INTRODUCTION

The lytic infection cycle of human cytomegalovirus (HCMV) is accompanied by a characteristic deregulation of the host cell cycle (Fortunato et al., 2000; Kalejta & Shenk, 2002). Cellular DNA synthesis is disturbed by the virus, resulting in cell-cycle arrest at or near the G1/S transition (Bresnahan et al., 1996; Dittmer & Mocarski, 1997; Lu & Shenk, 1996). At the same time, many S-phase-promoting activities are induced, even under conditions of growth factor deprivation, contact inhibition and differentiation (Bresnahan et al., 1996; Jault et al., 1995; Sinclair et al., 2000). In particular, cyclin E–cdk2 kinase activity, which drives G1/S transition in the normal cell cycle (Sauer & Lehner, 1995), is constitutively up-regulated during HCMV infection (Jault et al., 1995). Consistent with this, the cyclin E–cdk2 substrates pRb and p130 have been found to be hyperphosphorylated (Jault et al., 1995; McElroy et al., 2000; Sinclair et al., 2000) and Rb/E2F-controlled genes, such as dihydrofolate reductase and thymidylate synthase, are transcriptionally activated in infected cells (Gribaudo et al., 2000; Wade et al., 1992). All in all, this leads to an S-phase metabolism with greatly increased nucleotide pools in HCMV-infected cells (Biron et al., 1986). However, whereas cyclin E–cdk2 activity and biosynthesis of nucleotides are both essential host-cell functions for HCMV replication (Bresnahan et al., 1997a; Gribaudo et al., 2000), ongoing cellular replication appears to counteract progress of the infectious cycle (Salvant et al., 1998).

The HCMV 86 kDa immediate-early protein 2 (IE2) is not only an essential transcriptional activator of early viral gene expression (Gebert et al., 1997; Heider et al., 2002; Marchini et al., 2001) but also a cell-cycle regulator that can reproduce some important features of the deregulated cell-cycle phenotype in HCMV infection. It blocks cell-cycle progression at the beginning of S phase in various cell types (Murphy et al., 2000; Wiebusch & Hagemeier, 1999, 2001), including primary fibroblasts (Kronschnabl et al., 2002). This arrest resembles the specific inhibition of DNA replication by chemical inhibitors such as hydroxyurea (HU) or aphidicoline since it is dominant over cyclin–cdk activities and leaves the Rb–E2F pathway untouched (Wiebusch & Hagemeier, 2001). Moreover, cyclin E expression and activity are up-regulated in IE2-arrested cells (Wiebusch & Hagemeier, 2001) and the mRNA levels of numerous genes involved in nucleotide synthesis and DNA replication are elevated (Song & Stinski, 2002). Other findings also point towards IE2 as a factor with an ambivalent role in regulating cell proliferation: IE2 relieves...
the flat-cell phenotype of pRB-transfected Saos cells (Fortunato et al., 1997) and rescues the defect of ts13 cells in cyclin transcription (Lukac et al., 1997) – in both cases the cell cycle is still blocked. IE2 even forces quiescent REF-52 cells to re-enter the cell cycle and synthesize at least limited amounts of DNA (Castillo et al., 2000). Thus, there is considerable evidence that IE2 triggers both proliferative and anti-proliferative cellular activities but it is unknown how these functions integrate mechanistically on IE2.

Activation of cyclin E–cdk2 in HCMV-infected cells is mainly achieved by a strong increase in cyclin E transcription (Bresnahan et al., 1998; Salvant et al., 1998). Other HCMV-induced alterations that are known to contribute to the cyclin E–cdk2 activation are the transllocation of cdk2 from the cytoplasm into the nucleus (Bresnahan et al., 1997b) and the enhanced degradation of the cdk inhibitor p21WAF1,CIP1 (Chen et al., 1997b). How IE2 could contribute towards cyclin E–cdk2 regulation is not fully understood. One report investigating the requirements for cyclin E activation in infected cells questioned the importance of IE2. It demonstrated that cyclin E induction depended on early viral gene expression and was mainly associated with a change in cyclin E promoter occupancy concerning the composition of E2F–pocket protein complexes (McElroy et al., 2000). In contrast, in vitro experiments showing binding of IE2 to the cyclin E promoter (Bresnahan et al., 1998) and a functional IE2–p21WAF1,CIP1 interaction (Sinclair et al., 2000) have suggested a direct involvement of IE2 in cyclin E activation in infected cells questioned the importance of IE2. This could be an important point in understanding the role of IE2 in cell cycle regulation.

