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Infection of Primary Cells by Adeno-Associated Virus Type 2 Results in a Modulation of Cell Cycle-Regulating Proteins

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It has been demonstrated that infection of primary human cells with adeno-associated viruses (AAV) leads to a decrease in cellular proliferation and to growth arrest. We analyzed the molecular basis of this phenomenon and observed that infection with AAV type 2 (AAV2) had an effect on several factors engaged in the control of the mammalian cell cycle. In particular, all of the pRB family members, pRB, p107, and p130, which are involved in G_1 cell cycle checkpoint control, were affected. After infection, a shift from hyper- to hypophosphorylated forms was observed. Cyclins A and B1, which are required for G_1/S transition and progression into mitosis, respectively, were downregulated at the transcriptional level as well as at the protein level, whereas the G_1 cyclins D1 and E remained unaffected. In addition, the steady-state levels of cyclin-dependent kinases CDK1 and CDK2 and of transcription factor E2F-1 were diminished. Of all the factors known to be involved in phosphorylation of pRB family proteins, only the CDK inhibitor $p21^{WAF1}$ exhibited a response to AAV2 infection. $p21^{WAF1}$ mRNA was quickly and progressively upregulated in a p53-independent manner over at least 72 h. Consistent with the increased $p21^{WAF1}$ protein levels, cyclin E- and cyclin A-dependent kinase activities declined to low levels and E2F-p130-cyclin-CDK2 complexes were disrupted. From these data, we conclude that the major effect of AAV2 infection on primary human fibroblasts appears to be upregulation of $p21^{WAF1}$ gene expression and thus cell cycle arrest by the suppression of pRB family protein phosphorylation.

Nonlytic infection of tumor cells with members of the family *Parvoviridae* can lead to growth arrest and tumor suppression (66, 68). The molecular mechanism of this growth inhibition is presently not understood. Therefore, studies of parvoviruses and their interaction with host cells deserve interest with respect to the potential use of these agents in cancer treatment.

Parvoviruses are small nonenveloped viruses which can be divided into the following three groups: densoviruses, which multiply in insects; autonomous parvoviruses, some of which are causative agents of diseases in animals (74, 76); and adeno-associated viruses (AAV). As indicated by the name, AAV strongly depend on helper virus functions for productive infection (8, 10). The best studied representative, AAV type 2 (AAV2), has a 4.675-kbp single-stranded genome that encodes three capsid proteins and at least four regulatory proteins (Rep). Depending on the presence of helper virus, the REP proteins are able to act as both negative and positive regulators of all AAV2 promoters (6, 37, 46–49). Their negative regulation of heterologous promoters has been previously reported (34, 37, 48, 54), and recently an enhancing effect on the platelet-derived growth factor B c-*sis* promoter was demonstrated (84). The *rep* gene products have also been connected with tumor suppression due to their ability to interfere with simian virus 40 DNA (41)-, adenovirus E1A/*ras* (41)-, H-*ras* (34)-, and bovine papillomavirus (33)-mediated transformation of cells. In addition, the combination of high-titer AAV infection and genotoxic stress leads to increased cell death. In these experiments, the number of infectious particles used per cell varied from 10^2 (44, 83) to 10^5 (1, 4), thus exceeding the number needed for normal viral production by 2 to 3 orders of magnitude. Depending on the cell type used, infection with AAV may result in changes of proliferation rates (3) and even to complete growth arrest (1, 2, 4, 83), cell death (32), and tumor suppression (16, 18, 42, 43, 53, 63, 80).

Cell cycle progression in eukaryotes is tightly controlled at least at three different points, in G_1 before entering the S phase, in G_2 before entering mitosis, and during mitosis, where the correct spindle assembly is measured (60). The G_1 checkpoint is mainly controlled by the phosphorylation state of the pRB protein family, consisting of the retinoblastoma protein pRB, p107, and p130 (19, 35). The abilities of these proteins to bind to free E2F transcription factor in their hypophosphorylated forms and to liberate E2F in their hyperphosphorylated forms are responsible for E2F-mediated transcriptional upregulation of genes involved in the control of cell cycle progression (14, 59). The sequential expression of diverse cyclins and resultant activation of cyclin-dependent kinases (CDK) drive the cell cycle. In this respect, cyclin D-CDK4 and -CDK6 complexes, as well as cyclin E-CDK2 complexes, control G_1 phase progression, whereas cyclin A-CDK1 and -CDK2 complexes are responsible for G_1/S transition and S-phase progression. G_2/M transition and mitosis are controlled by cyclin B-CDK1 complexes (39). Within the cyclin gene activation cascade, at least the genes for cyclin E and cyclin A are responsive to E2F-mediated upregulation of transcription (27, 62, 70).

