B) Autoradiograph illustrating the results of the S1 nuclease mapping. The positions to which the input probe (916 nt) and protected fragments (418 nt) migrated are indicated by a filled circle and an arrow, respectively. The control reactions shown in lanes 1 and 2 contained no RNA; for lanes 3 to 6, the type of infection from which the RNA sample was prepared is indicated above the lanes. Lane M, DNA molecular weight standards, with sizes indicated at the left.

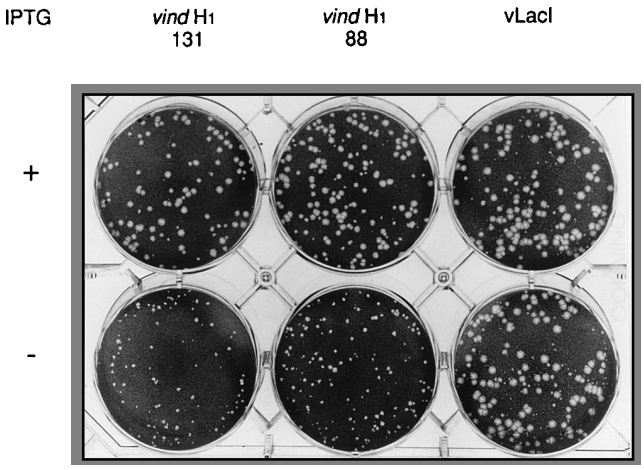

FIG. 3. Plaque morphology of v*ind*H1 isolates and vLacI titrated in the presence or absence of IPTG. BSC40 monolayers in a six-well cluster dish were inoculated with approximately 130 PFU of either v*ind*H1^{131}, v*ind*H1^{88}, or vLacI, and the cultures were maintained in the presence or absence of 5 mM IPTG as indicated. At 48 h p.i., the medium was removed and plaques were visualized by staining with crystal violet.

in plaque size in the absence of IPTG was observed for both v*ind*H1 isolates. Average plaque diameter in the absence of IPTG was approximately one-third of that seen under induced conditions.

Plaque formation requires both the formation of infectious viral progeny (predominantly intracellular mature virions [IMV]) and the maturation of a small subset of these virions to the cell-associated enveloped (CEV) and extracellular enveloped (EEV) forms. This subset of virions becomes doubly wrapped by membranes of the *trans* Golgi network and migrates to the cell surface. Upon fusion of their outermost lipid bilayer with the plasma membrane, the enveloped viruses are delivered to the extracellular environment. CEV particles remain tethered to the cell surface and are thought to mediate local cell-cell spread, whereas EEV particles are implicated in long distance dissemination (3). To determine whether the small-plaque phenotype of v*ind*H1 reflected a specific deficit in CEV formation and spread or a reduction in total virus production, the yield of infectious virus produced during a 24-h infectious cycle was determined. Cultures were infected with either vLacI or v*ind*H1 at various MOIs and in the presence or absence of IPTG, and total cell-associated virus (IMV plus CEV) was harvested at 24 h p.i. Viral yields were then titrated in the presence of IPTG. Representative results are shown in Table 1. The yields from vLacI-infected cultures were equivalent whether or not IPTG was present in the culture medium. However, the yields from v*ind*H1 infections performed in the absence of IPTG were only 10 to 20% of those obtained in the presence of IPTG.

This reduction in viral yield might reflect a reduction in the number of particles produced or in their specific infectivity (i.e., the particle/PFU ratio). To resolve this question, the yields of v*ind*H1 recovered from infections (MOI of 5) performed in the presence of IPTG ($H1^+$) and in the absence of IPTG ($H1^-$) were purified on sucrose gradients, quantitated by measuring the OD_{260}, and visualized by transmission electron microscopy. No significant difference was seen in the particle yields from repressed and induced v*ind*H1 infections (Table 2). However, when serial dilutions of these purified preparations were titrated in plaque assays, the particle/PFU ratio was five- to sevenfold higher for the $H1^-$ preparations than for the $H1^+$ preparations. A direct comparison of the number of plaques formed by inocula containing equivalent numbers of $H1^+$ and $H1^-$ particles confirmed this difference in specific infectivity (data not shown). We have prepared a number of $H1^+$ and $H1^-$ stocks in parallel; although the exact particle/PFU ratio varies with experiment, the relative ratios obtained for the two stocks are constant. We have also observed that the particle/PFU ratio of $H1^+$ stocks is higher than that seen for *wt* stocks prepared in parallel.

TABLE 1. Quantitation of virus harvested from BSC40 cells 24 h after infection with vLacI or v*ind*H1 in the presence or absence of IPTG[a]

Virus and MOI (PFU/cell)	Viral yield (PFU/ml)		−IPTG/+IPTG (%)
	+IPTG	−IPTG	
vLacI			
0.03	2.0×10^6	2.0×10^6	100
2	3.0×10^7	3.0×10^7	100
15	4.0×10^7	3.9×10^7	97.5
v*ind*H1			
0.03	1.0×10^6	1.0×10^5	10
2	1.0×10^7	2.0×10^6	20
15	8.0×10^6	1.5×10^6	18.8

[a] Confluent 35-mm-diameter dishes of BSC40 cells were infected with the virus shown at the indicated MOI and in the presence or absence of 5 mM IPTG; at 24 h p.i., cells were harvested and disrupted and viral yields were titrated in the presence of IPTG.

TABLE 2. Comparison of particle/PFU ratio in $H1^+$ and $H1^-$ virion preparations

Virions[a]	Particle yield[b] (particles/ml)	Infectious virus yield[c] (PFU/ml)	Particle/PFU ratio
$H1^+$	3.37×10^{11}	6×10^9	56
$H1^-$	2.36×10^{11}	7.5×10^8	315

[a] Confluent 150-mm-diameter dishes of BSC40 cells were infected with *vind*H1 at an MOI of 5 in the presence of 5 mM IPTG; cells were harvested at 24 h p.i., and virions were purified by ultracentrifugation through sucrose gradients. The resulting virions were designated as $H1^+$; $H1^-$ virions were similarly prepared except that IPTG was absent from the culture medium.

