Developmental and Cell-Cycle Regulation of *Caenorhabditis elegans* HCF Phosphorylation†

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ABSTRACT: HCF-1 is a mammalian protein required for cell proliferation. It is also involved in transcriptional activation of herpes-simplex-virus immediate-early gene transcription in association with the viral transactivator VP16. HCF-1 and a related protein called HCF-2 possess a homologue in *Caenorhabditis elegans* that can associate with and activate VP16. Here, we demonstrate developmental regulation of *C. elegans* HCF (CeHCF) phosphorylation: a hyperphosphorylated form of CeHCF is present in embryos, whereas a hypophosphorylated form is present in L1 larvae. The phosphorylation patterns of endogenous CeHCF in worms and ectopically synthesized CeHCF in mammalian cells are remarkably similar, suggesting that the way CeHCF can be recognized by kinases is conserved in animals. Phosphorylation-site mapping of endogenous CeHCF, however, revealed that phosphorylation occurs at four clustered sites in the region of the protein that is not highly conserved among HCF proteins and is not required for VP16-induced complex formation. Indeed, phosphorylation of either CeHCF or human HCF-1 appears to be dispensable for association with VP16. All four CeHCF phosphorylation sites match the consensus recognition site for the cell-cycle kinases CDC2 and CDK2. Consistent with this similarity and with the developmental phosphorylation of CeHCF in *C. elegans* embryos, CeHCF phosphorylation is cell-cycle-regulated in mammalian cells.

The human protein HCF-1 is a transcriptional regulatory protein involved in cell proliferation (*1*). Its transcriptional regulatory properties have been elucidated through its role in activation of herpes simplex virus (HSV)[1] immediate-early (IE) gene transcription. During HSV infection, the viral transcriptional activator VP16 (also referred to as αTIF and Vmw65) associates with the host cell factor HCF-1 (also called C1, VCAF, and CFF) and a second cellular factor called Oct-1 to form a multiprotein complex on IE-gene promoters, called the VP16-induced complex (for review, see ref *2*). The role of HCF-1 in cell proliferation has been elucidated through the analysis of the hamster cell line tsBN67, which contains a single missense mutation in HCF-1 that causes a temperature-induced cell proliferation arrest (*1*). This mutation also disrupts VP16 association with HCF-1, suggesting that the roles of HCF-1 in cell proliferation and HSV IE-gene transcription are linked.

HCF-1 is a large protein of over 2000 amino acids that undergoes an unusual form of proteolytic processing, which results in amino- and carboxy-terminal fragments that remain noncovalently associated (*3*−*5*)—only the amino-terminal 380 residues of HCF-1 are required, however, to associate with VP16 and promote transcription (*6, 7*). In mammals, there is a second smaller protein called HCF-2, which is related to HCF-1 (*8*). In the worm *Caenorhabditis elegans*, there is a single homologue of the human HCF-1 and HCF-2 proteins, called CeHCF (*9*). All three proteins share significant similarity within the region of HCF-1 that interacts with VP16; indeed, albeit with differing efficiencies, all three proteins can associate with VP16 and promote VP16-induced complex formation (*8, 9*). All three proteins also share significant sequence similarity within their carboxy-terminal regions, but the central region of these proteins, which in HCF-1 is involved in its proteolytic processing, is not conserved among the three proteins (*8, 9*).

C. elegans is a simple metazoan, which we are using to study the function of the HCF family of proteins in animals (*9*). Here, we have studied HCF protein phosphorylation in worms and mammalian cells. We demonstrate that CeHCF is developmentally phosphorylated in worms. By conducting parallel analyses in worms and mammalian cells, we show that CeHCF phosphorylation is remarkably similar in worms and mammalian cells, occurring at four consensus CDC2/CDK2 sites. Our data suggest a dynamic view of HCF modification during worm cell-cycle progression and development.

EXPERIMENTAL PROCEDURES

Synchronized C. elegans Liquid Culture, and Embryo and L1 Larvae Extract Preparation. Viable Bristol N2 strain *C.*

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[1] Abbreviations: CeHCF, *Caenorhabditis elegans* HCF; HuHCF, human HCF; CDK, cyclin-dependent kinase; CDC, cell-division cycle; HSV, herpes simplex virus; IE, immediate-early; VIC, VP16-induced complex; WT, wild type; PD, phosphorylation deficient; PPase, protein phosphatase.

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elegans embryos were prepared by treating mixed populations of worms with alkaline hypochlorite (*10*). Embryos were transferred to S medium (*11*) and allowed to hatch at 20 °C in the absence of nutrients. Subsequently, the worms were fed packed *E. coli* strain NA22 and incubated with vigorous shaking at 20 °C for 60 h. To obtain a large number of embryos, the resulting gravid adult worms were treated with alkaline hypochlorite. A portion of the embryos was washed, resuspended in French press buffer [10 mM HEPES, pH 7.6, 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM EGTA, and Complete protease inhibitor (Roche)], and passed through a French press at 15 000 psi. The homogenate was then centrifuged at 40000*g* for 30 min at 4 °C and the supernatant was taken as total worm extract. The remaining embryos were allowed to hatch overnight at 20 °C in the absence of nutrients and the resulting L1 larvae were either directly or after 3 h of feeding separated from contaminating unhatched embryos by passing through nylon mesh with 13 mm mesh opening (Spectra/Mesh, Spectrum). Pure L1 larvae were collected and resuspended in French press buffer, and total worm extract was prepared as described above.

CeHCF Immunoprecipitation and Immunoblotting. The N16 αCeHCF polyclonal peptide antiserum has been described previously (*9*). The mouse αCeHCF monoclonal antibody Y1 was produced against the central, nonconserved part of the CeHCF protein (residues 416−557) after synthesis in *E. coli* as a glutathione *S*-transferase fusion protein. Endogenous CeHCF was immunoprecipitated from total worm extract supplemented with 150 mM NaCl. CeHCF synthesized in mammalian cell culture was immunoprecipitated from a "whole cell nuclear" extract prepared by lysing cells in extraction buffer containing 0.1% NP40, 100 mM Tris-HCl, pH 8, 200 mM NaCl, 0.2 mM EDTA, 10% glycerol, and Complete protease inhibitor. Polyclonal N16 αCeHCF antibody cross-linked to protein A−Sepharose beads was used for immunoprecipitation. The resin was incubated with worm or cell extract, washed five times with extraction buffer lacking glycerol, and eluted with N16 peptide at a concentration of 0.5 mg/mL. Immunoblotting was performed by semidry transfer after SDS−PAGE as described (*3*) and detected by SuperSignal (Pierce) chemiluminescence reagent, with either the N16 polyclonal antiserum at 1:3000 dilution or the monoclonal antibody Y1 at 1:10 000 dilution.