In addition to the binding of cyclins, CDK activity is modulated by positive and negative interfering factors. Site-specific phosphorylation by CDK-activating kinase and dephosphorylation by CDC25 phosphatases lead to activation of CDK, whereas members of the $p16^{INK4}$ and $p21^{WAF1}$ families can inhibit kinase activities. The two families possess different specificities. Members of the $p16^{INK4}$ family are selective for cyclin D-CDK complexes and arrest cells in early G_1 (31). The

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importance of this type of CDK inhibitor is further supported by the fact that at least the $p16^{INK4}$ gene is frequently deleted or mutated in human cancers (71). The second family comprises $p21^{WAF1}$, $p27^{KIP1}$, and $p57^{KIP2}$, and inhibition by these proteins is not restricted to any type of CDK (31). In this respect, $p21^{WAF1}$ is one of the most interesting members of the family since it has multiple binding capacities not only for CDK (30, 85) but also for cyclins (26) and PCNA (79); it thus may be involved in cell cycle progression, cell cycle arrest, and DNA synthesis. All CDK inhibitors are targeted by various signal transduction cascades in such a way that each signal produces a defined pattern of upregulated inhibitors (31, 39).

The data presented here demonstrate that the phosphorylation of pRB family proteins is impaired in AAV2-infected primary human fibroblasts, resulting in a shutoff of genes that control cell cycle progression. The inability to phosphorylate pRB, p107, and p130 can be explained by AAV2-induced upregulation of the universal CDK inhibitor $p21^{WAF1}$.

MATERIALS AND METHODS

Cell culture. Primary human diploid MS28 fibroblasts were obtained by culturing materials obtained from a tonsillectomy. Cells were cultured in Dulbecco's modified Eagle's minimum essential medium as monolayers at 37°C in 5% CO_2. The growth medium was supplemented with glutamine, antibiotics, and 10% fetal calf serum.

Virus and virus infection. The titer of AAV2 was determined by dot blotting HeLa cultures, coinfected with AAV2 and adenovirus type 2, on nitrocellulose filters, followed by hybridization with radiolabeled AAV2 DNA by the method of Bantel Schaal and zur Hausen (5). Approximately 5×10^6 MS28 fibroblasts were grown on 165-cm^2 tissue culture plates (Greiner) and were either mock infected with heat-inactivated adenovirus type 2 or infected with AAV2 at a multiplicity of infection (MOI) of 2×10^2 to 1×10^3 infectious particles per cell. MOI values of 1 or 10 do not give rise to the described effects (data not shown). This is also consistent with previous reports demonstrating that MOI values have to exceed a critical limit to achieve cellular effects (1, 43). Infections were performed under low-serum (0.5%) conditions at 37°C for 1 h. Afterwards, nonadsorbed virus was removed by washing cells twice with phosphate-buffered saline (PBS) and further incubated under normal growth conditions.

Flow cytometry. Cells were trypsinized, pelleted by low-speed centrifugation, washed three times with ice-cold PBS, and fixed in 90% ice-cold ethanol for at least 12 h. After centrifugation, cells were stained with propidium iodide and counted in a Becton Dickinson FACScan. Cell cycle profile analyses were performed by using the SOBR model for the determination of cellular distribution and the CellFit program (Becton Dickinson).

Northern (RNA) blot analysis. RNA was harvested by guanidine isothiocyanate lysis (12). Equal amounts of RNA, as determined by measurement of optical density at 260 nm, were resolved by electrophoresis on a 1% agarose gel (50 mM morpholinepropanesulfonic acid [MOPS; pH 7.0], 1 mM EDTA [67]), transferred to a nylon membrane (Hybond N^+; Amersham) by vacuum blotting (Vacu Gene XL; Pharmacia) in 10× SSC (1.5 M NaCl, 0.15 M sodium citrate [pH 7.0]) for 3 h, washed with 2× SSC, and air dried. Before hybridization, filters were prewetted in 2× SSC–0.2% sodium dodecyl sulfate (SDS), hybridized, and washed to stringency at 65°C by previously described protocols (13). The expression levels of cyclins D1, E, A, and B1 and of $p21^{WAF1}$ were monitored with human probes. RNA loading was controlled by hybridization with the glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) gene.

