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Herpes Simplex Virus Induces Intracellular Redistribution of E2F4 and Accumulation of E2F Pocket Protein Complexes

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Accumulation of E2F-p107 and E2F-pRB DNA binding complexes occurred after herpes simplex virus infection of U2-OS cells. Accumulation of E2F-p107 also occurred by 4 h p.i. in C33 cells. This corresponded to a time when host DNA synthesis was reduced by 50%, and lagged by ≥1 h, the onset of viral DNA synthesis. To determine the basis for increased nuclear E2F complexes, we investigated the effects of virus infection on the intracellular distribution of the E2F-dependent DNA binding complexes and their protein constituents. Western blot analyses of whole cell extracts revealed that amounts of E2F4, E2F1, DP1, and p107 remained unchanged after infection of C33 cells. Analysis of cytoplasmic and nuclear fractions, however, revealed that cytoplasmic E2F4 decreased and nuclear E2F4 increased. This correlated with a loss of cytoplasmic E2F DNA-binding activity and a corresponding increase in nuclear DNA-binding activity. Concomitant with its redistribution, the apparent molecular weight of total and p107-associated E2F4 increased, at least partially as a result of protein phosphorylation. Increased nuclear E2F-pRB in U2-OS cells was accompanied by the conversion of pRB from a hyper- to a hypophosphorylated state. Infection of U2-OS cells with viral mutants indicated that viral protein IE ICP4 was necessary for the decrease in cytoplasmic E2F-p107, and that viral protein DE ICP8 was required for nuclear accumulation of p107-E2F. In contrast, ICP8 was not required for accumulation of E2F-pRB. These results indicate that the increase in E2F-p107 may be explained by the redistribution and modification of E2F4 and the increase in E2F-pRB by modification of pRB.

INTRODUCTION

After infection by herpes simplex virus (HSV), a regulated program of transcription ensues, which in its early stages results in the synthesis of immediate-early (IE) regulatory proteins and delayed-early (DE) DNA replication proteins. These two waves of gene expression promote the onset of viral DNA replication, the expression of late (L) structural proteins, and finally, the formation of progeny virions (Roizman and Sears, 1990). Coincident with these events, the virus triggers a set of profound changes in the structure and function of the host cell. Among these are the *vhs*-mediated inhibition of host protein synthesis (Schek and Bachenheimer, 1985; Kwong and Frenkel, 1987; Strom and Frenkel, 1987; Read, 1997), inhibition of cell cycle progression (de Bruyn Kops and Knipe, 1988), alterations to cellular mRNA cleavage and polyadenylation components (McLaughlan *et al.,* 1992; Sandri-Goldin and Mendoza, 1992; McGregor *et al.,* 1996), redistribution of cellular splicing components (Martin *et al.,* 1987; Phelan *et al.,* 1993; Sandri-Goldin *et al.,* 1995), inhibition of host DNA replication (Roizman and Roane, 1964), fragmentation of the Golgi apparatus (Campadelli-Fiume *et al.,* 1993), reorganization of cytoskeletal elements (Bedows *et al.,* 1983; Norrild *et al.,* 1986), and redistribution of the nuclear structure ND10 (Maul *et al.,* 1993; Maul and Everett, 1994). Additional alterations to host cell components include the accumulation of cellular transcription factors. Among those reported to increase after HSV infection are AP-1, Sp1, NF-κB and E2F (Gimble *et al.,* 1988; Jang *et al.,* 1991; Rong *et al.,* 1992; Hilton *et al.,* 1995; Patel *et al.,* 1998).

E2F is a family of heterodimeric factors, consisting of one of six different E2F proteins, E2F1–6, complexed with one of two DP proteins, DP1–2 (LaThangue, 1994; Dyson, 1998). The transcriptional *trans*-activation function of E2F, originally identified through its ability to bind and activate the adenovirus early region 2 promoter (Kovesdi *et al.,* 1986; Nevins, 1992), is negatively regulated through interactions with the A–B boxes, or pocket region of pRB, or related proteins p107 and p130 (Hiebert *et al.,* 1992; Qin *et al.,* 1992; Helin *et al.,* 1993; Zhu *et al.,* 1993; Hijmans *et al.,* 1995; Sellers *et al.,* 1995; Smith *et al.,* 1996; Starostik *et al.,* 1996; Weintraub *et al.,* 1992; Weintraub *et al.,* 1995). These interactions have been suggested to occlude E2F *trans*-activation domain interactions with TBP (Hagemeier *et al.,* 1993), and coupled with the ability of pocket proteins to bring histone deacetylase activity to the promoter (Brehm *et al.,* 1998; Ferreira *et al.,* 1998; Luo

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et al., 1998; Magnaghi-Jaulin *et al.,* 1998), to convert the complex into a transcriptional repressor.

Negative regulation of E2F also occurs when cyclin A/cdk2, binding directly to E2F1 and perhaps E2F2 and E2F3 (Xu *et al.,* 1994), phosphorylates both E2F and DP proteins, resulting in loss of DNA-binding activity (Dynlacht *et al.,* 1994; Xu *et al.,* 1994). This may represent the mechanism for downregulation of E2F1 during S phase (Krek *et al.,* 1995). Finally, a mechanism of negative regulation of E2F4 appears to depend on its cytoplasmic sequestration either alone or in a complex with p107 or p130 (Shirodkar *et al.,* 1992; Verona *et al.,* 1997).

The pattern of E2F-pocket protein interactions is regulated through the cell cycle. A complex of E2F and p130 is the predominant E2F-dependent DNA-binding activity detected in quiescent, G0 cells (Cobrinik *et al.,* 1993; Vairo *et al.,* 1995; Moberg *et al.,* 1996). As cells reenter the proliferative state and traverse G1, this complex is gradually replaced by one that additionally contains cyclin E/cdk2 (Cobrinik *et al.,* 1993). The amount of this complex declines as cells approach the G1/S boundary (Moberg *et al.,* 1996) coincident with the accumulation of a pentameric complex of E2F/DP, p107, and cyclin A/cdk2, as cells traverse S phase. This complex declines in abundance as cells traverse G2/M (Devoto *et al.,* 1992; Lees *et al.,* 1992; Chittenden *et al.,* 1993; Schwarz *et al.,* 1993; Beijersbergen *et al.,* 1994; Ginsberg *et al.,* 1994; Moberg *et al.,* 1996). A third DNA binding complex containing pRB and E2F is normally observed beginning in early G1 and persists until mid- to late-G1, or the restriction point, when the combined action of cyclin D/CDK4/6 and cyclin E/CDK2 results in hyperphosphorylation of pRB and release of E2F (Mudryj *et al.,* 1991; Lees *et al.,* 1992; Moberg *et al.,* 1996; Verona *et al.,* 1997; Leone *et al.,* 1998). There is at least one report that some E2F-pRB persists into S phase (Schwarz *et al.,* 1993). There appear to be preferential associations between pocket proteins and E2F activities. pRB preferentially forms complexes with E2Fs 1–3, (Helin *et al.,* 1992; Kaelin *et al.,* 1992; Lees *et al.,* 1992; Shan *et al.,* 1992; Ivey-Hoyle *et al.,* 1993), though an E2F4-pRB complex has also been reported (Ikeda *et al.,* 1996; Moberg *et al.,* 1996). E2Fs 4–5 are found preferentially in complexes with either p107 or p130 (Beijersbergen *et al.,* 1994; Ginsberg *et al.,* 1994; Hijmans *et al.,* 1995; Sardet *et al.,* 1995).

Endogenous E2F1 and E2F2 protein and associated DNA-binding activities are confined to the nucleus though their levels fluctuate with the cell cycle, reaching a maximum in mid to late G1 (Magae *et al.,* 1996; Lindeman *et al.,* 1997; Müller *et al.,* 1997; Sears *et al.,* 1997; Verona *et al.,* 1997). In contrast to other E2Fs, endogenous and overexpressed E2F4 and E2F5 protein and DNA-binding activities are predominantly cytoplasmic (Magae *et al.,* 1996; Lindeman *et al.,* 1997; Müller *et al.,* 1997; Verona *et al.,* 1997). Relocalization of overexpressed E2F4 protein to the nucleus occurs when co-expressed with p107 or p130 (Magae *et al.,* 1996; Lindeman *et al.,* 1997; Müller *et al.,* 1997), despite the finding that endogenous E2F-p107 DNA-binding activity is both nuclear and cytoplasmic (Verona *et al.,* 1997). Overexpressed E2F4 can also be translocated to the nucleus by co-expression of DP2 but not DP1 (Verona *et al.,* 1997). While the total amount of endogenous E2F4 protein remains relatively constant, its distribution between the nucleus and cytoplasm is regulated in a cell cycle-dependent manner. Levels of nuclear E2F4 protein and DNA-binding activity, the latter as an E2F-p130 complex are maximal in G0 cells (Verona *et al.,* 1997). In cycling cells, however, most E2F4 protein and DNA-binding activity, both free and bound to p107, is cytoplasmic during G1, whereas free and E2F-p107 can be detected in the nucleus as cells enter S phase (Lindeman *et al.,* 1997; Müller *et al.,* 1997; Verona *et al.,* 1997). Importantly, however, the majority of E2F4 protein and DNA-binding activity is cytoplasmic in G1 and S phase of cycling cells (Verona *et al.,* 1997). Finally, p107-associated E2F4 exists as a slower mobility, hyperphosphorylated form, in contrast to a faster mobility hypophosphorylated form in free E2F (Ginsberg *et al.,* 1994).

