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Modulation of Cyclin Gene Expression by Adenovirus E1A in a Cell Line with E1A-Dependent Conditional Proliferation

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To investigate how adenovirus E1A controls cell proliferation, we have fused E1A to the hormone-binding domain of the human estrogen receptor (ER) and introduced the E1A-ER chimeric gene together with an activated *ras* gene into primary rat embryo fibroblasts. Cell lines derived from this transfection proliferate in an estrogen-dependent manner. Estrogen-dependent activation of E1A-ER led to a rapid induction of both cyclin E and cyclin A gene expression. In contrast, levels of cyclin D1 were strongly reduced by activation of E1A-ER. Similar changes in cyclin gene expression were observed when primary human fibroblasts were infected with wild-type adenovirus and when adenovirus E1A was stably expressed in NIH 3T3 cells. Our findings suggest that activation of cyclin A and E, but not D1, gene expression by E1A precedes and may be responsible for E1A-dependent cell proliferation. In contrast, we found that quantitative disruption of complexes between the E2F transcription factor and the retinoblastoma protein is not required for E1A-dependent S-phase entry.

Recent evidence suggests a correlation between the development of certain cancers and infection with different DNA tumor viruses (for a review, see reference 42). One characteristic property of these viruses is their ability to stimulate proliferation in quiescent cells; to carry out this function, they have evolved specific genes, such as the E7 gene of human papillomaviruses (35 and references therein), the large T antigen of simian virus 40 (reviewed in reference 9), and the E1A gene of adenovirus (see below). The deregulation of cell cycle control achieved by such oncogenes may contribute to the immortalization and transformation of mammalian cells by these viruses.

The mechanism(s) by which these genes override cell cycle control is largely unknown. Adenovirus E1A protein may interfere with growth control by physically interacting with several cell cycle control proteins (39), including the retinoblastoma protein (pRB), p107, cyclin A, cyclin E, and *cdk2* (reviewed in reference 7). The interaction between E1A and pRB (38) has attracted particular attention, as pRB has been shown to negatively regulate cell cycle progression during the G_1 phase (10). One intracellular target for pRB is the E2F transcription factor (4, 5). E2F is involved in the control of several genes required for cell proliferation (reviewed in reference 23). In untransformed mammalian cells, E2F is complexed to pRB (2), and the activity of E2F is repressed by pRB in transient cotransfection experiments (11, 12, 41). Binding of E1A to pRB can disrupt the association of pRB with E2F in vitro (2). Disruption of pRB-E2F complexes by E1A might, therefore, contribute to the stimulation of DNA synthesis by the viral oncoprotein.

Progression through the mammalian cell cycle is thought to be regulated by a set of related protein kinases, termed cyclin-dependent kinases (*cdk* gene family [20, 36]), and their regulatory subunits, termed cyclins (reviewed in reference 32). Several classes of cyclins which differ in the timing of their expression in the cell cycle have been identified (for reviews, see references 6, 22, 26, and 34). Evidence that cyclins regulate cell cycle progression is provided by the observations (i) that injection of antibodies to several cyclins blocks progression through the cell cycle (1, 29), (ii) that overexpression of any one of the cyclin genes A, D1, or E can overcome the cell cycle block imposed by the RB gene (13), and (iii) that overexpression of cyclin E (25) as well as cyclin D1 (33) can accelerate the G_1 phase in mammalian fibroblasts and alter their growth factor requirements.

Since there is ample evidence that adenovirus E1A can act as a transcription factor (for a recent review, see reference 24), it is possible that cell cycle entry may be correlated to specific changes of cyclin gene expression mediated by E1A. However, such changes are not documented; in particular, it is not clear from previous data which cyclin genes, if any, may be targets for E1A. To address this question, primary rat fibroblasts were transfected with the EJ *ras* oncogene and a second vector encoding a chimeric protein (E1A-ER), in which the E1A protein was rendered conditionally active by fusing it to the hormone-binding domain of the human estrogen receptor (ER). We found that in cells expressing the chimeric protein E1A-ER (IREE-1 cells), proliferation is dependent on the addition of the appropriate steroid hormone. Expression of both the cyclin A and cyclin E genes is induced by E1A in this system, while expression of cyclin D1 is downregulated. E1A-dependent modulation of cyclin gene expression was con-

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firmed in control experiments in which E1A was delivered to fibroblasts by an adenovirus infection or, alternatively, by stable transfection.

The potential to manipulate E1A activity by an external stimulus, as in IREE-1 cells, offers the unique opportunity to perform kinetic experiments on the stability of E2F-pRB complexes when challenged by E1A. We found that indeed E1A interferes with E2F-pRB complexes in IREE-1 cells when grown in the presence of estrogen. However, complexes of E2F with pRB persisted through several cell cycles after the addition of hormone to quiescent cells, indicating that disruption of such complexes occurs with slow kinetics. Taken together, our data indicate that E1A-dependent S-phase entry is correlated to specific modulation of cyclin gene expression, whereas disruption of complexes of E2F with pRB may not be required for the onset of DNA synthesis.

MATERIALS AND METHODS

Construction of plasmid pE1AER. ER sequences from plasmid HE14 (17) were first subcloned into the *Bam*HI site of pSP64 to introduce a *Sma*I site upstream of the coding sequences. ER sequences excised from this intermediate plasmid were subsequently ligated to adenovirus-2 E1A sequences at the *Sma*I site in plasmid pm975 (31). In this construct expression of a chimeric protein containing the 150 amino-terminal amino acids of E1A (containing CR1 and CR2; see Results) fused to the ER hormone-binding domain is driven by the E1A promoter.

Cell lines. To establish cells expressing the E1A-ER construct, rat embryo fibroblasts were transfected with 5 μg of pE1AER and 5 μg of pEJras. Transfections were plated in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS), to which estrogen was added at a final concentration of 100 nM. In this experiment six foci of transformed cells appeared, whereas in a control experiment no transformed cells appeared in the absence of estrogen. Cells from one focus of transformation were established and gave rise to the cell line IREE-1. To obtain cells that constitutively express adenovirus E1A, NIH 3T3 fibroblasts were infected by a recombinant retrovirus (pMXSVneo-18) carrying the E1A 13S cDNA (a gift of R. Ralston). Subsequently, colonies were selected for growth in soft agar. One of the resulting clones was used to establish the NIH 3T3/13S cell line. Control cells were derived by infecting NIH 3T3 cells with the empty vector.

Cell cycle analysis. Cells were washed with phosphate-buffered saline (PBS), fixed in 70% ethanol, and stained by propidium iodide as described previously (8). Fluorescence-activated cell scanning (FACScan) analysis was performed by the cell-fit program, with a Becton-Dickinson FACScan system. Thymidine incorporation was measured as described previously (8).