[b] Determined by measuring the OD_{260}, assuming that 1 OD unit equals 1.2×10^{10} particles per ml.

[c] Determined by plaque assay. Serial dilutions of purified virion preparations were titrated on BSC40 cells in the presence of 5 mM IPTG.

A reduction in the level of H1 protein encapsidated within $H1^-$ virions is correlated with the decreased fraction of particles which are infectious. The data shown above indicated that, relative to $H1^+$ preparations, fewer $H1^-$ virions were capable of initiating productive infections. However, virion morphogenesis per se was not compromised. Equivalent numbers of particles with normal morphology and protein profiles were formed (data not shown). We hypothesized that either H1 must act late during infection to ensure the infectivity of the particles produced or that it is itself encapsidated within these particles. Virion proteins were fractionated by SDS-PAGE and analyzed by immunoblotting, using a polyclonal antiserum raised against a TrpE-H1 fusion protein. As shown in Fig. 4A, H1 could clearly be detected within virions and was shown to localize to the subviral core, an internal structure containing a subset of structural proteins, the nucleoprotein genomic complex, and an array of transcriptional enzymes. Comparison of the immunoreactive signals seen with virion preparations and infected cell lysates revealed that H1 is highly enriched during encapsidation (data not shown). From these data, we conclude that H1 is a bona fide virion component and not a fortuitous contaminant. Figure 4B presents an immunoblot analysis of serial dilutions of *wt*, $H1^+$, and $H1^-$ virions. *wt* virions appear to contain sixfold more H1 than an equivalent number of $H1^+$ particles, and the H1 content of $H1^-$ virions is reduced by a further sevenfold. Comparison of the immunoreactive signal seen in virions with that obtained with known amounts of purified, recombinant H1 (data not shown) indicates that approximately 200 molecules of H1 are encapsidated per *wt* virion.

Relative to an equivalent number of $H1^+$ virions, $H1^-$ virions contain, on average, one-seventh of the amount of H1 protein and one-seventh as many infectious particles. These data suggest that a reduction in the level of encapsidated H1 protein below a certain threshhold abrogates the ability of vaccinia virions to initiate a productive infection. It is worth reiterating that $H1^-$ virions show a quantal loss of infectivity; i.e., very few virions were infectious at all, rather than all virions showing a reduction in infectivity (for e.g., inducing slower infections or yielding reduced bursts). Moreover, the block to infectivity for $H1^-$ virions must reside at an early stage in infection, because titrations of the $H1^-$ virions were performed in the presence of IPTG. Under these conditions, those infections that progressed to the onset of late gene expression would then continue under conditions permissive for H1 expression, permitting virus production and plaque formation. To demonstrate this more clearly, $H1^+$ and $H1^-$ virions were applied to cells at a multiplicity of 28 particles per cell. This was equivalent to 0.5 PFU per cell for $H1^+$ and 0.07 PFU per cell for $H1^-$. $H1^+$ infections were maintained in the presence of IPTG; $H1^-$ infections were maintained in the absence of IPTG. One would predict that one-half of the cells would be productively infected with $H1^+$, yielding a sizeable burst of virus. On the contrary, we predicted that only in one-seventh as many of the cells infected with $H1^-$ would infection proceed until the onset of late gene expression, and then only one-seventh as much H1 would be produced and encapsidated in the absence of IPTG. Hence, the overall yield of infectious virus would be 1/49 of that obtained from the $H1^+$ infections. This prediction was borne out; as shown in Table 3, a 53.8-fold difference in infectious viral yield was obtained.

$H1^-$ virions are not deficient in cellular entry. To assess at what stage the infectious cycle initiated by the majority of $H1^-$ virions aborted, we first looked at the stage of cellular entry. Cultures were inoculated with an equivalent number of *wt*, $H1^+$, or $H1^-$ particles; after 40 min of adsorption, cultures were washed and trypsinized extensively to remove any extracellular virions, and cells were collected by centrifugation. Cell lysates were fractionated by SDS-PAGE and analyzed by immunoblotting; filters were incubated with an antibody raised against the core protein F18. F18 is an 11-kDa DNA-binding protein that is expressed only at late times after infection and is associated with the viral genome within the viral core (17, 18,

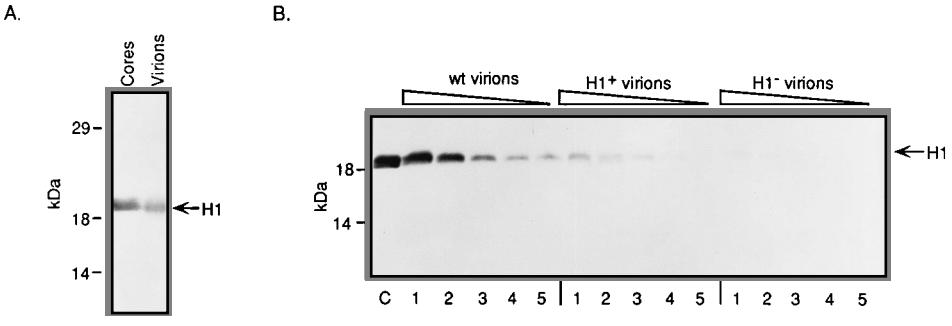

FIG. 4. H1 protein is encapsidated in vaccinia virions. (A) *wt* vaccinia virions (1.5×10^{10}) and subviral cores (2×10^{10}) were fractionated on an SDS–15% polyacrylamide gel and subsequently transferred to a nitrocellulose membrane for immunoblot analysis. The immunoblot shown was developed colorimetrically after incubation with a polyclonal antiserum directed against a TrpE-H1 fusion protein. The arrow on the right indicates the position of the 20-kDa H1 protein. The position to which protein standards migrated, and their molecular masses, are shown at the left. (B) *wt*, $H1^+$, and $H1^-$ virions were fractionated on an SDS–15% polyacrylamide gel and analyzed by immunoblot development as described for panel A. Descending triangles shown above the lanes portray twofold serial dilutions of the stock indicated. The numbers of virions in the lanes were as follows: lanes 1, 1.5×10^{10}; lanes 2, 7.5×10^9; lanes 3, 3.8×10^9; lanes 4, 1.9×10^9; lanes 5, 9.4×10^8. Lane C, subviral cores (2×10^{10}).

