

# Herpes Simplex Virus 1 $\alpha$ Regulatory Protein ICP0 Interacts with and Stabilizes the Cell Cycle Regulator Cyclin D3

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**The herpes simplex virus 1 (HSV-1) infected-cell protein 0 (ICP0) has the characteristics of a promiscuous transactivator of genes introduced into cells by infection or transfection. To identify cellular proteins interacting with ICP0, we used a domain of exon II of ICP0 that is known to be crucial for regulatory function of the protein as bait in the yeast two-hybrid screen. Our results were as follows. (i) A cDNA in a positive yeast colony was found to encode cyclin D3, a cell cycle regulator of G<sub>1</sub> phase. (ii) A purified chimeric protein consisting of glutathione S-transferase (GST) fused to cyclin D3 specifically formed complexes with ICP0 contained in HSV-1-infected cell lysate. (iii) To enhance the expression of cyclin D3, the gene was inserted into the viral genome and overexpressed in infected cells. The overexpressed cyclin D3 colocalized with ICP0 in nuclear structures characteristic of ND10 and which earlier have been reported to contain ICP0. (iv) The accumulation of cyclin D3 protein in Vero cells infected with an  $\alpha$ 0 deletion mutant was reduced relative to that of cells infected with wild-type virus or a recombinant virus in which the deleted  $\alpha$ 0 sequences were restored. (v) Lysates of *Spodoptera frugiperda* Sf9 cells doubly infected with baculoviruses genetically engineered to express cyclin D3 and cyclin-dependent kinase 4 (CDK4) phosphorylated GST fused to retinoblastoma protein (GST-pRb) but did not phosphorylate the GST- $\alpha$ 0<sub>20-241</sub> or GST- $\alpha$ 0<sub>543-768</sub> fusion protein or immunoprecipitated ICP0 proteins. Moreover, the chimeric GST-ICP0<sub>exon II</sub> protein shown to bind cyclin D3 had no effect on the activity of the kinase on GST-pRb when added to mixtures of lysates of Sf9 cells which coexpressed cyclin D3 and CDK4. These results indicate that ICP0 interacts with, colocalizes with, and stabilizes the cyclin D3 cell cycle regulator and does not affect its interaction with the cyclin-dependent kinase.**

To date, the herpes simplex virus 1 (HSV-1) genome has been found to encode at least 84 different proteins (38). The genes of HSV-1 have been shown to fall into several major classes whose expression is coordinately regulated and sequentially ordered in a cascade fashion (14, 15, 22). The  $\alpha$  genes are expressed first, and functional  $\alpha$  proteins are required for the expression of  $\beta$  genes, and both  $\alpha$  and  $\beta$  proteins and viral DNA synthesis mediated by  $\beta$  proteins are required for optimal expression of  $\gamma$  genes (14, 15). Six  $\alpha$  proteins, termed infected-cell protein 0 (ICP0), ICP4, ICP22, ICP27, ICP47, and U<sub>S</sub>1.5, have been identified and shown to perform regulatory functions or prevent a host response to infection (4, 39). Two of these, ICP4 and ICP27, are essential for viral replication (reviewed in reference 39). ICP4 plays a dual role as a transactivator and as a repressor (39), whereas ICP27 regulates the processing of viral mRNA (39). ICP22 and possibly U<sub>S</sub>1.5, a truncated version of ICP22, have been linked to several aspects of viral gene expression in cell-type-dependent manner (reviewed in reference 39). Last, ICP47 inhibits the presentation of antigenic peptides to CD8<sup>+</sup> cells (reviewed in reference 39).

ICP0, the subject of this report, has been shown to be a positive regulator of viral replication, based on the observations that ICP0 mutants grow in cell culture more slowly than wild-type virus, particularly at low multiplicities of infection (40, 44). Although the mechanism by which ICP0 acts in viral replication is unclear, the consensus of the last decade is that ICP0 mediates its activity through transcriptional machinery because ICP0 has been shown to be a promiscuous transacti-

vator of both HSV and non-HSV promoters in transient transfection assays (8, 11, 12, 34, 35, 37). However, no evidence that ICP0 interacts with cellular transcriptional machinery has been reported so far. Meanwhile, several lines of recent evidence, listed below, suggest that ICP0 is a multifunctional protein that interacts with a variety of cellular proteins and that the function of ICP0 results from the sum of these interactions.

(i) Maul et al. (26) reported that ICP0 colocalizes with and modifies ND10, a nuclear multiprotein domain of unknown function (1, 9, 25). Further, Everett et al. (10, 27, 28) reported that ICP0 associates with a transitory component of ND10, termed HAUSP and identified as a novel ubiquitin-specific protease whose function is postulated to stabilize target protein by removing ubiquitin adducts. This postulate, if proven, would imply that ICP0 modulates the ubiquitin-dependent pathway.

(ii) Cai and Schaffer (3) reported that a cellular activity expressed maximally 8 h after release of Vero cells from growth arrest in the G<sub>0</sub>/G<sub>1</sub> phase enhanced the replication of a mutant virus lacking ICP0, whereas replication of wild-type virus exhibited no dependence on the cell cycle. These results suggest that a cellular function associated with cell cycle progression is involved in productive infection and that ICP0 either substitutes for it or regulates its expression.

(iii) This laboratory reported that ICP0 accumulated in both the cytoplasm and the nuclei of infected cells (19). In addition, these studies showed that ICP0 interacted with the translation elongation factor 1 $\delta$  (EF-1 $\delta$ ). Furthermore, the domain of ICP0 which interacted with EF-1 $\delta$  affects translational efficiency in vitro. These results suggest that ICP0 has a significant cytoplasmic role and modulates viral gene expression at the translational level.

Taken at face value, these reports suggest that the functions of ICP0 are related to many aspects of cellular functions such

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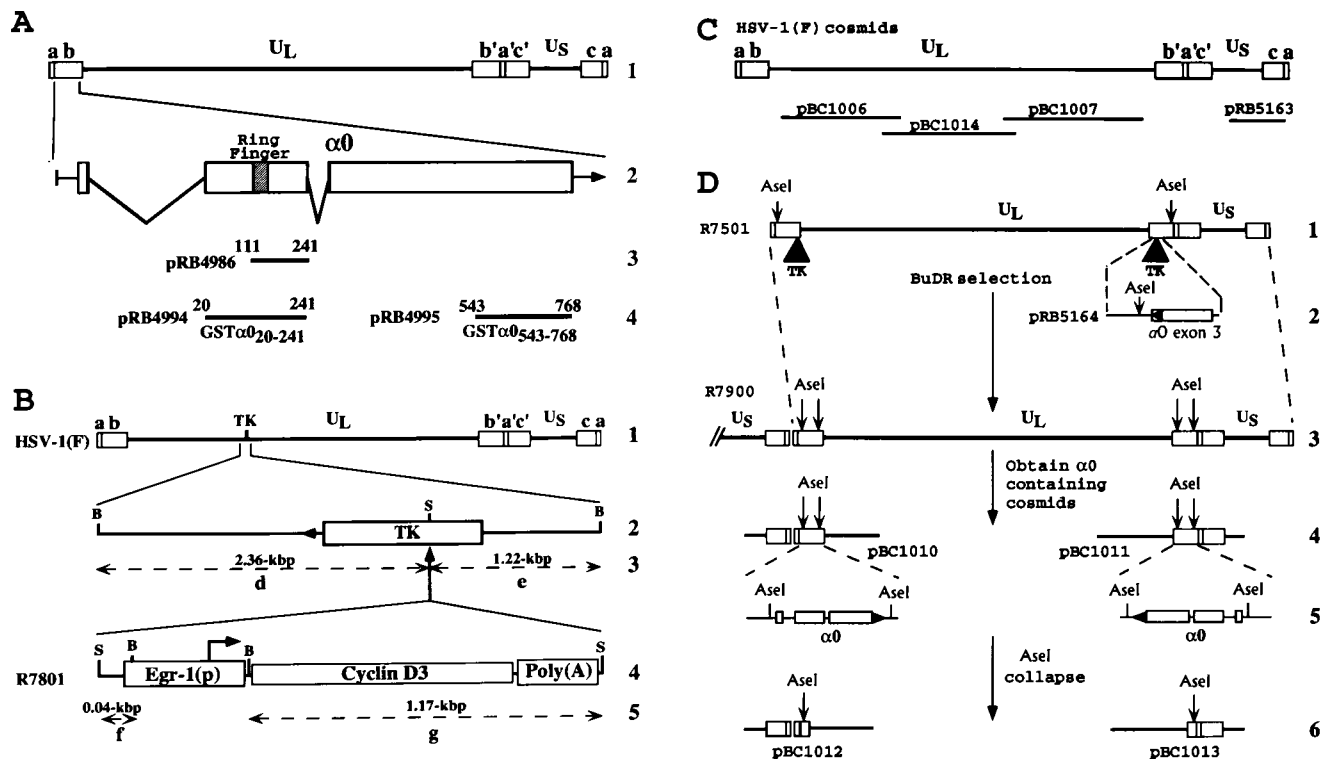