Therefore, the work presented here is aimed at examining whether IE2 can serve as a genuine activator of cyclin E gene expression and looking at the possible functional implications of such an activation on IE2-expressing cells.

**METHODS**

**Cells.** U373-MG cells and primary human embryonic lung fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% newborn calf serum, 5% foetal calf serum, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. Every 3 days, cells were split in a 1:3 ratio to maintain a subconfluent state.

**Plasmids.** pSG5 (Green et al., 1988) and the expression plasmids for CD20, IE2(195-579) and cyclin E [pSG5-CD20, pSG5-3HA-IE2(195-579) and pCMX-CycE] have been previously described (Wiebusch & Hagemeier, 2001). For IE2 expression, the vector pSGS-IE2-His was used containing full-length IE2 cDNA of AD169 origin C-terminally fused to a tag of six histidine codons.

**DNA transfections.** Cells were transfected using the calcium phosphate co-precipitation method, as previously described (Wiebusch & Hagemeier, 1999). The DNA precipitates were left on the cells for 14–16 h. The transfection medium was replaced with fresh culture medium supplemented with 1 mM mimosine, 1 mM HU or 10 μM 5-bromo-2'-deoxyuridine (BrdU) as indicated. Nocodazole was added at a final concentration of 50 ng ml⁻¹ 24 h after removal of the DNA precipitates. The time-points of cell harvest are specified in the figure legends.

**Cell sorting.** Transfected cells were labelled with a CD20 antibody (clone 2H7; Pharmingen) and separated from untransfected cells by magnetic affinity cell sorting (MACS), as previously described (Wiebusch & Hagemeier, 2001). To control the sorting efficiency, aliquots of sorted and unsorted cells were stained with an FITC-conjugated secondary antibody (F0313; DAKO) and analysed by flow cytometry for CD20 expression. To control for IE2 co-expression, cells were permeabilized by overnight incubation in 70% ethanol in PBS, blocked in 0.5% BSA in PBS and labelled with a primary IE1/2-specific antibody (clone E13; Argene) and a secondary FITC-conjugated mouse IgG1-specific antibody (clone A85-1; Pharmingen). Both antibody-binding reactions were carried out for 15 min at room temperature using 5 μg antibodies ml⁻¹ in 0.5% BSA in PBS, followed by a washing step with 0.5% BSA in PBS. Finally, cells were suspended in PBS and analysed by flow cytometry.

**Cell-cycle analysis.** The overall DNA content of transfected cells was determined by propidium iodide staining and flow cytometry, as previously described (Wiebusch & Hagemeier, 1999). The BrdU incorporation assay for detection of newly synthesized DNA was carried out as described by Wiebusch & Hagemeier (2001). Briefly, following CD20-directed cell sorting, BrdU-treated cells were permeabilized in 70% ethanol and stained by indirect immunofluorescence using an anti-BrdU antibody (clone 3D4; Pharmingen) and an isotype-specific secondary antibody (clone A85-1; Pharmingen) to avoid cross-reaction with the CD20 antibody used for cell sorting. Cells were then co-stained with propidium iodide and analysed by flow cytometry.

**Immunoblot analysis and kinase assay.** After sorting, cell extracts of CD20-positive cells were prepared as previously described (Wiebusch & Hagemeier, 2001). These were used for immunoblot analysis and cyclin E kinase assays. Both assays were performed exactly as previously described (Wiebusch & Hagemeier, 2001) employing the cyclin E monoclonal antibodies HE12 (Santa Cruz) for immunoblotting and HE111 (Santa Cruz) for immunoprecipitation of cyclin E kinase activity. Blots were routinely controlled for equal loading and protein transfer by Ponceau S staining. To control the expression level of IE2 and its mutant IE2(195-579), blots were probed with a rabbit antiserum recognizing the C-terminal region of IE2 (a kind gift from Thomas Stamminger, Erlangen, Germany).