Antibodies and Western immunoblotting. Antibodies to human cyclin D1 were obtained by immunizing a rabbit with purified cyclin D1 proteins as described previously (1). Monoclonal antibodies to cyclin E were produced by standard procedures after BALB/c mice were immunized with a glutathione-*S*-transferase-cyclin E fusion protein, as described previously (18). Polyclonal antibodies to cyclin A (29) and *cdk2* (27), respectively, were prepared as described previously. Monoclonal antibodies recognizing the C terminus of E1A (M73) were obtained from Dianova (Hamburg, Germany). Antibodies to the N terminus of E1A (M37) were obtained from Ed Harlow. Western blots were performed as described previously (27), with the enhanced chemiluminescence system (Amersham, Inc.).

Northern (RNA) blotting. Total cellular RNA was extracted by the guanidinium thiocyanate-acid phenol method. Total RNA (10 μg) was electrophoresed on 1% agarose formaldehyde gels and transferred to nylon membranes. Expression of E1A and the E1A-ER construct was analyzed with a 0.3-kb *Bst*XI fragment prepared from an E1A cDNA clone. Expression of cyclin A was monitored with a 1.6-kb mouse probe (15) or a 2.2-kb human probe, expression of cyclin E was monitored with a 1.5-kb mouse probe (15) or a 2.5-kb human (16) cDNA probe, and expression of cyclin D1 was monitored with the mouse *cyl1* probe (19). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was analyzed with a rat cDNA probe (15).

Band shift assays. A synthetic oligonucleotide encompassing the E2F-binding site of the adenovirus E2 promoter was incubated with extracts from different cell lines, as described previously (28). E2F-associated proteins were analyzed by incubation of the band shift reaction with specific antibodies on ice for 50 min prior to electrophoresis. For detection of cyclin A and cdk2 proteins, a polyclonal antiserum was used after affinity purification (28). Human and rat pRB were detected by monoclonal antibody XZ 55 (Dianova), which has been shown previously to recognize native rodent pRB (15).

Infection of fibroblasts with adenovirus. Primary human fibroblasts (MS107, provided by E.-M. deVilliers) (28) that were obtained from the oral cavity of a healthy human were infected at about 50% confluency by adenovirus 5 or the E1A-deficient mutant *dl*312, as described previously (14). For infection, cells were placed in serum-free medium and incubated for 1 h with virus at 10 PFU per cell. After 1 h the medium was replaced by DMEM supplemented by 10% FCS.

For detection of E1A in infected fibroblasts, immunofluorescence was performed on coverslips with infected MS 107 cells. Coverslips were rinsed twice in PBS, and cells were fixed for 6 min at room temperature in 3.7% formaldehyde in PBS. Coverslips were dipped in ice-cold acetone for 20 s and then washed in 70% ethanol and PBS. The monoclonal anti-E1A antibody M73, diluted 1:20 in PBS containing 5 μg of bovine serum albumin, was applied for 60 min at 37°C. After three PBS washes fluorescein-conjugated goat anti-mouse immunoglobulin G was applied. After further incubation for 45 min at 37°C, cells were washed three times with PBS and then counterstained with DAPI (4′,6-diamidino-2-phenylindole) (1 μg/ml) in PBS for 15 min at 37°C. Cells were washed twice with PBS, mounted on glass slides with Mowiol (Calbiochem), and examined with a Leitz fluorescence microscope.

RESULTS

Characterization of a cell line expressing E1A-ER fusion proteins. Primary rat embryo fibroblasts were transfected with a vector encoding the EJ *ras* oncogene together with an expression vector encoding a chimeric protein, in which E1A is fused in frame to a steroid receptor hormone-binding domain. Initial experiments using full-length E1A fused to the hormone-binding domain of the glucocorticoid receptor (31) revealed transforming activity even in the absence of hormone (data not shown). Since this result may reflect inappropriate spacing between the hormone-binding domain and the active domain of E1A (reviewed in reference 30), we constructed a vector coding for a fusion protein (E1A-ER) (Fig. 1A) in which the N-terminal 150 amino acids of adenovirus type 2 E1A are linked to the hormone-binding domain of the human ER. Both conserved regions 1 and 2 of E1A (CR1 and CR2 [21]) are retained in this construct. Two weeks after transfection, foci were observed on plates in which cultures were grown in the

