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Human Cells Arrest in S Phase in Response to Adenovirus 12 E1A

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It has previously been shown that following viral infection, Ad5 E1A induces cell cycle progression of quiescent rodent cells, leading to DNA synthesis and mitosis. Here we have examined the effect of Ad12 E1A on the cell cycle characteristics of human cells. Human tumor (A549, KB, and HeLa) cells were infected with Ad12 *dl*620, a mutant virus which has a lesion in the E1B gene and essentially expresses only E1A. These infected cells progressed from being largely in G_1 into S phase, where they arrested. Even up to 96 h postinfection (p.i.) the cells remained blocked in S phase. DNA synthesis did, however, proceed in Ad12 *dl*620-infected cells, giving rise to multiple copies of cellular DNA. Similar results were obtained when primary human skin fibroblasts were infected, although the polyploidy was less marked. The expression of cyclins A, B1, and E in the tumor cells increased appreciably in response to E1A. In contrast, there was a dramatic reduction in the levels of cyclin D1 and D3. Increases in cyclin D1 expression could be detected at very late times p.i. In those cell lines expressing low levels of cdc2 and cdk2 an appreciable increase in expression was seen soon after Ad12 E1A could be detected. The elevated levels of cyclins A, B1, and E were associated with increased protein kinase activity directed against histone H1. An increase in cyclin D1-associated kinase activity against Rb1 was also observed at late times. This deregulation of the cell cycle was not solely dependent on E1A inactivation of Rb, since similar effects were seen in Ad12 *dl*620-infected retinoblastoma (Y-79) cells, implicating p107 and p130 in E1A-mediated changes in cell cycle progression. We propose that the E1A-induced levels of cyclins A, B1, and E by Ad12 E1A in human cells may lead to an uncoupling of S phase from cell cycle progression.

INTRODUCTION

It is now well-established that the introduction of the adenovirus (Ad) early region (E1) DNA into mammalian cells is sufficient for complete transformation (reviewed Bernards and van der Eb, 1984; Gallimore *et al.,* 1985a; Boulanger and Blair, 1991). E1 comprises two transcription blocks: E1A encodes two homologous proteins which are identical except for the presence of a short region located toward the C-terminus of the larger molecule (Bayley and Mymryk, 1994; Williams *et al.,* 1995); the E1B gene encodes two distinct polypeptides of 19K and 58K/54K (in Ad5/Ad12) which are thought to play a major role in the protection of infected and transformed cells from E1A-induced apoptosis (White, 1995). Although Ad E1 DNA is usually considered to be the minimum region required for the production and maintenance of a fully transformed phenotype it is possible to isolate Ad E1A-only transformed rodent, but not human, cell lines (Gallimore *et al.,* 1985b; Byrd *et al.,* 1987). The precise biochemical role of E1A in this process is not clear but it has been shown that two regions of the proteins, which are highly conserved between different adenovirus serotypes together with the N-terminal 25 amino acids, are essential (reviewed Bayley and Mymryk, 1994; Williams *et al.,* 1995). The two highly conserved regions (termed CR1 and CR2; Moran and Mathews, 1987) are essential for binding of E1A to p105 Rb1 and the related proteins p107 and p130 (Whyte *et al.,* 1989; Dyson *et al.,* 1992). The N-terminal region is much less well conserved between Ad5 and Ad12 than CR1 and CR2 but in both cases is involved in the interaction with p300, which appears to function as a transcriptional regulator (Yaciuk and Moran, 1991; Eckner *et al.,* 1994).

The major effect of E1A on quiescent rodent cells is to cause them to progress from G_0/G_1 to S phase and subsequently to undergo mitosis (Murray *et al.,* 1982; Braithwaite *et al.,* 1983; Quinlan and Grodzicker, 1987; Howe and Bayley, 1992; Shepherd *et al.,* 1993). Binding of E1A to pRb and/or p300 is essential for this progression since viral mutants with impaired ability to bind either of these cellular proteins have a reduced ability to stimulate host cell DNA synthesis and mitosis (Howe *et al.,* 1990; Howe and Bayley, 1992). Binding of E1A to pRb causes it to dissociate from E2F with consequent activation of transcription from E2F-specific promoters (Nevins, 1992).

As well as these relatively direct effects on transcriptional regulators, E1A expression has repercussions for other proteins involved in the cell cycle. It forms a complex with cyclins A and E, its interaction being mediated through the pRb homologues p107 and p130 (Faha *et al.,* 1992; Howe and Bayley, 1992; Li *et al.,* 1993). In addition, the level of cyclin A is increased in response to Ad5 E1A in Ad-transformed rat cells (Buchou *et al.,* 1993) and in

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Ad5-infected human cells (Spitkovsky *et al.,* 1994; Zerfass *et al.,* 1996). It appears that the up-regulation of cyclin A expression, in this instance, is mediated at the transcriptional level and is probably attributable to binding to p107, thus relieving its repression of the cyclin A promoter (Zerfass *et al.,* 1996). Changes in the levels of other cyclins in response to Ad5 E1 have also been observed such that cyclin D levels are reduced in Ad-transformed and -infected cells (Buchou *et al.,* 1993; Spitkovsky *et al.,* 1994, 1995), while cyclin E is increased in response to Ad5 E1A (Spitkovsky *et al.,* 1994).

Adenovirus E1A also can direct the expression of, and interact with, cyclin-dependent kinases (cdks). Thus, the smaller Ad5 E1A protein has been shown to cause increases in the level of cdc2 in infected baby rat kidney cells, at least in part, by increasing the amount of translatable mRNA (Draetta *et al.,* 1988). Complexes of E1A with cyclins A and E together with cdk2 have been demonstrated in 293 cells (Faha *et al.,* 1993; Mal *et al.,* 1996). It seems reasonable to assume that such interactions will have effects on the cell cycle in addition to those attributable to direct binding to the pRb family.

The normal cell cycle is controlled by cdks in association with the cyclin regulatory subunits. The expression of the cyclins is closely regulated temporally—thus determining progression through the cycle. Briefly, in normal cells the G_1 cyclins, cyclin D and cyclin E, associate predominantly with cdk4/cdk6 and cdk2, respectively, to promote G_1 progression and S phase entry (reviewed Sherr, 1994). Cyclin E is degraded early in S phase, whence cdk2 associates with cyclin A. Initiated late in G_1, cyclin A-associated kinase activity is first detected in S phase and is required for DNA replication. Cyclin A also associates with cdc2, peaking in G_2, after which it is rapidly degraded (reviewed Graña and Reddy, 1995). Mitosis is stimulated by the maturation promoting factor (MPF), which comprises cdc2 and cyclin B (Dunphy *et al.,* 1988; Gautier *et al.,* 1988; Draetta *et al.,* 1989). Cyclin B is degraded predominantly at the end of mitosis (Pines and Hunter, 1989; Jackman *et al.,* 1995), proteolysis maintaining very low levels in the intervening G_1 phase of cycle, until the next round of DNA replication (Brandeis and Hunt, 1996).

As has been noted above interest in the relationship of E1A to the mammalian cell cycle has been intense. However, while these studies go a considerable way to elucidating the effect of Ad5 E1A on the cycle they also leave a number of unanswered questions which we felt were worthy of consideration. First, relatively few studies have examined the effects of Ad12 E1A rather than the Ad5 protein or whether E1A has the same effects on human cells as those which have been reported for rat cells. In addition most previous studies have considered the effects of E1A in the presence of E1B protein expression. Thus, in experiments presented here we have examined the effect of Ad12 E1A, in the absence of the larger E1B protein, on the cycle of human cells in the belief that this will provide additional insights to the way in which Ad E1 effects the cell cycle in the very early stages of *in vivo* infection.

RESULTS

It is now well established that infection of quiescent baby rat kidney cells with adenovirus 5 leads to induction of S phase entry, DNA synthesis, and eventually mitosis (Murray *et al.,* 1982; Braithwaite *et al.,* 1983; Quinlan and Grodzicker, 1987). More recently it was shown that a virus lacking the Ad5 E1B gene was still able to induce mitosis in these cells (Shepherd *et al.,* 1993). In the present study we were interested in whether adenovirus 12 E1A would have a similar stimulatory effect on the cell cycle as the Ad5 homologue. Unfortunately, rodent cells are nonpermissive for Ad12, with E1 proteins being virtually undetectable even after viral infection at very high multiplicities (our unpublished data). Therefore, the effect of Ad12 E1A on a variety of human cell lines was assessed. We have previously shown that infection of human cells with the mutant virus Ad12 *dl*620 results in expression of Ad12 E1A and small amounts of Ad12 E1B 19K protein but no other viral polypeptides (Byrd *et al.,* 1988; Grand *et al.,* 1994, 1996). This system has therefore been used in the present investigations as a means of "delivering" E1A to all of the cells in a particular culture.