We previously described the rapid increase of a pentameric DNA-binding activity containing E2F/DP, p107, cyclin A, and cdk2 after HSV infection of asynchronous C33 cells, which express a mutant form of pRB unable to form complexes with E2F (Hilton *et al.,* 1995). HSV infection of U2-OS cells, expressing normal pRB, resulted in the formation of an E2F-RB complex (Hilton and Bachenheimer, unpublished observations, and below). In the absence of any evidence for *de novo* synthesis of E2F or any of the constituents of the p107 complex including failure to immunoprecipitate ^{35}S-labeled proteins after infection, (Hilton and Bachenheimer, data not shown), we sought alternative explanations for the accumulation of the E2F-p107 complexes. The results of our experiments reported here indicate that (i) both E2F-p107 and E2F-pRB accumulate in the nucleus after infection of U2-OS cells, and this is accompanied by the accumulation of a hypophosphorylated form of pRB; (ii) accumulation of E2F-p107 and increased E2F4 protein in nuclei of infected C33 and U2-OS cells is mirrored by a depletion of a cytoplasmic pool of E2F4 protein; (iii) accumulation of E2F-p107 occurs after the inhibition of cell DNA synthesis has begun and after the onset of viral DNA synthesis; (iv) modifications to E2F4 including phosphorylation, occur after virus infection; and (v) viral mutants define distinct mechanisms for nuclear accumulation of E2F-p107 and E2F-pRB. These results suggest that HSV infection results in a novel accumulation of E2F-pocket protein complexes having characteristics of both G0/G1 and S phases. Changes in E2F location and association with pocket proteins are also consistent with the ability of HSV to prevent progression from G_1 into S phase and

to inhibit cellular DNA synthesis (deBruyn Kops and Knipe, 1988).

RESULTS

E2F4-p107 and E2F1-RB complexes accumulate in U2-OS cells after HSV infection

We previously described the accumulation in C33 cells, beginning at 3–4 h p.i., of an E2F-p107 complex containing cyclin A and cdk2 (Hilton *et al.,* 1995), similar if not identical to one accumulating as cells traverse S phase (Devoto *et al.,* 1992; Lees *et al.,* 1992; Schwarz *et al.,* 1993; Beijersbergen *et al.,* 1994; Ginsberg *et al.,* 1994; Moberg *et al.,* 1996). We have investigated the effects of HSV infection on E2F DNA-binding activities in U2-OS cells because this cell line expresses normal p107 and pRB, capable of forming complexes with E2F. Though this cell line has been reported to express an activity that can functionally substitute for ICP0, (Yao and Schaffer, 1995), we have determined that an ICP0-null mutant induces accumulation of the E2F-p107 complex as efficiently as WT virus (Hilton *et al.,* 1995). Replicate monolayers of U2-OS cells were either mock-infected or infected with HSV-1, harvested at 2-h intervals to 8 h p.i., and nuclear and cytoplasmic extracts prepared for analysis of E2F-dependent gel shift activities. Mock-infected nuclear extract contained two slow mobility complexes, designated E2F-p107 and E2F-pRB (Fig. 1A, lane 1), both of which increased in amount between 2 and 8 h p.i. (lanes 2–5). Analysis of the 6-h extract, by addition of specific antibodies to gel shift reactions containing either a wt E2F site competitor oligonucleotide (lanes 7–9) or a mutant oligonucleotide (lanes 10–12), confirmed the identity of these complexes as E2F-p107 and E2F-pRB and that the antibodies did not cause nonspecific supershifts. Specifically, a pRB antibody disrupted only the faster mobility activity (lanes 7 and 10), whereas a p107 antibody left this activity unaffected and supershifted the slower mobility activity (lanes 8 and 11). An antibody specific for E2F1, a pRB-associated E2F, also disrupted most of the E2F-pRB complex (lanes 9 and 12). Of interest, and in contrast to our observations with nuclear extracts prepared from C33 cells, was the inability to detect significant amounts of free E2F activities in nuclear extracts of U2-OS cells. Laser densitometry indicated that the amount of E2F-pRB increased 50% between the mock and 2-h samples, 2.5-fold by 6 h, and >7-fold by 8 h. The increase in E2F-p107 is 3.8-fold between the mock and 8-h samples. We conclude from these results that both types of E2F-pocket protein complexes, normally seen in cycling cells, increase in amount as early as 2 h after HSV infection.

Analysis of cytoplasmic E2F complexes from the same U2-OS time course (Fig. 1B), revealed that the small amount of cytoplasmic E2F-p107 seen in mock-infected cells declined after 4 h of infection, while a doublet of E2F4 activity decreased 80% over the 8-h course of infection (lanes 1–8). The identity of the E2F-p107 complex was confirmed by the ability of E2F but not mutant oligonucleotide to compete with the labeled probe (lanes 2 and 3) and depletion of the complex in the presence of a p107 antibody (lane 4); the identification of the free E2F as E2F4 was confirmed by antibody supershifts in the presence of either E2F or mutant oligonucleotide competitors (lanes 9–11). From these analyses, we conclude that the increase in nuclear E2F-p107 and E2F-pRB is accompanied by loss of free cytoplasmic E2F4 and the small amount of E2F-p107.

The increase in nuclear E2F-pRB DNA-binding activity prompted us to determine whether the distribution and amount of hypo- and hyperphosphorylated pRB protein changed during the course of infection (Fig. 1C). Analysis of cytoplasmic and nuclear extracts from the U2-OS time course revealed a loss of cytoplasmic and an increase in nuclear pRB protein. A comparison with pRB in whole cell extracts prepared from G0 and S phase normal human embryonic lung fibroblasts (HEL), revealed that most cytoplasmic, mock-infected U2-OS pRB was hypophosphorylated (lanes 1–3), whereas nuclear pRB was primarily hyperphosphorylated (lanes 8–10 and 15–17). The mobility of nuclear pRB that accumulated after virus infection appeared intermediate between the hypo- and hyperphosphorylated forms of pRB detected in G0 and S phase HEL extracts (lanes 15–18). The accumulation of a hypophosphorylated form of pRB after infection was consistent with the accumulation of E2F-pRB DNA binding complexes (Fig. 1A). Although significant amounts of hyperphosphorylated pRB can be extracted from nuclei at salt concentrations ~150 mM and above (Mittnacht and Weinberg, 1991), our cytoplasmic extracts were prepared in 65 mM KCl (see Materials and Methods). Because the majority of the pRB detected in the cytoplasmic extract of mock-infected U2-OS cells was hypophosphorylated, it remained possible that our extraction procedure resulted in contamination with a small amount of nuclear protein or that a fraction of pRB normally is distributed in the cytoplasm. The former possibility seems unlikely because another protein, E2F1, thought to be exclusively in the nucleus, was not detected in cytoplasmic fractions of U2-OS cells through 8 h of virus infection (lanes 15–24).