Phosphatase Treatment. Five microliters of purified CeHCF or HuHCF-1 protein eluate were diluted 10-fold in λ phosphatase buffer suplemented with 2 mM $MnCl_2$, and 400 units of the broad-specificity λ protein phosphatase (NEB) was added. The reaction was incubated for 1 h at 30 °C and stopped by addition of sodium vanadate to a final concentration of 10 mM.

In Vivo Labeling of C. elegans. E. coli NA22 strain culture (0.5 L) in midlogarithmic growth was labeled overnight with 30 mCi of [^{32}P]orthophosphate (NEN). After labeling, the bacteria were washed twice with S-basal medium and collected. A synchronized liquid culture of worms (60 mL) was started as described above with nonradioactive NA22 *E. coli*. At the young adult stage (56 h after feeding) worms were collected and purified from bacteria by a series of four differential centrifugations (100*g* for 2 min in S medium). The purified worms were fed the ^{32}P-labeled bacteria in S medium. After all radioactive bacteria were consumed, the ^{32}P-labeled worms were collected and total worm extract was prepared as described above.

Phosphoamino Acid Analysis and Phosphopeptide Mapping. Phosphoamino acid analysis was performed as described previously (*12*). Briefly, CeHCF immunoprecipitates were resolved by SDS−PAGE and the gel was dried. The radioactive CeHCF band was visualized by autoradiography and excised from the gel, and the protein was eluted from the gel slice, TCA-precipitated, and subjected to limited acid hydrolysis (6 M HCl) at 106 °C for 1 h. The resulting lysate was lyophilized and resuspended in 10 μL of formic acid/acetic acid buffer (pH 1.9) and unlabeled phosphoamino acid standards were added. Phosphoamino acids were resolved by two-dimensional thin-layer electrophoresis on cellulose plate. The phosphoamino acid standards were visualized with ninhydrin staining and the [^{32}P]phosphoamino acids by autoradiography.

Phosphopeptide mapping was performed as described (***12*).** Briefly, the radioactive CeHCF band was excised from a dried gel and the gel piece was rehydrated in 50 mM ammonium bicarbonate, ground into small pieces, and subjected to "in-gel" digestion with trypsin for 24 h at 37 °C. The supernatant was collected, lyophilized, and resuspended in 10 μL of pH 1.9 buffer. Phosphopeptides were spotted onto a 20-cm cellulose plate and resolved by thin-layer electrophoresis in pH 1.9 buffer in the first dimension and thin-layer chromatography in phosphobuffer in the second dimension. ^{32}P-Radiolabeled peptides were visualized by autoradiography.

Protein Purification for Phosphopeptide Sequence Analysis. Ten micrograms of CeHCF was purified from total worm extract obtained from a 40 mL pellet of synchronized gravid adult worms by α-N16 immunoprecipitation and N16 peptide elution. The preparative immunoprecipitate was resolved by SDS−PAGE, the gel was stained with Coomassie brilliant blue, and the CeHCF band was excised from the gel. ^{32}P-Labeled CeHCF tracer (62 000 cpm in less than 0.5 μg of protein) was purified by the same method from a "whole cell nuclear" extract prepared as described above from ^{32}P-labeled CeHCF-synthesizing hamster tsBN67 cells grown at 33.5 °C.

HPLC Analysis and Protein Sequencing. To obtain the N-terminal phosphopeptide amino acid sequence, purified endogenous CeHCF and tracer CeHCF (20:1 molar ratio) were mixed as gel fragments and digested in situ with LysC endopeptidase. The resulting peptide fragments eluted from the gel fragments were separated by HPLC (Model 1090, Hewlett Packard) on a Vydac C18 column (Separations Group). The fractions were collected by peaks, and radioactivity of each fraction was measured by Cerenkov counting. A portion of each radioactive fraction was analyzed in an automated protein sequencer (Procise 494, Applied Biosystems). The remaining portion was covalently bound to arylamine PVDF membrane (Sequelon-AA, Millipore) and subjected to Edman degradation analysis in which the radioactivity of extracted Edman degradation products was quantified after each cycle (*13*).

Mammalian-Cell CeHCF Expression Vector, Mutagenesis, and Transient Transfection. The CeHCF expression construct $pCGNCeHCF_{FL}$ has been described previously (*9*). A tsBN67 cell line stably synthesizing CeHCF was isolated from a population of cell colonies obtained by cotransfection of

pCGNCeHCF$_{FL}$ expression construct with the plasmid pSVneo followed by 2 weeks of selection in the presence of G418 under conditions described previously (*9*). Amino acid substitutions were generated as described previously (*14*) by single-strand oligonucleotide-directed mutagenesis of the pCGNCeHCF$_{FL}$ construct. COS-1 cells were transfected by electroporation on a Bio-Rad Genepulser with Extendor set at 200 V and 960 mF. Electrophoretic mobility retardation analysis was performed as described previously (*3*)

Human HCF-1 Immunoprecipitation and Immunoblotting. The N18 polyclonal antiserum (*1*) and M2 monoclonal antibody directed against the carboxy-terminal portion of HCF-1 (*15*) have been described previously. Endogenous human HCF-1 was immunoprecipitated from a 293-cell "whole cell nuclear" extract with the N18 antibody covalently linked to protein A beads. The beads were washed five times with extraction buffer without glycerol, and HCF-1 was eluted with the N18 peptide at a concentration of 0.5 mg/mL. A 1:10 000 dilution of M2 antibody was used to probe the immunoblot.

Cell Cycle Analysis. Unsynchronized populations of hamster tsBN67 cells synthesizing CeHCF were blocked for 24 h in DMEM medium containing 10% FBS supplemented with either mimosine or nocodazole. Cells were washed, released from the block in DMEM medium containing 10% FBS, and harvested at different time points. To determine cellular DNA content, cells were trypsinized, washed with PBS, fixed with 70% ethanol, and stained with propidium iodide (50 μg/mL). Cellular DNA content was then analyzed by FACS analysis (EPICS Elite, Coulter).

RESULTS

Developmental Regulation of CeHCF Phosphorylation in C. elegans. We have previously described by Northern hybridization analysis the presence of CeHCF-encoding mRNA in *C. elegans* embryos and adults, with lower levels in L1−L4 larvae (*9*). To study the presence of CeHCF protein, we prepared cell extracts of worms at embryonic, L1, L2, L3, L4, and adult stages of development and analyzed the extracts by immunoblotting with CeHCF-specific antibodies (see Experimental Procedures). In such an experiment, CeHCF protein was evident in extracts from adults, embryos, and L1 larvae with lower levels, if any, evident in L2−L4 larvae (data not shown and Figure 1A). The CeHCF protein present in adult and embryo extracts, however, migrated as a doublet, whereas the protein in L1 larvae extracts prepared from either starved L1 or L1 fed for 3 h migrated as a single species corresponding to the faster migrating of the two embryonic and adult species. This result is illustrated in Figure 1A, where the embryonic extract produces the doublet (lane 2) and the L1 larval extract produces the single CeHCF species (lane 4).