Cell cycle analysis

Following infection with Ad12 *dl*620, A549 cells were pulse-labeled with BrdU 2 h prior to harvesting. Cells were subsequently labeled with propidium iodide and subjected to FACS analysis as described under Materials and Methods. It can be seen from the data presented in Fig. 1A that in the normal cultures the cells were distributed throughout the cycle, with a slight preponderance in G_1. By 24 h postinfection (p.i.) a rightward shift of propidium iodide fluoresence was apparent, indicative of movement of a large proportion of cells into S phase (Fig. 1A). This was borne out by the dramatic increase in BrdU-positively stained cells at this time. At 48 h, approximately 90% of the cells were in S phase, though it was apparent that a small number of cells with a DNA content greater than 4 N was visible. Similarly, at 72 h p.i. the majority of cells were arrested in S phase, although there were approximately 20% of cells that had multiple copies of cellular DNA ($>$4 N). Ad12 *dl*620-infected A549 cells did not complete cell cycle and failed to undergo mitosis. This cessation of proliferative capacity was reflected in the lack of change in total cell number following infection with Ad12 *dl*620. The observation that the G_1 peak was depleted throughout the time course was also indicative of the cells failing to undergo mitosis.

To confirm that A549 cells at 72 h p.i. contained multiple copies of genomic DNA an equal number of infected and uninfected cells were harvested. After extraction, DNA from each sample was digested using *Bgl*II, frac-

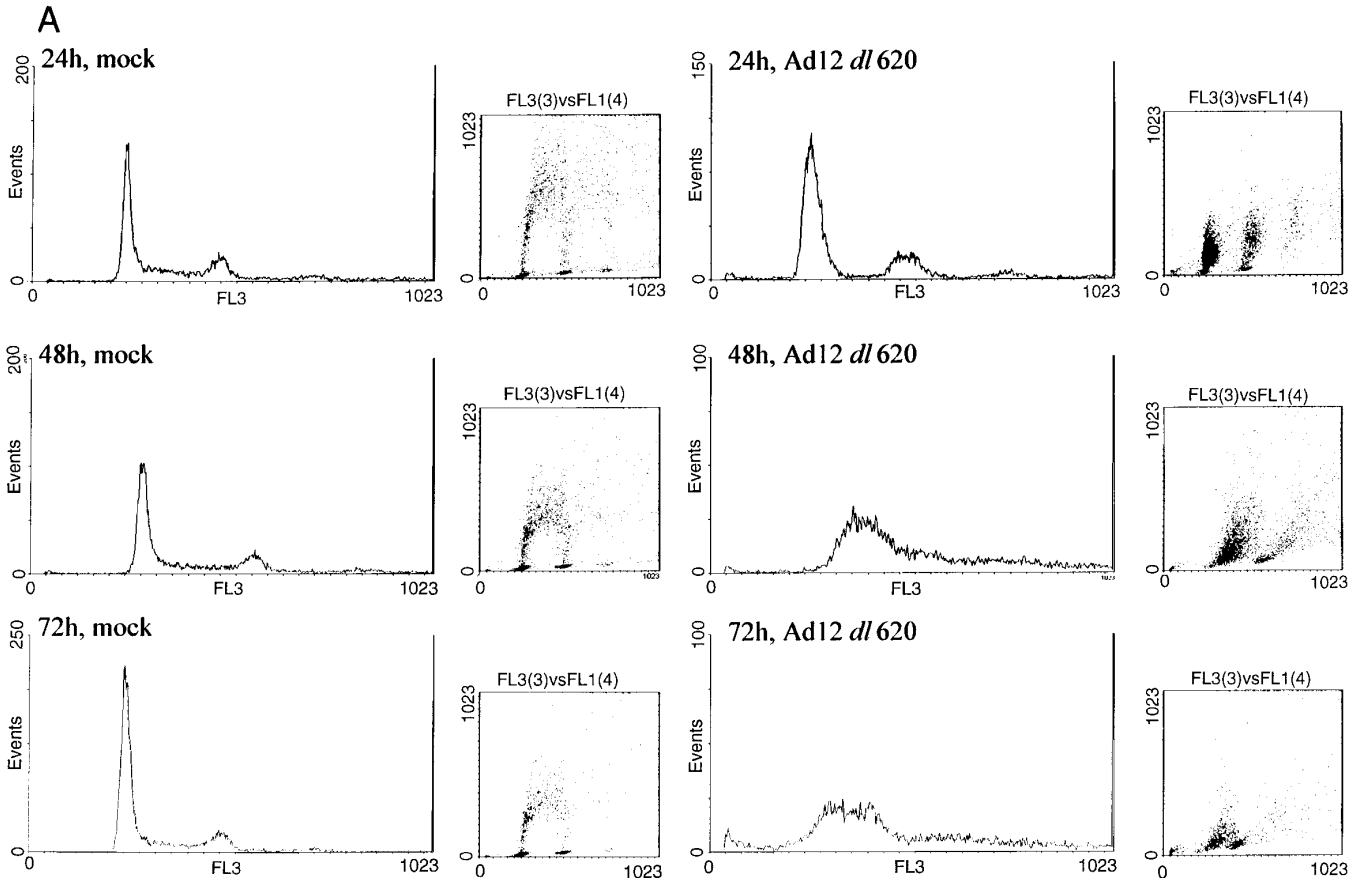

FIG. 1. Cell cycle analysis and DNA content of infected A549 cells. (A) Both Ad12 *dl*620-infected A549 cells and uninfected A549 cells were pulse-labeled with BrdU, 2 h prior to harvesting. Cells were subsequently probed with an anti-BrdU FITC antibody and counterstained with propidium iodide. FACS analysis was performed according to Materials and Methods. The corresponding histograms (for propidium iodide-stained cells) and dot plots (showing BrdU incorporation) are shown. (B) Southern blot analysis of infected and uninfected cells. At 72 h p.i. with Ad12 *dl*620 an equal number of infected and uninfected A549 cells were harvested and genomic DNA was extracted. After digestion with *Bgl*II the DNA was fractionated and transferred to a membrane which was probed with the ^{32}P-labeled *Hin*dIII–*Eco*RI fragment of the human J-γ region of the T cell receptor gene. Track 1, uninfected A549 cells; 2, cells infected with Ad12 *dl*620. Numbers under the two tracks represent the ratio of intensities of the signals determined by densitometric scanning.

tionated by agarose gel electrophoresis, and subjected to Southern blotting (see Materials and Methods). Blots were probed with the *Hin*dIII–*Eco*RI fragment of the human J-γ region of the T cell receptor gene (Fig. 1B). Densitometric scanning showed the relative intensities of the detected DNA fragments to be 1:2.9 for uninfected: infected A549 cells, clearly suggesting multiple copies of the genome in Ad12 *dl*620-infected cells.

Chromosome preparations were also made from uninfected and infected (24, 48, and 72 h) A549 cells. Uninfected metaphase A549 cells showed the presence of 60–65 chromosomes including the presence of readily recognizable marker chromosomes. In addition, occasional hypotetraploid cells were observed and also occasional cells with chromosome damage (chromatid gap or dicentric chromosomes). Uninfected A549 cells had a mitotic index of 0.75%. In the Ad12 *dl*620-infected cells (24 h p.i.) the most dramatic change was the increased size of the A549 cell nuclei (Fig. 2). The mitotic index was reduced to 0.25% at this time. About half of these cells showed high levels of chromatid-type chromosome damage compared with the uninfected cells, as has been noted previously (Murray *et al.,* 1982). At 48 h p.i. the frequency of mitoses was reduced further (0.04%) and at 72 h p.i. the mitotic index was only 0.03%. All these cells showed chromatid-type damage. At late times the enlarged nuclei remained prominent. Some evidence of a limited number of apoptotic cells was visible at 48 and 72 h p.i. Acridine orange staining of these samples showed 12% apoptosis at 72 h p.i.