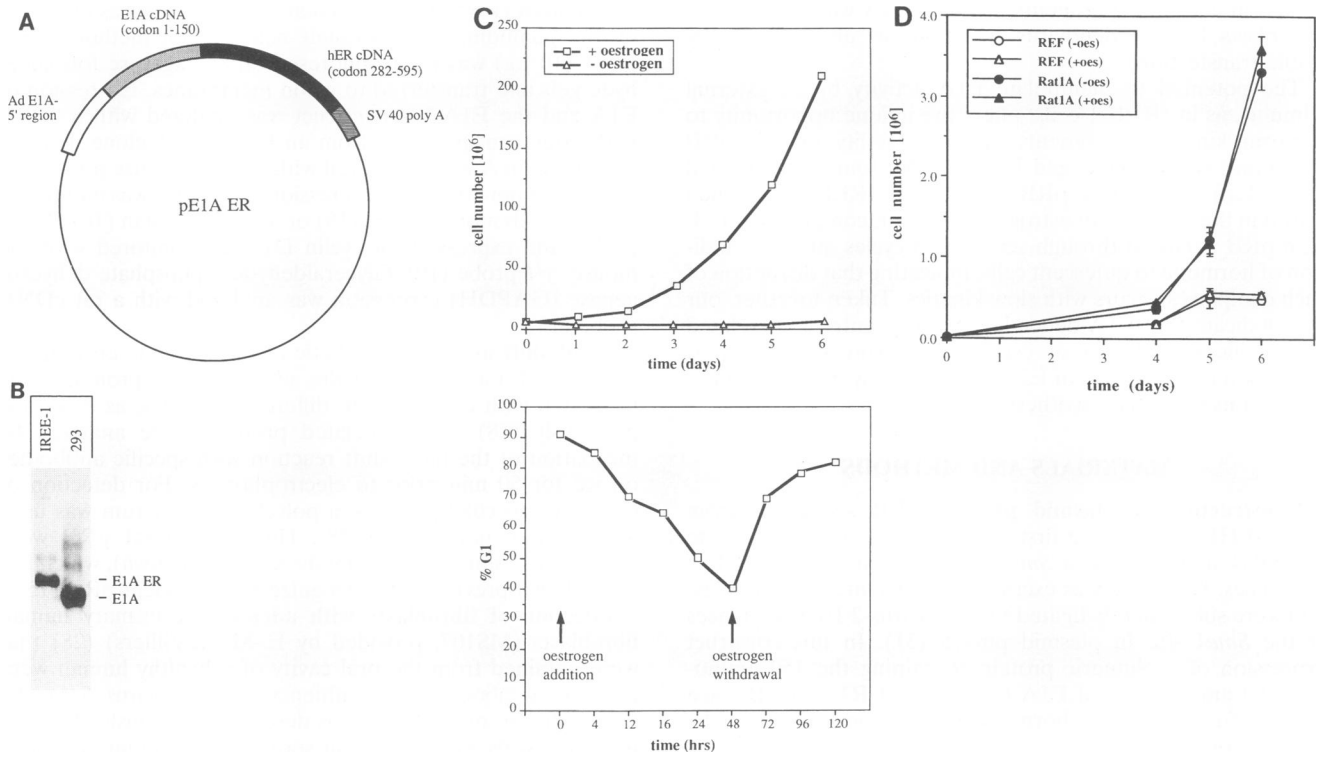

FIG. 1. Characterization of IREE-1 cells. (A) Structure of pE1AER. pE1AER contains the E1A promoter-enhancer driving expression of the E1A-ER fusion protein, comprising amino acids 1 to 150 of Ad2 E1A fused to amino acids 282 to 595 of the human ER. Furthermore, this pSP64-derived vector contains the simian virus 40 *Bam*HI-*Bcl*I fragment (nucleotides 2533 to 2770) comprising the simian virus 40 poly(A) site. (B) Expression of the E1A-ER gene in IREE-1 cells. RNA from 293 cells and IREE-1 cells was isolated and hybridized to a probe derived from E1A sequences. E1A expression in 293 cells is indicated by the appearance of a 0.8-kb mRNA, whereas in IREE-1 cells an mRNA with a length of about 1.6 kb was detected. (C) Growth properties of IREE-1 cells. (Upper panel) IREE-1 cells were seeded in duplicate in DMEM (10% FCS), containing either 100 nM estrogen or no estrogen. Cells were counted every 24 h. (Lower panel) Cells were kept in hormone-free medium for 1 week and restimulated by the addition of 100 nM estrogen. At the indicated time, samples of the cells were analyzed by FACScan. The proportion of cells in G_1 phase is shown. (D) Growth properties of control cells. Rat embryo fibroblasts (REF) and Rat1A cells were seeded in duplicate in DMEM (10% FCS), containing either 100 nM estrogen (+ oes) or no estrogen (− oes). Cells were counted every 24 h.

presence of estrogen but not on plates from which estrogen was omitted. Cell lines were established from individual foci and grown in DMEM supplemented with 10% FCS in the presence of 100 nM estrogen. One of these cell lines, IREE-1, was used for subsequent experiments. To monitor expression of the E1A-ER gene in IREE-1 cells, RNA was prepared from IREE-1 cells and hybridized to an E1A-derived labelled probe. The presence of an mRNA with a length of 1.6 kb in IREE-1 cells demonstrates expression of the transfected gene (Fig. 1B). Using a monoclonal antibody to the N-terminal part of E1A (M37), we detected a protein of the expected size (75 kDa) in extracts from IREE-1 but not from control cells (see below) (Fig. 2B).

As shown in Fig. 1C (upper panel), IREE-1 cells grow exponentially in the presence of serum and estrogen; there was no sign of growth arrest under these conditions for at least 12 months (data not shown). In the absence of estrogen, the cells do not grow but remain quiescent for several weeks. To analyze the kinetics of E1A-dependent S-phase entry, IREE-1 cell growth was arrested by withdrawal of estrogen for 1 week and then restimulated by the addition of estrogen. At several times after the estrogen addition, samples were taken and analyzed for their distribution in different phases of the cell cycle by FACScan. The results of this experiment are shown in Fig. 1C (lower panel). It appears that IREE-1 cell growth was arrested in G_1 in the absence of estrogen, and cells entered S-phase after readdition of hormone, reaching a peak after about 48 h. Withdrawal of estrogen after 48 h led to rearrest of cell growth into G_1. Estrogen-dependent S-phase entry of IREE-1 cells was confirmed in a parallel experiment measuring [^{3}H]thymidine incorporation (data not shown). To demonstrate that estrogen-dependent proliferation of IREE-1 cells is mediated by the E1A-ER chimeric gene, we established growth curves for nontransfected primary rat embryo fibroblasts and for an established rat fibroblast cell line, Rat1A, in the absence and presence of estrogen. For both cell types, the proliferation rate was not significantly altered by the addition of 100 nM estrogen to the medium (Fig. 1D). The results of these experiments indicate that estrogen-dependent proliferation of IREE-1 cells reflects their dependence on an E1A function for growth, rather than activation of the endogenous ER by the steroid. This conclusion is supported by our finding that rat fibroblasts do not express detectable levels of the rat ER (7a).

Activation of cyclin A and cyclin E gene expression by E1A. We determined whether activation of cell proliferation in IREE-1 cells was accompanied by changes in cyclin gene expression. RNA was prepared from quiescent IREE-1 cells and from IREE-1 cells at different times after readdition of estrogen and analyzed by Northern blotting. Estrogen-depen-