FIG. 1. (A) Schematic diagram of the sequence of the HSV-1 genome and the location of the  $\alpha 0$  gene. Line 1, a linear representation of the HSV-1 genome. The unique sequences are represented as the unique long ( $U_L$ ) and unique short ( $U_S$ ) regions. The terminal repeats flanking the unique sequences are shown as open rectangles with their designation letters given above. Line 2, an expanded section of the domain encoding the  $\alpha 0$  gene. The transcript and coding regions are shown for only one copy of the  $\alpha 0$  gene. A second, identical copy is located in the terminal inverted repeats flanking  $U_L$ . Line 3, the region used in the yeast two-hybrid screen. Line 4, the domains of the  $\alpha 0$  gene used for generation of GST-ICP0 fusion proteins. (B) Schematic diagram of the sequences of HSV-1(F) and of the recombinant virus, R7801, derived from it. Line 1, sequence arrangement of the HSV-1(F) DNA. Line 2, an enlarged portion of the *Bam*HI Q fragment of HSV-1(F) containing the *tk* gene. The open rectangle and arrow indicate the coding region of the *tk* gene and the direction of transcription, respectively. Line 4, sequence of the recombinant virus, R7801, which was selected, as described in Materials and Methods, from among the progeny of transfection of rabbit skin cells with intact HSV-1(F) DNA and plasmid pRB5162. The promoter sequence of Egr-1, the cyclin D3 coding sequence, and the sequence containing bidirectional poly(A) signals of  $U_L21$  and  $U_L22$  of HSV-1(F) are shown as open rectangles. The arrow indicates the site of transcription initiation and the direction of transcription. Lines 3 and 5 show expected sizes of DNA fragments generated by cleavage of the DNA. The fragment designations shown here are identical to those described in the text and Fig. 2A. Restriction sites: B, *Bam*HI; S, *Sph*I. Abbreviations: d, 2.36-kbp *Bam*HI-*Sph*I fragment; e, 1.22-kbp *Sph*I-*Bam*HI fragment; f, 0.04-kbp *Sph*I-*Bam*HI fragment; g, 1.17-kbp *Bam*HI-*Sph*I fragment. (C) Schematic diagram of the sequences of the HSV-1(F) DNA and the cosmid clones derived from it. The cosmids were used to construct the  $\alpha 0$  deletion mutant. Clone pRB5163 was created for the purpose of bridging the nonoverlapping cosmids shown in Fig. 1D, line 6. (D) Schematic diagram of the sequences of recombinant virus R7501, used to construct recombinant R7900, and of the cosmids derived from R7900 used for the construction of the  $\alpha 0$  deletion cosmids. Line 1, sequence of recombinant virus R7501 carrying single *Ase*I sites in inverted repeats and the HSV-1(F) *tk* gene inserted into a unique *Not*I site downstream of the  $\alpha 0$  coding domain. Line 2, organization of plasmid pRB5164, containing an *Ase*I restriction site cloned within the unique *Not*I restriction site as shown in line 1. Line 3, arrangement of recombinant virus R7900, which was selected from among the progeny of transfection of rabbit skin cells with intact recombinant R7501 viral DNA and plasmid pRB5164 as described in Materials and Methods. Line 4, delineation of the cosmids that possess the  $\alpha 0$  coding sequences. Line 5, the *Ase*I sites which flank the  $\alpha 0$  open reading frame. Line 6, sequences of cosmids which were derived following cleavage with *Ase*I and ligation of cosmids listed in line 4.

as regulation of protein decay, the expression of cell cycle-dependent proteins, and translational regulation as well as the as yet undefined functions related to promiscuous transactivation. Since only two cellular proteins, HAUSP (10) and EF-1 $\delta$  (19), have been identified as ICP0 cognate proteins, it is conceivable that additional ICP0 cognate proteins exist and that these include proteins associated with cell cycle, transcription, or cytoplasmic functions beneficial to viral life cycle.

In this report, we show that (i) ICP0 interacts with a  $G_1$  cell cycle regulator, cyclin D3, and (ii) the accumulation of cyclin D3 is reduced in ICP0 deletion mutant virus-infected Vero cells. These results suggest that ICP0 interacts with cyclin D3 and stabilizes the expression of the  $G_1$  cell cycle regulator and that this interaction plays a role in regulatory functions expressed by ICP0.

#### MATERIALS AND METHODS

**Cells and viruses.** HEP-2 and Vero cell lines were obtained from the American Type Culture Collection. Rabbit skin and 143TK<sup>-</sup> cell lines were obtained

from J. McClaren and C. Croce, respectively. All cell lines were grown in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum (HEP-2 cells) or 5% newborn calf serum (Vero and rabbit skin cells) and 40  $\mu$ g of bromodeoxyuridine per ml (143TK<sup>-</sup> cells). HSV-1(F), a limited-passage isolate, is the prototype strain used in this laboratory (7).

**Plasmids.** To construct pRB4986, a DNA fragment encoding  $\alpha 0$  codons 111 to 241, which was reported to be crucial for regulatory function of ICP0 (11), was amplified by PCR and inserted into pGBT9 (Clontech, Palo Alto, Calif.) in frame with the DNA-binding domain of GAL4 (Fig. 1A). pBH1004, containing a 1.9-kbp cDNA encoding human cyclin D3, was isolated in a yeast two-hybrid screen.

pBH1005 was constructed by inserting a PCR fragment containing the entire coding sequence of cyclin D3 into pGEX4T-3 (Pharmacia, Uppsala, Sweden), in frame with glutathione *S*-transferase (GST). The coding sequence of cyclin D3 from pBH1005 was subcloned into *Eco*RI and *Xho*I sites of pGADGH (Clontech) to yield pBH1006. pRB4994 and pRB4995, expressing GST fused to the amino-terminal and carboxyl-terminal domains of ICP0, respectively, were described previously (19). pRb-Ase-End, expressing GST-retinoblastoma protein (pRb) large pocket protein (46), was provided by J. Wang.

To construct pRB5160, a 530-bp DNA fragment containing the Egr-1 promoter region was inserted into a T4 DNA polymerase blunt-ended *Xba*I site of plasmid Bluescript II KS+ (Stratagene, La Jolla, Calif.), and then a 270-bp PCR fragment containing the bidirectional polyadenylation [poly(A)] signals of

HSV-1 U<sub>L</sub>21 and U<sub>L</sub>22 genes was cloned. pRB4867 (13) contains the *Bam*HI Q fragment of HSV-1(F). An *Sph*I fragment from pRB5160 containing the Egr-1 promoter region, multicloning sites, and poly(A) signals was cloned into the *Sph*I site of the viral thymidine kinase (*tk*) gene in pRB4867 to yield pRB5161. To generate pRB5162, a *Spe*I-*Xho*I fragment of pBH1006 was inserted into *Spe*I-*Xho*I sites of pRB5161. In this plasmid, the expression of cyclin D3 was driven by the Egr-1 promoter.

pRB5165, a plasmid used to construct a cell line expressing ICP0, contained the  $\alpha$ 0 coding sequence driven by an Egr-1 promoter. This was constructed by digesting pDS18 carrying the  $\alpha$ 0 cDNA (a gift from S. Silverstein) with *Bgl*II, blunt ending the fragment with T4 polymerase, and cloning it into the *Hinc*II site of pRB5160. pRB5164, a plasmid used for the construction of recombinant virus R7900, was constructed by cloning the self-complementary oligonucleotide 5' GGCTTATAATAA 3' into the *Not*I site of pRB4784. pRB4784 carries a *Sac*I-*Sal*I subclone of the HSV-1(F) *Bam*HI B fragment (pRB112) in a pGEM3Z(+)- (Promega, Madison, Wis.) vector. The resulting clone contains a unique *Ase*I site, whereas the *Not*I site was destroyed. The cosmid cloning vector pRB78 was constructed by replacing the *Eco*RI fragment containing multiple cloning site of Superco 1 (Stratagene) with an oligonucleotide containing an *Eco*RI/*Pac*I/*Sse*8387I/*Spe*I/*Bam*HI/*Nde*I/*Eco*RV/*Pac*I/*Eco*RI polylinker. In accordance with manufacturer's instructions, pRB78 was cleaved with *Xba*I and dephosphorylated before cleavage with an enzyme compatible with the fragment to be cloned. pRB5163, a plasmid derived from nucleotides 138344 to 152242, was used to bridge two nonoverlapping cosmids mapping to the HSV-1(F) unique short region (Fig. 1D, line 6). This plasmid was constructed by cloning the DNA fragment derived by double digestion of HSV-1(F) DNA with *Hind*III and *Dra*I into the *Hind*III-*Dra*I site of pBR322.