TABLE 3. Quantitation of virus harvested from BSC40 cells 24 h after infection with vLacI, H1$^+$, or H1$^-$ viral stocks in the presence or absence of IPTG[a]

Infection			Viral yield (PFU/ml)		+IPTG/−IPTG
Virus	Particles/cell	PFU/cell	+IPTG	−IPTG	
vLacI		0.5	5.0×10^7	3.8×10^7	1.3
H1$^+$	28	0.5	7.0×10^6		53.8
H1$^-$	28	0.07		1.3×10^5	

[a] Confluent 35-mm-diameter dishes of BSC40 cells were infected with the virus shown at the indicated MOI and in the presence or absence of 5 mM IPTG; at 24 h p.i., cells were harvested and disrupted and viral yields were titrated in the presence of IPTG.

60). As shown in Fig. 5, the three populations of input virions contained equivalent amounts of F18 (lanes 1 to 3). Likewise, there was no difference in the amount of F18 taken up by infected cells after 40 min of infection with the three virion preparations (lanes 5 to 7), strongly suggesting that H1$^-$ virions are not deficient in cellular entry. As expected, no cell-associated F18 was observed when a parallel culture was washed and harvested immediately after the inoculum was applied (lane 4).

Early gene expression is severely impaired in cultures infected with H1$^-$ virions at a low MOI. The next stage of the viral life cycle that we examined was early gene expression. Cultures were infected with an MOI of 28 particles per cell, such that H1$^+$-infected cells received 0.5 PFU per cell and H1$^-$ cultures received 0.07 PFU per cell. RNA was prepared at 3 h p.i., and S1 nuclease analysis was performed in order to quantitate the steady-state levels of transcripts expressed from three representative early genes: B1, I3, and E9. As shown in Fig. 6, transcript levels were highest in cultures infected with *wt* virus (lanes 3), somewhat reduced in cultures infected with H1$^+$ virions (lanes 4), and almost undetectable in H1$^-$-infected cultures (lanes 5). Comparison of the mRNA levels seen in *wt*- and H1$^+$-infected cultures suggested that expression of the I3 gene (Fig. 6B) was somewhat less sensitive to the reduction in H1 content than was expression of the B1 and E9 genes (Fig. 6A and C, respectively).

To determine whether the transcriptional deficit seen with H1$^-$ virions could be rescued in *trans* by coinfecting infectious

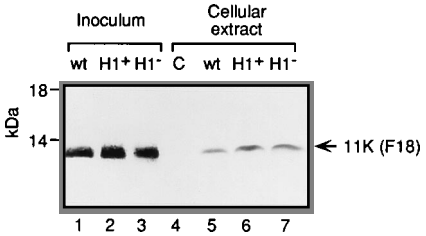

FIG. 5. H1$^-$ virions are not defective in cellular entry. Inocula and infected cell extracts were fractionated on an SDS–15% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was incubated with a polyclonal antiserum raised against a TrpE-F18 fusion protein and developed as described for Fig. 4. Lanes 1 to 3 contain 1.9×10^8 virions of the stock indicated; lanes 5 to 7 contain extracts from 10^6 infected BSC40 cells. Cells were infected with an equivalent number of particles (8.4×10^8) of either *wt*, H1$^+$, or H1$^-$ virus. At 40 min after infection, cells were washed and trypsinized extensively, and extracts were prepared. Lane 4 (C) contains a control extract in which cells were washed and harvested immediately after inoculation with *wt* virions. The position to which protein standards migrated, and their molecular masses, are shown at the left. The arrow on the right indicates the position of the 11-kDa F18 protein.

virions, a parallel experiment was performed at an MOI of 840 particles per cell. Under these conditions, H1$^+$-infected cells received 15 PFU and H1$^-$-infected cells received 2.1 PFU. If the defect in H1$^-$ virions could not be complemented, then one would predict a sevenfold difference in the RNA levels seen after the two infections; if complementation did occur, one would expect nearly equal levels of mRNA transcripts. As shown in Fig. 7, no significant difference was detected between the mRNA levels seen in cultures infected with *wt* or H1$^+$ virions (compare lanes 3 and 4); mRNA levels in cultures infected with H1$^-$ virions (lanes 5) were reduced by approximately twofold. It appeared, therefore, that the residual infectious particles within the H1$^-$ stock were complementing the transcriptional defect of the noninfectious particles in *trans*. To verify that a reduction in MOI from 15 to 2 PFU would normally lead to a corresponding decrease in the levels of early mRNA synthesized, we also analyzed RNA samples from cultures infected with vLacI at an MOI of either 15 or 2 (Fig. 7A and C, lanes 7 and 8). It is evident that in this case, a 7.5-fold reduction in the particle and PFU content of the inoculum did cause a significant reduction in the steady-state levels of early mRNA. We therefore conclude that the nearly normal levels of early transcription seen after infection with H1$^-$ virions at a higher MOI were achieved by complementation in *trans* by the minority of infectious particles.