Expression of cyclins in response to Ad12 E1A

It is apparent from the cell cycle analyses shown in Figs. 1 and 2 that in the system described here Ad12 E1A induces cells to progress into S phase and then arrest. In view of this the expression of the cyclins was examined. Actively cycling A549 cells were infected with Ad12 *dl*620 and harvested at times up to 80 h. Ad12 E1A was detected by Western blotting after 8 h (Fig. 3A). Around this time the expression of cyclins A, B1, and E was markedly

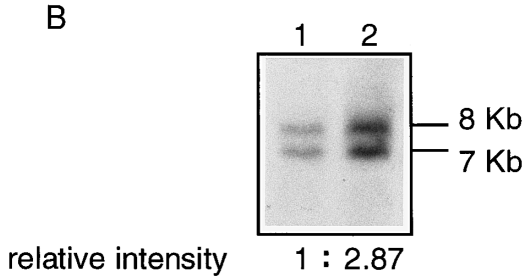

FIG. 1—*Continued*

increased (Fig. 3B). The levels of these proteins remained appreciably elevated throughout the time course of infection (up to 80 h), with no fluctuations which might be expected for cycling cells. In contrast, the level of the G_1 cyclin, cyclin D3, was rapidly and dramatically decreased (Fig. 3B). The level of cyclin B1 in Ad12 *dl*620-infected cells was somewhat in excess of that seen in a population of A549 cells collected by "mitotic shakeoff" (data not shown). Interestingly, the level of the G_1 cyclin, cyclin D1, was increased at very late times of infection (72 h, Fig. 3B). None of these changes are attributable to viral infection per se, since the mutant virus Ad5 ΔE1 had no effect on cyclin or cdk expression (data not shown). It is apparent therefore, that the variations in cyclin expression observed were the direct result of the presence of E1A. It should be noted that longer exposure of the autoradiographs presented in Fig. 3B shows the presence of low levels of cyclins B1 and E in the uninfected A549 cells consistent with a mixed population of cycling cells (Fig. 1). Reduction in the expression of cyclins D1 and D3 and increases in the expression of cyclins A and E are consistent with cell cycle progression from G_1 and arrest in S phase.

cdk expression in response to Ad12 E1A

Convincing evidence has previously been presented to support the proposition that there is a close relationship between E1A and cell cycle kinases. Ad E1A forms complexes with cyclin A–cdk2 and cyclin E–cdk2 (Faha *et al.,* 1992, 1993); in addition, E1A has been shown to be a substrate for cyclin-associated cdk2, cdk4, and cdc2 *in vitro* (Mal *et al.,* 1996), modulating its interaction with Rb (Mal *et al.,* 1996). The cdks were targets for regulation, following Ad12 *dl*620 infection (Fig. 4). Thus, cdk4 and cdk6, which associate with the D-type cyclins were slightly elevated early on during infection of A549 cells and were maintained at this level throughout the course of the experiment. More interesting were the changes which occurred in the expression of cdc2 and cdk2 in response to Ad12 E1A. Infection of A549 cells led to a rapid and marked induction of cdk2 and cdc2 (16 h p.i.) (Fig. 4).

Increase in cyclin-associated protein kinase activity in response to Ad12 E1A

While it is now clear from the data presented here, as well as those published earlier, that the expression of most, although not all, of the proteins regulating the cell cycle are increased when Ad5 E1A and Ad12 E1A are introduced into cells, until now there was little evidence indicating whether the cyclins were associated with active kinases. Therefore, to address this question A549 cells were infected with Ad12 *dl*620 and harvested at appropriate times. Proteins in these samples were immunoprecipitated with antibodies which recognize cyclins A, B1, D1, and E and the kinase activity in samples was assayed using histone HI (cyclins A, B1, and E) or truncated Rb protein (cyclin D1) as substrates. It can be seen from the data presented in Fig. 5 that increased levels of cyclins correlated well with increases in cyclin-associated kinase activity, although the increase in cyclin D1-associated kinase activity appeared to precede increases in overall level of cyclin D1 (Fig. 3). It is presumed that this reflects different titres in the two antibodies used to blot (Fig. 3) and immunoprecipitate (Fig. 5) cyclin D1. It is apparent that cyclin A-associated kinase activity is initially depressed in Ad12 *dl*620-infected cells. Overexposure of the cyclin A Western blot (Fig. 3B) suggests that this reduction in kinase activity reflects cyclin A protein levels (data not shown). The most likely explanation for this is that a subpopulation of cells already committed to mitosis, upon infection, have reentered G1, prior to a cell cycle block attributable to E1A.

In addition, it can be seen that histone H1-directed kinase activity can be immunoprecipitated with antibodies which recognize specific kinases (rather than cyclins) and these also show an increase in response to Ad12 E1A expression. Thus, the relatively high level of cdk2 (Fig. 4) seen in A549 cells before infection is not reflected in kinase activity, presumably due to the very low levels of cyclin A and/or cyclin E before Ad12 E1A expression (Fig. 3). It should be noted, however, that a proportion of newly synthesised cdc2 was phosphorylated on Y15, rendering it inactive (data not shown), although this is not sufficient to inhibit completely, kinase activity associated with G_2 cyclins (Fig. 5).

Infection of other human tumor cell lines with Ad12 *dl*620

To confirm that the results described above are not unique to the A549 cell line, KB and HeLa cells were also infected with Ad12 *dl*620. In both cases, FACS analysis showed that the cells arrested in S phase with multiple copies of cellular DNA (data not shown), as has been described for A549 cells. Furthermore, Western blotting of infected KB and HeLa cells clearly demonstrated that the Ad E1A had broadly similar effects on cyclin expression to those seen for A549 cells. Marked increases in cyclin B1 and E levels in both the cell lines were ob-

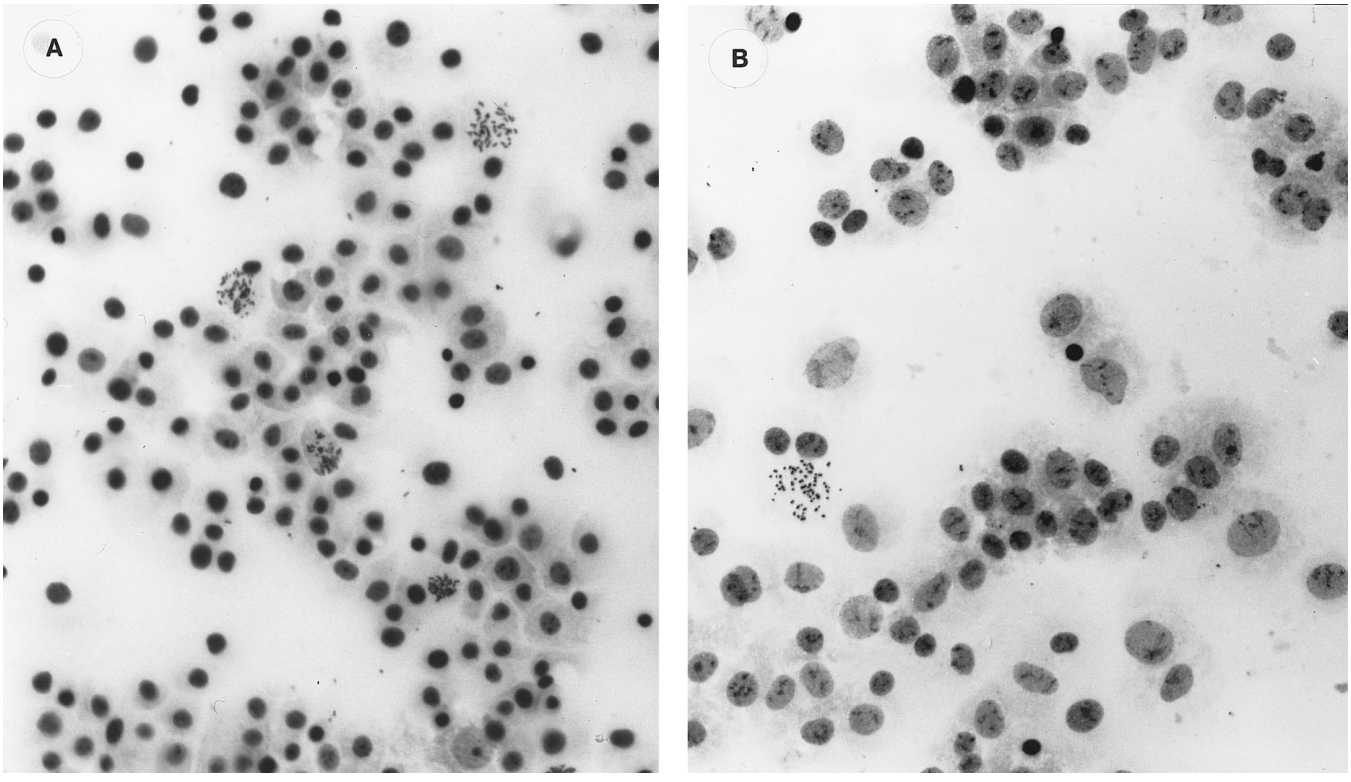

FIG. 2. Nuclear morphology following colcemid and hypotonic treatment of A549 cells either uninfected or infected with Ad12 *dl*620. All micrographs are at the same magnification. (A). Uninfected A549 cells showing the uniform size of cells and a high level of metaphases. (B). A549 cells 24 h after infection with Ad12 *dl*620, showing a dramatic increase in size of nuclei and lower frequency of mitotic spreads than uninfected cells. (C). A549 cells 48 h after infection with Ad12 *dl*620, showing the same increase in nuclear size as 24 h after infection, fewer mitotic spreads, and evidence of occasional apoptotic cells.