The embryonic and adult CeHCF doublet suggested that CeHCF is posttranslationally modified. To test this hypothesis, we assayed whether the embryonic CeHCF doublet is sensitive to treatment with phosphatase. CeHCF purified from both embryonic and L1 larvae extracts by immunoprecipitation followed by peptide elution was treated in the absence or presence of the broad-specificity λ bacteriophage phosphatase, and the effect of this phosphatase treatment was determined by immunoblot analysis, as shown in Figure 1A.

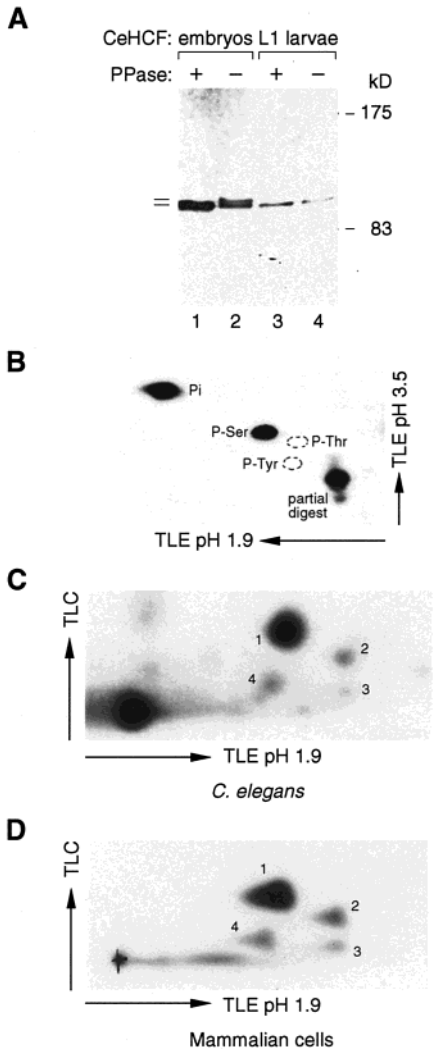

FIGURE 1: CeHCF phosphorylation is developmentally regulated and is similar in *C. elegans* embryos and when synthesized in mammalian cells. (A) Embryonic and L1 larval French press extracts were prepared as described under Experimental Procedures. CeHCF was immunopurified from both embryonic (lanes 1 and 2) and L1 larval (lanes 3 and 4) extracts. Eluted protein was then incubated either in the presence (lanes 1 and 3) or in the absence (lanes 2 and 4) of the phosphatase and analyzed by immunoblotting with anti-CeHCF monoclonal antibody. (B) CeHCF was purified by immunoprecipitation and SDS−PAGE from in vivo ^{32}P-labeled synchronized *C. elegans* adult extract and subjected to phosphoamino acid analysis by two-dimensional thin-layer electrophoresis as described under Experimental Procedures. The positions of phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) are indicated. The presence of ^{32}P-labeled phosphoserine was detected by autoradiography. (C) The remaining portion of in vivo labeled CeHCF immunoprecipitate used in panel B was resolved by SDS−PAGE. The tryptic peptides were prepared as described under Experimental Procedures and separated by electrophoresis in the first dimension and chromatography in the second dimension (indicated by arrows). Numbers 1−4 were assigned to the principal phosphopeptide spots. (D) CeHCF immunopurified from in vivo ^{32}P-labeled hamster tsBN67 cells stably transfected with pCGNCeHCF$_{FL}$ was subjected to two-dimensional tryptic phosphopeptide analysis as described under Experimental Procedures.

Consistent with phosphorylation of the slower-migrating embryonic form of CeHCF, the CeHCF doublet (lane 2) collapsed into the faster migrating form (lane 1) upon phosphatase treatment. In contrast, the mobility of the L1 larval CeHCF protein did not change upon phosphatase

treatment (compare lanes 3 and 4). These results indicate that there is a phosphorylation-induced shift in CeHCF electrophoretic mobility that is developmentally regulated, occurring in *C. elegans* embryos but not L1 larvae.

CeHCF Phosphorylation on Multiple Serine Residues in C. elegans. To characterize the phosphorylation of CeHCF, we performed phosphoamino acid and two-dimensional peptide map analysis. We incorporated radioactive phosphate into living worms by feeding a culture of synchronized young adults with ^{32}P-labeled *E. coli*. After consumption of the radioactive bacteria, we harvested gravid adult worms and prepared a French press extract. We subsequently purified CeHCF from this extract by immunoprecipitation and SDS−PAGE. A portion of the radiolabeled CeHCF was subjected to phosphoamino acid analysis, which revealed the exclusive presence of phosphoserine, as shown in Figure 1B. The remaining portion was digested with trypsin and analyzed by two-dimensional peptide mapping (*12*), which revealed the presence of four principal phosphopeptides (labeled 1−4, Figure 1C). These results suggest that CeHCF is phosphorylated at multiple serine residues.

Similar Patterns of CeHCF Phosphorylation after Ectopic Synthesis in Mammalian Cells. Using tsBN67 hamster kidney cells stably expressing the *C. elegans* HCF gene, we asked whether CeHCF is also phosphorylated in mammalian cells and, if so, whether the pattern of phosphorylation is similar to that in *C. elegans*. Phosphoamino acid analysis of CeHCF isolated from tsBN67 cells revealed the exclusive presence of phosphoserine in CeHCF from worms (data not shown), and, as shown in Figure 1D, tryptic peptide map analysis revealed the same pattern of four principal phosphopeptides (peptides 1−4) observed for CeHCF isolated from *C. elegans*. Strikingly, not only are the relative positions of the four phosphopeptides similar but their relative intensity is also remarkably similar (compare Figure 1 panels C and D). Thus, the manner in which CeHCF is recognized by kinases in very different organisms (nematodes vs mammalian cells) appears to be very similar, if not identical.

CeHCF Phosphorylation Site Determination. By designing the following strategy, we took advantage of the similarity of CeHCF phophorylation in *C. elegans* and tsBN67 cells, to determine the sites of CeHCF phosphorylation: We purified unlabeled CeHCF from synchronized gravid adult worms and mixed the purified protein with a small amount (less than 5% of the total) of radioactively labeled CeHCF tracer protein isolated from ^{32}P-labeled tsBN67 cells. The mixture of labeled and unlabeled CeHCF protein was used to determine the sites of phosphorylation by Edman degradation of peptides generated by digestion with the lysine-specific protease LysC and purified by HPLC (see Experimental Procedures). The HPLC fractionation revealed three radioactive fractions (numbered 1, 3, and 18) resulting from the tracer tsBN67-labeled CeHCF proteins. As indicated in Figure 2A, the peptide(s) in each fraction were subjected to repeated cycles of Edman degradation, which revealed the amino acid sequence of the unlabeled *C. elegans* CeHCF present in excess as shown in Figure 2B. The position(s) of phosphorylated residues in each peptide, which cannot be identified as a phenylthiohydantoin-amino acid derivative by HPLC (labeled "−" in Figure 2B), was confirmed by a parallel Edman degradation analysis in which the levels of ^{32}P present as a result of the tracer tsBN67 CeHCF were measured after each cycle, as shown in Figure 2C.