Analysis of the in vitro transcription capabilities of *wt*, H1$^+$, and H1$^-$ virions. The data shown above indicate that H1$^-$ virions enter cells normally but fail to direct significant early gene expression. Early gene expression takes place in the cytoplasm within the viral core, which remains intact for several hours until uncoating ensues and DNA replication initiates. In fact, the complete early transcriptional machinery is virally encoded and encapsidated in the viral core (32, 39). To address whether the phosphatase deficiency affected transcription per se or some step prerequisite for its onset in vivo, the ability of *wt*, H1$^+$, and H1$^-$ virions to direct transcription in vitro was assessed. Initially, we used the standard procedure of permeabilizing virions and initiating transcription by including NP-40, DTT, and ribonucleoside triphosphates in the reaction mixture. Under these conditions, we obtained variable results. With some preparations of H1$^+$ and H1$^-$ virions, no significant difference from *wt* virions was seen in time course studies of in vitro transcription. With other virion preparations, the levels of RNA synthesized by H1$^-$ virions were two- to threefold less than that seen with *wt* virions. It seemed possible that the residual infectious particles within the H1$^-$ preparation were complementing the H1-deficient, noninfectious particles in *trans*, making them transcriptionally competent. For this to be true, one would have to propose that the phosphatase (or a substrate) exited from the infectious viral cores and activated components within other particles. To test the latter possibility, we permeabilized the particles as before and then separated the supernatant fluid from the particulate cores. Both phases were assayed for phosphatase activity, using a colorimetric assay involving the artificial substrate pNPP (11). As shown in Fig. 8A, the encapsidated phosphatase was enzymatically active; after permeabilization, approximately half of the activity remained with the cores and half was released into the supernatant fluid. It thus seemed that our in vitro complementation model was plausible. As a further test, we performed in vitro transcription assays on virions permeabilized with *S. aureus* α-toxin. α-toxin forms pores of uniform size in lipid bilayers by assembling into ring-structured hexamers with a diameter of 2 to 3 nm and a molecular weight cutoff of 4,000 (9, 29). Hence, essentially all internal proteins and nucleic acids would be expected to remain within the viral particles under these con-

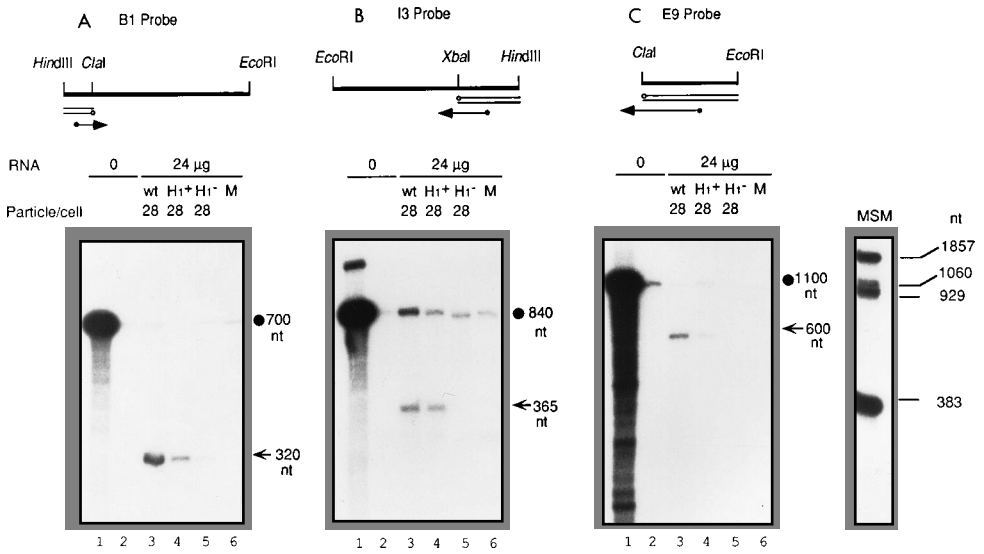

FIG. 6. Early gene expression is severely compromised in H1$^-$ infections. S1 analysis was performed on early mRNA populations prepared from cultures infected with either *wt*, H1$^+$, or H1$^-$ virions at a low MOI. The probes used for analysis of the B1, I3, and E9 genes are shown at the top. Probes are represented schematically beneath the plasmid map; the 5′ radiolabels are indicated by filled circles. The corresponding mRNA transcripts are represented by solid lines with arrowheads. Autoradiographs of the S1 mapping analyses are shown at the bottom. Cells were infected with the virus indicated at an MOI of 28 particles per cell, and RNA was prepared at 3 h p.i. For each panel, lanes 1 and 2 represent control reactions in which no RNA was included. For lanes 3 to 5, 24 µg of RNA from cells infected with the virus indicated above the lanes was analyzed; the reactions shown in lane 6 contained RNA from uninfected cells. Filled circles indicate the input probe, and arrows point to the protected fragments. Hybridizations involving the B1 probe were performed at 39°C; those involving I3 and E9 probes were performed at 42°C. Lane MSM contained DNA standards whose sizes are shown at the right.

ditions. Indeed, the data in Fig. 8B indicate that whereas the majority of phosphatase was released into the supernatant upon NP-40 permeabilization, <10% was released when α-toxin was used to permeabilize virions. With respect to in vitro transcription, although the amount of radiolabel incorporated into acid-precipitable material by α-toxin permeabilized *wt* virions is only 25% of that incorporated during the same period by NP-40-permeabilized virions, the size range of RNA species synthesized after the two treatments is indistinguishable (data not shown). In vitro transcription data obtained after α-toxin permeabilization of *wt*, H1$^+$, and H1$^-$ virions are shown in Fig. 8C. There is a striking difference between the activity of the *wt* particles and both the H1$^+$ and H1$^-$ virions, although the latter are the most impaired and show almost no transcriptional capability. These findings hold true for repeated analyses of different preparations of H1$^-$