served. The expression of cyclin A was affected rather less dramatically, however, with increases of 2- to 4-fold being observed (data not shown). Decreases in the expression of cyclins D1 and D3 were seen for both KB and HeLa cells. Interestingly, as for the A549 cells there was an increase in D1 expression at late times after infection (>48 h). Increases in the levels of cdc2 and cdk2 were also observed, although the change in expression of cdc2 in KB cells was less pronounced than that in A549 and HeLa cells. As with A549 cells noted previously (Grand *et al.,* 1994) infection with Ad12 *dl*620 leads to the overexpression of p53 (data not shown). We conclude that the data presented in Figs. 1, 3, and 4 represent a generalized effect of Ad12 E1A on human tumor cell lines.

Cell cycle analysis and cyclin expression in Ad12 *dl*620-infected HSFs

As the results detailed thus far have been obtained with tumor cell lines it was considered important to confirm that similar effects might occur with primary cells. In view of this the response of primary human skin fibroblasts (HSFs) to infection with Ad12 *dl*620 was examined. HSF cells were infected with Ad12 *dl*620, harvested at the appropriate times p.i., stained with propidium iodide, and DNA content was measured by FACS analysis. Uninfected HSFs were predominantly in G_1 phase of the cycle (80%) 24 h after commencement of the experiment, with a small proportion of cells in G_2/M (15%) (Fig. 6). There were very few cells in S phase at this time. In contrast, approximately 50% of HSFs had been stimulated to enter S phase in Ad12 *dl*620-infected HSFs by 24 h. At 48 h p.i. 80% of Ad12 *dl*620-infected HSFs were arrested in S phase, whereas mock-infected cells continued to proliferate. There was, however, little indication of Ad12 *dl*620-induced polyploidy in HSFs.

Analysis of the corresponding cyclin levels in Ad12 *dl*620-infected HSFs showed that there was some variation from Ad12 *dl*620-infected tumor cells. In each case a rapid induction of cyclin A and cyclin E expression was observed (Fig. 7). However, the increase in cyclin B1 levels was considerably less marked in HSFs than was observed for A549, KB, or HeLa cells (Figs. 3 and 7). It should be noted that cyclin A levels were massively induced in Ad12 *dl*620-infected HSFs, whereas cyclin E levels were only modestly induced in comparison. Cyclin D1 levels were initially reduced in Ad12 *dl*620-infected HSFs, as was seen in Ad12 *dl*620-infected tumor cells. Similarly, cyclin D1 levels were elevated at late times p.i. In contrast to Ad12 *dl*620 induction of cdc2 and cdk2 levels in A549 cells, Ad12 *dl*620 infection of HSFs had no discernable effect on cdc2 and cdk2 levels. However, it

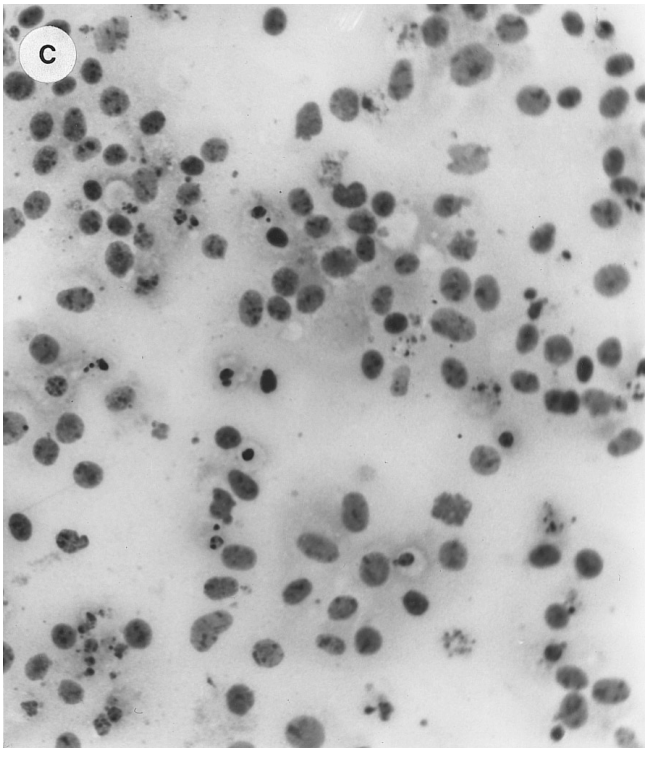

FIG. 2—*Continued*

must be stressed that the levels of cdc2 and cdk2 were initially high in uninfected HSF cells, whereas their levels in uninfected A549 cells were lower. p53 levels were also seen to increase following infection of HSFs, as has been noted for the tumor cell lines.

Cyclin expression and cell cycle analysis of Y-79 cells expressing Ad12 E1A

It has previously been suggested that the ability of Ad E1A to induce progression into S phase and subsequent DNA synthesis is dependent, at least partially, on its ability to bind pRb and thus dissociate E2F (Howe and Bayley, 1992). We therefore considered that it would be of interest to investigate the effect of E1A on cell cycle progression and cyclin expression in the absence of pRb. The Y-79, pRb-negative cell line was infected with Ad12 *dl*620 and analyses carried out as described above for A549 cells. It can be seen from Fig. 8 that the levels of p53 and cyclins A, B1, and E were appreciably increased in response to E1A, as has been seen for A549. In addition, elevation of expression of cdc2 and cdk2 was also observed.

Uninfected, cycling Y-79 cells showed a proportion of cells in each phase of the cell cycle (Fig. 9). BrdU incorporation indicated that these cells were actively synthesizing DNA. At 24 h p.i. with Ad12 *dl*620, an increased proportion of cells relative to their uninfected counterparts were stimulated to enter S phase. This was noted by the broad shoulder of propidium iodide fluoresence following the G_1 peak. Accordingly, there was an in-