Protein preparation. Up to 5×10^6 cells were washed twice in ice-cold PBS and extracted in 1 ml of lysis buffer containing 50 mM HEPES (pH 7.0), 150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 0.2 mM Pefabloc, 100 μM Na_3VO_4, 10 mM β-glycerophosphate, 1 mM NaF, and 10 μg (each) of aprotinin, pepstatin, and leupeptin per ml for 30 min at 0°C after sonication. Cell lysates were centrifuged for 10 min at 0°C.

Western blot analysis. Immunoblotting was performed after denaturing SDS-polyacrylamide gel electrophoresis (50) by using the enhanced-chemiluminescence Western blotting detection system (Amersham). Briefly, up to 100 μg of proteins was loaded on SDS–5 to 15% polyacrylamide gradient gels, electroblotted to polyvinylidene difluoride membranes (Immobilon P; Millipore), and stained with Ponceau Red to control equal transfer. Filters were blocked in PBS with 5% nonfat dry milk and 0.05% Tween 20 and incubated with the primary antibodies in blocking solution overnight at 4°C. After being washed, filters were incubated with the secondary antibodies (Promega) in blocking solution for 1 to 2 h at room temperature. In order to control for equal loading, each blot was incubated with a monoclonal antiactin antibody (C4) from ICN Pharmaceuticals Inc. On all gels, the standard deviation between lanes never exceeded 15%. Polyclonal antibodies for cyclin D1 (H-295), cyclin A (H-432), CDK2 (M2), CDK4 (C-22), CDK6 (C-21), $p27^{KIP1}$ (C-19), pRB (C-15), p107 (C-18), and p130 (C-20) were obtained from Santa Cruz Biotechnology, Inc. (Alameda, Calif.); polyclonal antibody for CDC2 (Ab-1) was obtained from Oncogene (Cambridge, Mass.); and polyclonal antibodies for $p21^{WAF1}$ (15431E) and $p16^{INK4}$ (15126E) were obtained from Pharmingen (San Diego, Calif.). Monoclonal antibodies for PCNA (Ab-1) and p53 (DO1) were obtained from Oncogene, and monoclonal antibody for cyclin E (HE12) was obtained from Santa Cruz Biotechnology, Inc. Polyclonal cyclin B1 antibody was produced as described elsewhere (36).

Immunoprecipitation. For each immunoprecipitation, 500 μg of protein was used and precleared for 1 h with protein A-Sepharose (Pharmacia). Precleared lysates were then subjected to immunoprecipitation by using antibodies for cyclin E (HE111; Santa Cruz Biotechnology Inc.) and cyclin A (Mitotix Inc.) with protein A-Sepharose as the precipitating agent. Immunoprecipitates were washed three times with ice-cold lysis buffer and twice with kinase buffer (50 mM Tris [pH 7.5], 1 mM dithiothreitol, 5 mM $MgCl_2$, 5 mM $MnCl_2$).

In vitro kinase assays. Immunoprecipitates were supplemented with 5 μg of histone H1 (Boehringer Mannheim) per ml, 0.1 μM rATP, and 100 μCi of [γ-^{32}P]rATP per ml, and the reaction mixtures were incubated for 15 min at room temperature in a thermomixer (Eppendorf). Reactions were terminated by adding equal volumes of 2× sample buffer, boiled, resolved on an SDS–12% polyacrylamide gel, and visualized by autoradiography.

Electrophoretic mobility shift assay. Bandshift experiments were performed as described previously (64). Probe E2wt, containing the E2F binding site of the adenovirus E2 promoter, has been described elsewhere (69). E2F-associated proteins were analyzed by incubation of the bandshift reaction mixture with specific supershifting antibodies on ice for 50 min prior to electrophoresis. p107 was detected by monoclonal antibody SD15 (a gift from N. Dyson, Charlestown, Mass.), p130 was detected by polyclonal antiserum C20 (Santa Cruz Biotechnology, Inc.), pRB was detected by monoclonal antibody clone XZ55 (Pharmingen), DP1 was detected by a polyclonal antibody (a gift from N. La Thangue, Glasgow, United Kingdom), cyclin E was detected by polyclonal antiserum M20 (Santa Cruz Biotechnology, Inc.), cyclin A was detected by a polyclonal antiserum (a gift from M. Pagano, New York, N.Y.), and CDK2 was detected by polyclonal antiserum M2 (Santa Cruz Biotechnology, Inc.).