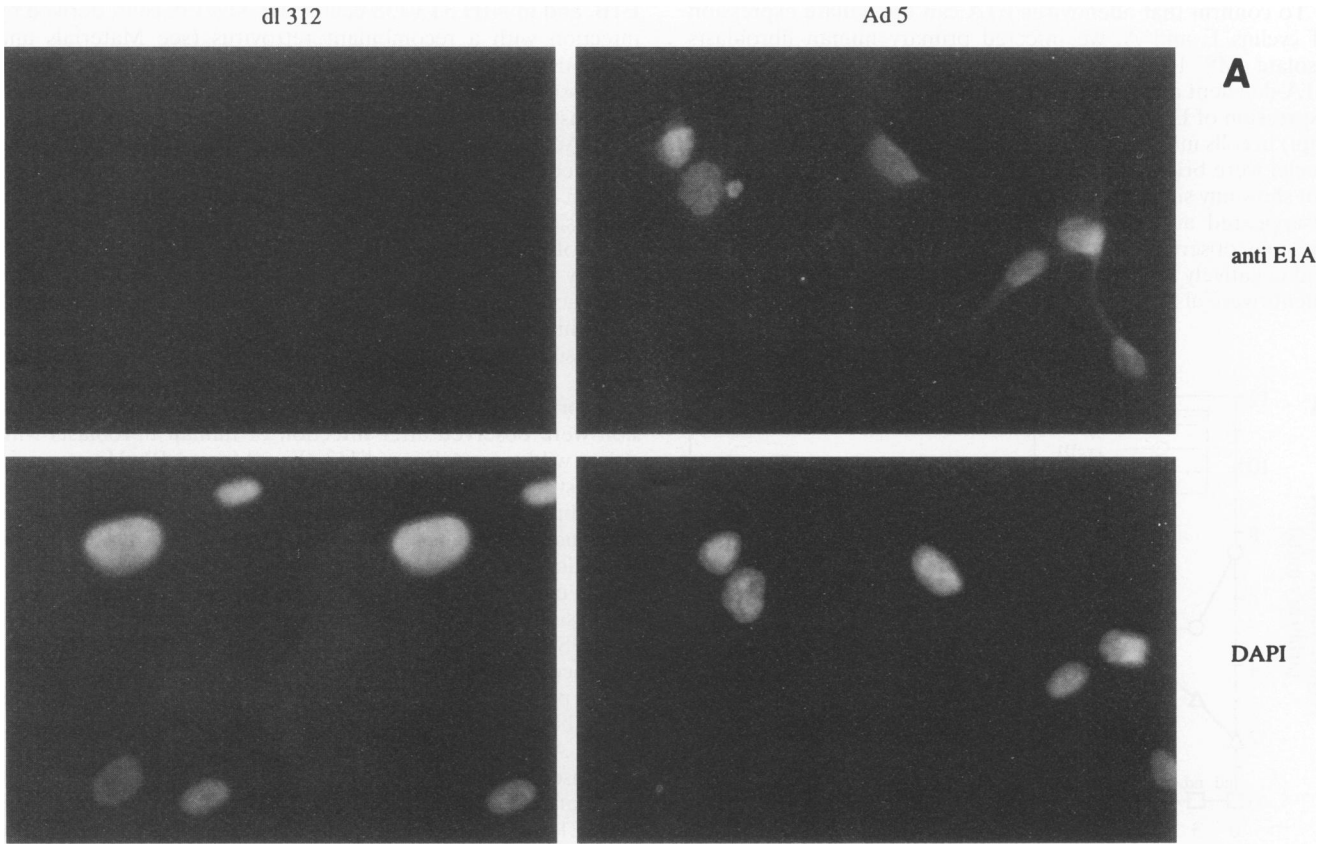

FIG. 2. E1A expression in different cell lines. (A) Expression of E1A in adenovirus-infected fibroblasts. The presence of E1A in cells infected by Ad5 or *dl*312 was analyzed by indirect immunofluorescence with the M73 antibody and counterstaining with DAPI. As shown here, a large proportion of Ad5-infected cells expressed E1A at 12 hpi (Table 1). (B) Comparison of E1A protein levels in different cell lines. Extracts were prepared from growing IREE-1 cells, NIH 3T3/13S cells, 293 cells, Ad5-infected MS 107 cells, and control cells (3T3, Rat1A, and REF), as indicated. Each extract (20 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The E1A and E1A-ER proteins were detected by Western blotting with the anti-E1A antibody M37.

dent proliferation was accompanied by a rapid induction of cyclin A and cyclin E mRNA levels (Fig. 3A). In additional experiments, we determined that maximal induction of cyclin E mRNA levels was reached at 8 h after the addition of estrogen, while by 4 h half-maximal activation was observed (Fig. 3A) (data not shown). To analyze whether this induction is reversible, RNA was also prepared from growing IREE-1 cells and from cells shifted to estrogen-free medium for 4 h. mRNA levels of both cyclins A and E were found to be much higher in cells grown in the presence of estrogen than in the cells withdrawn from estrogen (Fig. 3B). Higher levels of cyclin A mRNA were reflected in similar changes of the protein levels (Fig. 3B). Rat cyclin E was not detectable by antibodies to human cyclin E available to us.

Control experiments demonstrated that the addition of 100 nM estrogen did not induce significant changes in the level of cyclin A in rat embryo fibroblasts and Rat1A cells (Fig. 3C). Similarly, the level of cyclin E mRNA was not affected by estrogen in Rat1A cells (Fig. 3D). Taken together, these data indicate that activation of cyclin A and cyclin E gene expression, observed in IREE-1 cells after the addition of estrogen, reflects activation of the E1A-ER fusion protein and is not mediated by activation of the rat ER.

TABLE 1. E1A-positive nuclei were counted at different times of adenovirus infection, as outlined in the legend to Fig. 2A

Time (hpi)	E1A-positive nuclei (%)[a]
0	0
6	0
12	85 ± 5
16	80 ± 6
24	84 ± 3
31	3

[a] Results are averages ± standard deviations for two independent infection experiments.

To confirm that adenovirus E1A can upregulate expression of cyclins E and A, we infected primary human fibroblasts (isolate MS 107) with adenovirus type 5 (Ad5) and the E1A-deficient mutant *dl*312. Immunofluorescence showed that expression of E1A could first be detected at 12 h postinfection (hpi) in cells infected with Ad5, at which time about 85% of the nuclei were brightly stained (Fig. 2A); *dl*312-infected cells did not show any signal. During the Ad5 infection, nuclear staining disappeared at about 30 hpi (Table 1), consistent with the previous observation that E1A proteins have a short half-life and negatively regulate their own synthesis (3). Control experiments were also performed with 293 cells, expressing E1A and E1B, and in NIH 3T3/13S cells. NIH 3T3/13S cells, derived by infection with a recombinant retrovirus (see Materials and Methods), express the 289-amino-acid (13S) E1A protein. Expression of E1A in the cell lines used in this study was demonstrated by Western blotting (Fig. 2B).

In Ad5-infected, but not *dl*312-infected MS 107 cells, we observed a strong increase in cyclin E mRNA (Fig. 4A) and protein (Fig. 4B) at 18 hpi, demonstrating that cyclin E gene expression is induced in an E1A-dependent fashion. High levels of cyclin E were also observed in 293 cells (Fig. 4A). As already suggested by the rapid induction of cyclin E mRNA upon activation of E1A-ER in IREE-1 cells, these data indicate that E1A acts as a potent inducer of cyclin E gene expression, independent of the proliferative status of the target cells.