To construct a transfer plasmid for making a recombinant baculovirus, an *Eco*RI-*Not*I fragment from pBH1005 including the entire coding sequence of cyclin D3 was cloned into pRB4996. The resulting plasmid was designated pRB4997. pRB4996 is identical to pAcSG-His BT-C (Pharmingen, San Diego, Calif.) except that a *Bam*HI-*Xho*I fragment containing a six-histidine tag, a protein kinase A site, and a thrombin cleavage site was deleted by cleavage. The transfer vector encoding human cyclin-dependent kinase 4 (CDK4) (29) was provided by M. Meyerson.

**Cosmids.** R7900 viral DNA described below was partially digested with *Sau*3AI, dephosphorylated, and ligated to a prepared, *Bam*HI-digested pRB78 cosmid vector. This linear, ligated DNA was then packaged into lambda phage with Gigapack XLII (Stratagene) according to manufacturer's instructions. *Escherichia coli* XL-1 Blue MR was then infected, and Amp<sup>r</sup> colonies were screened by restriction mapping for cosmids containing the *Bam*HI B or *Bam*HI E fragment, each of which contain  $\alpha$ 0 coding sequences. Cosmids pBC1010 carrying *Bam*HI-E and pBC1011 carrying *Bam*HI-B were shown by end sequencing to contain R7900 nucleotides 146073 to 33013 and 107003 to 143403, respectively. To eliminate the  $\alpha$ 0 coding sequences, cosmids pBC1010 and pBC1011 were digested with *Ase*I to remove the *Ase*I fragment, religated, and repackaged. The resulting cosmids, pBC1012 derived from pBC1010 and pBC1013 derived from pBC1011, were restriction enzyme mapped and end sequenced.

HSV-1(F) viral DNA was partially digested with *Sau*3AI, ligated to *Bam*HI-digested pRB78, packaged, and infected as described above. All cosmids obtained were screened by restriction mapping. Cosmids of interest (pBC1006, pBC1014, and pBC1007) were later end sequenced and shown to contain nucleotides 2945 to 45035, 40617 to 80454, and 77933 to 116016, respectively.

**Yeast two-hybrid screen.** A yeast two-hybrid system as employed previously (19) was used to isolate cDNAs encoding proteins able to interact with ICP0. In brief, the bait plasmid (pRB4986) (Fig. 1A) and an Epstein-Barr virus-transformed human peripheral blood lymphocyte cDNA library (a gift of Aviron Inc.), which was fused to the GAL4 transcriptional activation domain in pACT, were sequentially cotransformed into yeast strain HF7c (Clontech). Plasmids were isolated from colonies which grew on SD synthetic medium lacking histidine and expressed  $\beta$ -galactosidase. The purified plasmids were cotransformed with pGBT9, pRB4986, pLAM5' encoding human lamin C (Clontech), or pVA3 encoding a murine p53 (Clontech) into HF7c cells, and transformants were assayed for  $\beta$ -galactosidase activity to eliminate false positives.

**Production and purification of GST fusion proteins.** GST fusion proteins were expressed in *E. coli* BL21 transformed with either pRB4994, pRB4995, pRB-Ase-End, or pGEX4T-1 and purified on glutathione-agarose beads (Sigma, St. Louis, Mo.) as described previously (19). GST-cyclin D3 fusion protein was also purified as described previously (19) except that the bacterial cells transformed with pBH1005 were lysed in phosphate-buffered saline (PBS) containing 1% Triton X-100 and 1% Sarkosyl by sonication. The eluted proteins were quantified with the aid of a Bio-Rad (Hercules, Calif.) protein assay kit. The proteins immobilized on the glutathione-agarose beads were quantified by Coomassie blue staining. Bovine serum albumin (BSA) was used as the protein standard.

**Affinity precipitation with GST-cyclin D3 fusion protein.** HEP-2 cells infected with HSV-1(F) were lysed in HEPES buffer (50 mM HEPES [pH 7.4], 250 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.1 mM TLCK [*N* $\alpha$ -*p*-tosyl-L-lysine chloromethyl ketone], 0.1 mM TPCK [tolylsulfonylethyl phenylalanine] chloromethyl ketone) containing 1% Triton X-100. A 200- $\mu$ l aliquot of lysate corresponding from  $2 \times 10^7$  cells was incubated with GST or GST-cyclin D3 protein immobilized on glutathione-agarose beads. After the beads were washed four times with HEPES buffer containing 1% Triton X-100,

the bound protein complexes were subjected to electrophoresis on an 8% polyacrylamide gel containing sodium dodecyl sulfate (SDS), transferred to a nitrocellulose sheet, and reacted with a mouse monoclonal antibody to ICP0 (H1083).

**Construction of the ICP0-expressing cell line N3.** pRB5165 containing the  $\alpha$ 0 coding sequences under the control of an Egr-1 promoter was cotransfected into rabbit skin cells with pMAMneo (Clontech) at a molar ratio of 10:1, respectively (10  $\mu$ g, total), using a calcium phosphate-glycerol shock method as described previously (6). Under G418 selection, cells expressing neomycin resistance were made clonal by limiting dilution. Stock of each isolate were then screened for the expression of ICP0 by immunoblotting.

**Construction of recombinant viruses.** Recombinant virus R7801 was constructed by cotransfection of rabbit skin cells with intact HSV-1(F) viral DNA and pRB5162. *tk*<sup>-</sup> recombinant viruses were selected on 143TK<sup>-</sup> cells overlaid with Dulbecco's modified Eagle medium containing 5% newborn calf serum and 40  $\mu$ g of bromodeoxyuridine per ml of medium as described previously (36). Viral DNAs were extracted from infected cells and purified on a 5 to 20% potassium acetate gradient as described previously (16). The recombinant was verified by hybridization of electrophoretically separated restriction digests of progeny viral DNA (Fig. 2).

R7900 was constructed using viral DNA from R7501 (21), a virus containing the HSV-1(F) *tk* gene inserted within a unique *Not*I site 3' to the  $\alpha$ 0 coding domain. R7501 DNA was cotransfected with pRB5164 on rabbit skin cells, and a recombinant virus containing two copies of the *Ase*I site flanking the  $\alpha$ 0 gene was selected in 143TK<sup>-</sup> cells overlaid with medium containing bromodeoxyuridine.

The ICP0 deletion virus R7901 was constructed by homologous recombination of overlapping cosmids pBC1012, pBC1006, pBC1014, pBC1007, and pBC1013 and plasmid pRB5163. Two micrograms of each cosmid was pooled and digested with *Pac*I to release the HSV-1 fragments from the cosmid vector. Two micrograms of pRB5163 was linearized by *Nde*I digestion and added to the pool. All enzymes were heat inactivated, and the pool was purified on a 12 to 25% sucrose gradient to separate the vector and cosmid fragments. Fractions were analyzed for the presence of high-molecular-weight DNA, dialyzed, and concentrated by ethanol precipitation. Serial dilutions of the purified pool were transfected into N3 cells as previously described (6). Within 72 h, plaques were isolated and plaque purified on N3 cells.

In recombinant virus R7902, the  $\alpha$ 0 sequences deleted from R7901 were restored by cotransfection of R7901 DNA with a *Bsr*GI fragment containing  $\alpha$ 0 coding sequences from the HSV-1(F) genome. Plaques were isolated, plaque purified, and analyzed for wild-type protein expression.

All viruses except R7901 were grown in Vero cells. The R7901 recombinant was grown in ICP0-expressing cell line N3. All titrations of infectivity were done on Vero cells.