RESULTS

AAV2 infected cells are arrested in G_1 and G_2/M. A comparison of corresponding cell cycle profiles of mock- and AAV2-infected primary human MS28 fibroblasts revealed striking differences between the two cultures after 3 to 7 days (Fig. 1). Whereas mock-infected cells over the 7 days of the experiment accumulated in the G_0/G_1 phase of the cell cycle, indicating that they became contact inhibited due to increasing cell density, the profiles of AAV2-infected cells showed no change. In addition, after 24 h, an increase in neither cell number nor cell death was noted. Thus, without obviously proliferating cells in AAV2-infected cultures, fluorescence-activated cell sorter profiles indicated a growth arrest in both the G_0/G_1 and G_2/M phases of the cell cycle. Consistent with the cell cycle profiles of MS28 cells, analyses of embryonic human AAV-infected and Syrian hamster fibroblasts have led to similar results (4, 83).

Inhibition of pRB family protein phosphorylation by AAV2 infection. Since escape from restriction point control in G_1 phase of the cell cycle is controlled by pRB family proteins, the status of pRB family proteins in response to AAV2 infection was analyzed. Protein preparations of infected cultures were separated on high-resolution denaturing gels and examined in time course experiments (Fig. 2). All three members of the pRB family clearly responded to AAV2 infection. Whereas at time zero mostly hyperphosphorylated forms of pRB, p107, and p130 were visible, a shift to faster-migrating hypophosphorylated forms became apparent at about 12 h after infection and was completed after 24 h. In the case of p130, dephosphorylation in the mock control was visible after 24 h but it was preceded by that of AAV2-infected cultures. For each of these proteins, the effects were observable for at least 48 h (data not shown). Since it appeared that in cultures arrested due to infection with AAV2 the overall amount of pRB protein was reduced, we analyzed the pRB contents in cells arrested by serum starvation. As a control, extracts from cells harvested 24 h after serum addition were used. Since the pRB protein contents in cells arrested by serum starvation (0% serum, 4

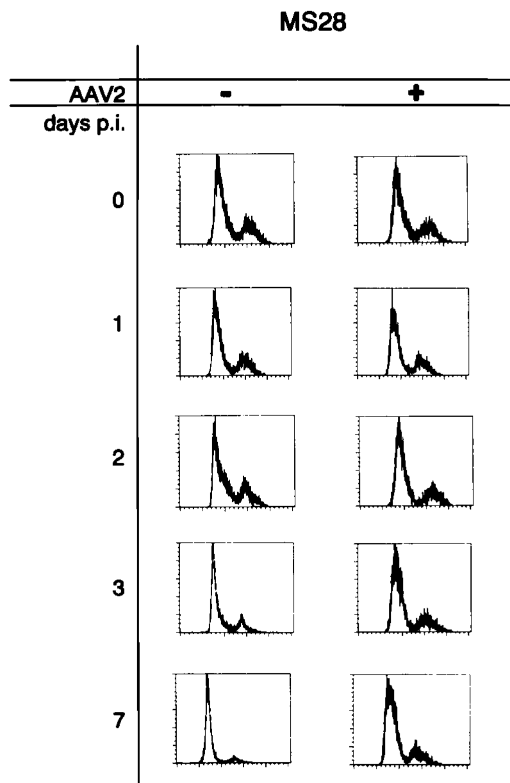

FIG. 1. Cell cycle profiles of AAV2-infected human MS28 fibroblasts. Human MS28 fibroblasts were either mock infected with heat-inactivated adenovirus type 2 (−) or infected with AAV2 (+) (MOI = 500). At the indicated time points postinfection (p.i.), aliquots of cells were processed for fluorescence-activated cell sorter analysis to determine cell cycle distribution.

days) were also reduced, it was obvious that the differences in the amount of pRB were due to cell cycle arrest and were not a consequence of AAV2 infection itself. Similar observations have been made for p107, whose levels are also significantly reduced in growth-arrested fibroblasts (7).