Sequence analysis of fraction 1 revealed the presence of two amino acid derivatives in Edman degradation cycles 1−4, suggesting a mixture of two phosphopeptides. Indeed, the paired amino acid residues resulting from the cycles of Edman degradation can be matched to two LysC digestion products deduced from the CeHCF sequence—ETEQSPK and NETASPK—which differ in positions 1−4 but are identical in positions 5−7. The site of phosphorylation was confirmed by the finding that the fifth Edman degradation cycle was also the cycle containing the radioactive ^{32}P tracer (Figure 2C). These results suggest that fraction 1 contained two phosphorylated heptapeptides.

The analysis of fraction 3 revealed the LysC peptide sequence RATT−PRK (Figure 2B), in which the fifth position, which corresponds to a serine residue in the CeHCF peptide sequence RATTSPRK, was radiolabeled (Figure 2C), indicating that this serine is a site of CeHCF phosphorylation. The analysis of fraction 18 revealed the LysC peptide sequence EQSV−PIK (Figure 2B), which probably corresponds to the CeHCF sequence EQSVSPIK. We failed, however, to identify the position of tracer ^{32}P labeling for this peptide (Figure 2C). Nonetheless, only the fifth Edman degradation cycle did not reveal a phenylthiohydantoin-amino acid derivative by HPLC (Figure 2B), suggesting that the serine at the fifth position in the deduced peptide sequence EQSVSPIK is the site of phosphorylation.

The four phosphoserines in the four phosphopeptides identified in CeHCF correspond to positions 423 (fraction 18, EQSV*S*PIK), 431 (fraction 3, RATT*S*PRK), 449 (fraction 1, ETEQ*S*PK), and 498 (fraction 1, NETA*S*PK). As shown in Figure 2D,E, all four deduced serine phosphorylation sites lie within an 80 amino acid region of CeHCF that is in the nonconserved central region of the CeHCF protein. The region of CeHCF just amino-terminal of sites 423 and 431, however, is highly conserved and corresponds to a region of human HCF-1 involved in amino-terminal and carboxy-terminal HCF-1 fragment association (*16*; see Discussion).

Confirmation of Phosphorylation Sites by Site-Directed Mutagenesis. To confirm the identity of the CeHCF phosphorylation sites, we engineered four individual serine-to-alanine substitutions: S423A, S431A, S449A, and S498A. To assay phosphorylation of the mutant proteins, we (i) transfected cDNA constructs encoding the wild-type and mutant CeHCF proteins into COS-1 cells, (ii) labeled the transfected cells with ^{32}P 24 h after transfection, (iii) purified the labeled CeHCF proteins by immunoprecipitation, and (iv) analyzed the purified proteins by SDS−PAGE and two-dimensional peptide mapping as shown in Figure 3. In monkey COS-1 cells (Figure 3B) and human 293 cells (data not shown), CeHCF displays a similar phosphorylation pattern as in tsBN67 cells (Figure 1C), indicating that CeHCF phosphorylation is generally similar in mammalian cells. We used COS-1 cells to label wild-type and mutant CeHCF proteins because CeHCF is synthesized at very high levels in these cells. Immunoblot analysis showed that the wild-type and mutant proteins were synthesized at similar levels (Figure 3A, lanes 2−6). Like the wild-type protein (lane 2), the singly mutated S423A, S431A, and S498A proteins appeared as doublets (lanes 3, 4, and 6), but the S449A mutant protein appeared as a single species corresponding