**Antibodies.** To generate polyclonal antibodies for ICP0, purified GST- $\alpha$ 0<sub>20-241</sub> (19) was used for immunization of rabbit by standard protocol at Josman Laboratories (Napa, Calif.). Specifically, the rabbits were administered five subcutaneous injections of 2.5 mg of the purified GST fusion protein at 14-day intervals. The serum used in the studies reported here was collected 4 weeks after the first immunization. The mouse monoclonal antibody to ICP0 (H1083) and the rabbit polyclonal antibody to EF-1 $\delta$  were described elsewhere (19). Mouse monoclonal antibody to cyclin D3 (G107-565) and rabbit polyclonal antibody to CDK4 (H-22) were purchased from Pharmingen and Santa Cruz Biotechnology Inc. (Santa Cruz, Calif.), respectively.

**Immunoblotting.** The electrophoretically separated proteins transferred to nitrocellulose sheets were reacted with antibodies as described elsewhere (19). The final dilutions of the antibodies were 1:500 for the mouse anti-ICP0 monoclonal antibody (H1083) and 1:1,000 for the rabbit polyclonal antibody to ICP0 or the rabbit polyclonal antibody to EF-1 $\delta$ . To detect cyclin D3 and CDK4, nitrocellulose membranes were blocked with 5% skim milk in PBST (PBS containing 0.1% Tween 20) for 1 h or overnight, rinsed twice, washed once for 15 min and twice for 5 min each time in PBST, and reacted for 2 h with primary antibodies in PBST containing 1% BSA. The final dilutions were 1:1,000 for a mouse monoclonal antibody to cyclin D3 (G107-565) and 1:250 for a rabbit polyclonal antibody to CDK4 (H-22). The blots were then washed as before, reacted for 1 h with a 1:1,000 dilution of goat anti-mouse immunoglobulin G (IgG) or 1:3,000 dilution of anti-rabbit IgG conjugated to peroxidase (Sigma) in PBST containing 3% skim milk, rinsed twice, washed once for 15 min and four times for 5 min each time in PBST, and developed by using the Amersham (Buckinghamshire, England) ECL chemiluminescence reagent.

**Immunofluorescence.** HEP-2 cells seeded on glass slides (Cell-Line, Newfield, N.J.) were infected with 10 PFU of virus R7801 per cell and incubated for 8 h at 37°C. Cells were fixed in ice-cold methanol for 20 min, blocked in PBS containing 1% BSA and 20% normal human serum for 30 min, rinsed once with PBS, reacted for 2 h with a 1:250 dilution of a mouse monoclonal antibody to cyclin D3 and a 1:1,000 dilution of a rabbit antiserum to ICP0 in PBS containing 10% normal human serum and 0.2% BSA, rinsed three times in PBS, reacted for 1 h with a 1:400 dilution of a goat anti-mouse IgG conjugated to Texas red (Molecular Probes, Eugene, Oreg.) and a 1:140 dilution of a goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (Sigma) in PBS containing 10% normal human serum and 0.2% BSA, rinsed with PBS as before, and mounted in PBS containing 90% glycerol and 1 mg of *p*-phenylenediamine per ml. The slides were examined in a Zeiss confocal fluorescence microscope. Digitized images of the

fluorescent antibody-stained cells were acquired with software provided with the Zeiss confocal microscope.

**Generation of recombinant baculoviruses.** *Spodoptera frugiperda* Sf9 cells were maintained in Grace's medium supplemented with 10% fetal calf serum. Each transfer vector was cotransfected with linearized BaculoGold viral DNA (Pharmingen) into Sf9 cells by using Lipofectin (GibcoBRL, Gaithersburg, Md.), and the recombinant viruses were subsequently amplified. Expression of the protein products was confirmed by immunoblotting.

**In vitro kinase assay.** Sf9 cells ( $10^7$ ) were infected with wild-type (*Autographa californica* nuclear polyhedrosis virus) or cyclin D3 recombinant baculovirus or coinfecting with both cyclin D3 and CDK4 recombinant baculoviruses at 50 PFU/cell. After 48 h, cells were washed with PBS once, resuspended in 250  $\mu$ l of kinase buffer (50 mM HEPES [pH 7.5], 10 mM  $MgCl_2$ , 1 mM dithiothreitol, 1 mM NaF, 2.5 mM EGTA, 10 mM  $\beta$ -glycerophosphate, 0.1 mM sodium orthovanadate, 20  $\mu$ M ATP) containing 0.1 mM PMSF and 10  $\mu$ g of leupeptin per ml, and lysed by passage through 26-gauge, 1/2-in needle six times. The cleared lysates were aliquoted and stored at  $-80^\circ C$ . To test whether ICP0 is a substrate for the cyclin D3-CDK4 complex, GST- $\alpha_{0_{20-241}}$  and GST- $\alpha_{0_{543-768}}$  fusion proteins and immunoprecipitated ICP0 from HEP-2 cells infected with HSV-1(F) were used as substrate proteins in kinase assay. For immunoprecipitation, HEP-2 cells grown in four 150-cm<sup>2</sup> flasks were infected with 10 PFU of HSV-1(F) per cell. At 6 h after infection, the cells were scraped into PBS and washed with PBS and lysed in 2 ml of Nonidet P-40 (NP-40) buffer (50 mM Tris-HCl [pH 8.0], 120 mM NaCl, 0.5% NP-40, 1 mM NaF, 1 mM PMSF, 0.1 mM TLCK, 0.1 mM TPCK). Centrifuged lysates were reacted with the monoclonal antibody to ICP0 (H1083) for 2 h at  $4^\circ C$ , and protein A-Sepharose beads (Sigma) were added and allowed to react for an additional hour. Immunoprecipitates were collected by centrifugation, washed four times with NP-40 buffer and twice with kinase buffer, and subjected to kinase assay. The reaction were done with 0.2  $\mu$ g of GST-pRb fusion protein, 1.0  $\mu$ g of GST-ICP0 fusion proteins, or immunoprecipitate from two-thirds of a 150-cm<sup>2</sup> culture of infected HEP-2 cells at  $30^\circ C$  for 20 min in a total volume of 50  $\mu$ l of kinase buffer containing 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP and 2  $\mu$ g of Sf9 cell lysates. To investigate whether ICP0 affects the activity of cyclin D3-dependent kinase, the indicated amount of purified GST or GST- $\alpha_{0_{20-241}}$  protein was mixed with 4  $\mu$ g of lysates from Sf9 cell coinfecting with cyclin D3 and CDK4 recombinant baculoviruses in 10  $\mu$ l of kinase buffer and allowed to react for 2 h at  $4^\circ C$ . The kinase activity was assayed in a 50- $\mu$ l reaction mixture at  $30^\circ C$  for 20 min in the same buffer to which 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP and 0.2  $\mu$ g of GST-pRb large pocket protein were added. The phosphorylated proteins were subjected to electrophoresis on 9% polyacrylamide gels containing SDS. The gels were then stained with Coomassie blue and exposed to X-ray film.

## RESULTS

**Preparation of reagents used in this study.** In this report, we describe the interaction between ICP0 and cyclin D3. To characterize this interaction fully, a number of reagents had to be constructed and tested. This section summarizes the preparation of reagents used in this study.