Downregulation of cyclins A and B1 in response to dephosphorylation of pRB-like proteins. Potentially due to the change in phosphorylation of the pRB family proteins, the steady-state level of cyclin A was downregulated after 18 h postinfection, whereas those of cyclin B1 and PCNA were downregulated after 24 h postinfection (Fig. 3A). In contrast, the G_1 cyclins D1 and E were not significantly affected (14, 35). For cyclin D1, comparable results were obtained with two different antibodies. A significant decrease in E2F-1 protein levels was detected in AAV-infected cultures at 36 h after infection; this might reflect an E2F-mediated shutoff of the E2F-1 gene (38). For cyclins A and B1, decreases in gene expression were also reflected at the mRNA level, where differences became apparent at 6 h after infection for cyclin A and at 12 h after infection for cyclin B1 (Fig. 3B), suggesting that the transcription of these genes is inhibited in AAV-infected cultures. Cyclin E mRNA levels were unaffected by AAV2 infection (data not shown). Interestingly, cyclin D1 mRNA levels reproducibly started to decrease in mock-infected cells after 36 h, whereas they remained constant in cultures infected with AAV2. This phenomenon cannot yet be explained.

Inhibition of CDK activity by AAV2. Since it appeared that pRB phosphorylation was impaired in AAV2-infected cultures, some factors involved in this process were analyzed in detail. Under normal conditions, pRB and its family members are phosphorylated by active cyclin D1-CDK2, -CDK4, and -CDK6 complexes in collaboration with cyclin E-CDK2 and cyclin A-CDK complexes (59). In AAV2-infected cultures, the histone H1-phosphorylating activity of cyclin E-dependent kinases was drastically reduced compared to that of the mock control (Fig. 4). At 24 h after infection, a reduction to a basal level was already visible. Similar results were obtained when cyclin A-dependent kinase activity was analyzed (Fig. 4). Whereas depletion of cyclin A protein (Fig. 3A) may account for the decline in cyclin A-dependent kinase activity, this does not appear to be the case for cyclin E-dependent kinase activity. Therefore, the steady-state protein levels of the corresponding candidate kinases were examined. As shown in Fig. 5A, the protein levels of the cyclin D-dependent kinases, CDK4 and CDK6, were not significantly affected by AAV2 infection, whereas the protein levels of CDK2, which binds to cyclins E and A, and of CDK1 (CDC2), which binds to cyclin A, started to decline at 36 h after infection.

AAV2-dependent inhibition of pRB phosphorylation is correlated with an increase in $p21^{WAF1}$. The activities of CDK are further regulated by positive and negative interfering factors (58). In this respect, CDK-activating kinase and CDC25 phosphatases act as positive regulators, whereas members of the $p16^{INK4}$ family, which interact specifically with CDK4 and CDK6 (72), and the more universally acting members of the $p21^{WAF1}$ family inhibit CDK (24, 25, 31). As can be seen in Fig. 5B, the abundances of $p16^{INK4}$ and $p27^{KIP1}$, a member of the $p21^{WAF1}$ family, were unaffected by infection with AAV2. In contrast, as early as 6 h after infection, the $p21^{WAF1}$ protein level increased, reaching a maximum between 12 to 18 h, and remained this high for at least 24 h (Fig. 6). Since the steady-