Surprisingly, no significant changes in cyclin A gene expression were observed after infection of human fibroblasts with either wild-type Ad5 or *dl*312 (Fig. 4A and B). However, in contrast to IREE-1 cells kept in the absence of estrogen, MS 107 fibroblasts were actively proliferating when E1A was introduced into these cells by adenovirus infection. Thus, activation of cyclin A gene expression by E1A may be detectable in quiescent cells only. To test this hypothesis, we analyzed expression of cyclin A in control NIH 3T3 cells and in NIH 3T3/13S cells, both in the absence (0.5% FCS) and in the presence (10% FCS) of external growth factors. In cells grown in the presence of 10% FCS, no difference in cyclin A levels could be detected, regardless of E1A expression. However, cyclin A expression was downregulated in control cells in response to serum deprivation, whereas cells that expressed E1A failed to downregulate cyclin A (Fig. 4C). We conclude that E1A-dependent upregulation of cyclin A reflects the

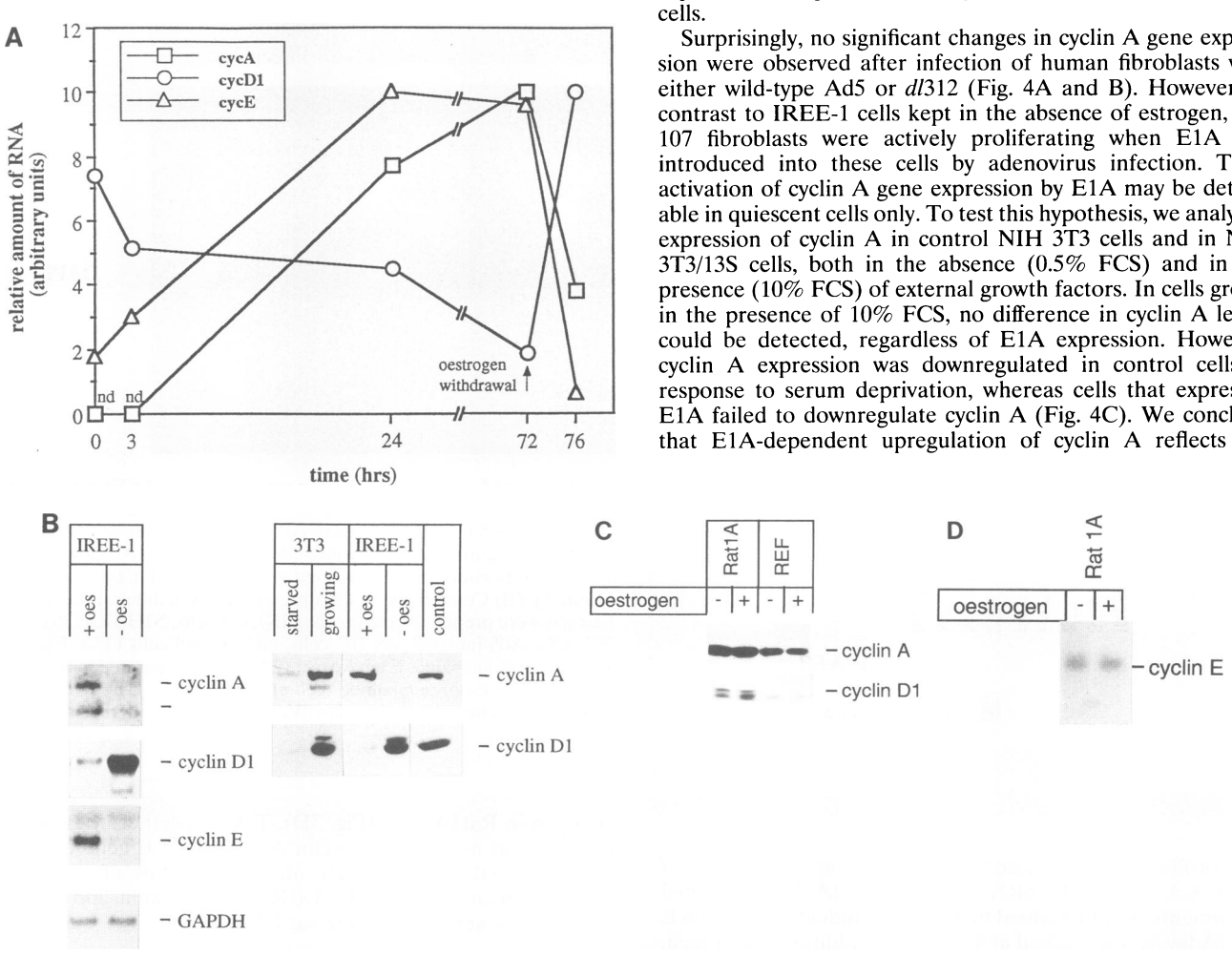

FIG. 3. Estrogen-dependent changes of cyclin gene expression in IREE-1 cells. (A) Changes of cyclin mRNAs during estrogen stimulation of IREE-1 cells by Northern blot. IREE-1 cell growth was arrested by hormone-free medium and restimulated by the addition of estrogen. After 72 h the medium was replaced by hormone-free medium, and the cells were kept for 4 h in the absence of estrogen. At the time indicated RNA was prepared and hybridized to probes derived from cyclin A, cyclin D1, and cyclin E, as indicated (also see panel B). mRNA levels were quantitated by densitometric scanning. (B) Changes of cyclin gene expression induced by estrogen are reversible. (Left panel) RNA was prepared from IREE-1 cells growing in the presence of estrogen (+ oes) or from cells shifted to estrogen-free medium for 4 h (− oes). Total cellular RNA was hybridized to probes derived from cyclin A, cyclin D1, cyclin E, and GAPDH, as indicated. (Right panel) Extracts were prepared from IREE-1 cells growing in the presence of estrogen (+ oes) or from cells shifted to estrogen-free medium for 24 h (− oes). Extracts were also prepared from growing NIH 3T3-cells (10% FCS) or serum-starved NIH 3T3 cells (0.5% FCS). Control lanes contained either cyclin A, in vitro translated from a bacterial expression vector (29), or extracts from RT112 cells, derived from a human tumor expressing cyclin D1 (18a). Cyclin D1 appears as a double band in Western blots performed with extracts from rodent cells, whereas only one band is visible in extracts of human cells (Fig. 4B). (C) Expression of cyclin A and cyclin D1 is not changed by estrogen in rat embryo fibroblasts (REF) and Rat1A cells. Rat embryo fibroblasts and Rat1A cells were grown in the absence of oestrogen (−) or treated with 100 nM estrogen for 48 h (+). Levels of cyclin A and cyclin D1 were analyzed by Western blotting. (D) Expression of cyclin E is not changed by estrogen in Rat1A cells. Rat1A cells were grown as described for panel C; expression of cyclin E was analyzed by Northern blotting.