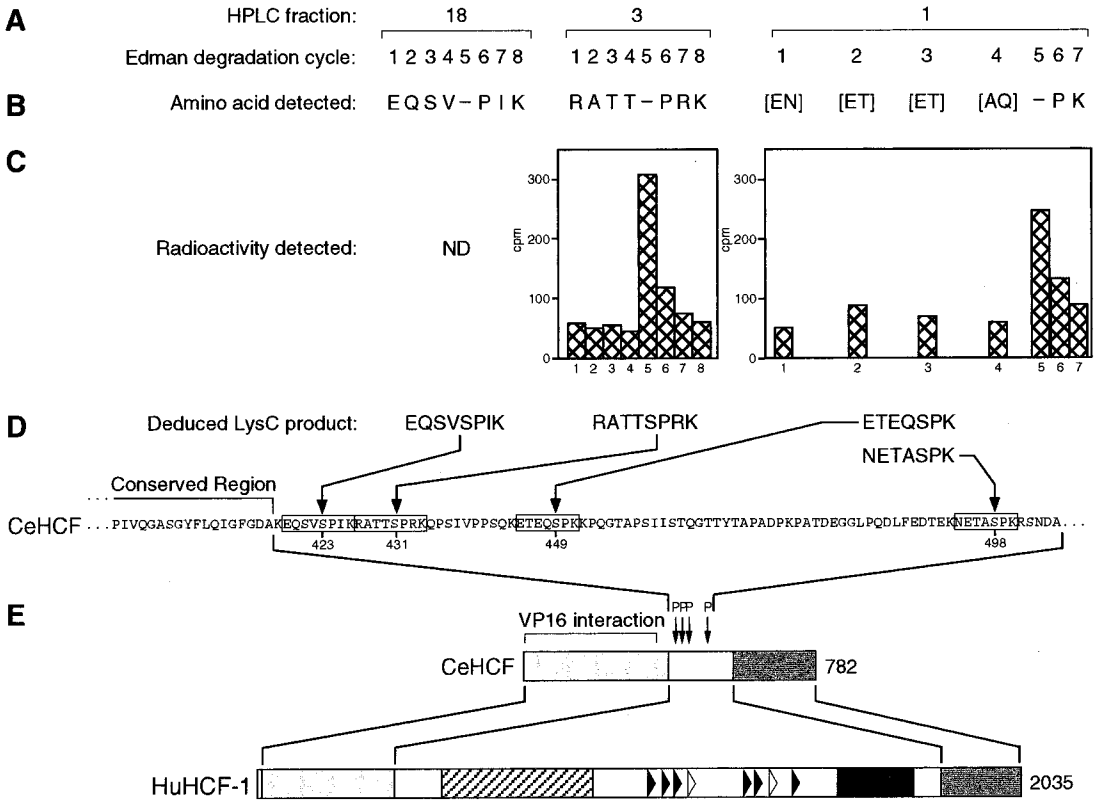

FIGURE 2: CeHCF phosphorylation-site determination. Endogenous CeHCF was purified from a synchronized *C. elegans* adult extract and mixed with radioactively labeled CeHCF tracer protein isolated from ^{32}P-labeled pCGNCeHCF$_{FL}$ transfected tsBN67 cells (see Experimental Procedures). The mixture of labeled and unlabeled proteins was digested with lysine-specific endopeptidase LysC, and the resulting peptides were fractionated by HPLC. Cerenkov counting of HPLC fractions revealed the presence of three radioactive fractions (1, 3, and 18). (A) The number of Edman degradation cycles performed on fractions 18, 3, and 1 is indicated. (B) Amino acid derivative detected in each Edman degradation cycle shown in panel A; (−) no derivative detected. (C) Results of parallel radioactivity measurements of Edman degradation cycles with the phosphopeptides from each fraction in panel A covalently bound to PVDF membrane. Results are represented as bar graphs with the cycle number indicated on the horizontal axis and radioactivity indicated on the vertical axis. ND, not determined. (D) Deduced sequences of the phosphorylated CeHCF LysC digest products in each fraction are shown and positioned in the amino acid sequence of the central region of CeHCF. The phosphopeptide sequences are boxed, phosphorylated serines are identified by arrows, and their positions within the CeHCF amino acid sequence are indicated. The extent of the amino-terminal conserved region of CeHCF is denoted by the bracket. (E) Schematic structure comparison of human and *C. elegans* HCF. The schematic representations of CeHCF and HuHCF-1 are as shown previously (*3*, *9*). Within the diagram, structural and functional features of HCF-1 are indicated from left to right as follows: VP16-interaction domain (gray box), basic region (hatched), functional (filled triangles) and nonfunctional (open triangles) HCF-1 processing repeats, acidic region (black), and fibronectin type 3 repeats (dark gray). Phosphorylation sites in CeHCF are indicated by vertical arrows.

to the faster migrating form (lane 5), suggesting that it is phosphorylation of serine 449 that is responsible for the CeHCF phosphorylation-induced mobility shift seen in Figure 1. Consistent with multiple CeHCF phosphorylation sites, each of the singly mutated CeHCF proteins was labeled with ^{32}P (data not shown).

To test whether the four mutated sites are responsible for all CeHCF phosphorylation, we prepared a putative phosphorylation-deficient (PD) mutant CeHCF protein with all four of these serines substituted with alanine. After synthesis in COS-1 cells, this protein comigrated with the S449A mutant (Figure 3A, lane 7). When it was labeled with ^{32}P, however, very little ^{32}P was incorporated into this quadruple-mutated protein relative to the wild-type protein (compare lanes 9 and 10). Therefore, we conclude that the four identified phosphorylation sites are responsible for the large majority of CeHCF phosphorylation. We refer to this phosphorylation-deficient mutant as CeHCF$_{PD}$.

To map the phosphorylation sites to the spots resulting from the two-dimensional peptide maps (see Figure 1), we digested each mutant protein with trypsin and prepared peptide maps. In data not shown here, these studies revealed that the two-dimensional peptide map spots 1−4 represent phosphorylation at sites 431, 423, 498, and 449, respectively, as indicated in Figure 3B. As examples, the two-dimensional peptide map of the CeHCF mutant S449A lacks spot 4 (compare Figure 3 panels B and C, see arrow) and that of CeHCF$_{PD}$ lacks all four spots (Figure 3D). For the experiment shown in Figure 3D, we used a 5-fold larger amount of the CeHCF protein; even at these higher levels we were unable to identify any phosphopeptides of the two-dimensional peptide map. Nevertheless, phosphoamino acid analysis of CeHCF$_{PD}$ showed that the residual ^{32}P labeling is of serine residues (data not shown). This residual phosphorylation may be due to low-level phosphorylation of different serine residues. These results of CeHCF phosphorylation site mapping and subsequent mutagenesis validate the two-pronged approach of mixing unlabeled *C. elegans* protein and labeled tracer protein synthesized in mammalian cells that we have developed here.

Native CeHCF Phosphorylation Is Not Required for VP16-Induced Complex Formation. A remarkable feature of

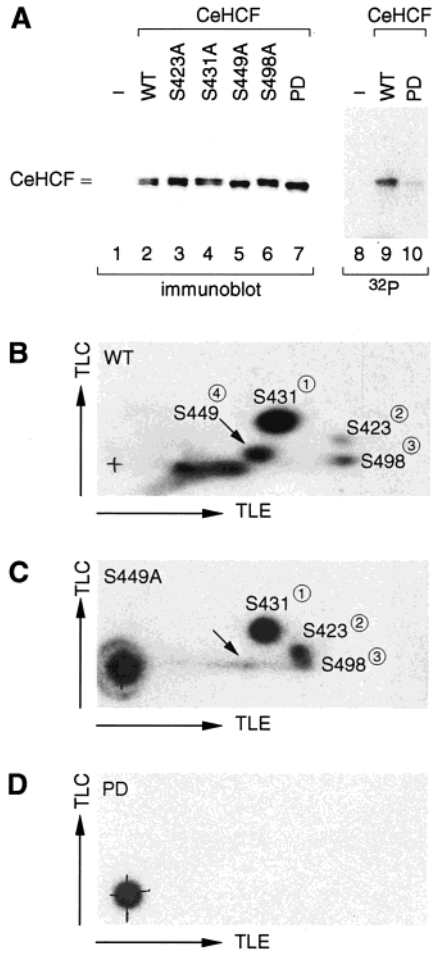

FIGURE 3: Mutagenesis of four identified phosphorylation sites blocks specific CeHCF phosphorylation. (A) Immunoblot analysis of wild-type (WT) and mutant CeHCF after transient synthesis in COS-1 cells. WT and mutant proteins were immunopurified from COS-1 cells transfected with CeHCF-encoding cDNAs, which were ^{32}P-labeled 24 h after transfection, as follows: untransfected COS-1 cells (lane 1); $CeHCF_{WT}$ (lane 2); CeHCF proteins containing individual serine to alanine substitutions at positions 423 (lane 3), 431 (lane 4), 449 (lane 5), and 498 (lane 6); and quadruple mutant S[423,431,449,498]A ($CeHCF_{PD}$; lane 7). Immunoprecipitates were resolved by SDS−PAGE and probed by immunoblotting with anti-CeHCF monoclonal antibody Y1. An autoradiogram of samples from untransfected cells (lane 8), or cells synthesizing $CeHCF_{WT}$ (lane 9), or $CeHCF_{PD}$ (lane 10) is shown. (B−D) Effects of selected serine-to-alanine substitutions on the patterns of CeHCF phosphorylation in COS-1 cells. ^{32}P-Labeled $CeHCF_{WT}$ (B), CeHCF S449A (C), and $CeHCF_{PD}$ proteins (D) were isolated from transiently transfected COS-1 cells and digested with trypsin. Resulting phosphopeptides were analyzed as described under Experimental Procedures. The identity of phosphoserines is indicated by each peptidic spot.