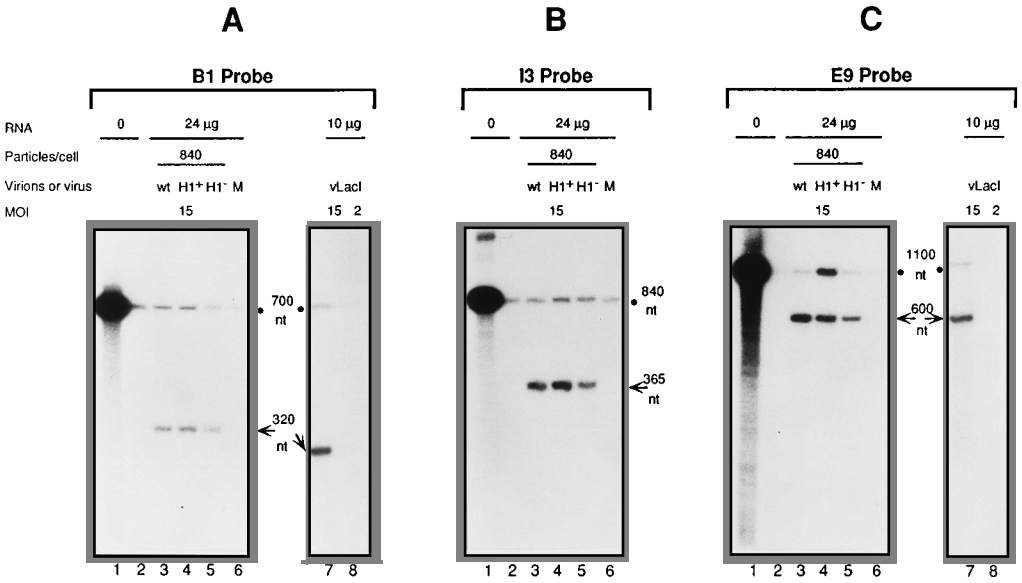

FIG. 7. The transcriptional defect of H1$^-$ virions can be partially rescued in *trans*. The experimental design is comparable to that shown in Fig. 6 except that cultures were infected with 840 particles of either *wt*, H1$^+$, or H1$^-$ virus per cell. This inoculum corresponds to an MOI of 15 PFU per cell for H1$^+$ virus and 2.1 PFU per cell for H1$^-$ virus. The probes used are the same as shown in Fig. 6, as are the descriptions of lanes 1 to 6. Lanes 7 and 8 represent reactions performed with 10 µg of RNA isolated from cells infected with vLacI at MOIs 15 and 2, respectively. The data shown in lanes 7 and 8 were obtained from an experiment different from those shown in lanes 1 to 6.

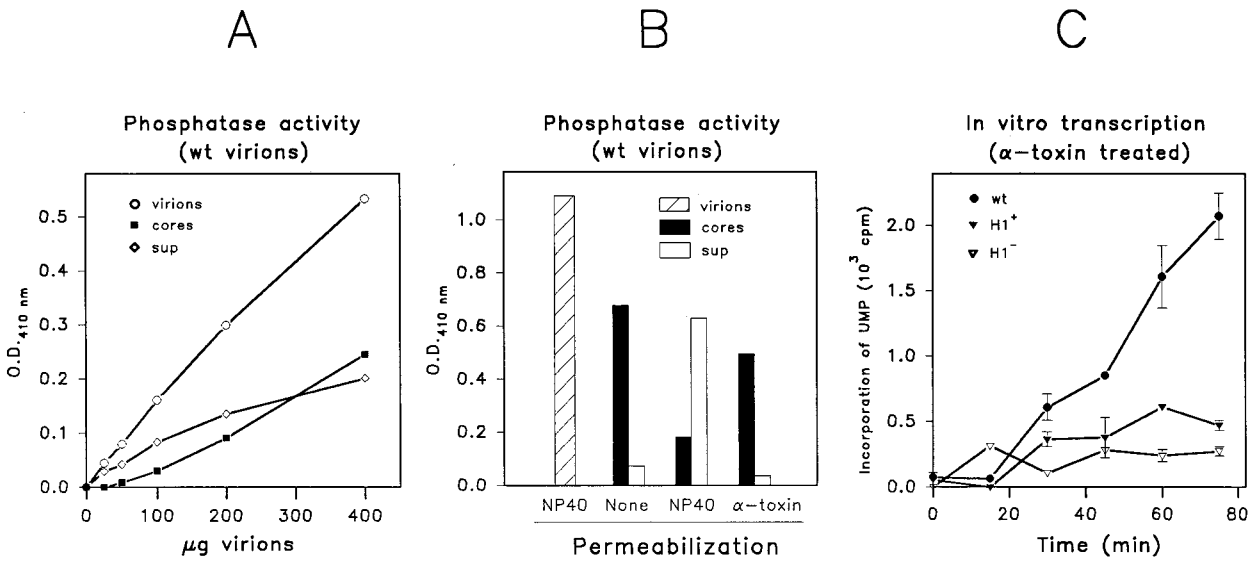

FIG. 8. H1$^-$ virions are impaired in the ability to direct in vitro transcription. (A) Permeabilized *wt* virions (25, 50, 100, 200, or 400 μg, representing a range of 6.3 $\times$ 10^9 to 10^{11} particles) or the soluble and particulate (core) fractions recovered after NP-40 permeabilization of equivalent samples were assayed for phosphatase activity (30 min, room temperature) in the presence of the colorimetric substrate pNPP. Phosphatase activity was quantitated by measuring the OD_{410}. (B) Intact virions (220 μg, or 5.5 $\times$ 10^{10} particles) or the soluble and particulate (core) fractions recovered after NP-40, α-toxin, or mock permeabilization were assayed for phosphatase activity (5.5 h) as described above. (C) Virions (2.8 $\times$ 10^8 particles) were treated with *S. aureus* α-toxin (30 U/ml) for 30 min at 37°C, and then in vitro transcription was performed as described in Materials and Methods. Aliquots were removed at the indicated time points, and RNA synthesis was quantitated by measuring the incorporation of [^{32}P]UMP into acid-precipitable material.

virions; we see a highly reproducible six- to sevenfold difference between *wt* and H1$^-$ virions. We therefore conclude that encapsidated H1 phosphatase is essential for transcription of early genes.