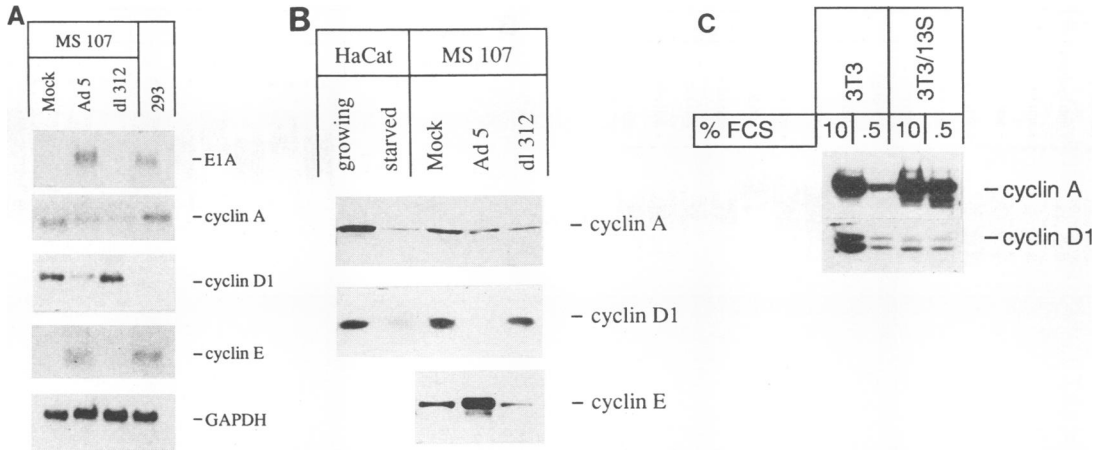

FIG. 4. E1A-dependent changes of cyclin gene expression in adenovirus-infected human fibroblasts. (A) E1A-dependent changes of cyclin mRNA levels in infected MS 107 cells. MS107 cells were infected by Ad5 or *dl*312 or mock infected. RNA was prepared at 18 hpi and probed with cyclin A-, D1-, and E- and E1A-specific probes, as indicated. For a control, RNA was also prepared from 293 cells and included as a reference. (B) E1A-dependent changes of cyclin protein levels in infected MS 107 cells. Extracts were prepared from MS 107 cells infected as described for panel A. The relative amount of cyclin A, D1, and E was determined by Western blotting. For a control, extracts were also prepared from HaCaT cells, either growing in the presence of 10% FCS or serum starved (0.5% FCS). (C) Modulation of cyclin A and cyclin D1 gene expression by serum starvation in NIH 3T3/13S cells and control cells. Extracts were prepared from NIH 3T3/13S cells that were either grown in the presence of 10% FCS or kept in the presence of 0.5% FCS for 48 h, as indicated. Extracts were also prepared from control NIH 3T3 cells that had been treated in the same way. Each extract (20 μg) was analyzed by Western blotting.

proliferative status of the target cells and may, therefore, be an indirect consequence of E1A-induced cell proliferation.

Repression of cyclin D1 gene expression by E1A. In contrast to cyclins A and E, activation of E1A-ER in quiescent IREE-1 cells led to a gradual decrease of cyclin D1 mRNA (Fig. 3A) and protein (data not shown; also see below). Full repression of cyclin D1 mRNA required exposure to estrogen for at least 72 h (Fig. 3A). Subsequent removal of estrogen for 4 h led to a rapid increase in cyclin D1 mRNA and protein (Fig. 3B). In other rodent fibroblasts, e.g., NIH 3T3, cyclin D1 is barely detectable in serum-starved cells but highly expressed in growing cells (Fig. 3B) (40). Furthermore, the addition of estrogen did not affect the level of cyclin D1 in rat embryo fibroblasts and Rat1A cells (Fig. 3C), indicating that repression of cyclin D1, as observed in IREE-1 cells, depends on the presence of the E1A-ER protein. These data suggest that expression of E1A can downregulate expression of the cyclin D1 gene.

This conclusion was confirmed by the observation that infection of MS 107 fibroblasts by Ad5 led to a substantial reduction of cyclin D1 mRNA (Fig. 4A) and protein (Fig. 4B). Infection with *dl*312 did not affect cyclin D1 expression in MS 107 cells, suggesting that repression requires an active E1A protein. To investigate the possibility that expression of E1A might change the cell cycle profile of an infected culture, FACScan analyses were performed with Ad5-infected and mock-infected MS107 cells. From these experiments it appears that at 16 hpi a slight increase in the G_1 fraction resulted from adenovirus infection (52% in G_1) compared with mock-infected cells (45% in G_1) (data not shown). This observation rules out the formal possibility that the changes in cyclin E and D1 levels which we observed are due to a major alteration in the cell cycle profile of cells at 16 hpi during an Ad5 infection.

Cyclin D1 expression was not detectable in 293 cells, further supporting the hypothesis that this gene may be repressed by an adenovirus early gene (Fig. 4A). In NIH 3T3 control cells, as well as in established human keratinocytes (HaCaT) (Fig. 4B), cyclin D1 is highly expressed when the cells are grown in medium containing 10% FCS but is reduced upon serum starvation. In contrast to this observation, cyclin D1 expression was strongly downregulated in proliferating NIH 3T3/13S cells, compared with proliferating control NIH 3T3 cells (Fig. 4C), indicating that E1A-expressing cells fail to upregulate cyclin D1 expression upon cell proliferation. Taken together, our data suggest that expression of the cyclin D1 gene is repressed by E1A.

E1A-dependent changes of E2F multiprotein complexes. We next addressed the question of whether complexes of E2F and pRB would be sensitive to E1A-dependent progression of IREE-1 cells to S phase. First, we monitored the status of E2F in rapidly growing IREE cells (with estrogen) and after the removal of estrogen for 2 days. Complexes between E2F and pRB were not detected in extracts from cells which were growing in the presence of estrogen and, hence, contained an active E1A protein. The removal of estrogen led to the appearance of a new complex which contains the RB protein (Fig. 5A). The protein composition of the other complexes seen in Fig. 5A remains obscure. However, the results shown in Fig. 5A rule out the association of either cyclin A or *cdk2* with E2F in any of the complexes observed. The antibodies to cyclin A and *cdk2*, which were used in this experiment, have been shown previously to recognize the relevant antigen in E2F bandshift experiments using extracts from rat cells (15) (Fig. 6). These results demonstrate that activation of E1A-ER by estrogen can indeed disrupt E2F-pRB complexes. Similar changes in the E2F composition were observed during adenovirus infection of MS 107 fibroblasts (Fig. 6), in agreement with current hypotheses (23). In a control experiment, we found that E1A-ER protein is coprecipitated when pRB is immunoprecipitated from IREE-1 cells (data not shown), indicating association of both proteins.