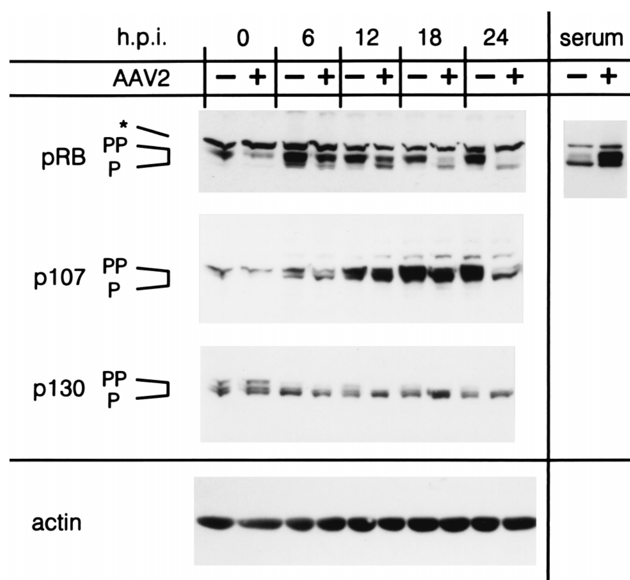

FIG. 2. Repression of pRB family protein phosphorylation by AAV2 infection. MS28 cells were either mock infected with heat-inactivated adenovirus type 2 (−) or infected with AAV2 (+) (MOI = 500). Whole-cell extracts were prepared from parallel samples and analyzed for the expression of pRB family proteins by Western blotting. To control for equal loading, filters were incubated with antiactin antibody; equal loading was independently confirmed by a nonspecific band (*) observed on top of the pRB bands. To verify whether the reduction of pRB protein in AAV2-infected cells was due to AAV2 infection itself or due to cell cycle arrest, extracts of serum-starved cells (−) and extracts of cells 24 h after serum addition (+) were included. PP, hyperphosphorylated; P, hypophosphorylated; h.p.i., hours postinfection.

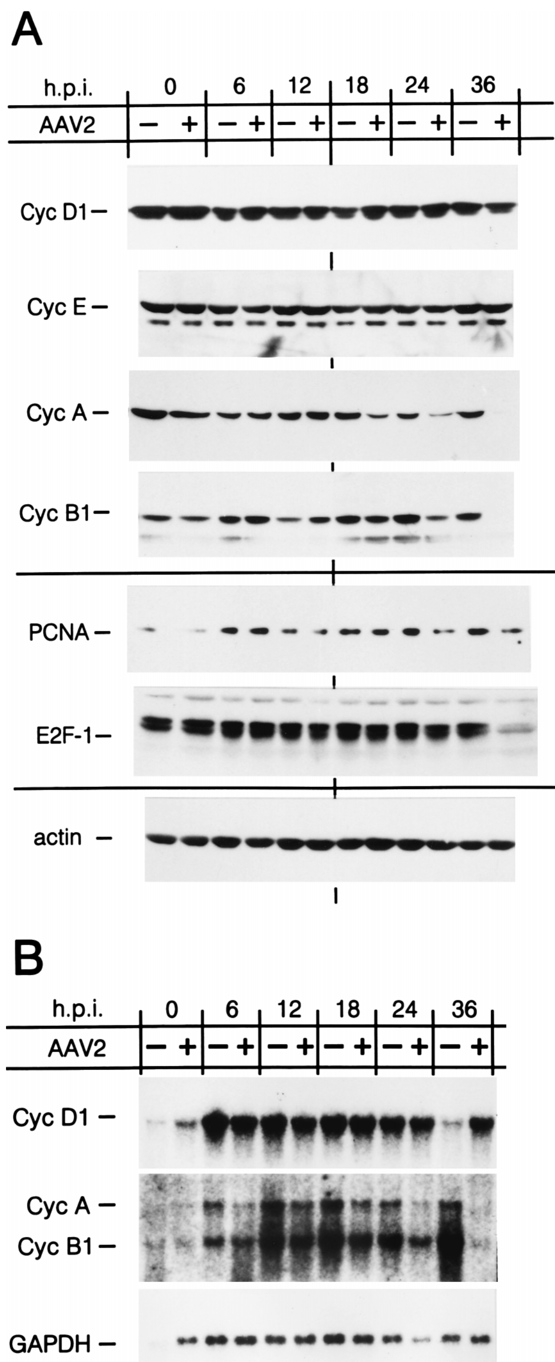

FIG. 3. Expression of genes involved in cell cycle progression in response to AAV2 infection. MS28 cells were either mock infected with heat-inactivated adenovirus type 2 (−) or infected with AAV2 (+) (MOI = 500). (A) Western blot analysis of cyclins D1, E, A, and B1 and of PCNA and E2F-1. To control for equal loading, filters were incubated with antiactin antibody. The 18-h time point is emphasized by a broken line. (B) Northern blot analysis of transcripts of cyclins D1, A, and B1 and of GAPDH. h.p.i., hours postinfection.

state levels of p21^{WAF1} are normally regulated transcriptionally (25, 31) and its promoter is responsive to the p53 protein, a tumor suppressor responsible for cell cycle arrest and induction of apoptosis by genotoxic stress (45), the levels of p21^{WAF1} and p53 proteins and p21^{WAF1} mRNA were examined (Fig. 6). As shown in Fig. 6B, the amount of p21^{WAF1} mRNA steadily increased over time (Fig. 6B), resulting in fivefold induction. In contrast, the protein level already reached its maximum at 12 h after infection (threefold induction). The means of three independent experiments are plotted in the scheme of Fig. 6A. Since the p53 protein level was not affected by AAV infection, transcriptional activation of the p21^{WAF1} gene apparently occurs via a p53-independent pathway.