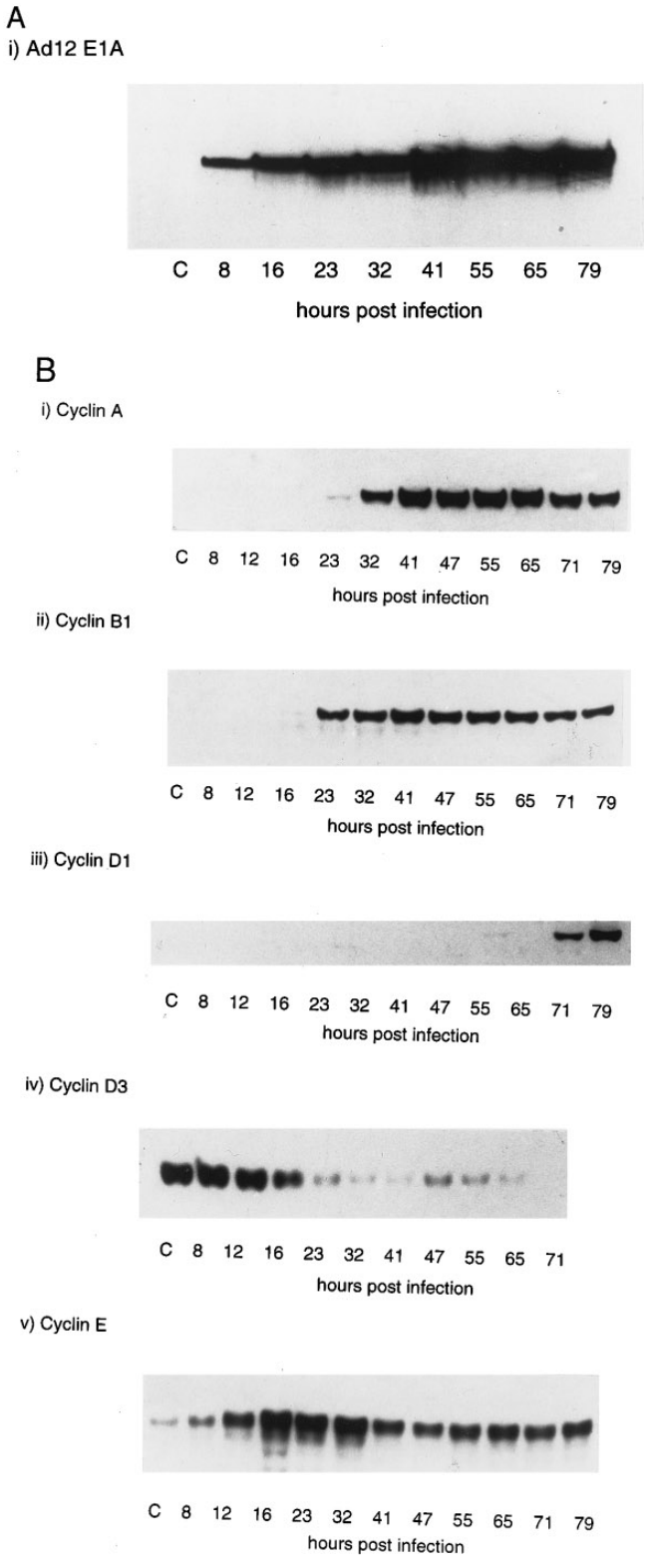

FIG. 3. Expression of cyclins in human cells in the presence of Ad12 E1A. A549 cells were infected with Ad12 *dl*620 (200 PFU per cell) and harvested at the times indicated. Aliquots, containing 50 μg protein, were subjected to SDS–PAGE and Western blotting using the antibodies indicated: (A i) Ad12 E1A; (B) cyclin A, cyclin B1, cyclin D1, cyclin D3, and cyclin E. No cyclin D2 could be detected. C, uninfected cells.

creased number of cells incorporating BrdU into their DNA at this time. Interestingly, and in accordance with the Ad E1A effects observed in A549 cells, there appeared to be a cessation in cells passing from S phase and entering G_2 in Ad12 *dl*620-infected Y-79 cells, as noted by the discrete loss of BrdU staining in the appropriate area of the chromatogram. By 48 h p.i. this effect was more dramatic. Approximately 75% of Ad12 *dl*620-infected Y-79 cells were in S phase at this time, compared with 25% of their uninfected counterparts. There was also a small amount of polyploidy at this time, which was also seen in Ad12 *dl*620-infected A549 cells.

DISCUSSION

The adenovirus E1A proteins appear to have evolved to allow the virus to take control of the cell cycle of the infected cell. This is primarily to facilitate replication of large quantities of viral DNA (greater than 10^6 viral genome equivalents per cell). Ad E1A exerts its influence on the cycle by interacting with a number of cellular regulatory components. The interaction with pRb is well characterized (Whyte *et al.,* 1988) and results in the release of E2F/DP1, allowing the transcription of other genes including those required for DNA synthesis. Also important is the interaction with p300 which appears to be necessary for E1A-induced cell cycle progression.

As well as forming a complex with pRb and its homologues p107 and p130, and p300 E1A impinges on the cell cycle in other ways. It binds indirectly to cyclins A and E through p107 and/or p130 (Faha *et al.,* 1993). cdk2 is found in multimolecular complexes with E1A and it is this enzyme which is responsible for E1A-associated kinase activity. Additionally, Ad E1A has been shown to affect expression of cyclins (Zerfass *et al.,* 1996), possibly at the transcriptional level. However, these previous studies are not all-encompassing. Adenoviruses which express the larger E1B protein down-regulate the expression of cellular polypeptides and therefore are not really ideal for the examination of the role of E1A in the regulation of cyclin expression. Following infection with Ad12 *dl*620, E1A is free to influence expression of cellular genes without triggering the Ad E1B-mediated reduction in processing of cellular mRNAs (Babbiss and Ginsberg, 1984; Babbiss *et al.,* 1985), which might have an influence on the results observed by other workers following *w.t.* infection.

Results of the studies presented here have indicated appreciable differences between the effect of Ad5 E1A on rodent cells and Ad12 E1A on human cells. In both cases there is an induction of S phase and DNA synthesis, but in the former case cells then go on to divide, while in the latter case there are repeated rounds of DNA replication giving rise to multiple copies of cellular DNA. There are differences in the extent of the polyploidy dependent on infected cell type (particularly prevalent in A549 cells but less obvious in

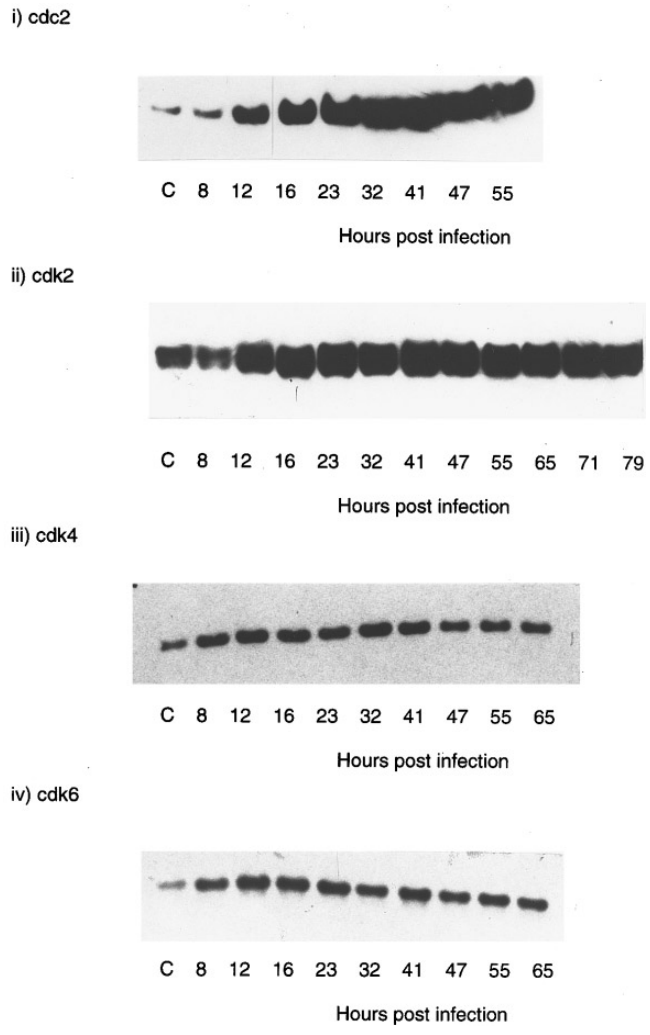

FIG. 4. Expression of cdks in human cells in the presence of Ad12 E1A. A549 cells were infected with Ad12 *dl*620 (200 PFU per cell) and harvested at the times indicated. Aliquots, containing 50 μg protein, were subjected to SDS–PAGE and Western blotting using the antibodies indicated: cdc2, cdk2, cdk4, and cdk6. C, uninfected cells.

HSFs) but in all three tumor cell lines and primary cells there is very little evidence for entry into G_2 and mitosis. It is, of course, possible that the cell cycle could have been greatly slowed in the presence of Ad E1A such that within the timescale of the experiment mitosis was not observed. However, the accumulation of cells with a DNA content greater than 4 N would tend to argue against this possibility.

It has been suggested that p53 can have an effect on Ad5-infected rat cells in the absence of the larger E1B protein, complementing pRb in the suppression of DNA synthesis after Ad5 E1A expression (Shepherd *et al.,* 1993). On the basis of previous reports it might be expected that the high level of p53 produced as a result of Ad E1A would cause the cells to arrest in G_1. This did not happen in the report by Shepherd *et al.* (1993) nor in any of the cell lines examined in the present study. We assume that this is attributable to the overriding influence of Ad E1A.