Second, we asked whether dissociation of E2F-pRB complexes would be an early event during restimulation of proliferation in cells in which growth was arrested by hormone

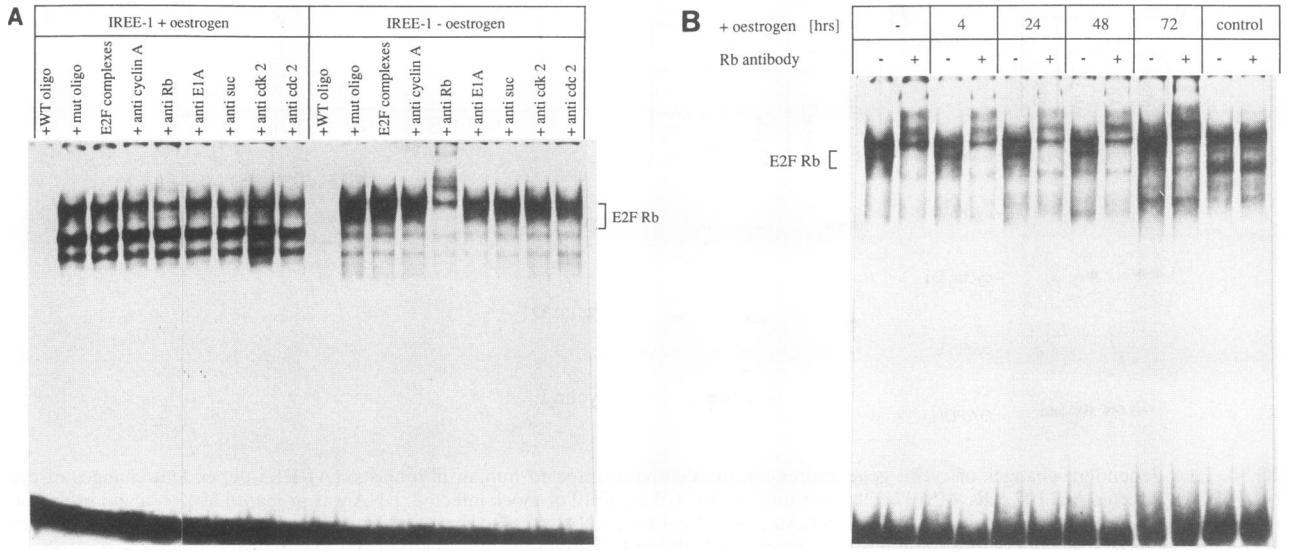

FIG. 5. E2F complexes in IREE-1 cells. (A) E2F-RB complexes were induced by estrogen withdrawal from growing IREE-1 cells. IREE-1 cells were grown in the presence of estrogen. Half of the culture was kept in the absence of estrogen for 48 h, while control cells were further grown in the presence of estrogen. Extracts were prepared from the cells and used for band shift experiments. Multiprotein complexes formed on the labelled DNA fragment are designated "E2F-complexes"; the composition of such complexes was analyzed by the addition of wild-type (WT) or mutant (mut) competitor oligonucleotides (oligo) and antibodies to pRB, p107, cyclin A, *cdk2*, E1A, *cdc2*, and $p13^{suc}$, as indicated (see Materials and Methods). (B) Disruption of E2F-pRB complexes by addition of estrogen to quiescent IREE-1 cells occurred slowly. IREE-1 cell growth was arrested by hormone-free medium and restimulated by the addition of estrogen. At the times indicated, samples were taken. Extracts of these cells were analyzed in band shift experiments in the presence or absence of pRB antibodies, as indicated. In the control lanes, extracts of IREE-1 cells grown in the presence of estrogen for several weeks were analyzed.

withdrawal. Cell growth was arrested by keeping the cells in hormone-free medium for 1 week. Estrogen was added, and the cells were harvested at several times after the addition of the hormone. The status of E2F was analyzed by the band shift technique. As expected, E2F-pRB complexes were detected in cells in the absence of estrogen. Surprisingly, such complexes persisted for at least 3 days after the hormone addition (Fig. 5B), although at that time the cells had gone through the first cell cycles (Fig. 1). We conclude that quantitative disruption of E2F-pRB complexes by E1A is not required for E1A-mediated entry into S phase.

DISCUSSION

We have analyzed the effects of adenovirus E1A on the expression of cyclin genes and the composition of E2F multiprotein complexes in two experimental systems. First, we constructed a cell line expressing EJ *ras* together with a chimeric gene (E1A-ER) encompassing the N-terminal 150 codons of the E1A cDNA fused in frame to the hormone-binding domain of the human ER. Cells that express this chimeric gene proliferate in a hormone-dependent manner. As discussed in Results, appropriate controls were included to rule out effects of estrogen that are not related to the presence of the E1A-ER protein. Second, we developed a protocol for quantitative infection of growing primary human fibroblasts by adenovirus 5, expressing E1A. Conclusions derived from such experiments were controlled by additional experiments involving the E1A-deficient virus *dl*312 as well as two different cell lines, expressing either E1A and E1B (293 cells) or only the 13S form of E1A (NIH 3T3/13S cells). From these experiments we make the following conclusions.

(i) expression of the cyclin E gene is rapidly induced in adenovirus-infected growing fibroblasts but not in cells in-

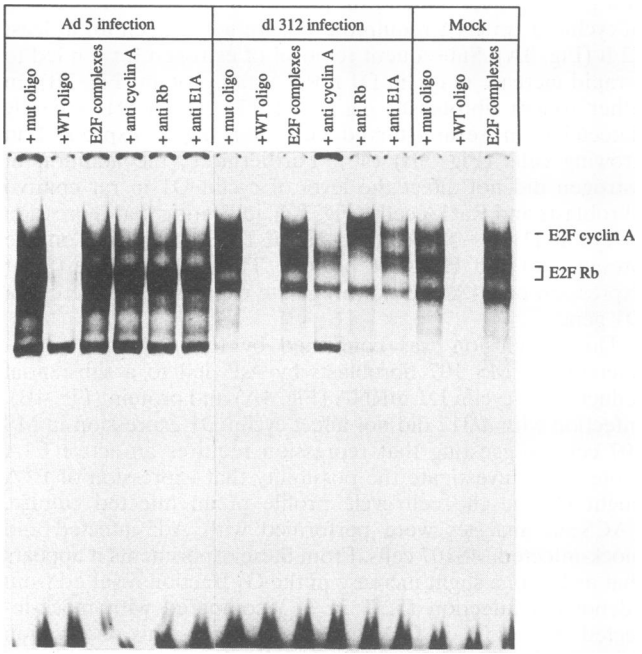

FIG. 6. E1A-dependent changes in E2F multiprotein complexes in adenovirus-infected MS 107 cells. Extracts were prepared from Ad5-infected cells, *dl*312-infected cells, or mock-infected cells at 16 hpi. E2F complexes were analyzed as described in the legend to Fig. 5. mut oligo, mutant oligonucleotide; WT oligo, wild-type oligonucleotide.