Redistribution of E2F4 DNA-binding activities in C33 cells

C33 cells express a mutant form of pRB that cannot bind E2F, and thus in an asynchronously growing culture, only free E2F and E2F-p107 complex would normally be detected. Our previous characterization of E2F complexes in C33 cells (Hilton *et al.,* 1995) involved supershifting DNA-binding activities with antibodies specific for p107, cdk2, and cyclin A and, by demonstrating that

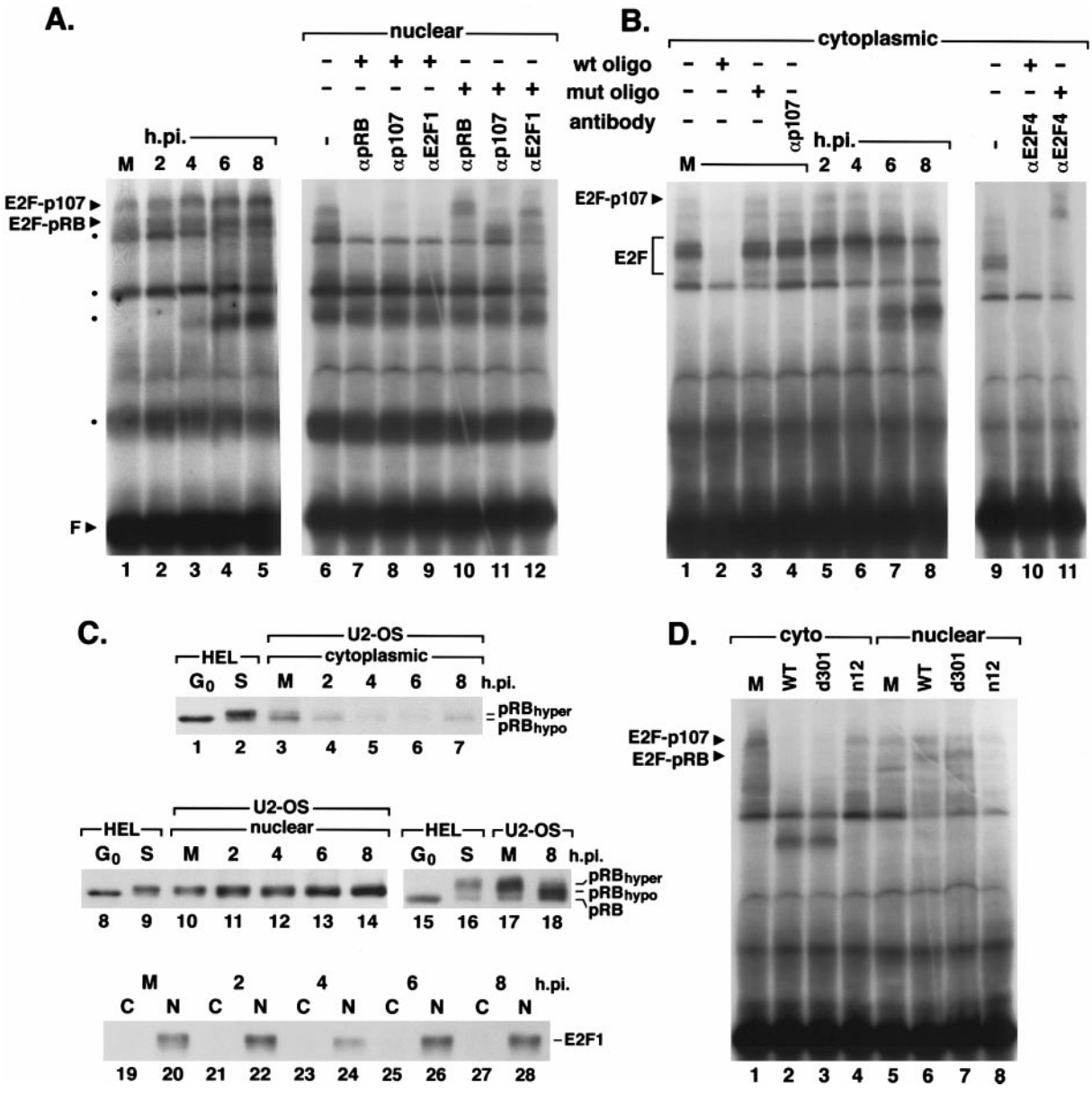

FIG. 1. E2F-RB and E2F-p107 complexes accumulate after infection of U2-OS cells. (A) Time course of nuclear U2-OS E2F complexes. Nuclear extracts were prepared from mock-infected and HSV-infected U2-OS cells harvested at 2, 4, 6, and 8 h p.i. Cell equivalent amounts of protein (3.0%, ~4 μg) were analyzed in a standard, E2F-dependent gel shift assay as described under Materials and Methods (lanes 1–5). Aliquots of the 6-h-infected extract (4 μg) were incubated with the indicated antibodies in the presence of 2.8 pmoles of E2F (wt) or mutant (mut) competitor oligonucleotides (a 100-fold excess over the probe) and then subjected to a standard E2F-dependent gel shift analysis (lanes 6–12). The αRB antibody was Ab2 (Oncogene Sciences); the remaining antibodies were obtained from Santa Cruz Biotechnology as TranzCruz reagents: αp107 (SC318); αE2F-1 (SC251); αE2F-4 (SC1082). ●: nonspecific activities based on the inability to compete with either an E2F oligonucleotide (A, lane 7, and B, lane 2) or with unlabeled probe DNA (not shown); F, free probe. (B) Time course of cytoplasmic U2-OS E2F complexes, Cell-equivalent amounts of the corresponding cytoplasmic extracts (3%, ~10 μg) from cells described in (A) were subjected to a standard E2F-dependent gel shift assay (lanes 1 and 5–8). Samples of mock-infected extract were incubated with E2F or mutant oligonucleotide (lanes 3 and 4) or with p107 antibody (lane 4) or with E2F4 antibody in the presence of either E2F or mutant oligonucleotide (lanes 10 and 11). (C) Western blot analysis of cytoplasmic and nuclear pRB and E2F1. Cell equivalent amounts of U2-OS cytoplasmic (lanes 3–7, 12%, ~40 μg) and nuclear (lanes 10–14, 18%, ~40 μg) extract were fractionated by 8% SDS–PAGE, transferred to PVDF paper, and probed for pRB with polyclonal rabbit antiserum (14001A, Pharmingen). Whole cell extracts, prepared from human embryonic fibroblasts (HEL) either in G0 (serum starved, lanes 1 and 8) or S phase (18 h after addition of serum, lanes 2 and 9), served as controls to determine the mobility of hypo- and hyperphosphorylated pRB. Additional samples of M and 8-h nuclear extract, along with HEL cell control extracts, were fractionated by 6% SDS–PAGE and probed for pRB (lanes 15–18). Alternating cytoplasmic (C) and nuclear (N) extract (lanes 19–28) were fractionated by SDS–PAGE and probed for E2F1 (SC251). (D) E2F complexes after WT and mutant virus infection. Nuclear and cytoplasmic extracts were prepared from replicate monolayers of U2-OS cells that were either mock-infected or infected with WT KOS strain of HSV-1, the ICP4 mutant n12, or the ICP8 mutant, d301. Cell equivalent amounts of cytoplasmic and nuclear extract (2.5%, ~12 μg cytoplasmic and ~5.0 μg nuclear) were subjected to a standard E2F-dependent gel shift assay. Arrowheads indicate the position of E2F-p107 and E2F-pRB complexes.

detection of the complex, was dependent on E2F binding sites in the probe DNA. E2F4 is the most abundant E2F protein in C33 cells (Moberg *et al.,* 1996) preferentially binding p107 and p130 (Beijersbergen *et al.,* 1994; Ginsberg *et al.,* 1994; Hijmans *et al.,* 1995; Sardet *et al.,* 1995), and unique among the E2Fs, to be distributed in both the nucleus and cytoplasm. For example, both free and p107-associated E2F4 DNA-binding activity is exclusively cytoplasmic in G1 cells, whereas E2F-p107 is detectable in both nuclear and cytoplasmic fractions during S phase (Magae *et al.,* 1996; Lindeman *et al.,* 1997; Verona *et al.,* 1997). In light of our previous observation of increased nuclear E2F-p107 complex after infection, it was of interest to determine whether the intracellular distribution of E2F DNA-binding activities, and in particular E2F4, was altered after infection of asynchronous cell cultures with HSV. Cytoplasmic and nuclear extracts were prepared from uninfected and HSV-infected C33 cells and analyzed for E2F-dependent gel shift activity. A time course analysis of C33 cell extracts (Fig. 2A) revealed both free E2F4 and E2F4-p107 binding activities in the cytoplasm of mock-infected cells (C lanes), and predominantly E2F-p107 in the corresponding nuclear fractions (N lanes). After HSV infection, cytoplasmic E2F activities gradually decreased over the next 8 h (lanes 3, 5, 7, and 9), and we observed the previously described increase in nuclear E2F-p107 (lanes 4, 6, 8, and 10). We confirmed the identity of these complexes by performing antibody supershift experiments in the presence of either E2F or mutant competing oligonucleotides (Fig. 2B). The E2F4 antibody efficiently supershifted the upper band of free cytoplasmic E2F to a position just above where E2F-p107 would normally migrate (compare lanes 10 and 14) and shifted the E2F-p107 to an even higher position (compare lanes 6 and 14). At present, the identity of the E2F activity migrating below free E2F4 remains unclear, but it could represent E2F5 as observed in cytoplasmic extracts by others (Leone *et al.,* 1998). The identity of the nuclear E2F activities was confirmed by supershift with antibodies against p107, cyclin E, and cyclin A (lanes 6, 8, and 9). Though we were unable to supershift the nuclear E2F-p107 with the E2F4 antibody in this analysis, perhaps reflecting the masking of a critical epitope in the nuclear complex, we have been able to supershift this complex with other E2F4 antibodies (data not shown). Competition with the E2F-site oligonucleotide also revealed a diffuse distribution of free E2F activities (lanes 2–5). The difference in the extent of the decline of cytoplasmic E2F4 between U2-OS and C33 at 8 h (compare Figs. 1B, 2A, and 6C) most likely reflects a difference in rate because we have observed complete loss of cytoplasmic E2F4 from 12 and 16 h infected U2-OS cells (data not shown). We conclude from the results presented in Fig. 2 that after virus infection, E2F4 DNA-binding activities are lost from the cytoplasm of C33 cells, whereas nuclear E2F4-p107 activity increases. The resulting distribution of