**Ribonuclease protection assay (RPA).** Total RNAs from CD20-positive cells were isolated using Trizol reagent (Gibco) according to the manufacturer’s instructions. Two μg of each preparation was taken as input in a multiprobe RPA (Riboquant; Pharmingen). A defined pool of radioactively labelled probes was generated by *in vitro* transcription from the template set hCyc-2 (Pharmingen).
and hybridized with the input RNA. The samples were subsequently digested with an RNase A/T1 mixture, purified by ethanol precipitation, separated by denaturing PAGE and subjected to autoradiography.

RESULTS

We set up an experimental system to determine whether IE2 was a genuine activator of cyclin E or whether cyclin E up-regulation was a mere consequence of the early S-phase block in IE2-expressing cells. This system allowed us to study the effect of IE2 on cyclin E gene expression prior to the physiological and cell-cycle-dependent induction of cyclin E. This distinction is important because it would allow us to assign a proliferative capacity to IE2 (and suggest a mechanistic explanation of it) in addition to the previously shown cell-cycle arrest function of this essential virus regulator.

Thus, we applied an experimental system that we have successfully used before (Wiebusch & Hagemeier, 2001), which relies on the co-transfection of cDNAs for IE2 and the cell-surface marker CD20 into U373 cells, a cell line in which HCMV can replicate. This enabled us: (i) to determine the cell-cycle stage of IE2-expressing cells, and (ii) to physically separate IE2-expressing cells via MACS for biochemical analysis (see Methods; Wiebusch & Hagemeier, 2001). In addition, we made use of two drugs that are known to arrest cell-cycle progression (Fig. 1A): mimosine, which can block cells in a dose-dependent manner in G1, and hydroxyurea (HU), which inhibits ongoing DNA synthesis in early S phase (Krude, 1999, Wiebusch & Hagemeier, 2001). Since cells take up transfected DNA primarily during mitosis at times when the nuclear membrane breaks down, these cells become functionally synchronized by the transfection procedure itself (Adams et al., 1997; Rodriguez et al., 1999; unpublished observations). Therefore, in the presence of mimosine or HU, control-transfected, i.e. CD20-positive, cells synchronously ran into a cell-cycle block that appeared to be characterized by a G1-like DNA content (Fig. 1B, top panel). However, HU-treated cells had elevated levels of cyclin E protein (Fig. 1C, lane 2) reflecting the fact that these cells were arrested right at the beginning of S phase, which could be directly demonstrated by these cells having incorporated a small amount of BrdU (data not shown; Wiebusch & Hagemeier, 2001). In contrast, mimosine-arrested cells were truly blocked in G1 (for BrdU incorporation analysis, see below) and importantly, these cells had non-elevated levels of cyclin E protein (Fig. 1C, lane 3). Although mimosine has also been reported to block cells in early S phase (Hughes & Cook, 1996), this was clearly not the case in the transfected, CD20-positive population, since after transfection these cells synchronously leave mitosis and pass into G1 before becoming arrested in this phase.

The validity of our experimental approach was further supported by analysing CD20-negative, i.e. non-transfected, cells, which reflected the non-synchronized population. Here, HU treatment resulted in arrested cells not only at the beginning of S phase but also, as expected, during later stages of S (Fig. 1B, lower panel). Equally, the mimosine-treated culture not only contained cells in G1 but also cells in early S, as one would expect from the mode of action of this drug on a proliferating cell population. Importantly, the analysis of cyclin E protein levels was consistent with this notion. As expected, CD20-negative HU-treated cells had high levels of cyclin E, whereas the mimosine-arrested population had intermediate levels of cyclin E, reflecting both the majority of G1-phase cells and cells that were hit by this drug in early S phase (Fig. 1C, lanes 4–6).