Hyperphosphorylation of the 11-kDa F18 protein in H1$^-$ virions can be reversed by exogenous H1 phosphatase. We hypothesized that the transcriptional block in H1$^-$ virions might be due to the encapsidation of hyperphosphorylated, and hence inactive or inhibitory, proteins. We therefore analyzed the relative phosphorylation state of the proteins encapsidated in *wt* and H1$^-$ virions. The majority of radiolabeled proteins identifiable within ^{32}P-labeled virions were comparable, but two proteins of 16 and 11 kDa were significantly hyperphosphorylated in H1$^-$ virions (Fig. 9B, lanes 1 and 2). The apparent molecular weight of the latter protein raised the possibility that it might be the product of the F18 gene; this DNA-binding protein has been shown previously to be a prominent phosphoprotein within infected cells and virions. Immunoprecipitation, immunoblot, and virion fractionation assays confirmed that, indeed, the 11-kDa protein was the F18 gene product. F18 immunoprecipitated from H1$^-$ infections was hyperphosphorylated, as detected by the extent of ^{32}P labeling; when ^{32}P-labeled virions were fractionated, the hyperphosphorylated 11-kDa species copartitioned with the anti-F18 immunoreactive species (data not shown). Moreover, the altered electrophoretic behavior of the F18 encapsidated in H1$^-$ virions can be seen in the immunoblot shown in Fig. 9C. Whereas the F18 protein within *wt* virions migrates as a relatively sharp band, the immunoreactive species encapsidated within H1$^-$ virions migrate more diffusely. The hyperphosphorylation of the 16- and 11-kDa proteins was suggestive of their being substrates of the H1 phosphatase; to confirm this more directly, NP-40-permeabilized virions were treated with purified recombinant phosphatase or a catalytically inactive form of this enzyme carrying a *cys*110*ser* mutation in the active site (Fig. 9A). In the presence of NP-40 and DTT, *wt* recombinant phosphatase completely removed the ^{32}P radiolabel from both the 16- and 11-kDa proteins (Fig. 9B, lanes 4 and 5). Interestingly, radiolabel was not removed from those phosphoproteins not hyperphosphorylated in H1$^-$ virions. These data confirm that the 16- and 11-kDa proteins are bona fide H1 substrates and that the phosphatase shows clear substrate specificity. Dephosphorylation of these proteins was not seen when permeabilization of H1$^-$ virions was performed without the addition of phosphatase (Fig. 9B, lane 3), when either NP-40 or DTT was omitted (lanes 6 and 7), or when catalytically inactive phosphatase was added (lane 8). Although dephosphorylation of the 11-kDa F18 protein required the addition of 50 mM DTT (which is optimal for H1 activity in vitro), the lesser amount of radiolabel in the 16-kDa protein was removed in the absence of exogenous reducing agent. The immunoblot shown in Fig. 9C confirms that the dephosphorylated 11-kDa protein was still intact (although a mobility shift can be seen), that equivalent amounts of exogenous phosphatase were added where indicated, and that the treatments had no effect on the encapsidated L4 protein. Exogenous phosphatase was incapable of removing the radiolabel on either the 16- or 11-kDa protein when permeabilization was performed with α-toxin (data not shown); these data confirm the gentler nature of α-toxin permeabilization and imply that neither phosphoprotein is exposed on the external surface of virions.

DISCUSSION

This report represents the first functional analysis of the role of the dual-specificity H1 phosphatase in the vaccinia virus life cycle. Our inability to recover a viable viral recombinant containing only an H1 null allele strongly suggested that the phosphatase played an essential role in the viral life cycle. The homologous genes in Shope fibroma and myxoma viruses, two members of the leporipoxvirus family, have also been recently shown to be essential (33). To facilitate a dissection of the