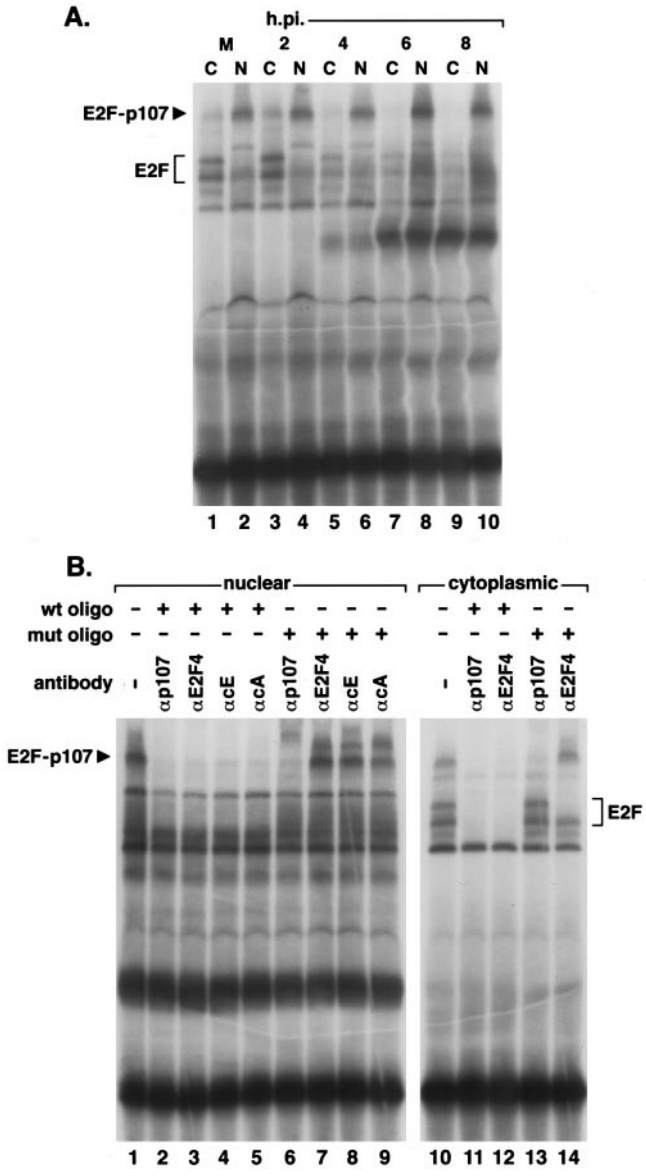

FIG. 2. E2F complexes are redistributed after HSV infection. (A) Cytoplasmic (C) and nuclear (N) extracts were prepared from mock-infected or HSV-infected C33 cells harvested at the indicated times p.i. Cell equivalent amounts of extract (1.5%) were analyzed in an E2F-dependent gel shift assay (lanes 1–10). (B) The identity of E2F-p107 and free E2F complexes was determined, based on the ability of specific antibodies [E2F4 (SC1082); p107 (SC-318); αcE (SC248); αcA (BF683)] to shift or disrupt gel shift activities in the presence or either E2F or mutant competitor oligonucleotides (lanes 1–14).

E2F4 DNA-binding activities is reminiscent of that observed for E2F4 protein in G0/G1 phase cells (Lindeman *et al.,* 1997).

Nuclear E2F-p107 accumulation and infected cell DNA synthesis

The increase in nuclear E2F-p107 complex after HSV infection did not occur in cells infected in the presence of PAA, an inhibitor of viral DNA synthesis. In addition,

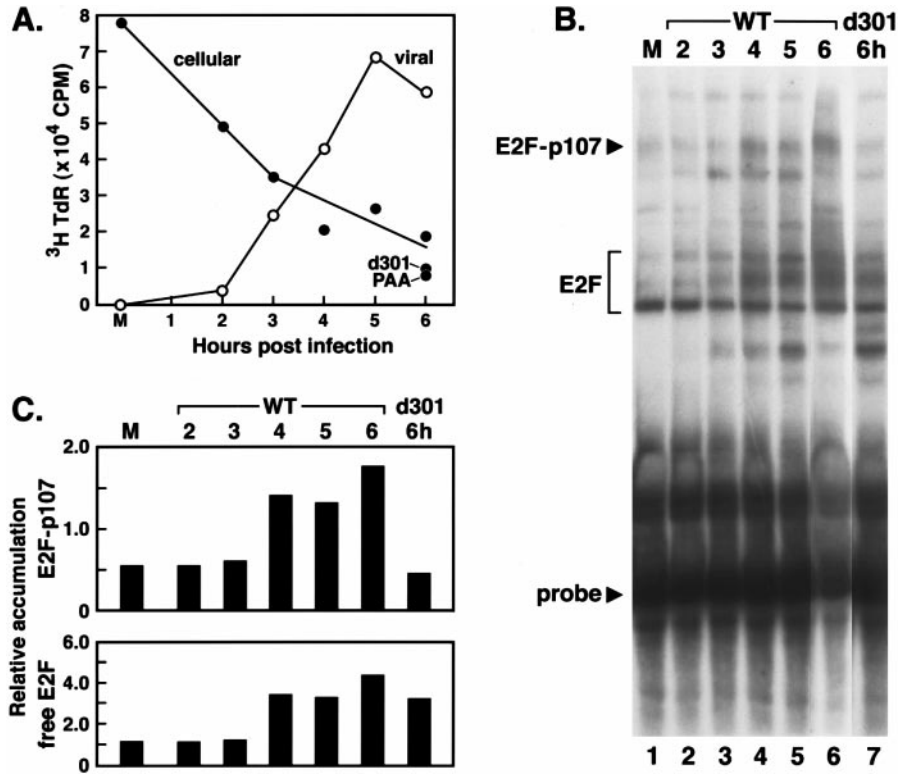

FIG. 3. Decrease in cellular DNA synthesis and onset of viral DNA replication precede accumulation of E2F-p107. Replicate cultures of C33 cells were either mock-infected or infected with HSV and at the indicated times, three-fourths of each cell monolayer was harvest and processed to yield soluble nuclear extracts. The remainder of each monolayer was pulse labeled with [^{3}H]thymidine for 30 min, then harvested and processed for DNA as described under Materials and Methods. (A) Time course analysis of infected cell DNA synthesis. Amounts of [^{3}H]thymidine-labeled DNA corresponding to cellular and viral DNA were determined after separation by isopycnic centrifugation on CsCl, as described under Materials and Methods, and plotted as a function of time p.i. ●: cellular DNA; ○: viral DNA; d301, cellular DNA in d301-infected cells; PAA, cellular DNA in PAA-treated, WT-infected cells. (B) Time course analysis of nuclear E2F activities. Equal amounts (3 μg) of nuclear extract were subjected to a standard E2F-dependent gel shift assay as described under Materials and Methods. WT, wild-type-infected cell extracts; d301, mutant d301-infected cell extract. The 5- and 6-h WT lanes and the WT 6-h and d301 6-h lanes were not adjacent to each other on the original autoradiograph. (C) Accumulation of free and E2F-p107 complexes. The relative amounts of p107-E2F and free E2F in extracts was determined by laser densitometry of the autoradiograph shown in (B).