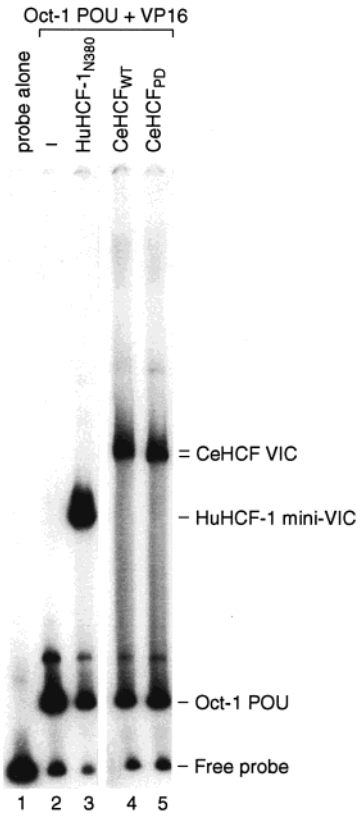

FIGURE 4: The phosphorylation-deficient mutant CeHCF is able to promote VP16-induced complex formation. $CeHCF_{WT}$ and $CeHCF_{PD}$ were synthesized in COS-1 cells. "Whole cell nuclear" extract from these cells was assayed for VP16-induced complex formation by using an electrophoretic mobility retardation assay with an ICP0 TAATGARAT DNA probe (*3*). The samples contain probe alone (lane 1) and probe with the Oct-1 POU domain and GST-VP16Δ*C* proteins (lanes 2−5). Samples contained in addition $HuHCF\text{-}1_{N380}$ (lane 3) or extract from COS-1 cells containing $CeHCF_{WT}$ (lane 4) or $CeHCF_{PD}$ (lane 5). The positions of free probe, the Oct-1 POU domain−DNA complex (Oct-1 POU), and the VP16-induced complex with $HuHCF\text{-}1_{N380}$ (HuHCF-1 mini-VIC) and CeHCF (CeHCF VIC) are indicated.

CeHCF is that it has retained the ability to associate with the HSV VP16 protein and promote VP16-induced complex formation even though the VP16-induced complex is involved in activation of the lytic program of a human viral pathogen (*9*). We believe that this conservation is a reflection of VP16 mimicry of an important HCF-1 interaction with a cellular effector molecule that has been conserved during metazoan evolution. Phosphatase treatment of purified human HCF-1 protein disrupts VP16 association with HCF-1 in a Far-Western blotting assay, suggesting that HCF-1 phosphorylation is important for VP16 association (*17*). Given the conserved ability of VP16 to associate with CeHCF, we asked whether CeHCF phosphorylation is important for CeHCF to form a VP16-induced complex.

For this purpose, we assayed the ability of $CeHCF_{PD}$ to stabilize the VP16-induced complex: (i) The $CeHCF_{WT}$ and $CeHCF_{PD}$ proteins were synthesized in COS-1 cells by transient expression, (ii) cell extracts were prepared, (iii) the levels of CeHCF protein were normalized, and (iv) the extracts were used to assay VP16-induced complex formation in an electrophoretic mobility retardation assay. As shown in Figure 4, although generating a complex possessing a slightly faster electrophoretic mobility, $CeHCF_{PD}$ formed a VP16-induced complex as well as the $CeHCF_{WT}$ protein (compare lanes 4 and 5). Phosphatase treatment of endogenous CeHCF also fails to influence the efficiency of VP16-induced complex formation (data not shown). These results indicate that CeHCF phosphorylation is dispensable for VP16-induced complex formation.

In Vitro Phosphatase Treatment of Human HCF-1 Does Not Inhibit VP16-Induced Complex Formation. The ability of $CeHCF_{PD}$ to stabilize the VP16-induced complex led us to investigate the role of human HCF-1 phosphorylation in VP16-induced complex formation. We purified endogenous HCF-1 from ^{32}P-labeled human 293 cells by immunopre-

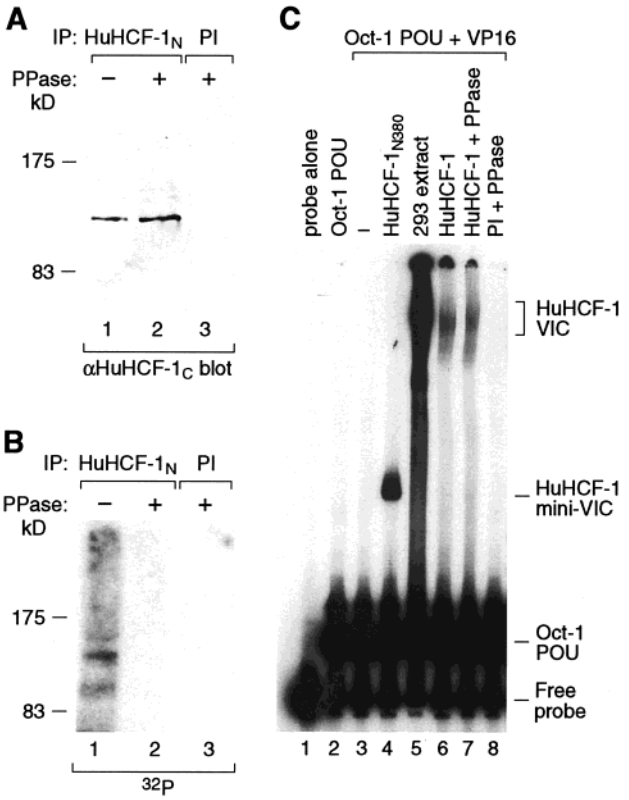

FIGURE 5: Phosphatase treatment of HuHCF-1 does not affect VP16-induced complex formation. Endogenous HuHCF-1 was purified from ^{32}P-labeled 293 cells by immunoprecipitation with the N18 antisera and elution with excess N18 peptide. The eluted protein was than treated in the absence or presence of the broad-specificity λ protein phosphatase and analyzed by (A) immunoblot with the anti-HuHCF-1 M2 monoclonal antibody (*15*), (B) autoradiography, and (C) VP16-induced complex formation assay. (A) Mock-treated (lane 1) and phosphatase-treated (lane 2) purified HuHCF-1 and phosphatase-treated extract purified with preimmune serum (lane 3). (B) Autoradiogram of the same samples used for immunoblot analysis in panel A. (C) Samples corresponding to $^1/_{30}$ of each sample analyzed in panels A and B were assayed for VP16-induced complex formation by electrophoretic mobility retardation assay: DNA probe alone (lane 1), probe with Oct-1 POU domain (lane 2), and probe with Oct-1 POU domain and GST-VP16Δ*C* (lanes 3−8). Samples contained in addition purified HuHCF-1$_{N380}$ (lane 4), 293 cell extract (lane 5), mock-treated (lane 6) and phosphatase-treated (lane 7) purified HuHCF-1, or phosphatase-treated extract purified with preimmune serum (lane 8). The positions of free probe, the Oct-1−POU domain complex (Oct-1 POU), and the VP16-induced complex with HuHCF-1$_{N380}$ (HuHCF-1 mini-VIC) and endogenous HuHCF-1 (HuHCF-1 VIC) are indicated.