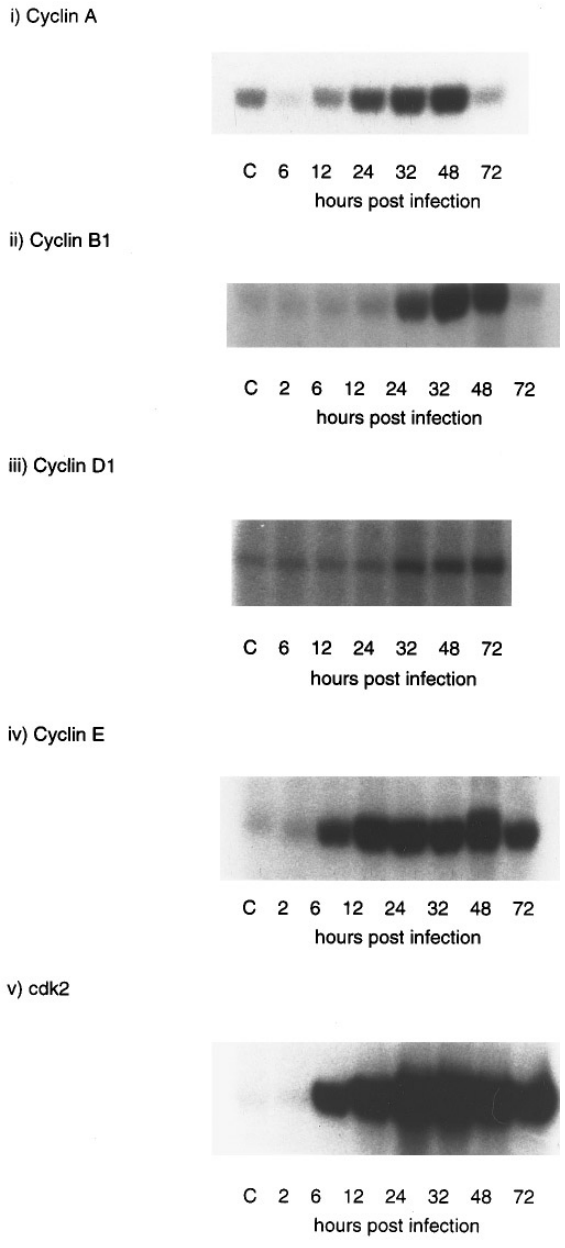

FIG. 5. Cyclin-associated kinase activity in human cells in the presence of Ad12 E1A. A549 cells were infected with Ad12 *dl*620 (200 PFU per cell) and harvested at the times indicated. Aliquots from each sample were immunoprecipitated with the antibodies shown. Associated kinase activity was assayed as described under Materials and Methods. Phosphorylated substrates (in each case histone H1 except for cyclin D1-associated kinase activity, which was directed against a fragment of Rb1) were subjected to SDS–PAGE. Dried gels were exposed to X-ray film. Antibodies used for immunoprecipitation were as follows: (i) cyclin A; (ii) cyclin B1; (iii) cyclin D1; (iv) cyclin E; and (v) cdk2. C, uninfected cells.

In previous studies of induction of cell cycle progression by Ad E1A, little attention was paid to the role of the cyclins. However, on the basis of the data presented here it is apparent that there is a rapid induction of cyclin A and E expression as well as some increases in the level of cyclin B1 and associated kinases. The increases in A and E are consistent with rapid entry into, and arrest in, S phase, and it seems reasonable to suppose that these observed changes could be responsible for the cell cycle effects noted (Figs. 1, 5, and 8). Presumably, the inactivation of pRb by binding to Ad E1A and release of E2F will produce similar results in stimulating S phase entry.

The rapid decrease in level of cyclin D3 appears to be directly attributable to the effect of E1A and would not normally be expected for cycling cells passing from G_1 to S. The reduction in detectable protein appears to be attributable to a reduction in cyclin D3 mRNA rather than a decrease in rate of degradation (data not shown). These observations are, however, consistent with previous reports (and our unpublished data) of very low levels of cyclin D in Ad-transformed cells (Spitkovsky *et al.,* 1995). Reasons for this are not clear at present but it might be attributable to the binding of E1A to Rb1, since it has previously been demonstrated that cyclin D expression is very low in Rb-negative cells (Lukas *et al.,* 1994; Müller *et al.,* 1994; Tam *et al.,* 1994). It might, therefore, be supposed that Rb is required for cyclin D3 expression or stabilization, as there is a lack of Rb function in Ad-infected cells, even though the protein is still expressed. No D cyclins were observed in Y-79 (pRb-negative) cells either before or after infection.

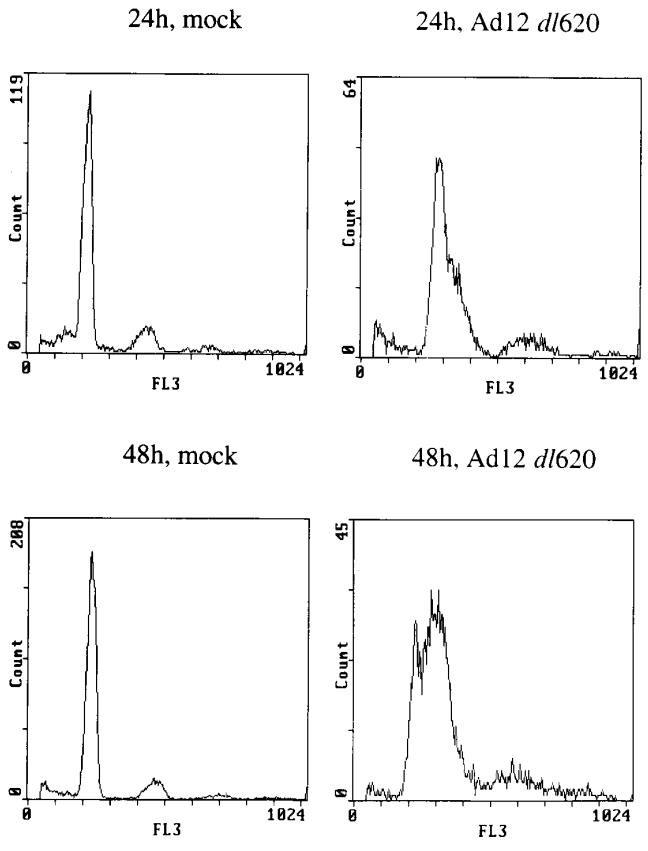

FIG. 6. DNA profiles from propidium iodide-stained HSFs, either uninfected or infected with Ad12 *dl*620. FACS analysis was performed according to Materials and Methods. The corresponding histograms are shown.

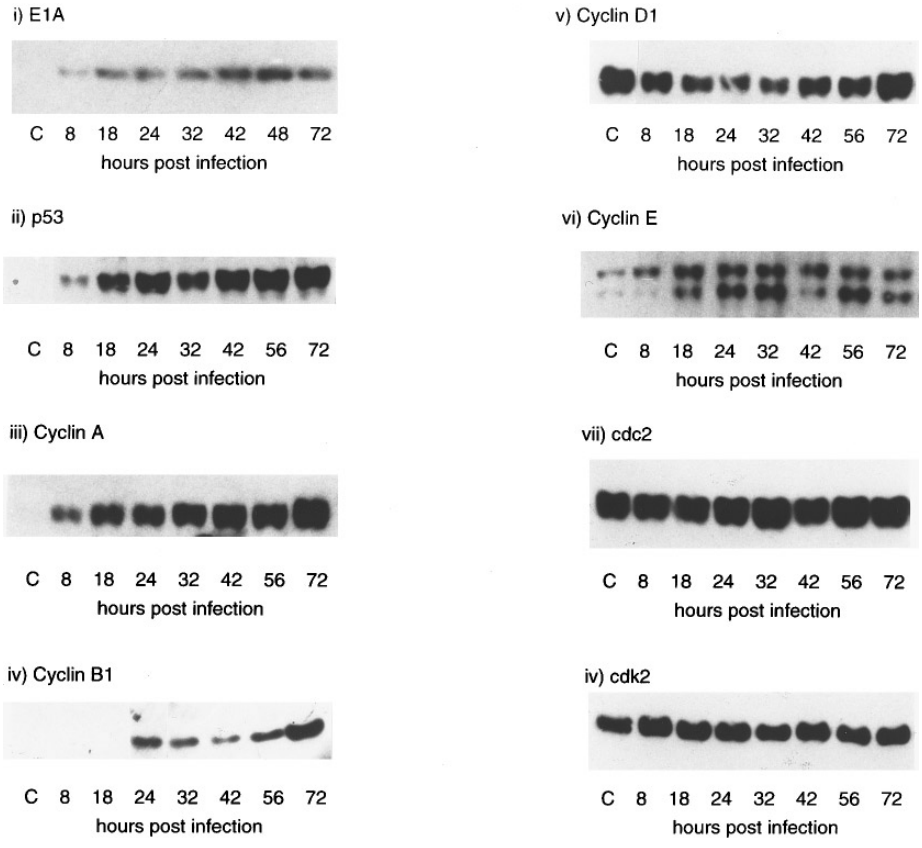

FIG. 7. Expression of cyclins and cdks in HSF cells in the presence of Ad12 E1A. HSFs were infected with Ad12 *dl*620 (200 PFU/cell) and harvested at the times indicated. Aliquots, containing 50 μg protein, were subjected to SDS–PAGE and Western blotting using the antibodies indicated: Ad12 E1A, p53, cyclins A, B1, D1, and E, and cdc2 and cdk2. C, uninfected cells.