viruses with mutations in genes that regulate (ICP4, ICP27) or mediate (ICP8, pol, UL5) viral DNA replication fail to induce nuclear accumulation of E2F-p107, whereas an ICP0 mutant virus induced accumulated E2F-p107 normally (Hilton *et al.,* 1995). We were interested in determining whether accumulation of nuclear E2F occurred coincident with or lagged behind the onset of viral DNA replication. The accumulation of E2F-p107, a complex that normally occurs as cells traverse S, was at odds with the ability of HSV to inhibit cellular DNA synthesis (Roizman and Roane, 1964). Therefore we also determined how the kinetics of E2F accumulation related to the inhibition of cellular DNA synthesis. Figure 3 presents time course analyses for both viral and cellular DNA synthesis, and E2F DNA-binding activities, after HSV infection. Replicate monolayers of asynchronously growing C33 cells were infected, and at the indicated times, three-quarters of each monolayer was harvested for nuclear extract preparation and the remainder of the monolayer was labeled for 30 min with [^{3}H]thymidine, followed by harvesting and isolation of DNA. Cellular and viral DNA were fractionated by isopycnic centrifugation in CsCl gradients, as described under Materials and Methods. By 3 h p.i., cellular DNA synthesis had decreased ~50%, and by 6 h p.i., 75–80%, regardless of whether cells were infected with WT virus, an ICP8 mutant, d301, or with WT virus in the presence of PAA (Fig. 3A). The onset of viral DNA synthesis began between 2 and 3 h p.i., and the rate of synthesis appeared to reach a maximum between 5 and 6 h p.i. The increase in free and complexed nuclear E2F DNA-binding activities began at 3–4 h p.i. in WT-virus-infected cells (Fig. 3B, lanes 3 and 4), whereas no increase was detectable in d301-infected cells (lane 7), consistent with our previous results (Hilton *et al.,* 1995). Quantitation by laser densitometry of the gel shift autoradiogram is presented in Fig. 3C. These results indicate that the onset of nuclear E2F accumulation corresponds to a time post infection when cellular DNA synthesis was already reduced ~50%, and when viral DNA replication had been detectable for ≥1 h. Thus the

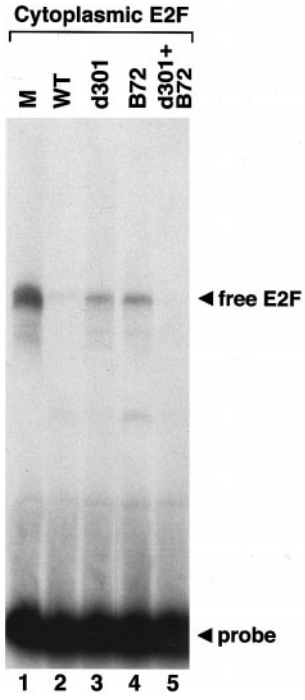

FIG. 4. Loss of cytoplasmic E2F occurs in both WT and DNA replication mutant-infected cells. Cytoplasmic extracts were prepared from replicate C33 cell cultures, which were mock-infected (lane 1); infected with WT KOS HSV-1 (lane 2), the ICP8 mutant d301 (lane 3), or the UL5 helicase mutant B72 (lane 4); or co-infected with d301 and B72 (lane 5) (see Figure 5, Hilton *et al.,* 1995). After treatment with DOC (0.8%) and NP-40 (1.2%), 6-μg aliquots were subjected to E2F-dependent gel shift analysis.

accumulation of nuclear E2F complexes does not trigger the decrease in cellular DNA synthesis, though it may have a role in sustaining an S phase inhibition (see below).

Loss of cytoplasmic E2F DNA-binding activity does not require viral DNA replication

We previously reported that accumulation of nuclear p107-E2F DNA-binding activity required the onset of HSV DNA replication. This conclusion was based on experiments with mutant viruses that did not express functional ICP8, or DNA polymerase, or infection with WT virus in the presence of PAA (Hilton *et al.,* 1995). We also demonstrated that coinfection of cells with ICP8 and polymerase mutants complemented for the increase in nuclear E2F-p107. We next determined whether viral DNA replication was required for loss of cytoplasmic E2F DNA-binding activity (Fig. 4). We analyzed the cytoplasmic extracts corresponding to the nuclear extracts we had previously analyzed, which were prepared from C33 cultures, either mock- or WT-virus-infected or infected with viruses containing mutations in either ICP8 (d301) or polymerase (B72) or infected simultaneously with both mutant viruses (d301 + B72). Gel shift analysis demonstrated loss of cytoplasmic E2F activity regardless of the potential of the cell to initiate viral DNA synthesis, though some residual E2F activity was present in extracts from d301- and B72-infected cells (compare lanes 1–5). We also determined the effect of mutant virus infection on the pattern of cytoplasmic and nuclear E2F DNA-binding activities in U2-OS cells (Fig. 1D). Similar to results observed in C33 cells, infection of U2-OS cells with the WT virus or the ICP8 mutant d301 resulted in loss of cytoplasmic E2F-p107 (lanes 2 and 3). Infection with the ICP4 mutant n12 resulted in only partial reduction of cytoplasmic E2F-p107 compared with WT- and d301-infected cells (lane 4). A more complex pattern of E2F activities, as a result of WT and mutant virus infection, was observed in nuclear extracts (lanes 5–8). E2F-p107 increased approximately fourfold, and E2F-pRB was induced after WT infection (lane 6). The latter result is consistent with our observation that nuclear pRB protein is converted to a hypophosphorylated form after infection (Fig. 1C, lanes 17–18). After infection with d301 (ICP8$^-$, lane 7), the amount of E2F-p107 remained unaffected, whereas E2F-pRB accumulated to the level seen during WT infection. All nuclear E2F activities decreased in n12 (ICP4$^-$, lane 8) infected cells compared with mock levels. The basis for this decrease is not known but Western blot analysis of the n12 nuclear extract revealed levels of E2F1 and E2F4 identical to that of mock-infected cell extract suggesting that the decrease cannot be attributable to *vhs* function (data not shown). We conclude from these results that the mechanism underlying the loss of cytoplasmic E2F DNA-binding activity is independent of the onset of viral DNA replication because this occurred after infection with the ICP8 mutant d301. This is in contrast to the mechanism for the increase in nuclear E2F-p107 activity, which requires IE and DE gene expression and the onset of viral DNA replication (lanes 7 and 8, and Hilton et al., 1995). The failure to detect E2F-pRB in the absence of ICP4 expression raises the possibility that this protein may play a direct role in formation of certain E2F-pocket protein complexes, and the distinct roles that pRB and p107-E2F complexes may play in cell cycle regulation (Dyson, 1998; Nevins, 1998). The precise role of IE proteins in the loss of cytoplasmic E2F activities and increase in nuclear E2F-pRB is currently under investigation.

Nuclear translocation of cytoplasmic E2F4 protein after HSV infection

Loss of cytoplasmic E2F4-dependent DNA-binding activities is mirrored by an increase in nuclear E2F-p107. To determine first how the total amount of E2F-associated protein was affected by virus infection, we prepared C33 whole cell extracts at 6, 10, and 16 h p.i. and probed equivalent amounts of protein for E2F4, E2F1, DP1, and p107 by immunoblotting (Fig. 5). Amounts of E2F4, E2F1, DP1, and p107 remained unchanged in comparisons of

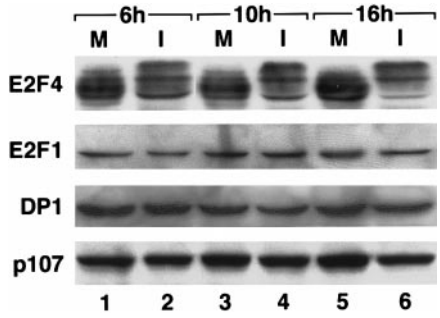

FIG. 5. Total E2F4, E2F1, DP1, and p107 remain constant after HSV infection. Replicate cultures of C33 cells were either mock-infected or infected with HSV and harvested at 6, 10, and 16 h p.i. Whole cell extracts were prepared as described under Materials and Methods. Aliquots (60 μg) of soluble extract were separated by SDS–PAGE and transferred to PVDF paper, and individual proteins were assayed by Western blot, all as described under Materials and Methods. The αDP1 antibody was SC-610 (Santa Cruz); the other antibodies were described in the legend to Fig. 1.

mock- and infected-cell extracts throughout the course of the 16-h infection period. Of interest was the shift of E2F4 to relatively slower mobility forms in extracts from infected cells (lanes 2, 4, and 6) when compared with E2F4 from uninfected cells (lanes 1, 3, and 5, and see below).