cipitation with an amino-terminal αHCF-1 peptide antiserum under native conditions followed by peptide elution; a control extract was treated in parallel with preimmune serum. We then divided the purified protein into two fractions, one of which was treated in vitro with λ bacteriophage phosphatase. The levels of HCF-1 in the phosphatase treated and untreated samples were compared alongside the preimmune precipitate control by immunoblotting as shown in Figure 5A. This analysis showed that phosphatase treatment did not affect the levels of purified HCF-1 (compare lanes 1 and 2) and no HCF-1 was recovered in the preimmune precipitate (lane 3). An autoradiogram of the same samples (Figure 5B) showed that the phosphatase treatment of the HCF-1 sample removed all of the ^{32}P label (compare lanes 1 and 2), indicating that the HCF-1 preparation was effectively dephosphorylated. When used in an electrophoretic mobility retardation assay, however, with Oct-1 and VP16 purified from *E. coli* as shown in Figure 5C, the phosphatase-treated HCF-1 (HuHCF-1 + PPase) promoted complex formation (HuHCF-1 VIC) as well as the untreated HCF-1 (compare lanes 6 and 7). Thus, although HCF-1 phosphorylation may be important for VP16 association in a Far-Western assay (*17*), it is not apparently important for VP16-induced complex formation. Thus, both human and *C. elegans* HCF have conserved the ability to stabilize the VP16-induced complex independently of phosphorylation.

Cell-Cycle-Dependent Phosphorylation of CeHCF in Mammalian Cells. Although phosphorylation of CeHCF is not required for VP16-induced complex formation, the highly conserved pattern of CeHCF phosphorylation in *C. elegans* embryos and mammalian cells (see Figure 1) permits a functional analysis of CeHCF phosphorylation in mammalian cells. As shown in Figure 6A, the four CeHCF phosphorylation sites share an extended region of sequence similarity (K$\psi\psi\psi$XSP$^{K}/_{R}{}^{K}/_{R}$ where Ψ indicates a hydrophilic residue and X denotes a variable residue). This extended similarity contains the consensus target sequence *S*P$^{K}/_{R}{}^{K}/_{R}$ for the cell-cycle regulated cyclin-dependent kinases CDC2 and CDK2 (*18*), which are active in the S and M phases. This sequence similarity suggests that CeHCF phosphorylation may be cell-cycle-regulated. To test this hypothesis, we asked whether CeHCF phosphorylation is cell-cycle-dependent after CeHCF synthesis in mammalian cells.

Unsynchronized populations of hamster tsBN67 cells synthesizing CeHCF were blocked for 24 h with either mimosine, to arrest cells at the G_1 to S transition, or nocodazole, to arrest cells in mitosis. Cells were released from the block, harvested at different time points, and analyzed for DNA content by staining with propidium iodide followed by flow cytometry. Cell extracts corresponding to each time point were probed for CeHCF by immunoblotting. The phosphorylation status of CeHCF at position S449 was monitored by the appearance of the phosphorylation-induced change in CeHCF electrophoretic mobility (see Figure 1A).

As shown in Figure 6B, after 24 h of mimosine treatment, 80% of the cells possessed G_1 DNA content (lane 2), and only the faster migrating form of CeHCF is evident (lane 2). As more cells enter S phase (lanes 3−5; up to 48% after 18 h), the slower migrating species, corresponding to phosphorylated CeHCF, is more prominent. In contrast, after 24 h of nocodazole treatment, 89% of the cells possessed G_2/M DNA content (lane 6) and only the slower migrating phosphorylated CeHCF form is apparent (lane 6), but cells progressing into G_1 3 h after release from the nocodazole block (69% of the total) contained the faster migrating hypophosphorylated form, indicating CeHCF dephosphorylation (lane 7).

Thus, CeHCF phosphorylation is cell-cycle-regulated in mammalian cells: At least one of the CeHCF sites (S449) is phosphorylated in late G_1/early S phase. It appears to remain phosphorylated through S to M phase and to be dephosphorylated at the mitosis to early G_1 phase boundary. Although the mobility shift assay only probes the phosphorylation status of residue S449, considering the high degree of similarity between S449 and the other three phosphorylation sites (Figure 6A), it is likely that they are all substrates for the same kinase and that therefore S449

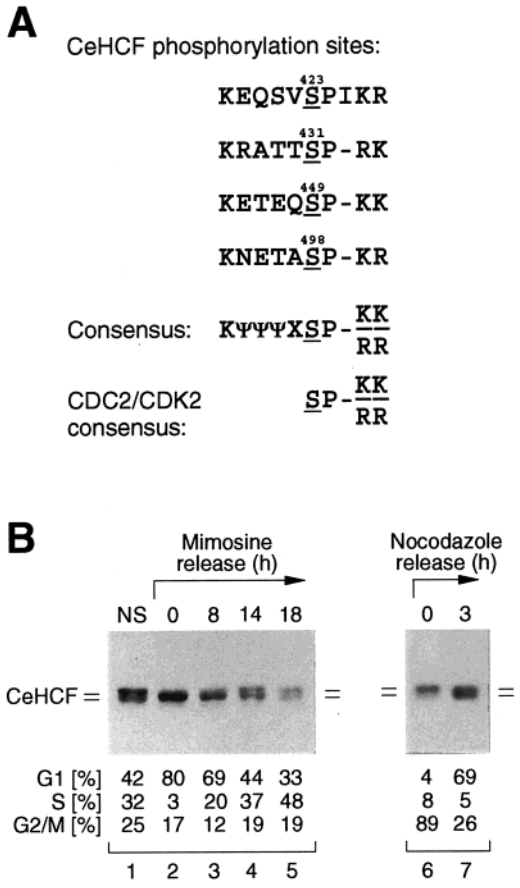

FIGURE 6: CeHCF phosphorylation site similarity to the CDC2/CDK2 recognition-site consensus sequence and cell-cycle dependent phosphorylation of CeHCF in mammalian cells. (A) CeHCF phosphorylation site sequences are shown aligned together with a deduced consensus sequence. A consensus recognition site sequence for CDC2/CDK2 kinases is shown at the bottom. $^{K}/_{R}$, either lysine or arginine; Ψ, hydrophilic amino acid; X, variable residue; hyphen, gap introduced to maximize similarity; underline, phosphorylated serine. (B) An asynchronous population (lane 1) of tsBN67 cells synthesizing CeHCF was blocked for 24 h with mimosine or nocodazole as described under Experimental Procedures. Extracts corresponding to 0 (lane 2), 8 (lane 3), 14 (lane 4), and 18 (lane 5) h post tsBN67-cell release from mimosine treatment and 0 (lane 6) and 3 (lane 7) h post tsBN67-cell release from nocodazole treatment were probed for CeHCF by immunoblot analysis. DNA content of the cells was analyzed in parallel by propidium iodide staining followed by flow cytometry. The percentage of cells in each cell population with G1, S, or G2/M DNA content is listed.

phosphorylation reflects phosphorylation status of CeHCF in general. Given the conserved pattern of CeHCF phosphorylation in *C. elegans* and mammalian cells, we suggest that CeHCF phosphorylation is also cell-cycle-regulated in worms. If true, then CeHCF phosphorylation in worms is both cell-cycle and developmentally regulated.