Together these data showed that in our experimental system of functionally synchronized cells, mimosine not only arrested transfected cells in G1 but, furthermore, the time point of arrest within G1 was prior to the physiological boost of cyclin E induction late in G1. This assay system should therefore allow us to measure any genuine effect of IE2 on the expression level of cyclin E.
**Cyclin E activation by IE2 is independent of the cell-cycle stage**

We next transfected cells with either an IE2 expression plasmid or its parental empty plasmid, both in the presence and absence of mimosine, to test for IE2-mediated cell-cycle-independent activation of cyclin E. First, we asked whether IE2 expression would interfere with the mimosine-dependent cell-cycle block in G1. Whereas in the absence of mimosine control-transfected cells were evenly distributed over S phase, IE2-expressing cells were blocked at the beginning of S (Fig. 2A, plots 1 and 2; B) as shown by BrdU incorporation assays. These results are consistent with previous data (Wiebusch & Hagemeier, 2001). In contrast, mimosine treatment, as expected from the data presented in Fig. 1, led to a clear-cut block of cell-cycle progression in G1, irrespective of the presence or absence of IE2 (Fig. 2A, plots 3 and 4; B). Thus, IE2 expression did not overcome the mimosine-induced G1 arrest.

We then used aliquots of these cells to look for cyclin E gene expression. Consistent with previously published results (Wiebusch & Hagemeier, 2001), IE2 up-regulates cyclin E in the absence of mimosine, which can be observed by increased steady-state levels of mRNA (Fig. 3A, lanes 2 and 3), protein (Fig. 3B, lanes 1 and 2) and kinase activity (Fig. 3C, lanes 1 and 2), the latter measured as cyclin E-dependent phosphorylation of histone H1. A virtually identical picture of an IE2-mediated cyclin E induction was observed when the experiment was performed in the presence of mimosine (Fig. 3A, lane 5; B, C, lane 4), although mimosine itself clearly arrested cells in G1 (Fig. 2A, plot 3) prior to cyclin E induction (Fig. 3A, lane 4; B, C, lane 3). This demonstrates that IE2 can actively (and independently from G1/S transition) up-regulate cyclin E protein levels, which results in the untimely induction of cyclin E-associated kinase activity under the experimental conditions. The activation appears to occur primarily at the level of transcription, although additional post-transcriptional mechanisms of activation cannot be excluded.

**Cyclin E up-regulation by IE2 also occurs in primary fibroblasts**

To exclude the possibility that IE2 up-regulation of cyclin E is cell-type-specific or a consequence of the transformed phenotype of U373 cells, we extended our analysis to primary lung fibroblasts. We co-transfected the CD20 surface marker together with IE2 and prepared extracts for immunoblot and ribonuclease protection analysis from transfected and MACS-sorted fibroblasts. Since the transfection efficiency of primary fibroblasts was very low (3 % in Fig. 4A, top right-hand panel), we first wanted to ensure that transfected cells were expressing both the CD20 marker and IE2. Fig. 4(A) shows that MACS allowed a very significant enrichment of transfected cells (compare top and bottom right-hand panels) and that most CD20-sorted cells indeed also expressed IE2 (89 %, bottom right-hand panel). In these cells, IE2 expression led to a marked increase in cyclin E expression at both the mRNA (Fig. 4B, lane 3) and protein levels (Fig. 4C, lane 2) when compared with sorted control-transfected primary fibroblasts (Fig. 4B, lane 2; C, lane 1). In contrast, a transactivation-deficient mutant of IE2 (Wiebusch & Hagemeier, 1999) lacking the first 194 amino acids, IE2(195-579), was not able to up-regulate cyclin E (Fig. 4B, lane 4; C, lane 3), although it was expressed to the same extent as full-length IE2 (see Fig. 5C; Wiebusch & Hagemeier, 1999). This mutant also failed to activate cyclin E in U373 cells (Wiebusch & Hagemeier, 2001), but was still competent in inducing a cell-cycle block (Wiebusch & Hagemeier, 1999; see below). These data support the view of transcriptional activation of cyclin E gene expression by IE2, which is consistent with the result shown in Fig. 3 and Fig. 4A.
in agreement with other reports (Bresnahan et al., 1996; Song & Stinski, 2002). Moreover, and in contrast to a previous study (McElroy et al., 2000), it has been shown here for the first time that this activation also resulted in elevated cyclin E protein levels in primary fibroblasts. Thus,
IE2-mediated up-regulation of the cyclin E protein is neither cell-type-specific nor dependent on cells with a transformed phenotype.