Because the amount of E2F-associated proteins did not change after infection, we next determined how infection altered the distribution of E2F4, DP1, DP2, and p107 proteins between the cytoplasm and the nucleus of C33 cells (Fig. 6A). Cell-equivalent amounts of mock and 8- and 16-h-infected whole cell extracts (lanes 1–3), were compared with nuclear and cytoplasmic fractions from mock, 8- and 16-h-infected cells (lanes 4–11). Detection of E2F4 in whole cell extracts from 8- and 16-h-infected cells again revealed the shift to relatively slower mobility forms (lanes 1–3). Fractionated extracts probed for E2F4 revealed a virtually complete loss of E2F4 from infected cell cytoplasmic fractions (compare lanes 6 and 7, 10 and 11). Laser densitometry confirmed increases of 26 and 43% in nuclear E2F4 (compare lanes 4 and 5, 8 and 9) in two independent experiments at 8 and 16 h p.i., respectively, compared with the corresponding levels in mock-infected cells. Analysis of the E2F binding partner DP1 revealed a decrease in cytoplasmic protein (lanes 6, 7, 10, and 11) at 8 and 16 h p.i., but no change or a slight increase in the amount of DP1 protein in nuclear fractions from mock and infected cells (lanes 4, 5, 8, and 9). Because cytoplasmic DP1 appears to represent a minority of cellular DP1 (compare lanes 4 and 6 and lanes 8 and 10), the apparent decrease in DP1 observed in this analysis of whole cell extracts after infection (lanes 1–3) remains unexplained. Detection of DP2, in contrast, revealed equivalent amounts of strictly nuclear protein at both 8 h (lanes 4–7) and 16 h p.i. (lanes 8–11). The total amount of p107 present in uninfected and infected cells remained constant (lanes 1–3) though because the great majority of p107 is nuclear, it was difficult to determine whether the amount of nuclear p107 increased after infection (lanes 4, 5, 8, and 9). The amount of p107 in the 8-h infected cytoplasm declined relative to the mock sample (lanes 6 and 7) but did not completely disappear, suggesting that either some p107 exists in the cytoplasm independent of E2F or that some p107 dissociated from E2F4 during the translocation process. From this analysis, it is apparent that the majority of p107 in asynchronous uninfected C33 cells is located in the nucleus,

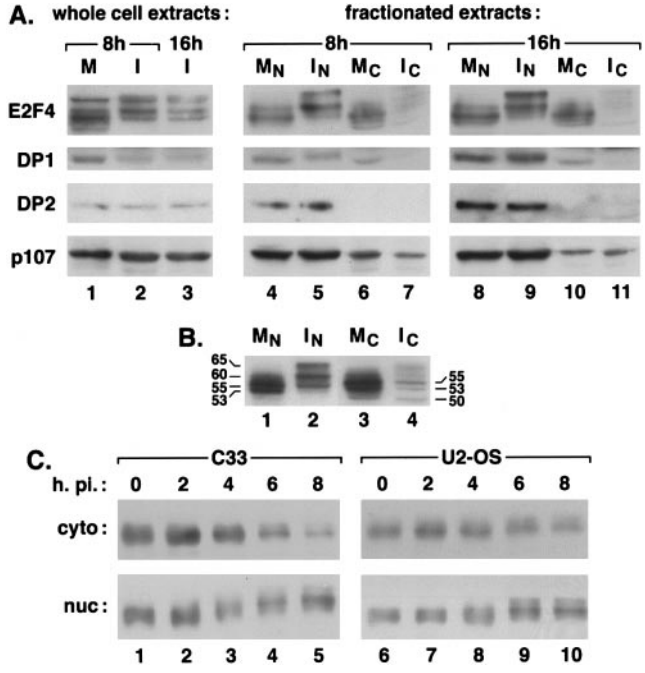

FIG. 6. The intracellular distribution and mobility of E2F4 is altered after HSV infection. (A) Western blot analysis of fractionated extracts from C33 cells. Whole cell extracts were prepared from either a mock-infected (M) C33 cells or cells infected with HSV (I) for 8 or 16 h (lanes 1–3). Parallel cultures were harvested, and nuclear (M_N, I_N) and cytoplasmic (M_C, I_C) fractions were prepared (lanes 4–11). Cell equivalent fractions of whole cell extracts (18%) and nuclear and cytoplasmic fractions (10%) were separated by SDS–PAGE and transferred to PVDF paper, and individual proteins were assayed by Western blot, all as described under Materials and Methods. Antibodies (Santa Cruz) for E2F4, DP1, and p107 are described in the legends to Figs. 1 and 6; αDP2 was SC-829 (Santa Cruz). (B) Western blot analysis of E2F4. Nuclear and cytoplasmic extracts were prepared from mock-infected and 16-h-infected C33 cells, and 40-μg aliquots, representing 3% of nuclear protein and 1.3% of cytoplasmic protein, were fractionated by SDS–PAGE, transferred to PVDF paper, and underwent Western blotting for E2F4. Molecular weight determinations were based on mobilities relative to Kaleidoscope prestained protein standards (Bio-Rad). (C) Time course analysis of C33 and U2-OS nuclear and cytoplasmic E2F4. Replicate C33 (lanes 1–5) and U2-OS (lanes 6–10) cultures were mock-infected (0 h pi) or infected with HSV and harvested at 2, 4, 6, and 8 h p.i. Cell equivalent amounts of cytoplasmic and nuclear extract were separated by SDS–PAGE, electroblotted to PVDF paper, and probed for E2F4. For C33 cells, the aliquots represented 2.6% of each fraction: ~45 μg of cytoplasmic protein and ~27 μg of nuclear protein. For U2-OS cells, the aliquots represented 6.7% of each fraction: ~48 μg of cytoplasmic protein and ~22 μg of nuclear protein.

whereas E2F4 is initially evenly distributed between the cytoplasm and the nucleus. This is consistent with detection of both free E2F and E2F-p107 DNA-binding activities in the cytoplasm (Fig. 2).

We have detected as many as five distinct mobility forms of E2F4 in mock-infected and 16-h-infected C33 cells (Fig. 6B): two fast mobility forms (~50 and 53 kDa apparent molecular weight) in the cytoplasm (lanes 3–4); an abundant 55-kDa form in cytoplasmic and nuclear extracts from uninfected cells (lanes 1 and 3); a major and minor 60-kDa form in uninfected and infected nuclear samples, respectively (lanes 1 and 2); and a 65-kDa form exclusively in nuclear extracts from virus-infected cells (lane 2).

We compared the kinetics of loss of cytoplasmic E2F4 with the decrease in apparent mobility of nuclear E2F4 in both C33 and U2-OS cells (Fig. 6C). By 4 h p.i. (lanes 3 and 8), levels of cytoplasmic E2F4 in both cell types had begun to decline and apparent mobilities of nuclear E2F4 had begun to decrease. By 8 h p.i. (lanes 5 and 10), both cell types had shown significant loss of cytoplasmic E2F4 (68 and 91% in U2OS and C33 cells, respectively) and a shift in mobility of nuclear E2F4. The change in the apparent molecular weight of E2F4 by SDS–PAGE between 4 and 6 h p.i. was not accompanied by any observable change in the mobility of the E2F4 by gel shift analysis (see Fig. 1B). From these results we conclude that (i) total amounts of E2F-associated proteins DP1, DP2, and p107 remain constant after virus infection, (ii) E2F4 protein is translocated to the nucleus, and (iii) concomitant with or after nuclear translocation, E2F4 undergoes a modification that alters its apparent mobility.

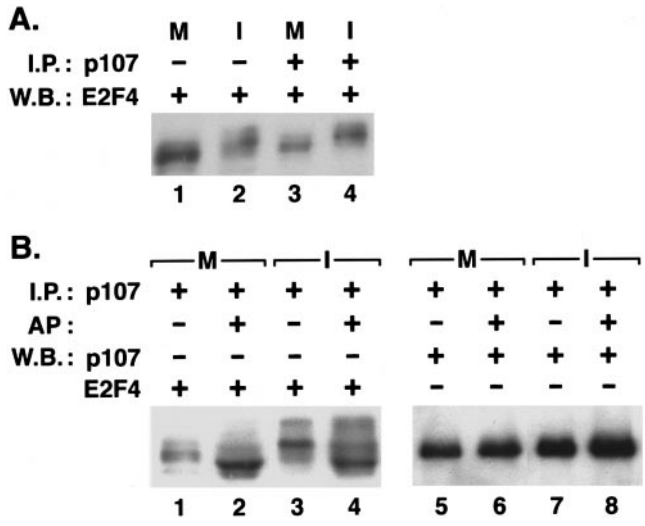

FIG. 7. Posttranslational modifications to E2F4 after HSV infection. (A) Analysis of E2F4 in whole cell p107 complexes. Whole cell extracts were prepared as described under Materials and Methods from mock and 16-h-infected C33 cells. Aliquots (300 μg) were immunoprecipitated with a mixture of mouse monoclonal antibodies to p107 (SD2, 4, 6, 9, and 15) as described under Materials and Methods, separated by SDS–PAGE, transferred to PVDF paper, and underwent Western blotting for E2F4 (lanes 3 and 4). For comparison, 30-μg aliquots of total cell protein were fractionated and probed for comparison (lanes 1 and 2). (B) Alkaline phosphatase treatment of p107-asociated E2F4. Whole cell extracts were prepared from mock (M) and 8-h-infected (I) C33 cells. Immunoprecipitates of p107 complexes were prepared as described under Materials and Methods, and equal portions incubated in the presence (lanes 2, 4, 6, and 8) or absence (lanes 1, 3, 5, and 7) of alkaline phosphatase, as described. After denaturation, separation on SDS–PAGE, and transfer to PVDF, filters were probed for E2F4 (lanes 1–4) or p107 (lanes 5–8).