The considerable emphasis previously placed on the role of pRb in the induction of cell cycle by Ad E1A led us to examine what happened in the absence of that protein. In fact, there appeared to be relatively little difference between the pRb-positive and -negative cells in cyclin expression or in their cell cycle profiles. These observations support the view that if the pRb family of proteins play a role in the E1A-mediated induction of cycle (which certainly seems to be the case) it is more likely attributable to p107 and p130 rather than pRb itself. This is consistent with the view that p107 and p130 play a large part in regulating activity of the E2F family of proteins in the progression from G_1 to S (Lees *et al.,* 1992; Shirodkar *et al.,* 1992; Schwarz *et al.,* 1993; Cobrinik *et al.,* 1993). None of these results are inconsistent with previous reports which stressed the importance of pRb binding to E1A since it shares binding sites with other family members in CR1 and CR2. Some caution must, of course, be exercised in the interpretation of these experiments, as Y-79 cells may compensate for the lack of pRb, perhaps through anomalous activities of other members of the pRb family. However, it is clear that Ad E1A can stimulate S phase entry regardless of the pRb status. Interestingly, when rodent cells are infected with Ad5 viruses with deletions in E1A CR2 (but not CR1) a cell cycle block ensues, with an increase in cells with DNA content greater than that at G_2/M (Moran and Zerler, 1988; Howe and Bayley, 1992). This has been attributed to the inability of E1A to bind pRb and/or p107. In the system described here other factors must play a role, as the *w.t.* Ad12 E1A proteins are able to bind both of these cellular factors and yet the cell cycle block with concomitant DNA replication still occurs.

The data presented here may have relevance to what happens when *w.t.* adenoviruses infect cells in the natural host. Human cells in culture are more likely to be cycling, albeit slowly if they are primary cells. When these cells are infected, even at low viral multiplicities, they will be stimulated to enter S phase even before the E1B proteins have been able to down-regulate the synthesis of cellular proteins. However, when human cells are infected *in vivo,* multiplicities of infection will be very low (probably only a few virus particles) and the target cells are most likely to be quiescent. In this case E1A expression will preceed E1B expression by some considerable time, a scenario that (in its early stages) is very close to the experimental model described here. Human cells infected *in vivo* will be stimulated to initiate cell cycle entry and progress into S phase, to allow for viral DNA synthesis. A subsequent Ad E1A-induced cell cycle

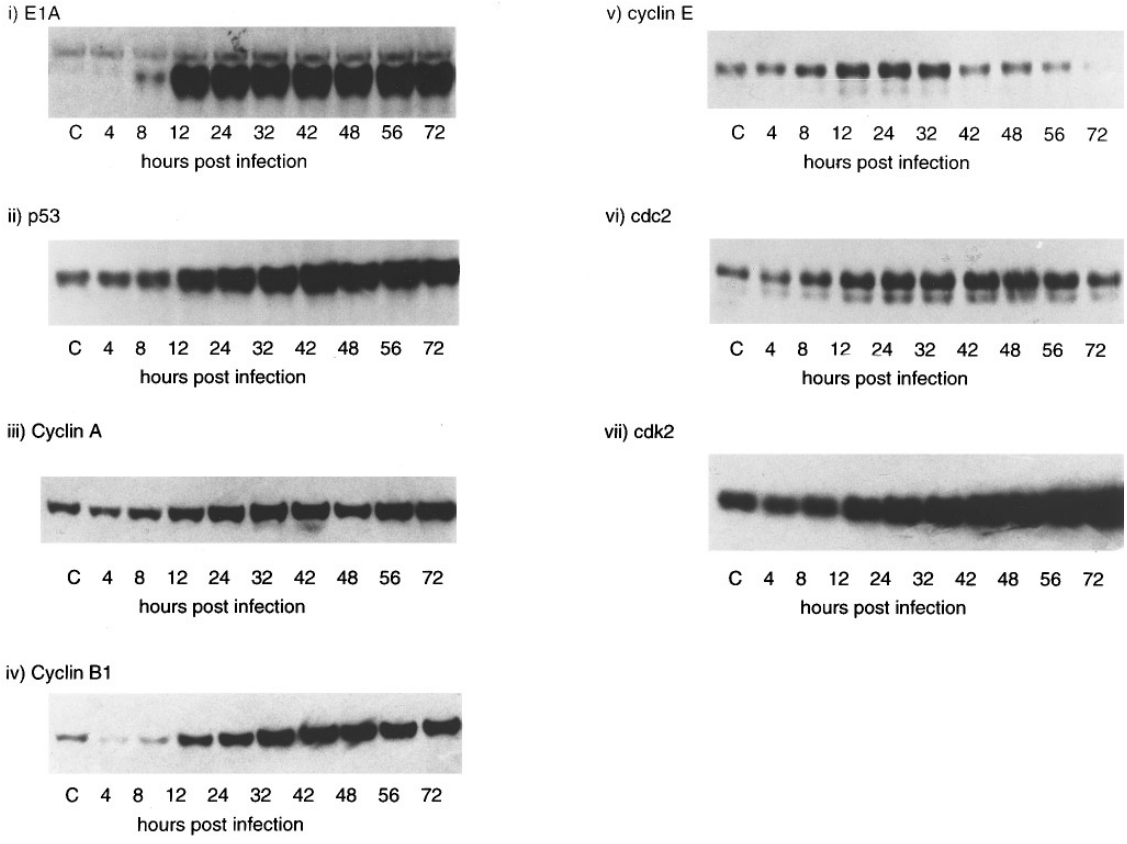

FIG. 8. Expression of cyclins and cdks in Y-79 cells in the presence of Ad12 E1A. Y-79 cells were infected with Ad12 dl620 (200 PFU/cell) and harvested at the times indicated. Aliquots, containing 50 μg protein, were subjected to SDS–PAGE and Western blotting using the antibodies indicated: Ad12 E1A, p53, cyclins A, B1, and E, cdc2, and cdk2. No cyclins D1 and D3 were detected. C, uninfected cells.

arrest in S phase, as observed in our experimental system, would be advantageous to the virus, allowing for the continuous synthesis of viral DNA, whereas an Ad E1A-induced mitosis might disrupt viral DNA synthesis until the next round of host cell S phase. This might explain why human cells are permissive for adenovirus infection, while rodent cells are semipermissive or nonpermissive.

The different responses of human and rodent cells suggest significant differences in cell cycle regulation by Ad E1A. Why human cells arrest in S phase while rodent cells go through the cycle is not at all clear, particularly as there is no evidence for differences in the cycle of human and rat cells. It is tempting to speculate that the factors which give rise to the different cell cycle responses of the cells to Ad E1A may also render human cells refractory to Ad E1A-mediated transformation but allow transformation of rat cells, albeit at low frequency (Gallimore *et al.,* 1985b, 1986; Byrd *et al.,* 1987). Presumably if Ad E1A causes cell cycle arrest (as in human cells) it is less likely to give rise to transformants than if it causes the cells to undergo mitosis (as in rat cells).

At the molecular level, it is not clear why the cells infected with Ad12 *dl*620 arrest in S phase. However, cyclin E overexpression has previously been implicated in stimulating repeated rounds of DNA synthesis in the absence of mitoses. Thus, ectopic expression of the human cyclin E gene in *Saccharomyces cerevisiae,* in contrast to cyclin D1, deregulates DNA synthesis such that DNA levels >2 N were observed (Mumberg *et al.,* 1996). Moreover, these cells had characteristically enlarged nuclei. Overexpression of cyclin E also stimulated DNA synthesis when these cells were growth-arrested in G_1 or G_2/M, suggesting that cyclin E can uncouple DNA replication from cell cycle progression (Mumberg *et al.,* 1996). It is also possible that the anomalous continued expression of high levels of cyclin B1 and associated kinase activity (Figs. 3 and 5) at the same time as cyclins A and E may play a part in preventing entry of cells into mitosis by an, as yet, undefined mechanism. This will have to await further investigation.

In summary we have shown that the expression of Ad12 E1A in human cells results in induction of cell cycle and passage into S phase, where arrest then occurs. Progress through G_1 and into S is accompanied by, or is attributable to, large increases in cyclins A, B1, and E and reduction in cyclin D3. Arrest in S phase then follows, with synthesis of cell DNA to give cells with DNA copy number >4 N. We attribute this cell cycle arrest to the disruption of normal cyclin expression in the wake of Ad12 E1A expression.