fected with the E1A-deficient mutant *dl*312. High levels of cyclin E gene expression were also found in 293 cells. Similarly, the addition of estrogen to quiescent IREE-1 cells led to a strong increase of cyclin E mRNA, detectable only a few hours after the addition of the hormone. Taken together, these data suggest that the cyclin E gene is rapidly turned on in an E1A-dependent pathway, consistent with the assumption that only very few intermediary events, if any, are required before activation of the cyclin E gene. Furthermore, activation of this gene does not merely reflect E1A-mediated proliferation, since it is observed also in growing cells.

(ii) Like cyclin E, cyclin A expression was also substantially increased upon activation of the E1A-ER protein in IREE-1 cells. However, in contrast to the results obtained with cyclin E, expression of the cyclin A gene was not changed by E1A in growing fibroblasts infected with adenovirus. Since cyclin A is already expressed to a considerable extent in such cells, we reasoned that induction of cyclin A by E1A may be restricted to quiescent cells. Consistent with this hypothesis, we found that in NIH 3T3/13S cells the cyclin A level is similar to that found in control cells when cells are grown in 10% FCS. However, unlike in control cells, cyclin A gene expression was not reduced by serum starvation in NIH 3T3/13S cells, indicating that E1A can mimick serum-dependent cell cycle activation. We conclude that the E1A-dependent increase in cyclin A gene expression, observed selectively in quiescent cells, may represent a consequence of cell cycle modulation by E1A (21), rather than direct activation of the cyclin A gene by the viral oncoprotein.

(iii) In contrast to the results obtained with cyclins A and E, adenovirus infection of human fibroblasts led to a severe reduction of cyclin D1 levels in the cells. This effect apparently depends on E1A expression, since it is also observed in 293 cells and NIH 3T3/13S cells, whereas *dl*312-infected fibroblasts express normal levels of cyclin D1. Cyclin D1 expression is also drastically reduced upon activation of the E1A-ER protein in IREE-1 cells. Taken together, our data show that expression of cyclin D1 is downregulated by E1A. This activity of E1A overrides the normal regulation of cyclin D1 by growth factors (40) (Fig. 3C and 4B and C) and may reflect E1A's ability to specifically repress transcription from several genes (3).

(iv) Complexes between E2F and pRB were disrupted at 16 hpi in the adenovirus infection (Fig. 6) as well as in 293 cells (data not shown) (18). Disruption of E2F-pRB complexes was also observed in IREE-1 cells grown in the presence of estrogen (Fig. 5A). These data confirm that also in IREE-1 cells, complexes between pRB and E2F are disrupted by E1A. However, disruption of complexes between E2F and pRB in these cells occurs too slowly to account for the rapid E1A-dependent S-phase entry observed in estrogen-treated IREE-1 cells. Consistent with this interpretation, Wang et al. (37) had previously demonstrated that E1A's pRB-binding domain is not required for the stimulation of DNA synthesis in quiescent rat fibroblasts. As proposed by these investigators, the interaction of E1A with pRB may prevent immortalized cells from reentering the G_0 phase. The experiments reported here extend these findings and raise the question whether the disruption by E1A of pRB-E2F complexes plays any role in progression to S phase. Although at present the conclusions rely on experiments with a single cell line expressing E1A-ER chimeric proteins, the data clearly argue against a model which invokes disruption of E2F-pRB complexes as a key step for E1A-mediated S-phase entry, at least in the cells characterized in this study. The absence of any detectable changes in the level of E2F-pRB complexes in estrogen-treated cells for up to 72 h, equivalent to at least two population doublings (Fig. 1), is most easily reconciled with the notion that the stability of such complexes is not related to the position of the cells in the cycle. This view is further supported by a recent report by Schwarz et al. (33a), demonstrating that E2F remains bound to hypophosphorylated pRB throughout S phase in synchronized human cells, irrespective of the overall phosphorylation level of pRB. Similarly, the pRB species found in complexes with E2F in growing IREE-1 cells may correspond to residual hypophosphorylated pRB. This assumption is supported by our finding that the addition of estrogen to IREE-1 cells does not result in significant changes of pRB phosphorylation, a considerable degree of pRB phosphorylation being detected also in the absence of estrogen (data not shown). We conclude that, by hormone withdrawal, IREE-1 cell growth is arrested in a stage at which pRB is already partially phosphorylated. This finding is unexpected but may actually be correlated to the remarkably high level of cyclin D1 in resting IREE-1 cells, as cyclin D1 is known to contribute significantly to pRB phosphorylation (15a).

The data reported here are consistent with the hypothesis that adenovirus E1A mediates its mitogenic effect by activating the expression of the genes for cyclin E and cyclin A, both of which were shown to contribute to cell cycle progression. The alternative possibility, that E1A-mediated proliferation would involve the known capability of another G_1 cyclin, cyclin D1, to facilitate progression through G_1 (1, 33), is not compatible with our findings. On the contrary, E1A-dependent S-phase entry coincides even with repression of cyclin D1, a property that E1A shares with another transforming oncogene, c-Myc (15). While the significance of this repression is not clear, recent data indicate that continuous expression of cyclin D1 at the G_1-S boundary prevents the onset of DNA synthesis (25a), indicating that cyclin D1 may block progression through the cell cycle at the G_1-S boundary. Whereas such function might provide a clue to the as-yet-unexplained repression of cyclin D1 by transforming oncogenes, further experiments are clearly required to address this question.

Our data provide a new example for the use of the inactivation domain of steroid receptors to block the function of a heterologous protein (reviewed in reference 30). The E1A-ER fusion protein described in this report contains the functions of the oncoprotein necessary for immortalization of rodent fibroblasts, including the capability to modulate expression of the three cyclin genes analyzed in this study. These findings may provide a clue to understand the interference of E1A with cellular growth control, by identifying known cell cycle regulatory genes as potential mediators of E1A's oncogenic activity. The experimental system presented here should enable us to reveal some details of the molecular mechanism underlying the observed biological effects.

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