Cyclin D3 is present in relatively small amounts in infected cells. To increase the amount of cyclin D3, we constructed recombinant virus R7801, in which a cyclin D3 expression cassette was inserted into the context of viral genome. As described in Materials and Methods and shown schematically in Fig. 1B, the coding sequence of cyclin D3 fused to the Egr-1 promoter was inserted into the *Sph*I site of the *Bam*HI Q fragment of HSV-1(F) DNA. The resulting plasmid, pRB5162, was cotransfected with intact HSV-1(F) viral DNA on rabbit skin cells, and the progeny of transfection were plated on 143TK<sup>-</sup> cells in the presence of bromodeoxyuridine. The *tk*<sup>-</sup> progeny viruses were isolated and plaque purified, and their viral DNAs were analyzed for the presence of the cyclin D3 expression cassette by Southern blotting. Viral DNAs prepared from cells infected with HSV-1(F) or the recombinant virus were digested with *Bam*HI, electrophoretically separated in an agarose gel, transferred to a Zeta-probe membrane (Bio-Rad), and probed with *Bam*HI-Q. As could be predicted from the details of the construction of these viruses (Fig. 1B), the <sup>32</sup>P-labeled probe hybridized to fragment d+e (3.6 kbp) in HSV-1(F) (Fig. 2A, lane 2) and to a doublet of fragments d+f and e+g (2.4 kbp) in R7801 (Fig. 2A, lane 1). To assay for expression of cyclin D3, HEP-2 cells were mock infected or infected with 10 PFU of HSV-1(F) or R7801 per cell. The lysates of cells harvested at 18 h after infection were electrophoretically

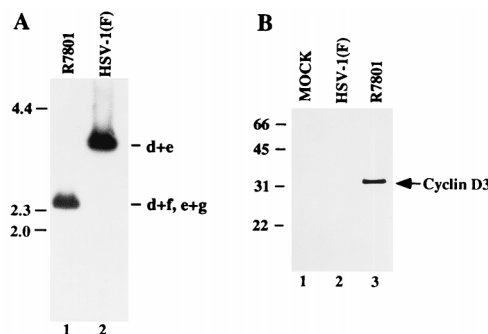


FIG. 2. Characterization of an HSV-1 recombinant virus expressing the cyclin D3 gene. (A) Autoradiographic images of electrophoretically separated *Bam*HI digests of parent HSV-1(F) or R7801 DNA (depicted in Fig. 1B), hybridized to the labeled *Bam*HI Q fragment. Lane 1, digest of DNA of recombinant virus R7801. Lane 2, digest of DNA of parent HSV-1(F). The letters on the right refer to the designations of the DNA fragments generated by restriction endonuclease cleavage. Molecular weights ( $10^3$ ) are shown on the left. (B) Photographic image of an immunoblot of electrophoretically separated lysates of mock (lane 1)-, HSV-1(F) (lane 2)-, or R7801 (lane 3)-infected HEP-2 cells harvested 18 h postinfection. The blot was probed with the monoclonal antibody to cyclin D3. Molecular weights ( $10^3$ ) are shown on the left.

separated in a 12% polyacrylamide gel containing SDS, transferred to a nitrocellulose sheet, and reacted with the cyclin D3 monoclonal antibody. As shown in Fig. 2B, lane 3, the antibody reacted with a protein with an  $M_r$  of 33,000 corresponding to cyclin D3 in R7801-infected cells, whereas cyclin D3 expression was not detected in lysates of mock-infected or HSV-1(F)-infected cells.

A rabbit polyclonal antiserum to ICP0 was generated for use in colocalization studies with the mouse monoclonal antibody to cyclin D3. The antibody produced following inoculation of rabbits with the GST- $\alpha_{0_{20-241}}$  fusion protein specifically reacted with a protein of approximately 110 kDa present in HSV-1(F)-infected cell lysate (Fig. 3, lane 2). The electrophoretic mobility of the protein detected by the polyclonal antibody was identical to that detected by the mouse monoclonal antibody to ICP0 (Fig. 3, lane 4).

Cell lines expressing ICP0 were constructed as described in Materials and Methods. Of the three clonal cell lines selected in medium containing G418, cell line N3 expresses ICP0 most efficiently (Fig. 4A) and was therefore used in further studies. To construct the  $\alpha 0$  deletion mutant, a mixture of wild-type cosmids and a bridging plasmid (Fig. 1C) and cosmids that have been deleted of  $\alpha 0$  coding sequences (Fig. 1D) were

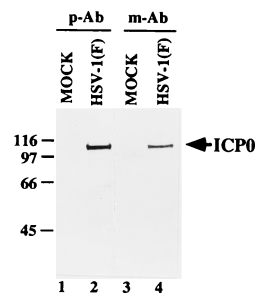


FIG. 3. Photographic image of an immunoblot of electrophoretically separated lysates of HEP-2 cells mock infected (lanes 1 and 3) or infected with HSV-1(F) (lanes 2 and 4) harvested 18 h postinfection. The blots were probed with a rabbit antiserum (p-Ab) raised against the GST-ICP0 fusion protein (lanes 1 and 2) or with the monoclonal antibody (m-Ab) to ICP0 (H1083) (lanes 3 and 4). Molecular weights ( $10^3$ ) are shown on the left.

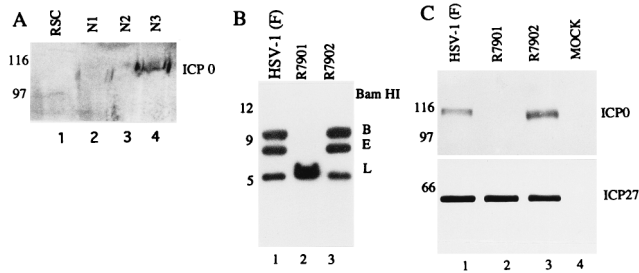


FIG. 4. (A) Photographic image of an immunoblot of electrophoretically separated lysates of clonal rabbit skin cell lines selected from progeny of cells cotransfected with a selectable marker and a plasmid capable of expressing ICP0. Confluent normal rabbit skin cells (lane 1) and clonal cell lines (lanes 2 to 4) were harvested, subjected to electrophoresis on an SDS-7% polyacrylamide gel, transferred to a nitrocellulose sheet, and reacted with mouse monoclonal antibody H1083 to ICP0. Molecular weight markers ( $10^3$ ) are shown on the left. (B) Autoradiographic images of the *Bam*HI digests of viral DNAs hybridized with specific probes. Lane 1, digests of HSV-1(F) viral DNA; lane 2, digests of the DNA of the R7901 ( $\alpha 0^-$ ) mutant; lane 3, digests of R7902 mutant DNA. The blot was probed with a mixture of  $^{32}$ P-labeled  $\alpha 0$  open reading frame containing pDS18 and  $^{32}$ P-labeled HSV-1(F) *Bam*HI L fragment (pRB123), located proximal to the  $\alpha 0$  gene, which served as a DNA loading control. (C) Photographic image of an immunoblot of electrophoretically separated lysates of HEP-2 cells infected with HSV-1(F) (lane 1), R7901 (lane 2), or R7902 (lane 3) or mock infected (lane 4), harvested 18 h after infection. The cells were harvested, solubilized, subjected to electrophoresis in an SDS-8.5% polyacrylamide gel, transferred to nitrocellulose, and reacted with either the mouse monoclonal antibody to ICP0 (H1083) or the mouse monoclonal antibody to ICP27 (H1113). Molecular weights ( $10^3$ ) are shown on the left.

transfected into the N3 cell line as described in Materials and Methods. Viral plaques apparent after 72 h were harvested, plaque purified on N3 cells, and examined for the absence of  $\alpha 0$  coding sequences in R7901. Figure 4B, lane 2, shows that  $\alpha 0$  sequences were absent in R7901 DNA but present in the R7902 DNA in which the  $\alpha 0$  sequences had been restored as described in Materials and Methods. In this experiment, the probe was specific for the  $\alpha 0$  sequence and *Bam*HI-L, which served as a DNA loading control. Similarly, R7902-infected cells made wild-type levels of ICP0, whereas no ICP0 was detected in cells infected with R7901 at an equivalent ratio of virus per cell (Fig. 4C). In this experiment, ICP27 served as a loading control.

Recombinant baculoviruses which overexpress cyclin D3 and CDK4 were generated as described in Materials and Methods so as to obtain enough activity of cyclin D3-dependent kinase. To confirm the expression of the protein products, lysates of Sf9 cells mock infected or infected with cyclin D3 or CDK4 baculovirus were electrophoretically separated in denaturing gels and stained with Coomassie blue or subjected to immunoblotting. As shown in Fig. 5A, overexpressed protein products of cyclin D3 (lane 3) and CDK4 recombinant (lane 4) were detected by Coomassie blue staining. Specificity of these products was confirmed by immunoblotting using as probes the monoclonal antibody to cyclin D3 (Fig. 5B) and the polyclonal antibody to CDK4 (Fig. 5C).