DISCUSSION

We have characterized the phosphorylation patterns of *C. elegans* CeHCF in both worms and mammalian cells and shown that stabilization of the VP16-induced complex by either CeHCF or human HCF-1 is apparently independent of phosphorylation. We investigated the possible role of HCF phosphorylation in VP16-induced complex formation because a previous report had indicated that VP16 binding to HCF-1 is phosphorylation-dependent (*17*). Phosphorylation of either one or both of the two cellular components of the VP16-induced complex, Oct-1 and HCF-1, could be a mechanism to regulate HSV pathogenesis in an infected cell, and both proteins are indeed phosphorylated. In the case of Oct-1, phosphorylation has been shown to regulate DNA-binding activity during mitosis (*19*).

In our initial studies of HCF in *C. elegans*, we observed the developmental regulation of CeHCF phosphorylation (Figure 1). We pursued the phosphorylation of CeHCF because its phosphorylation is much easier to study than that of human HCF-1: First, it is a much smaller protein that does not undergo the complicated pattern of human HCF-1 proteolytic cleavage, and second, its phosphorylation can be easily detected by the change in CeHCF electrophoretic mobility (see Figure 1). Thus encouraged, we developed an endogenous ^{32}P-labeling protocol for proteins in worms and established a mammalian cell line expressing CeHCF.

To our surprise, the pattern of CeHCF phosphorylation in *C. elegans* and mammalian cells is very similar, both in the identity of the four sites phosphorylated and in their respective levels of phosphorylation. We took advantage of this similarity to identify the CeHCF phosphorylation sites by combining unlabeled and radiolabeled CeHCF from different sources. Consistent with the role of mammalian HCF-1 in cell proliferation (*1*), the four phosphorylation sites share similarity to CDC2/CDK2 phosphorylation sites. Contrary to our expectations, however, the four phosphorylation sites are all clustered in the nonconserved central segment of CeHCF that is not required for VP16-induced complex formation (*9*). Indeed, mutagenesis of the four phosphorylation sites in CeHCF showed that CeHCF phosphorylation is not necessary to stabilize the VP16-induced complex.

Because CeHCF phosphorylation is not required to stabilize the VP16-induced complex, we asked whether phosphorylation of human HCF-1 is important for its role in stabilization of the VP16-induced complex. We found that efficient dephosphorylation of native human HCF-1 has no effect on its ability to stabilize the VP16-induced complex. We do not know at present why in other experiments phosphatase treatment of human HCF-1 inhibited its ability to associate with VP16 in a membrane-blotting Far-Western assay (*17*).

Our results suggest that phosphorylation of HCF-1 is not a mechanism to stringently regulate the assembly of the VP16-induced complex during HSV infection. These results are consistent with the studies of Schaffer and colleagues (*20*), who showed that although a CDK inhibitor can interfere with VP16-activated transcription, it does not interfere with VP16-induced complex formation.

CeHCF Phosphorylation Occurs at Sites Near a Conserved Region of HCF Proteins That Is Involved in Protein−Protein Contact. Although phosphorylation of CeHCF is not necessary for VP16-induced complex formation, two of the sites of phosphorylation, 423 and 431, lie just carboxy-terminal of a region that is highly conserved between CeHCF and the two human HCF-1 and HCF-2 proteins (see Figure 2D). In HCF-1 and HCF-2, this region is involved in association with the carboxy-terminal region of the protein (*16*). In HCF-1, this association tethers the amino-terminal and carboxy-terminal fragments of HCF-1, which result from its proteolytic cleavage (*16*). The role of such association in HCF-2, which is not proteolytically cleaved (*8*), is not

known, but the internal association of amino- and carboxy-terminal regions is also likely to occur in CeHCF because the sequence of the amino-terminal and carboxy-terminal association elements of the human HCF proteins is conserved in the CeHCF protein. The presence of phosphorylation sites so close to this predicted internal association element suggests a hypothesis in which phosphorylation of CeHCF and internal CeHCF association can influence one another.

Similarity of CeHCF Phosphorylation in Worms and Mammalian Cells. An unexpected result of our studies is the very similar pattern of CeHCF phosphorylation in worms and mammalian cells in a region of the protein that is not itself highly conserved. This result suggests that the kinase(s) that regulates CeHCF activity has been highly conserved during animal evolution but that the precise modes of regulation of HCF activity by phosphorylation have varied during animal evolution. The similarity of CeHCF phosphorylation in worms and mammalian cells allowed us to examine the role of the cell cycle in CeHCF phosphorylation because, in contrast to worms, easily manipulated mammalian cell culture systems are available. These studies showed that CeHCF is phosphorylated during S phase and probably dephosphorylated after mitosis (see Figure 6B). Owing to the similarity in CeHCF phosphorylation in worms and mamalian cells, we suggest that CeHCF is also phosphorylated during S phase in worms. Consistent with this hypothesis, CeHCF is hyperphosphorylated in embryos where cells are actively dividing and hypophosphorylated in larvae where there is much less active cell division. These results are consistent with the finding that mammalian HCF-1 is involved in cell proliferation (*1*).

Little is known about CDK phosphorylation during the S phase in *C. elegans*. In yeast, there is a single CDK (CDC28/Cdc2) that regulates the cell cycle, whereas in mammals, there is a family of CDKs that regulates different aspects of the cell cycle including S phase and mitosis. Boxem et al. (*21*) have shown that, as in mammals, worms possess a family of CDKs, one of which (NCC-1) regulates mitosis (*21*) and another of which regulates postembryonic cell-cycle progression (*22*). Probably, another *C. elegans* CDK(s) regulates CeHCF phosphorylation in the S phase of embryonic cells. Our analysis of CeHCF phosphorylation may provide a well-characterized CDK substrate to study embryonic S-phase-dependent CDK phosphorylation in future studies.

Very little is known about either the endogenous patterns of *C. elegans* protein phosphorylation or its conservation in other animal species. The high degree of conservation of CeHCF phosphorylation in worms and mammalian cells in a region of the protein that is not itself highly conserved suggests that the interspecies approach we have described here may be amenable to the study of other *C. elegans* phosphoproteins. Furthermore, the biochemical analysis of *C. elegans* protein phosphorylation described here suggests that, in addition to being a powerful genetic model to study the function of proteins in animals, *C. elegans* may also prove to be a powerful biochemical model.

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