Infected cell E2F4 is hyperphosphorylated

p107-associated E2F4 is hyperphosphorylated (Ginsberg *et al.,* 1994), and after HSV infection, E2F4 species of decreased mobility accumulated (Figs. 5 and 6). E2F4 of 53- to 60-kDa apparent molecular weight could be found in nuclear extracts, while E2F4 of 50- to 60-kDa apparent molecular weight were present in cytoplasmic extracts. In addition, a 65-kDa E2F4 was uniquely associated with nuclear extracts from infected cells (Fig. 6B). Analysis of total and p107-associated E2F4 from equivalent amounts of whole cell extracts of infected and mock-infected C33 cells (Fig. 7A) revealed a gradient of E2F4 species of increasing apparent molecular weight. E2F4 from uninfected cells migrated in the 50- to 53-kDa range, whereas p107-associated E2F4 migrated in the 53- to 55-kDa size range (Fig. 7A, lanes 1 and 3). The bulk of E2F4 in HSV-infected cells migrated in the range of 55–60 kDa, whereas p107-associated E2F4 migrated in the 55- to 65-kDa size range (lanes 2 and 4). To determine the basis for the shift in apparent molecular weight after virus infection, p107 immunoprecipitates were subjected to treatment with alkaline phosphatase followed by detection of E2F4 by Western blot analysis (Fig. 7B). The amount of both the abundant uninfected cell 55 kDa and infected cell 60-kDa form of E2F4 decreased after alkaline phosphatase treatment, consistent with both of these representing differentially phosphorylated E2F4 species. The amount of 65-kDa, infected cell-specific form of E2F4 was unaffected by phosphatase treatment, and the biochemical basis for its altered mobility is currently unknown. When the blot was stripped and reprobed for p107, no change in mobility after alkaline phosphatase digestion was detected.

DISCUSSION

HSV infection induces a novel intracellular distribution of E2F-pocket protein complexes

We have accumulated evidence that after infection of asynchronously dividing cells by HSV, elements of the E2F-pocket protein arm of the cell cycle regulatory machinery are reorganized in a novel way that combines aspects of both the G0/1 and S phases of the cell cycle. Specifically, in C33 cells the ratio of nuclear to cytoplas-

mic E2F4 increased (Fig. 2), reminiscent of G0/G1 (Lindeman *et al.,* 1997), while the amount of E2F-p107 complexed with cyclin A/CDK2 increased, reminiscent of S phase (Schwarz *et al.,* 1993; Moberg *et al.,* 1996). However, the increase of E2F-p107-kinase complex in viral-infected cells occurs in the nucleus and in the face of inhibition of cellular DNA synthesis. This is different from the normal situation in an uninfected cell where E2F-p107 complexes are found in both the cytoplasm and the nucleus during S phase (Verona *et al.,* 1997). In U2-OS cells that are $RB^{wt/wt}$, HSV infection results in accumulation of E2F-p107 and E2F-pRB (Fig. 1), the latter normally occurring in mid to late G1 in cycling cells. This is accompanied by the accumulation of a hypophosphorylated form of pRB (Fig. 1). We hypothesize that nuclear accumulation of E2F-p107 has some necessary role in virus replication, despite cessation of cellular DNA synthesis. Indeed, infection of murine 3T3 cells derived from $p107^{-/-}$; $p130^{-/-}$ knockout embryos resulted in a 2.5 log drop in virus yield compared with either normal 3T3 cells or cells derived from $RB^{-/-}$ knockout embryos (Ehmann, McLean, and Bachenheimer, manuscript in preparation).

Accumulation of nuclear E2F4 was accompanied by its hyperphosphorylation (Figs. 5 and 6). While this modification is consistent with previous reports of hyperphosphorylation of p107-associated E2F4 (Ginsberg *et al.,* 1994), we observed at least one infection-specific form of E2F4 that appears not to be due to increased phosphorylation (Fig. 7B). The total amount of p107 and E2F4 protein did not decline after HSV infection, but their subcellular distribution was altered, decreasing in the cytoplasm and increasing in the nucleus (Fig. 6A). The E2F binding partner, DP2 was exclusively nuclear and unchanged in amount after infection. DP1, however, was detectable in the cytoplasm but decreased after infection (Fig. 6A). Other studies have indicated that E2F4 may require association with either DP2, p130, or p107 to localize in the nucleus (Magae *et al.,* 1996; Lindeman, *et al.,* 1997). Because we have observed that the majority of cytoplasmic E2F4 was free rather than complexed with p107, the mechanism of nuclear translocation of E2F4 during HSV infection remains unclear. One possibility is that its altered phosphorylation state is sufficient to drive nuclear localization.

Effects of viral gene expression and DNA replication on alterations to E2F

Nuclear translocation of E2F4 and its association with p107 in the nucleus can be uncoupled. Mutations in viral genes such as ICP8, polymerase, and UL5 or treatment of cells with drugs that directly affect viral DNA replication prevented the accumulation of nuclear E2F-p107 (Hilton *et al.,* 1995). However, no decline in cytoplasmic E2F4 or increase in nuclear E2F-pRB DNA-binding activities occurred in cells infected with the ICP4 mutant n12 (Fig. 1). The intracellular distribution of ICP0 is exclusively cytoplasmic in n12-infected cells (Zhu *et al.,* 1994), likely due to the overexpression of ICP27 in the absence of ICP4 (Zhu *et al.,* 1996). In contrast, ICP0 is exclusively nuclear in ICP27-mutant-infected cells (Zhu *et al.,* 1994), possibly the result of aberrant phosphorylation of ICP4 in the absence of ICP27 (Su and Knipe, 1989). Previous experiments indicated a role for ICP27, but not ICP0, in the accumulation of E2F-p107 (Hilton *et al.,* 1995), and it remains to be determined how ICP4, ICP22, and ICP27 may affect these processes.

Accumulation of E2F-pocket protein complexes

During discrete stages of the cell cycle, E2F can complex with hypophosphorylated forms of pRB, p107, and p130. Also, there is evidence that pRB, among other cellular proteins, can colocalize with ICP8, a component of the viral replication complex (Wilcock and Lane, 1991). We have observed a rapid decline in the level of cyclin E/cdk2 activity after infection (Ehmann, McLean, and Bachenheimer, manuscript in preparation). These results suggest at least two models to explain the increase in E2F-pocket protein complexes. In one, decreased cyclin kinase activity, or an increase in phosphatase activity that targets pocket proteins, results in an increase in hypophosphorylated pRB and p107 capable of forming complexes with E2F1 and E2F4, respectively. A second possibility, not mutually exclusive of the first, is that the onset of viral DNA replication and the resulting alterations to one or more nuclear structures may release a pool of hypophosphorylated pocket proteins available for interaction with E2F. Our observations that the amount of nuclear p107 does not increase substantially after infection whereas the amount of E2F-p107-cyclin kinase does increase, suggest that E2F-p107-kinase accumulation may occur in a step-wise fashion involving p107-kinase formation followed by association with translocated E2F4. The accumulation of a hypophosphorylated pRB in the nucleus may be sufficient to explain the increase in nuclear E2F-pRB. We are currently evaluating the role of viral DNA replication on the localization and phosphorylation state of pRB, p107, and p130 and the association of cdk2 with p107.