**Cyclin E activation is responsible for S-phase entry of IE2-expressing cells**

The fact that IE2 and IE2(195-579) can both induce a cell-cycle arrest but only full-length IE2 activates cyclin E opens up the question of the biological significance of the cyclin E activation by IE2. In order to address this question, we further characterized cells arrested by IE2 and IE2(195-579) using BrdU incorporation assays (Fig. 5A, B). Control-transfected cells were evenly distributed over S phase, whereas IE2-arrested cells were found to be located right at the beginning of S phase (Fig. 5A, plots 1 and 2), as previously shown. Surprisingly, lack of BrdU incorporation in cells expressing the mutant form of IE2 suggested that this mutant blocks cell-cycle progression in G₁ rather than early S phase (Fig. 5A, plot 3). The IE2(195-579)-expressing population contained only half as many cells in early S phase compared with cells expressing IE2, which was primarily due to a respective increase of cells in G₁ (Fig. 5B).

Given that the IE2 mutant protein failed to activate cyclin E (see above), this suggested to us that IE2-mediated cyclin E induction is a prerequisite for S-phase entry. Conversely, lack of cyclin E expression in IE2(195-579)-transfected cells appeared to go hand in hand with a lack of S-phase entry. To test this hypothesis directly, we co-expressed cyclin E and IE2(195-579) and asked whether, under these conditions, cells would now enter S phase as measured by BrdU incorporation. As predicted, cyclin E was found to completely rescue the S-phase entry of IE2(195-579)-expressing cells (Fig. 5A, plot 4). At the same time, full-length IE2 was also able to rescue S-phase entry when co-expressed with the IE2 mutant form (Fig. 5A, plot 6), also implying that IE2(195-579) does not function as a dominant negative form of IE2. Quantification of the rescue experiments showed that co-expression of cyclin E induced a more pronounced S-phase entry in IE2(195-579)-transfected cells than co-expression of IE2 (49 % versus 38 %, Fig. 5B). This was most likely due to the significantly higher levels of cyclin E protein in these cells (data not shown). In accordance with this notion, a significantly higher proportion of S phase cells was also seen after the sole transfection of cyclin E (59 %) versus IE2 (40 %). These findings further support the view that IE2-mediated cyclin E
induction drives IE2(195-579)-arrested cells across the G1/S border.

Although S-phase entry in IE2(195-579)-expressing cells could be rescued by raising cyclin E protein levels as demonstrated, these cells did not progress through S phase and into G2 (Fig. 5A, B). Instead, they remained in an early S phase compartment, like cells arrested by IE2 [Fig. 5B, compare the G0/G1 : early S ratios between cells expressing IE2 (1:1:2), IE2(195-579) plus cyclin E (1:2:3) and IE2(195-579) plus IE2 (1:1:1)]. Again, the direct overexpression of cyclin E appeared to empty the G1 compartment more rapidly than IE2. As demonstrated by immunoblot analysis, we could exclude cross-regulation of the IE2 mutant by cyclin E (Fig. 5C, left-hand panel, lanes 3 and 4) and of cyclin E by the IE2 mutant (Fig. 5C, right-hand panel, lanes 4 and 5) to account for the differences in BrdU incorporation.

Together these data suggest that IE2, by transcriptionally up-regulating cyclin E, promotes G1/S transition before cells finally arrest in early S phase. Thus, in addition to the demonstrated cell-cycle-arrest function of IE2, we can also assign an independent and genuine proliferative capacity to IE2. This view is consistent with the observation of the untimely induction of cyclin E in mimosine-arrested IE2-expressing cells shown in Fig. 3.