#### ICP0 interacts with cyclin D3 in a yeast two-hybrid screen.

The results of the yeast two-hybrid studies described in Materials and Methods were as follows. Of  $4.6 \times 10^6$  transformants, 31 displayed strong expression of  $\beta$ -galactosidase activity. Of these, the cDNA of one plasmid (pBH1004) was isolated and characterized further. The cDNA retransformed with various plasmids into yeast yielded evidence of positive interactions of cDNA products with ICP0 and negative interaction with human lamin, murine p53, or the GAL4 DNA-binding domain alone. Partial sequence analysis and restriction endonuclease

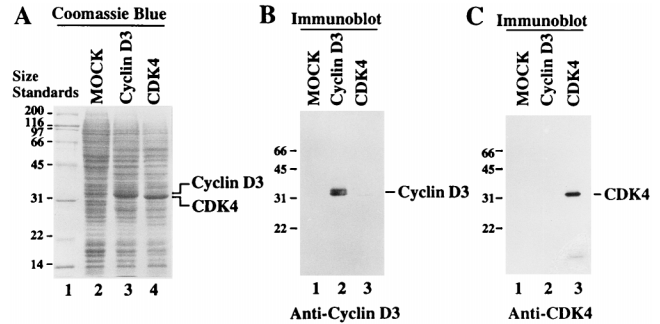


FIG. 5. Generation of recombinant baculoviruses. (A) Photographic image of a Coomassie blue-stained denaturing gel of electrophoretically separated lysates of Sf9 cells mock infected (lane 2) or infected with cyclin D3 recombinant baculovirus (lane 3) or CDK4 recombinant baculovirus (lane 4). Lane 1, molecular weight markers ( $10^3$ ). (B and C) Photographic images of immunoblots of electrophoretically separated lysates of Sf9 cells mock infected (lane 1) or infected with cyclin D3 (lane 2) or CDK4 (lane 3) recombinant baculovirus. The blots were reacted with the monoclonal antibody to cyclin D3 (B) or the polyclonal antibody to CDK4 (C). Molecular weights ( $10^3$ ) are shown on the left of each panel.

analysis of the plasmid revealed that the plasmid contains the cDNA of cyclin D3, a cell cycle regulator of  $G_1$  phase (30).

**Cyclin D3 specifically forms complexes with ICP0 in HSV-1-infected cell lysate.** To verify and extend the observed interaction between ICP0 and cyclin D3 in yeast, a GST-cyclin D3 fusion protein was expressed in *E. coli*. The GST-cyclin D3 or GST protein bound to glutathione-agarose beads was reacted with an extract from HSV-1(F)-infected HEP-2 cells. After extensive rinsing, protein complexes captured on the beads were solubilized, subjected to electrophoresis in a denaturing gel, transferred to a nitrocellulose sheet, and reacted with the monoclonal antibody to ICP0 (H1083). As shown in Fig. 6, the GST-cyclin D3 fusion protein was able to pull down ICP0, whereas GST alone did not. The electrophoretic mobility of the ICP0 that complexed with GST-cyclin D3 fusion protein was similar to that found in extracts of whole infected cells. The results indicate that cyclin D3 can interact with native, full-length ICP0.

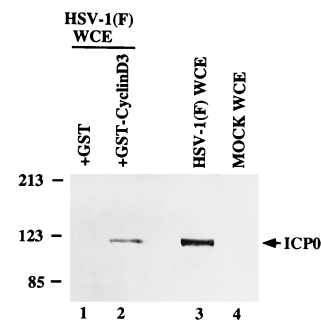


FIG. 6. Photographic image of an immunoblot of infected-cell proteins bound to GST or chimeric GST-cyclin D3 fusion protein, electrophoretically separated in a denaturing gel, and reacted with a mouse monoclonal antibody to ICP0 (H1083). Lysates of infected HEP-2 cells were reacted with GST or GST-cyclin D3 chimeric protein immobilized on glutathione-agarose beads. The beads were pelleted, rinsed extensively, subjected to electrophoresis on an SDS-8% polyacrylamide gel, transferred to a nitrocellulose sheet, and reacted with the ICP0 antibody. Lanes 1 and 2, whole-cell extracts (WCE) from HSV-1(F)-infected HEP-2 cells bound to GST and GST-cyclin D3, respectively; lanes 3 and 4, whole-cell extracts from HSV-1(F)-infected or mock-infected HEP-2 cells, respectively. Molecular weights ( $10^3$ ) are shown on the left.

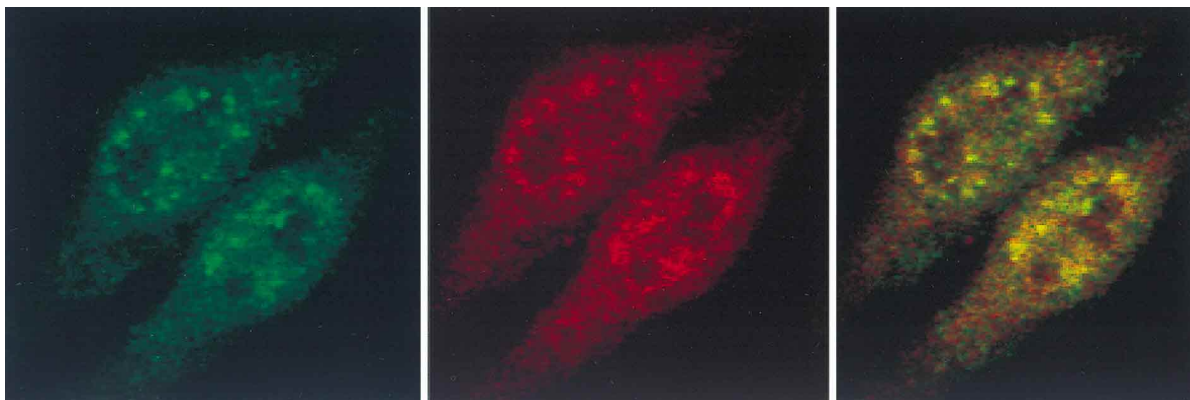


FIG. 7. Digital images of R7801-infected HEp-2 cells reacted with antibodies to ICP0 and cyclin D3. The infected cells were fixed 8 h after infection, double labeled with a combination of the mouse monoclonal antibody to cyclin D3 and the rabbit polyclonal antibody to ICP0, and then reacted with anti-mouse IgG conjugated to Texas red (red fluorescence) and anti-rabbit IgG conjugated to fluorescein isothiocyanate (green fluorescence). Single-color images were captured separately and are shown in the left (ICP0) and middle (cyclin D3) panels; the right panel (overlay) represents simultaneous acquisition of both colors. The yellow color visualized in the overlaid image represents colocalization of cyclin D3 and ICP0. The images were captured with software provided by Zeiss and printed by a Codonics CP210 printer. The digitized images were not modified subsequent to capture.

#### ICP0 and cyclin D3 colocalize in the nuclei of infected cells.

To determine whether ICP0 interacts with cyclin D3 at the cellular level, double-label immunofluorescence assays were done. A problem that emerged during the preliminary experiments was that clear fluorescence of cyclin D3 was not detectable even in normal cultured cells (data not shown). Therefore, we constructed a recombinant virus which overexpressed cyclin D3 as described above. HEp-2 cells were infected with recombinant virus R7801, fixed at 8 h after infection, reacted with a cyclin D3 monoclonal antibody and a polyclonal antibody to ICP0, and then examined with a Zeiss confocal microscope. As shown in Fig. 7, cyclin D3 colocalized with ICP0 in infected cells in granular structures strongly reminiscent of the ND10 structures reported earlier (1, 9, 25, 26).

**Reduction in the level of expression of cyclin D3 in cell infected with the  $\alpha 0$  deletion mutant virus.** D-type cyclins are known to associate with CDKs to form active holoenzymes which phosphorylate appropriate cellular substrates to facilitate cellular entry into S phase (41, 42). Like other cyclins, they are short-lived proteins (half-lives of <25 min), and free forms of D-type cyclins are degraded faster than the species bound to associating proteins (2). Our demonstration of the interaction between ICP0 and cyclin D3 raised three nonmutually exclusive, testable possibilities: (i) ICP0 affects the steady-state level of expression of cyclin D3, (ii) ICP0 is a substrate for the cyclin D3-dependent kinase, and/or (iii) ICP0 affects the activity of the kinase.

To test the first possibility, we constructed an  $\alpha 0$  null mutant, R7901, as described above (Fig. 1C and D and 4) and determined the level of expression of cyclin D3 protein in replicate Vero cell cultures mock-infected or infected with 1 PFU of HSV-1(F), R7901, or R7902 per cell. The cells were harvested at 3, 8, or 19 h after infection, solubilized, subjected to electrophoresis in a denaturing gel, transferred to a nitrocellulose sheet, and reacted with monoclonal antibody to cyclin D3. The results (Fig. 8A) were as follows. At 3 h after infection, the expression levels of cyclin D3 in HSV-1(F)-, R7901-, and R7902-infected cells were similar. At 8 h after infection, however, the amount of cyclin D3 in cells infected with the R7901 ( $\alpha 0^-$ ) mutant was much less than cells infected with wild-type virus or the recombinant R7902 in which the  $\alpha 0$  sequences were restored. At 19 h after infection, cyclin D3 was

detected in mock-infected cells but not in any of the infected cell cultures.