A role for nuclear translocation of E2F4 in the HSV replication cycle

Does the novel rearrangement of E2F4 protein and accumulation of E2F-pocket protein complexes described here suggest any relationship to effects of virus infection on host DNA synthesis or to the mechanism of viral DNA replication? Comparisons of the kinetics of decrease in host DNA synthesis with alterations to E2F4 location, indicate that loss of cytoplasmic E2F4 precedes increases in nuclear E2F-p107 but that the latter lags behind the decline in host cell DNA

synthesis. Furthermore the onset of viral DNA replication occurs ≥1 h prior to the first indication of E2F-p107 accumulation. Ongoing cellular DNA synthesis is sensitive to the rate of protein synthesis (Stimac *et al.,* 1977). Thus the decline in cellular DNA synthesis after infection may reflect the activity of virus-encoded *vhs* function in rapidly inhibiting host cell protein synthesis (Read, 1997). Krek *et al.* (1995) have suggested that unscheduled occupancy of E2F binding sites could delay or inhibit S phase. Because many E2F responsive genes appear to function directly in DNA synthesis and replication origin function (LaThangue, 1994; DeGregori *et al.,* 1995; Ohtani, 1996; Leone *et al.,* 1998), the large increase in nuclear E2F, in the form of transcriptional repressor complexes with pRB or p107, may represent an additional mechanism to ensure that expression of cellular genes required for S phase is silenced and that cellular DNA synthesis is suppressed.

MATERIALS AND METHODS

Cells and virus

C-33 cells (ATCC HTB31) were derived from a human cervical carcinoma, lack HPV sequences, and express a mutated pRB (Scheffner *et al.,* 1991). U2OS cells (ATCC HTB-96) were derived from a human osteogenic sarcoma and contain wild type pRB and p53. Monolayer cultures were maintained in Dulbecco's modified Eagle medium plus glucose (DMEM-H) supplemented with 10% fetal calf serum. Herpes simplex virus type 1 (HSV-1) strain KOS, and strain mutants were used at a multiplicity of 5–10 plaque forming units (PFU) per cell. The ICP8 mutant, d301 (Gao and Knipe, 1991) was originally obtained from D. Knipe, Harvard University; the UL5 mutant, B72 (Zhu and Weller, 1992) was originally obtained from S. Weller, Connecticut Health Science Center; and the ICP4 mutant n12 (DeLuca and Schaffer, 1988) was obtained from N. DeLuca, U. of Pittsburgh.

Preparation of cell extracts

Cytoplasmic and nuclear extracts were prepared by a rapid lysis method. Cells were released from culture dishes by trypsinization, washed twice in PBS, and resuspended in three packed cell volumes (PCV) of CE buffer (10 mM HEPES, pH 7.8; 1 mM EDTA; 60 mM KCl; 1 mM PMSF; 0.1% NP-40; 25% glycerol; 0.4 mM NaF; 0.4 mM Na_3VO_4; 10 μM pepstatin; and 4% Complete Protease Inhibitor Cocktail [Boehringer Mannheim]). After 4-min incubation on ice, nuclei were pelleted by a 10-s spin in a bench top microcentrifuge. The supernatant, representing the cytoplasmic extract, was further clarified by centrifugation at 14,000 rpm for 10 min at 4°C. Nuclei were resuspended in CW buffer (CE buffer without NP-40 or glycerol), subjected to 10–20 strokes in a 1-ml Dounce homogenizer and repelleted. After discarding the CW supernatant, nuclei were resuspended in 2 PCV of NE buffer (20 mM Tris–HCl, pH 8.0; 420 mM NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 0.5 mM PMSF; 25% glycerol; and phosphatase and protease inhibitors as described above). After incubation for 10 min on ice, nuclei were pelleted at 60,000 rpm for 20 min at 4°C. The supernatant, representing the nuclear extract, was carefully removed, and both cytoplasmic and nuclear extracts were stored at −70°C.

Whole cell extracts were prepared according to the method of Ginsberg *et al.* (1994). Briefly, cells were rinsed twice in ice cold PBS, incubated on ice for 20 min in TNN buffer (50 mM Tris–HCl, pH 7.4; 120 mM NaCl; 5 mM EDTA; 0.5% NP-40; 50 mM NaF; 0.2 mM sodium orthovanadate; 1 mM DTT; 1 mM PMSF; and 20 μg/ml aprotinin. Cellular debris were removed by centrifugation for 10 min at 10,000 g, and the supernatant was recovered as the soluble extract.

Analysis of cellular and viral DNA synthesis

Infected cell DNA synthesis was monitored essentially as described previously (Sherman and Bachenheimer, 1987). Briefly, replicate cultures of C33 cells were infected with HSV and at various times p.i., monolayers were washed in serum-free medium and three-fourths of the monolayer was removed with a rubber scrapper and processed to yield soluble protein extracts as described above. The remaining fourth of the monolayer was labeled for 30 min with [^{3}H]thymidine (50 μCi/ml). Cells were collected, washed in PBS and resuspended in TE (10 mM Tris, 1 mM EDTA, pH 7.6). Sarcosyl and proteinase K were added to final concentrations of 0.5% and 250 μg/ml, respectively, and incubated overnight at 37°C. The viscosity of the DNA was reduced by passing the solution through a Pasteur pipet several times. CsCl was added until the solution reached a refractive index of 1.4010 and the DNA was centrifuged to equilibrium in a TV-865 (Sorvall) rotor at 35,000 rpm for 16 h. Gradient fractions were collected and the distribution of labeled cellular and viral DNA determined by precipitating aliquots of samples with 5% trichloroacetic acid onto GFA glass fiber paper, drying in acetone and counting in a liquid scintillation counter.

E2F gel mobility assay

Formation of DNA protein complexes, their fractionation on nondenaturing acrylamide gels and detection by autoradiography was performed as previously described (Hilton *et al.,* 1995). Each reaction contained ~0.03 pmoles of a double site E2F probe (top strand sequence: CGTAGTTTTCGCGCTTAAATTTGAGAAAGGGCGCGAAACTAGTC). Competitions with single site E2F (AATTTGAGAAAGGGCGCGAAACTAGTC) or mutant (AATTTGAGAAAC-

TAGAGTCTGCTAGTC) oligonucleotides were performed in 100-fold excess of the labeled probe.

Detection of proteins

Aliquots of whole cell, cytoplasmic or nuclear extracts were denatured by boiling in sample buffer (Harlow and Lane, 1988), electrophoresed on SDS–PAGE gels, and electroblotted onto PVDF paper. After blocking in 3% milk, proteins were detected by incubation with rabbit polyclonal or mouse monoclonal antibodies, an appropriate secondary antibody, followed by incubation with a protein A-horseradish peroxidase conjugate, and detection on X-ray film by light emission from the oxidation of luminol (Du Pont NEN Renaissance).

Immunoprecipitation of p107 complexes

Aliquots of cytoplasmic and nuclear extract were precleared with protein A beads (Boehringer-Mannheim) and then incubated for 18 h at 4°C. with rabbit anti-p107 antibody (C-18, Santa Cruz). Immune complexes were incubated with protein A beads for 1 h at 4°C, collected by centrifugation, washed three times in lysis buffer, and resuspended in sample buffer (Harlow and Lane, 1988) for electrophoretic separation.

Alkaline phosphatase treatment

Whole cell extracts were prepared from mock-infected and 8-h HSV-infected C33 cells, as described above. Aliquots (1 mg) were precleared with protein A agarose beads, then incubated with a cocktail of p107 monoclonal antibodies (20 μl each SD2, 4, 6, 9, and 15, provided by N. Dyson, MGH Cancer Center), followed by further incubation with protein A agarose to collect immune complexes. The beads were washed four times with TNN, and resuspended in 40-μl alkaline phosphatase buffer (50 mM Tris–HCl, pH 8.0; 1 mM $MgCl_2$; 0.1 mM $ZnCl_2$). One half of each immune complex was incubated with 20 U alkaline phosphatase at 37°C for 60 min. Proteins were denatured and released from beads by addition of an equal volume of 2× sample buffer (Harlow and Lane, 1988) by boiling for 3 min. Proteins were fractionated by SDS–PAGE, and electroblotted onto PVDF paper. E2F4 and p107 were detected with rabbit polyclonal antibodies (Santa Cruz) by procedures described above. Alternatively, 150-μg aliquots of TNN whole cell extracts from cultures of mock- or HSV-infected C33 cells were dialyzed against 50 mM Tris–HCl, pH 8.0, and 0.1 mM EDTA, divided into equal fractions, and either left untreated, treated with 20 U alkaline phosphatase, or treated with 20 U alkaline phosphatase in the presence of 5 mM NaH_2PO_4/Na_2HPO_4, pH 7.9, and 380 mM NaF at 37°C for 15 min. E2F4 and p107 were detected by Western blotting as described above.

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