**IE2(195-579) retains the ability to arrest cells in early S phase**

The rescue experiments shown in Fig. 5 suggested that cells finally arrest in early S phase after traversing the G1/S border. In order to test further whether the cell-cycle arrest imposed by the mutant form of IE2 is dominant over cdk activity, as for IE2, we used nocodazole treatment in the experimental set-up described in Fig. 5. Nocodazole is a spindle poison and blocks cells at the beginning of mitosis. Under this treatment, cycling control-transfected cells primarily accumulated in G2/M as expected (Fig. 6, plot 1). In contrast, a large proportion of IE2- and IE2(195-579)-expressing cells remained arrested in S and G1, respectively (Fig. 6, plots 2 and 3). Importantly, the same was true for cells over-expressing cyclin E in addition to the mutant form of IE2 (Fig. 6, plot 4). This demonstrated that, although cyclin E expression drives cells arrested by IE2(195-579) in G1 into S phase (see Fig. 5A, plot 4), the majority of these cells do not progress further through S phase and do not enter G2. Cyclin E overexpression alone first accelerated G1/S transition and then slowed down S-phase progression (Fig. 6, plot 5). This resulted in a high proportion of a pan-S cell population that slowly emptied into G2, which is consistent with previously published observations (Resnitzky et al., 1994; Spruck et al., 1999). As expected, cells expressing both IE2 and the IE2 mutant remained to a great extent in the G1/S compartment, demonstrating the cell-cycle arrest suggested from the findings shown in Fig. 5.

In conclusion, our data suggest that IE2 has the capacity to block cells in G1 but this cell-cycle block is counteracted by the intrinsic activity of IE2 to up-regulate cyclin E. Consequently, cells enter S phase where they finally arrest due to an as yet unknown mechanism, despite high cyclin E-associated kinase activity.

**DISCUSSION**

We have presented experimental evidence to demonstrate for the first time that the IE2-mediated up-regulation of endogenous cyclin E is cell-cycle independent, i.e. not a consequence of the cell-cycle state of IE2-expressing cells but rather actively promoted by the viral regulator. This activation of cyclin E is necessary to push IE2-expressing cells into S phase where they finally arrest since, rather surprisingly, an IE2 mutant defective in cyclin E activation was shown to block cell-cycle progression in G1. Therefore, IE2 appears to integrate two opposing cell-cycle regulatory activities: a proliferating activity promoting G1/S progression via cyclin E activation and an anti-proliferating activity leading to an early S-phase arrest, as previously shown (Murphy et al., 2000; Wiebusch & Hagemeier, 2001).

The initial question of this study, whether IE2 is a genuine activator of cyclin E or not, was answered by experiments employing the plant amino acid mimosine to arrest IE2-expressing cells in G1 before they entered into the IE2 block and the time window of periodic cyclin E induction around the G1/S transition phase (Figs 1–3). Mimosine is known to arrest cells 2–4 h before the onset of S phase by as yet undefined mechanisms (Krude, 1999). Since cyclin E does not start to accumulate until 2 h before S-phase entry (Ekholm et al., 2001), our finding of low cyclin E expression...
in mimosine-arrested cells is fully consistent with what one would expect from published evidence. Therefore, the fact that IE2 promotes up-regulation of cyclin E in these cells without having any obvious effect on the mimosine arrest itself demonstrates that IE2 can act as a cell-cycle-independent activator of cyclin E.