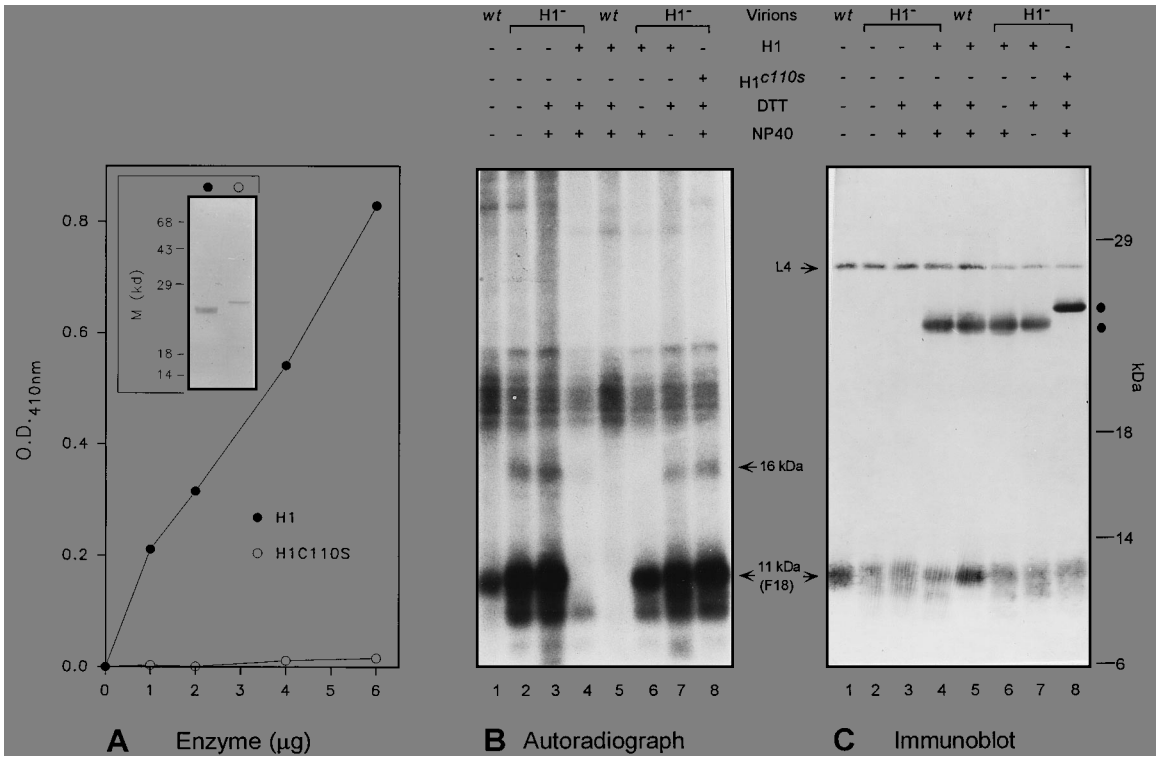

FIG. 9. The hyperphosphorylation of the 11-kDa F18 protein and a 16-kDa protein in $H1^-$ virions can be reversed by recombinant H1 phosphatase. (A) Enzymatic activity and electrophoretic profile of recombinant H1 phosphatase and $H1^{c110s}$ phosphatase. Purified, recombinant H1 phosphatase and the mutant H^{c110s} phosphatase were assayed for enzymatic activity by using the colorimetric substrate pNPP; activity was determined by measuring the OD_{410}. A Coomassie blue-stained gel demonstrating the purity of the recombinant proteins (2 and 1.5 μg for H1 and $H1^{c110s}$, respectively) is shown in the inset. (B and C) ^{32}P-labeled *wt* or $H1^-$ virions (4×10^8) were left untreated or subjected to the treatments shown above the panels and described in more detail in Materials and Methods. Samples were then fractionated on an SDS–17% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. An autoradiograph of the membrane is shown in panel B; the same membrane is shown in panel C after immunodevelopment with antisera directed against the 11-kDa F18 protein, H1 phosphatase, and, as an internal control, the L4 protein. The positions of the hyperphosphorylated 11- and 16-kDa proteins as well as the immunoreactive F18 (11 kDa) and L4 proteins are indicated with arrows. The recombinant phosphatase proteins are indicated with filled circles; in this experiment, the endogenous H1 phosphatase within the virions analyzed was below the level of detection. The electrophoretic migration of protein standards, and their molecular masses, are shown to the right of panel C.

biological role of the vaccinia virus phosphatase, a conditional mutant of H1 was constructed by placing the endogenous gene under the regulation of the *lac* operator and repressor. Analysis of the steady-state levels of H1 mRNA and protein indicated that the level of H1 expressed in the absence of IPTG (repressed) was <15% of that seen in the presence of IPTG (induced). The level of H1 protein synthesized under induced conditions was only 17% of that seen in *wt* infections, reflecting our utilization of a suboptimal promoter in the recombinant virus. Transcription and translation of the wild-type gene normally initiate at a TAAATG motif. This ATG is disrupted during the insertion of the downstream *lac* operator to prevent constitutive translation. We chose a TAAATC motif as a replacement in order to reduce intrinsic promoter strength fivefold (32), hoping to ensure a mutant phenotype when expression from the weakened promoter was further restrained by the binding of the *lac* repressor (59). Because our functional comparisons were largely drawn between induced and repressed infections performed with the v*ind*H1 recombinant (sevenfold difference in H1 expression), the phenotypes reported are underestimates of the impact of H1 repression. When the comparisons of particle/PFU ratios or early mRNA synthesis are made between *wt* and repressed v*ind*H1 infections (42-fold difference in H1 expression), the contrasts are even more striking.

Repression of H1 expression led to a reduction in plaque size; this reduction could be attributed to a diminished burst of infectious virus in each individual round of infection. Quantitation of the virion particles produced, irrespective of their infectivity, revealed that production of virions per se was not compromised under conditions of reduced H1 expression. Moreover, repeated analyses of the protein composition of $H1^-$ virions (by both Coomassie blue and silver staining) revealed no striking deficiencies. However, the fraction of v*ind*H1 particles capable of inducing productive infections (the PFU/particle ratio) was reduced sevenfold when H1 expression was repressed. Since titrations of viral yield were performed in the presence of IPTG, only the early phase of the first round of infection could be affected by an absence of H1. These data suggested that H1 was required for an early event in the viral life cycle. Analysis of low-multiplicity infections confirmed that, relative to virions produced in the induced condition ($H1^+$), only one-seventh as many of the virions produced in the absence of IPTG ($H1^-$) could initiate productive infections. These data correlated well with our observation that $H1^-$ virions contained only one-seventh of the amount of encapsidated H1 protein as did $H1^+$ virions and implied that virions must contain a threshold level of H1 in order to be infectious. We estimate that, on average, the numbers of H1 molecules encapsidated in *wt*, $H1^+$, and $H1^-$ virions are 210, 35, and 5, respectively. Because the majority of $H1^-$ virions are not infectious, while a minority remain fully infectious, it is unlikely that the residual H1 synthesized under repressed conditions is distributed evenly among all virions. It is therefore difficult to

precisely define the number of encapsidated H1 molecules that represents the threshold value required for infectivity. Further analysis of the stage at which the infection initiated by the majority of $H1^-$ virions aborted indicated that entry into target cells was apparently not impaired, but that the subsequent phase of early gene expression was virtually absent. During high-multiplicity infections, these noninfectious particles were complemented in *trans* and did contribute to the synthesis of early mRNA.