An earlier publication described the rabbit polyclonal antibody to EF-1 $\delta$  (19). This antibody also reacts with an  $M_r$ -50,000 cellular protein of unknown function. Reaction of the same immunoblot with this antiserum revealed that the  $M_r$ -50,000 protein was present in approximately equal amounts in all lanes (Fig. 8B).

These results indicate that the cellular proteins are not down-regulated nonspecifically and suggest that ICP0 modulates the expression level of cyclin D3, possibly by stabilization of cyclin D3 in infected cells.

Inasmuch as  $\alpha 0^-$  mutants do not multiply and spread efficiently in cells infected at low multiplicities of infection, the

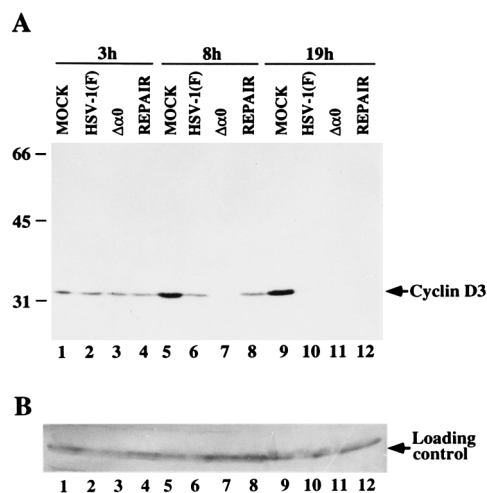


FIG. 8. Photographic image of electrophoretically separated lysates of Vero cells mock infected or infected with 1 PFU of HSV-1(F), R7901 ( $\Delta\alpha 0$ ), or R7902 (REPAIR) per cell and reacted with the monoclonal antibody to cyclin D3 (A) or with the rabbit polyclonal antibody to EF-1 $\delta$  (B). The infected cells were harvested at indicated times, solubilized, subjected to electrophoresis on an SDS-9% polyacrylamide gel, transferred to nitrocellulose, and reacted with antibodies as described in Materials and Methods. Molecular weights ( $10^3$ ) are shown on the left.



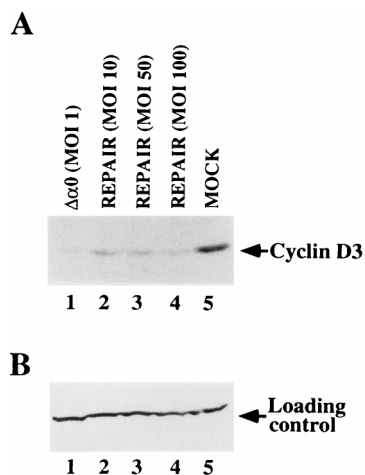


FIG. 9. Photographic image of electrophoretically separated lysates of Vero cells mock infected or infected with R7901 ( $\Delta\alpha 0$ ) or R7902 (REPAIR) and reacted with the monoclonal antibody to cyclin D3. Panel A and B shows levels of expression of cyclin D3 and the loading control, respectively. Vero cells were mock infected (lane 5) or infected with R7901 ( $\Delta\alpha 0$ ) at a multiplicity of infection (MOI) of 1 (lane 1) or R7902 (REPAIR) at a multiplicity of infection of 10 (lane 2), 50 (lane 3), or 100 (lane 4). The infected cells were harvested at 8 h after infection, solubilized, subjected to electrophoresis on an SDS-10% polyacrylamide gel, transferred to nitrocellulose, and reacted with the cyclin D3 antibody as described in Materials and Methods.

titers of these mutants may understate the actual number of physical particles contained in the virus stock. In the case of the stock used in these studies, the titer of the  $\alpha 0$  mutant was  $1.6 \times 10^7$  PFU/ml whereas that of the repaired virus was  $2.1 \times 10^9$  PFU/ml. To test the hypothesis that the disappearance of cyclin D3 in cells infected with the  $\alpha 0^-$  mutant is not due to the particle/cell ratio, we compared the stability of cyclin D3 in cells infected with 1 PFU of the  $\alpha 0^-$  mutant with that of cells infected with 10, 50, or 100 PFU of the repaired virus. The results, shown in Fig. 9, indicate that cyclin D3 is detectable in cells infected with the repaired virus at multiplicity of infection of as high as 100 PFU/cell. In this experiment, the protein loading control was a protein with an apparent  $M_r$  of 68,000 that was not affected by the multiplicity of infection.

Given the capacity of cells to produce a finite amount of virus, the particle yield of repaired virus at 100 PFU/cell should approximate or exceed the particle load of the  $\alpha 0^-$  mutant.

**ICP0 is not a substrate for cyclin D3-dependent kinase, and the amino-terminal domain of ICP0 does not affect the activity of cyclin D3-dependent kinase in vitro.** To address the question whether cyclin D3-dependent kinase enables the phosphorylation of ICP0, we constructed recombinant baculoviruses which overexpressed cyclin D3 and CDK4 as described above (Fig. 5). Studies described elsewhere have shown that a high level kinase activity is readily demonstrable in the baculovirus system (18). GST-pRb-Ase-End, GST- $\alpha 0_{20-241}$ , GST- $\alpha 0_{543-768}$ , and immunoprecipitated ICP0 from HEP-2 cells infected with HSV-1(F) were used as substrate proteins and reacted in the kinase buffer containing  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  with lysates of Sf9 cells infected with cyclin D3 recombinant baculovirus alone or lysates of Sf9 cells coinfecting with cyclin D3 and CDK4 recombinant baculoviruses, separated on a denaturing gel, and subjected to autoradiography. The results (Fig. 10A) were as follows.

(i) The lysate of Sf9 cells singly infected with cyclin D3 recombinant baculovirus did not phosphorylate the GST-pRb fusion protein (Fig. 10A, lane 9). The lysate of Sf9 cells coin-

fecting with cyclin D3 and CDK4 recombinant baculoviruses efficiently phosphorylated the GST-pRb fusion protein, indicating that the cell lysate contains high activity of the cyclin D3-CDK4 complex (Fig. 10A, lane 10). The Rb protein is a known substrate of cyclin D3 and CDK4 (45), and therefore the results indicate that the baculovirus-derived reagents were active and specific.

(ii) GST- $\alpha 0_{20-241}$  (Fig. 10A, lane 11) and immunoprecipitated ICP0 (Fig. 10A, lane 15) were phosphorylated in reaction mixtures containing lysates of Sf9 cells infected with cyclin D3 recombinant baculovirus. Cell lysates from Sf9 cells mock infected or infected with wild-type baculovirus also showed endogenous kinase activity for ICP0 (Fig. 10B). Addition of lysate of Sf9 cells which coexpressed cyclin D3 and CDK4 did not affect the level of phosphorylation of ICP0 (Fig. 10A, lanes 12 and 16). GST- $\alpha 0_{543-768}$  was not phosphorylated by lysates of Sf9 cells infected with baculoviruses expressing either cyclin D3 alone or both cyclin D3 and CDK4 (Fig. 10A, lanes 13 and 14). These results indicate that ICP0 is not a substrate for the cyclin D3-CDK4 complex.

Last, to test whether the interaction of ICP0 with cyclin D3 affects the activity of the kinase, increasing amounts of either GST alone or GST- $\alpha 0_{20-241}$  fusion protein were added to a mixture of GST-pRb and lysates of Sf9 cells expressing cyclin D3 and CDK4 in kinase buffer containing  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . As shown above, GST- $\alpha 0_{20-241}$  binds cyclin D3, and if this interaction affected the functional interaction of cyclin D3 and CDK4, we could expect interference with the phosphorylation of GST-pRb protein. Phosphorylated proteins were separated on a denaturing gel, stained with Coomassie blue, and subjected to autoradiography. The results shown in Fig. 10C indicate that the level of phosphorylation of GST-pRb was not affected by the presence of increasing amounts of GST alone or GST- $\alpha 0_{20-241}$ .