Disruption of E2F complexes in AAV-infected cells. Recently, it was demonstrated that p21^{WAF1} is able to disrupt E2F complexes which consist of cyclins, CDK, and p107 or p130 (73, 88). We therefore investigated the status of E2F complexes in AAV2-infected cells. Extracts were prepared from mock- and AAV-infected cells at 12 h postinfection and analyzed in E2F bandshift experiments. In addition to some nonspecific bands which cannot be supershifted by the DP1 antibody, three different E2F complexes were visible in mock-infected dividing cells (Fig. 7). Complex A appeared to be mainly formed by p130, cyclin A, and CDK2, as indicated by the complete disappearance of the complex when supershifting antibodies for p130, cyclin A, and CDK2 were used. Since the amounts of p107 and cyclin E supershifted by the corresponding antibodies were rather small, they cannot clearly be attributed to any complex. E2F and pRB appeared to form complex B, as indicated by the complete supershifts upon the addition of pRB and DP1 antibodies. Complex C corresponded, most probably, to free E2F, as it was supershifted only by the DP1 antibody. Of the two major complexes (A and B), with complex B consisting of pRB and E2F and complex A consisting of p130, cyclin A, CDK2, and E2F, only the pRB-E2F complex (complex B) can be found in AAV2-infected cells. Complex A was disrupted and shifted to a position of higher mobility directly above complex B. Neither cyclin A, cyclin E, nor CDK2 can be supershifted from this complex. Thus, already at 12 h after infection with AAV, a time point at which dephosphorylation of pRB family proteins has started but is still incomplete, E2F-p107 and E2F-p130 complexes, which are specific for S-phase cells (15, 73, 78) and correspond to a transcriptionally

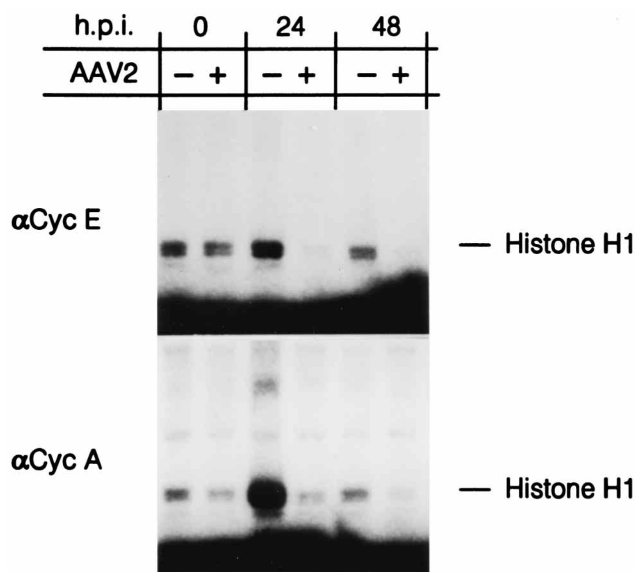

FIG. 4. Cyclin E- and cyclin A-dependent kinase activities are reduced in AAV2-infected cells. Extracts from mock (−)- and AAV2 (+) (MOI = 500)-infected cells were immunoprecipitated by using monoclonal anti-cyclin E (αCyc E) and polyclonal anti-cyclin A (αCyc A) antibodies. The immunoprecipitates were assayed for histone H1 kinase activity, and the products were resolved on 12% polyacrylamide gels. h.p.i., hours postinfection.