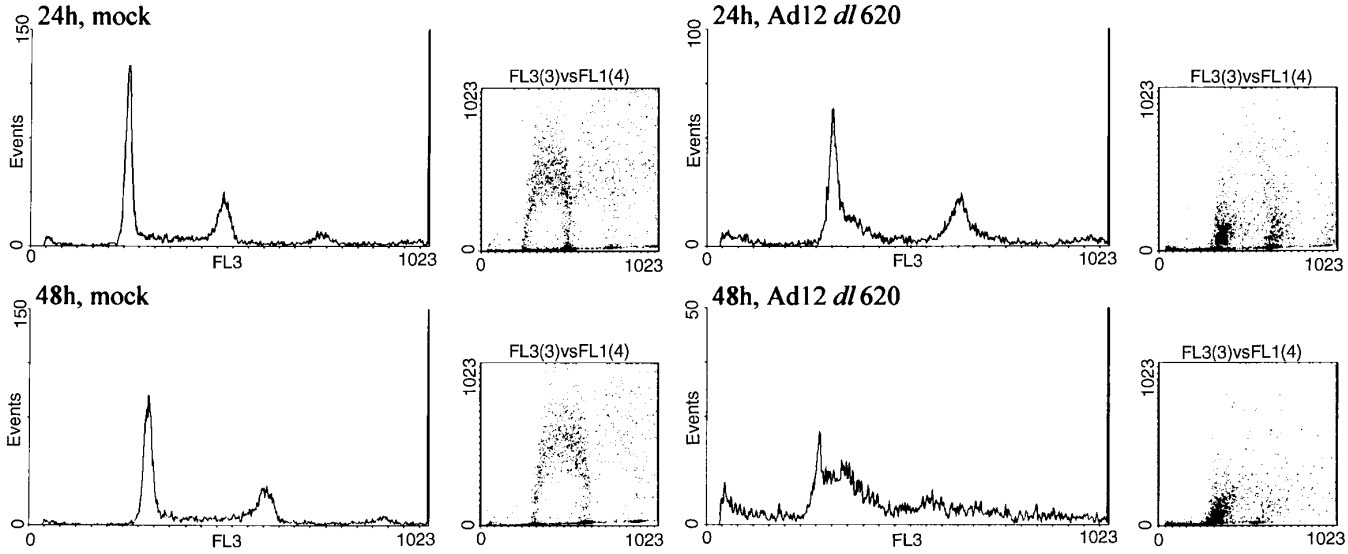

FIG. 9. Both Ad12 *dl*620-infected Y-79 cells and uninfected Y-79 cells were pulse-labeled with BrdU, 2 h prior to harvesting. Cells were subsequently probed with an anti-BrdU FITC antibody and counterstained with propidium iodide. FACS analysis was performed according to Materials and Methods. The corresponding histograms (for propidium iodide-stained cells) and dot plots (showing BrdU incorporation) are shown.

MATERIALS AND METHODS

Cell lines

Four human tumor cell lines were used in these studies. A549 cells derive from a small cell lung carcinoma, KB cells derive from an oral epidermoid carcinoma, HeLa cells derive from a cervical carcinoma, and Y-79 cells derive from a human retinoblastoma. Human skin fibroblasts (HSF) were obtained from an adult donor. All cells, except Y-79s, were grown in Hepes-buffered DME supplemented with 8% FCS and 2 mM glutamine. Y-79 cells were grown in RPMI supplemented with 15% FCS and 2 mM glutamine.

Adenovirus infections

Subconfluent A549, HeLa, KB, and HSF cells were infected (200 PFU per cell) with the mutant adenovirus Ad12 *dl*620 which has a lesion in the E1B gene (Byrd *et al.,* 1988) such that the only viral proteins expressed are E1A and a small amount of the E1B 19K polypeptide. Y-79 cells were infected at a similar ratio in suspension. Cells were harvested in ice-cold saline at designated times after infection and stored as a pellet at −70°C until required. In addition, A549 cells were infected with Ad5 ΔE1—a mutant virus lacking the entire early region 1 (a generous gift from Dr. Frank Graham, McMaster University, Ontario).

Western blotting

For Western blotting studies cell pellets were resuspended in 9 M urea, 50 mM Tris/HCl (pH 7.4), 0.15 M β-mercaptoethanol, sonicated, and analyzed for protein concentration. Aliquots containing 50 μg of protein were fractionated by polyacrylamide gel electrophoresis in the presence of SDS (PAGE). Proteins were electrophoretically transferred to nitrocellulose membranes which were probed with appropriate antibodies. Antigens were visualized using enhanced chemiluminescence (ECL).

Kinase assays

Pellets of A549 cells infected with Ad12 *dl*620 were resuspended in equal volumes of low [20 mM Tris/HCl (pH 7.2), 1% NP-40, and 0.15 M NaCl]- and high [20 mM Tris/HCl (pH 7.2), 1% NP-40, and 1.25 M NaCl]-salt extraction buffer and sonicated. Cyclin-associated kinase activities were immunoprecipitated from 400 μg of soluble extract using the appropriate antibodies and collected on Protein G–Sepharose (Sigma). Bound cyclin–cdk complexes were washed four times in wash buffer [20 mM Tris/HCl (pH 7.2) 1% NP-40, 5% sucrose, 0.5 M NaCl, and 5 mM EDTA], washed once in kinase buffer [20 mM Tris/HCl (pH 7.2), 1 mM Na_4VO_3, and 5 mM EDTA], and finally resuspended in 20 μl kinase buffer containing 1 mg/ml histone H1 (Calbiochem) or 50 μg/ml of a bacterially produced GST-Rb fragment (pRb amino acids 773–928; Santa Cruz). To this mixture was added 10 μl kinase buffer containing 10 μCi [γ-^{32}P]ATP, 100 μM ATP, and 75 mM $MgCl_2$. Samples were incubated at 30°C for 30 min. The reaction was stopped by adding 2 vol of SDS sample buffer and the mixture subjected to polyacrylamide gel electrophoresis. Gels were briefly stained with Coomassie blue, destained, dried, and exposed to X-ray film.

Antibodies

Antibodies against cyclins A, D, and E for use in Western blotting studies were obtained from Santa Cruz. A mouse monoclonal antibody against cyclin B1 was

obtained from U.B.I. Kinase assays were performed after immunoprecipitation with antibodies against cyclin A (Dr. Julian Gannon, ICRF Clare Hall Laboratories), cyclin B1 (UBI), cyclin D1 (Dr. Jiri Bartek, Danish Cancer Society, Copenhagen), cyclin E (Santa Cruz), and cdk2 (Santa Cruz).

Cell cycle analysis

Asynchronous, cycling cells were either mock-infected or infected with Ad12 *dl*620 for 2 h with occassional agitation. Two hours prior to harvesting, cells were labeled with 10 μM bromodeoxyuridine (BrdU). Cells were harvested by trypsinization and fixed in 70% ethanol at −20°C for at least 30 min. Cells were then treated at room temperature with 2 M HCl (containing 2 mg/ml pepsin) for 30 min and then neutralised with 0.1 M sodium borate (pH 8.5), prior to incubation with an anti-BrdU-FITC antibody (Becton–Dickinson), which was used according to manufacturer's instructions. Cells were subsequently treated with RNase A and labeled with propidium iodide (final concentration 5 μg/ml), prior to analysis on a Coulter Epics XL flow cytometer. The percentage of cells in each phase of cycle was determined using multicycle version 2.53 (Pheonix). DNA profiles were processed using WinMDI version 2.5.

Cytogenetics analysis of uninfected and Ad12 *dl*620-infected A549 cells was carried out following incubation for 4 h in the presence of colcemid. Cells were harvested by trypsinization and centrifuged, and the pellet resuspended in 75 mM KCl for 10 min at 37°C. Cells were again centrifuged, fixed in 3:1 methanol:acetic acid, dropped onto a microscope slide, and stained with Giemsa.

DNA copy number in Ad12 *dl*620-infected A549 cells

A549 cells were harvested 72 h after infection with Ad12 *dl*620. DNA was extracted from an equal number of infected and uninfected cells and was digested with *Bgl*II. The resulting fragments were fractionated by formaldehyde agarose gel electrophoresis. Following Southern blotting membranes were probed with the 0.7-kb ^{32}P-labeled *Hin*dIII–*Eco*RI fragment of the human J-γ region T cell receptor gene. After appropriate washing the membranes were subjected to autoradiography at −70°C. The relative abundance of signals in the two samples was determined by densitometric scanning.

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