How does IE2 activate cyclin E? An earlier study (Bresnahan et al., 1998) suggested a model based on transient transfection assays employing cyclin E promoter constructs whereby IE2 directly transactivates cyclin E transcription. Two of our experimental results supported such a view. Firstly, IE2 expression led to an increase in endogenous cyclin E mRNA abundance (Fig. 3A, Fig. 4A), and secondly, the IE2 mutant IE2(195-579), which lacks the transcriptional activator function of IE2, was deficient in cyclin E activation (Fig. 4; Wiebusch & Hagemeier, 2001). Since the same mutant still contains the major binding regions for pRb and p21\textsuperscript{WAF1,CIP1} (Fortunato et al., 1997, 2000; Sinclair et al., 2000), it seems unlikely that an IE2 interaction with one of these proteins causes the untimely up-regulation of cyclin E that we observed in U373 cells. Furthermore, p21\textsuperscript{WAF1,CIP1} expression levels are very low in this p53-negative cell line, arguing against the functional relevance of IE2 targeting p21\textsuperscript{WAF1,CIP1} in these cells. However, considering the fact that cyclin E–cdk2 activity is a convergence point for multiple negative control mechanisms in G\textsubscript{1}/S (Bartek & Lukas, 2001), it cannot be excluded that IE2 generally exerts additional functions at a post-transcriptional level contributing to the constitutive activation of cyclin E. For instance, IE2 activates cyclin E–cdk2, even in p16\textsuperscript{INK4a}-arrested cells (unpublished observation), where cyclin E–cdk2 is normally held inactive by p21\textsuperscript{WAF1,CIP1} that has been set free from cyclin D–cdk4 complexes (Jiang et al., 1998). This supports the view that under conditions of high p21\textsuperscript{WAF1,CIP1} availability, as in differentiated cells, an IE2-mediated inactivation of this cdk inhibitor may become important for cyclin E activation (Sinclair et al., 2000). Similarly, it should be interesting to investigate whether IE2 has an influence on SCFCDC4-mediated proteolysis of cyclin E, which is normally initiated shortly after S-phase entry (Strohmaier et al., 2001).

A number of reports have shown that premature activation of cyclin E–cdk2 by constitutive overexpression of cyclin E induces S phase, even in growth-arrested cells (Leone et al., 1999; Lukas et al., 1997). Accordingly, the ability of IE2 to up-regulate cyclin E expression independent of the cell-cycle state may be one explanation of how IE2 expression leads to S-phase entry in quiescent cells (Castillo et al., 2000). Since cyclin E–cdk2 is a key regulator of the pocket protein/E2F pathway, which controls the transcription of numerous genes coupled to proliferation and DNA synthesis (DeGregori et al., 1995), it is feasible that the activation of E2F-responsive gene expression observed in IE2-transfected fibroblasts (Song & Stinski, 2002) is, in addition to the direct transactivation of certain genes by IE2, the consequence of more general IE2 functions, such as cyclin E–cdk2 activation or pRb inactivation (Hagemeier et al., 1994).

There is considerable evidence that cyclin E overexpression contributes to the development of many types of human cancer (Donnellan & Chetty, 1999). Therefore, the finding that IE2 activates cyclin E constitutively also suggests a latent oncogenic activity for this viral regulator. Given the functional relationship between IE2 and viral oncogenes such as E1A and SV40 large T antigen [namely the interactions with pRb (Hagemeier et al., 1994) and p53 (Speir et al., 1994)] and regarding the mutagenic (Shen et al., 1997) and anti-apoptotic (Yu & Alwine, 2002; Zhu et al., 1995) capabilities of IE2, one could predict that a – still undiscovered – IE2 species specifically lacking the cell-cycle-arrest function could have considerable oncogenic potential.

Intriguingly, the lack of cyclin E activating function in IE2(195-579) revealed a true G\textsubscript{1} arrest activity that is inherent to IE2. This activity normally appears to be overcome by the full-length IE2-mediated induction of cyclin E, which pushes these cells into early S phase where they finally arrest (Fig. 7). This underlines the importance of the IE2-mediated cyclin E up-regulation.

Although moderate cyclin E overexpression has been reported to be associated with a delay in S-phase progression (Resnitzky et al., 1994), the constitutive cyclin E activity
in IE2-expressing cells cannot explain the long-term cell-cycle arrest in those cells. Even when we overexpressed cyclin E by transient transfection in U373 cells leading to cyclin E protein levels nearly two orders of magnitudes higher than in normal cells (Fig. 5C), we could easily distinguish S-phase prolongation in these cells from S-phase arrest in IE2-transfected cells (Fig. 6). This clearly demonstrates that IE2 has an activity capable of arresting cells in S phase that is independent of cyclin E expression. Is the same activity of IE2 responsible for the IE2(195–579)-mediated G1 arrest and the full-length IE2-induced block in S phase? In principle, two alternative models could explain this finding: either IE2 possesses two independent arrest functions or the same arrest function is active in G1 and S but sensitive to high cyclin E–cdk2 activity in G1. Elucidation of the molecular mechanism(s) underlying the IE2 arrest phenotype should decide which of these models is valid.

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