## DISCUSSION

The results presented in this report are a strong endorsement of the hypothesis that ICP0 is a multifunctional protein and that the apparent role of ICP0 in the HSV-1 replicative cycle may include control of viral gene expression through stabilization of cell cycle-dependent proteins, modulation of protein degradation pathways, translational regulation, and as yet undefined control of transcriptional processes. In this report, we show that ICP0 interacts with the cell cycle regulator cyclin D3. The salient features of this study are as follows.

(i) In the yeast two-hybrid system, the domain encoded by exon II of ICP0 interacted with cyclin D3. The interaction of cyclin D3 with ICP0 was also demonstrated by using GST-cyclin D3 fusion protein in pull-down experiments that are in effect a reciprocal of the yeast two-hybrid system and which reinforced the evidence of physical interaction between the two proteins. Furthermore, consistent with the binding data obtained in vitro, ICP0 colocalized with cyclin D3 in infected cells. These results suggest that the interaction between ICP0 and cyclin D3 occurs at the cellular level.

(ii) D-type cyclins including D1, D2, and D3 govern the rate of progression of mammalian cells through the  $G_1$  phase of the cell cycle and enforce the commitment of cells to replicate their chromosomal DNA (41, 42). In general, D-type cyclins which are highly induced by growth factors assemble with CDKs, mainly CDK4 and CDK6, to form holoenzymes that facilitate exit from  $G_1$  phase by phosphorylating key substrates including pRb (41, 42, 45). The latter protein controls gene expression mediated by a family of transcriptional regulators, collectively termed the E2Fs (23). Phosphorylation of pRb by

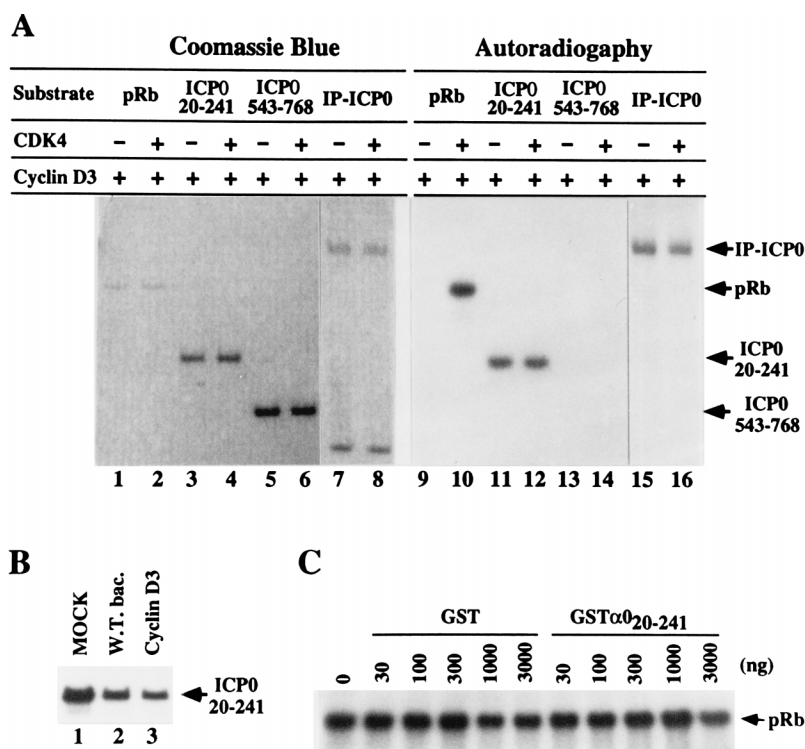


FIG. 10. In vitro kinase assays. (A) Photographic image of Coomassie blue-stained denaturing gels of electrophoretically separated GST fusion proteins and immunoprecipitated proteins (lanes 1 to 8) and autoradiographic images of the gels of the electrophoretically separated [ $\gamma$ - $^{32}$ P]ATP-labeled proteins (lanes 9 to 16). Lysates of Sf9 cells singly infected with cyclin D3 recombinant baculovirus or coinfecting with cyclin D3 and CDK4 recombinant baculoviruses were incubated with the indicated GST fusion proteins (pRb, ICP0<sub>20-241</sub>, and ICP0<sub>543-768</sub>) or immunoprecipitated ICP0 (IP-ICP0) for 20 min at 30°C in kinase buffer containing [ $\gamma$ - $^{32}$ P]ATP. The tested proteins were separated on denaturing gels, stained with Coomassie blue, and subjected to autoradiography. (B) Autoradiographic image of electrophoretically separated [ $\gamma$ - $^{32}$ P]ATP-labeled GST- $\alpha$ 0<sub>20-241</sub> fusion protein. Lysates of Sf9 cells mock infected (lane 1) or infected with wild-type baculovirus (lane 2) or cyclin D3 recombinant baculovirus (lane 3) were incubated with the GST- $\alpha$ 0<sub>20-241</sub> protein in kinase buffer containing [ $\gamma$ - $^{32}$ P]ATP. The proteins were electrophoretically separated and subjected to autoradiography. (C) Autoradiographic image of electrophoretically separated [ $\gamma$ - $^{32}$ P]ATP-labeled GST-pRb fusion protein. Lysates of Sf9 cells infected with recombinant baculoviruses carrying both cyclin D3 and CDK4 were reacted with the indicated amounts of GST or GST- $\alpha$ 0<sub>20-241</sub> protein, and the extracts were then assayed for pRb kinase activity in a kinase buffer containing [ $\gamma$ - $^{32}$ P]ATP. The products were separated on a denaturing polyacrylamide gel, stained with Coomassie blue, and subjected to autoradiography.

cyclin D-dependent kinases frees E2Fs trapped by hypophosphorylated forms of pRb and enables them to activate genes whose products are required for S-phase entry (23, 32, 45). Therefore, cyclin D3 plays an important role in cell cycle progression of G<sub>1</sub> phase. D-type cyclins are often called sensors of extracellular signals (41, 42). Because both their mRNAs and proteins turn over very rapidly, withdrawal of growth factors results in a precipitous decline in the levels of D-type cyclins (2, 41, 42). This dependency on extracellular signals is a key property of D-type cyclins, as opposed to those of cyclins E, A, and B. This singular feature of cyclin D3 renders it an ideal target of a viral protein such as ICP0, inasmuch as the latter has already been shown to be functionally associated with cell cycle-dependent functions (3).

(iii) In this report, we show that in cells infected with the ICP0 deletion mutant virus, the amount of cyclin D3 is less than in wild-type virus- or  $\alpha$ 0-repaired virus-infected cells. Taken together with the binding data also described in this report, these results suggest that ICP0 interacts with cyclin D3 and stabilizes the expression of the protein. Three lines of evidence support our conclusions.

First, ICP0 also interacts with a novel ubiquitin-specific protease, HAUSP, which is supposed to be involved in stabilization of target proteins by removing ubiquitin adducts (10). Although the mechanism by which D-type cyclins turn over is

unknown at present, decay of many other cyclins is regulated by the ubiquitin pathway (20, 31).

Second, Cai and Shaffer previously reported that cellular functions expressed 8 h after release of Vero cells from growth arrest in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle enhanced replication of an ICP0 deletion mutant, whereas replication of wild-type virus was independent of the cell cycle (3). In light of our results that ICP0 stabilizes cyclin D3, the hypothesis that ICP0 merely substitutes for a cell cycle-dependent function is less tenable than the alternative that cellular functions associated with the cell cycle promote viral replication and that the level of expression of the cellular function is regulated by ICP0.

Third, the need for cyclin D3 appears to be shared by other herpesviruses. Thus, the simian herpesvirus saimiri and its distant cousin human herpesvirus 8 both encode functional homologs of D-type cyclins (5, 17, 24, 33). Epstein-Barr virus encodes proteins which induce expression of a D-type cyclin (43). Herpesviruses thus employ different mechanisms to obtain D-type cyclin activity; some encode a homolog in their own genomes, whereas other herpesviruses encode viral proteins which modulate the level of D-type cyclins.

The scenario that we would like to propose is that one function of ICP0 is to bring HAUSP in apposition to cyclin D3 to protect the latter from degradation but not to impede its function in priming the cell for expression of events that would



be necessary for entry into S phase. This scenario is consistent with the published evidence that ICP0 colocalizes with HAUSP in the ND10 nuclear structures and with our evidence that cyclin D3 colocalizes with ICP0 in structures strongly reminiscent of ND10.

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