The transcriptional deficit in $H1^-$ particles was also evident in vitro when assays were performed under conditions that preserved virion integrity. $H1^+$ particles, which contain one-sixth of the H1 content of *wt* virions, were severely compromised in transcriptional ability, and $H1^-$ particles were essentially inactive. This deficiency was less severe after NP-40 permeabilization, which causes greater disruption of virion structure. We conclude that the transcriptional deficit in $H1^-$ virions is of a regulatory nature and that permeabilization conditions can alter the accessibility of the encapsidated nucleoprotein template, lead to the mobilization of residual phosphatase from the minority of infectious virions, or permit the release of an inhibitory, hyperphosphorylated species. The conclusion that the basic transcriptional apparatus per se is intact within the $H1^-$ virions is further strengthened by our observation that soluble extracts prepared in parallel from $H1^-$ and *wt* virions support comparable levels of nonspecific RNA synthesis when incubated with an M13 template (data not shown) (58). $H1^-$ virions clearly encapsidate *wt* levels of catalytically active RNA polymerase. On the basis of reports of a hierarchical encapsidation of the transcriptional machinery (26, 58), it is almost certain that the encapsidation of vETF and RAP94 are also unperturbed.

There are numerous precedents for the modulation of eukaryotic transcription by the phosphorylation state of the proteins involved (14). In most cases, phosphorylation appears to play a stimulatory role. Phosphorylation regulates both the activation and the action of numerous transcription factors, beginning with the formation or dissolution of cytoplasmic protein-protein complexes that in turn control nuclear translocation. Within the nucleus, both DNA binding and the hierarchy of protein-protein interactions that drive transcriptional activation can be affected by phosphorylation. The impact of phosphorylation on the carboxy-terminal tail of RNA polymerase II has also been the focus of significant attention (4, 16, 35); the unphosphorylated IIa form appears to be essential for the assembly of preinitiation complexes, whereas the phosphorylated IIo form is implicated in elongation. In this case, phosphorylation of promoter-bound polymerase II is thought to be accomplished by TFIIH-associated cdk-activating kinase (41, 42, 44); presumably, a cycle of dephosphorylation and rephosphorylation must accompany every round of RNA synthesis. Finally, it remains unclear how phosphorylation of either the histone or nonhistone components of chromatin affects gene expression. A suggested role for histone H1 phosphorylation in the down-regulation of transcription during mitosis is but one example of how phosphorylation might link chromatin configuration to transcriptional activity.

Although the role of protein kinases in modulating transcription factors and RNA polymerase is receiving significant attention, the opposing phosphatases that must participate in order to make this regulation meaningful are still obscure. In most cases, the impact of phosphatase inhibitors such as vanadate or okadaic acid has been to stimulate the induction of a pathway of gene expression. An exception to this rule is found in maize, in which case phosphatase action is required for the activation of light-inducible genes (43). Similarly, it has been shown that phosphatase action stimulates two facets of *Xenopus laevis* ribosomal gene transcription in vitro (25). These latter two examples are more reminiscent of the situation described in this report, in which a phosphatase plays an essential role in enabling transcription.

Vaccinia virus encodes two serine/threonine kinases (B1 and F10) (2, 27, 28, 36, 54, 55) as well as the dual-specificity H1 phosphatase. Both the F10 kinase and the H1 phosphatase are present in approximately 200 molecules per virion. It is tempting to speculate that their presence in the virion provides a regulatory tension which controls virion activities during morphogenesis and before and after entry into a target cell. Which essential component(s) of the transcriptional apparatus is inoperative in phosphatase-deficient virions has not yet been identified. The known components of the early transcription machinery include a heterodimeric transcription factor (vETF), a seven-subunit RNA polymerase, the RAP94 polymerase accessory protein, the heterodimeric capping enzyme/termination protein, and the heterodimeric poly(A) polymerase, one of whose subunits also functions as a methyltransferase during mRNA cap maturation (32). An encapsidated RNA helicase is also essential for transcription (45), and two other encapsidated nucleoside triphosphatases are reported to affect transcription in vivo and/or in vitro (5, 15, 24, 47). None of the proteins involved in transcription have been reported to be phosphorylated in vivo. By analogy with the nuclear transcriptional apparatus, however, it remains an obvious possibility that one or more of these proteins is packaged in a hyperphosphorylated, inactive form in $H1^-$ virions.

Other encapsidated proteins which might well affect transcription are the DNA-binding proteins presumed to form the viral chromatin. The 11-kDa histone-like F18 protein is an especially compelling candidate; it is known to be phosphorylated and is required for DNA encapsidation (60). That a phosphorylated DNA-binding protein might regulate the transcriptional competence of the viral chromatin is an attractive hypothesis. There is a precedent for the regulation of vaccinia virus gene expression by the conformation of the genomic nucleoprotein complex: intermediate genes cannot be expressed from encapsidated genomes until they have undergone DNA replication (1, 19, 56). Analysis of $H1^-$ virions indicates that the F18 protein is dramatically hyperphosphorylated when H1 expression is repressed. Moreover, F18 within permeabilized $H1^-$ virions can be dephosphorylated by exogenous recombinant H1 phosphatase, confirming that these two proteins have a substrate-enzyme relationship.

In recent years, poxviruses have served as experimentally tenable systems that have elucidated a variety of basic biochemical processes and provided invaluable insights into virus-host interactions. Among other effectors, these viruses encode an inhibitor of the interleukin-1-converting enzyme, soluble forms of the interleukin-1β, gamma interferon, and tumor necrosis factor receptors, an epidermal growth factor-like growth factor, inhibitors of both the classical and alternate complement cascades, and two inhibitors of the double-stranded RNA-activated protein kinase that mediates interferon-induced RNA breakdown and translational arrest (48). Given this mimicry of host biology, it is perhaps not surprising that the utilization of networks of phosphorylation and dephosphorylation has also been appropriated for the regulation of the viral life cycle. Our directed genetic approach has yielded a clear demonstration that the H1 phosphatase is essential for early transcription. A mechanistic dissection of this regulatory network, made easier by the relative simplicity of the viral system, should be broadly applicable to our under-

standing of eukaryotic phosphatases and their biological significance.

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