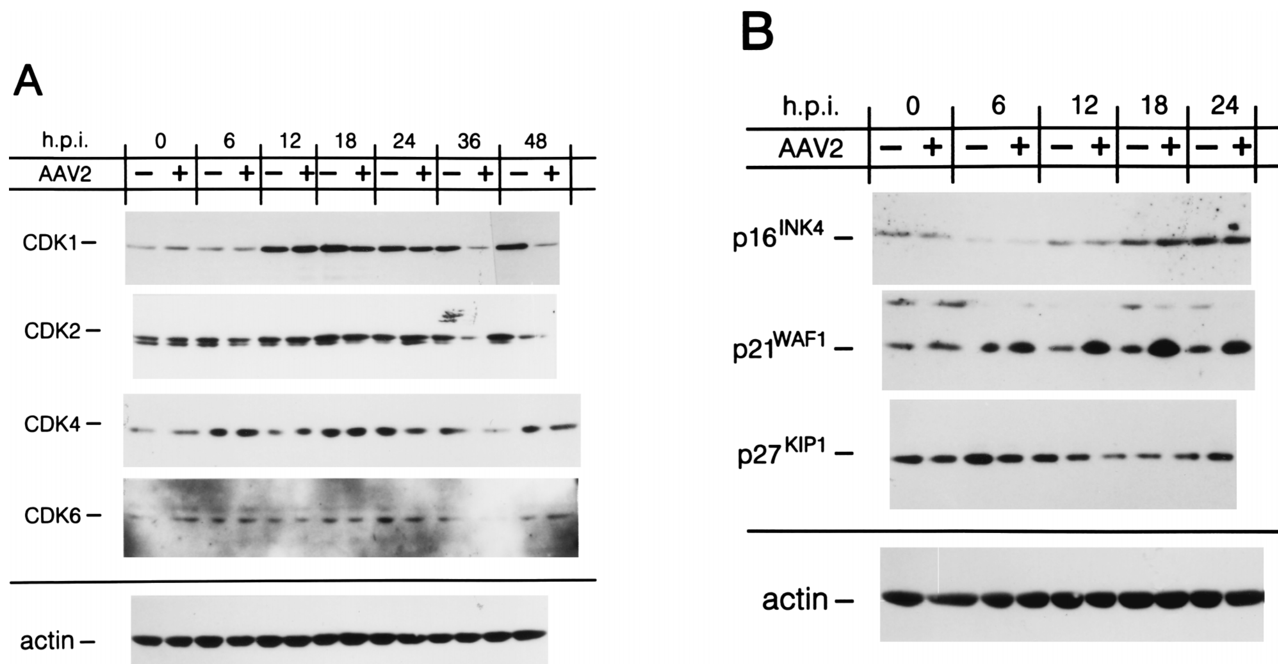

FIG. 5. The universal CDK inhibitor $p21^{WAF1}$ is upregulated in response to AAV2 infection. Whole-cell extracts of mock (−)- and AAV2 (+) (MOI = 500)-infected MS28 cells were analyzed for their contents of cell cycle regulators. (A) Western blot analysis of cyclin D-dependent kinases CDK4 and CDK6 and cyclin E- and A-dependent kinases CDK1 and CDK2. (B) Western blot analysis of CDK inhibitors $p27^{KIP1}$, $p21^{WAF1}$, and $p16^{INK4}$. To control for equal loading, filters were incubated with antiactin antibody. h.p.i., hours postinfection.

active form of E2F (87), were depleted of cyclin-CDK heterodimers.

DISCUSSION

The ability of AAV2 to inhibit cellular proliferation of presenescent fibroblasts has previously been demonstrated to result in perturbation of and arrest in G_0/G_1 and G_2/M phases of the cell cycle (4, 83). The data presented here indicate that the G_1 block correlates with the inhibition of pRB family protein phosphorylation. Phosphorylation of pRB is a key event needed for cell cycle progression in mammals (14, 35). Hypophosphorylated pRB binds to the transcription factor E2F in a way that E2F-responsive genes are transcriptionally repressed (82). Upon phosphorylation of pRB by CDK, E2F is thought to be released and is available as a transcriptional activator of several genes involved in cell cycle progression, e.g., cyclin A and E2F-1 genes (51). The finding that the transcription of the cyclin E gene was not affected by AAV2, although it is regulated by E2F (9, 27, 87), suggests that additional regulatory mechanisms are targeted by AAV. The presence of the G_1 cyclins D1 and E in AAV2-arrested cells indicates that the gene activation cascade which drives the cell cycle is disrupted before transcriptional activation of the cyclin A gene occurs. This situation is reminiscent of that observed in senescent fibroblasts, in which there is also downregulation of proteins involved in late cell cycle progression (20). In these fibroblasts, CDK activities are reduced due to upregulation of the CDK inhibitor $p21^{WAF1}$ (61). A second mechanism by which AAV2 mediates cell cycle arrest appears to be the disruption of E2F complexes which are specific for dividing cells. In nondividing cells, E2F remains associated with p130, forming a two-member complex (15, 78), whereas CDK2 and a cyclin component are added to the complex in dividing cells (15). The latter complex was shown to be susceptible to disruption by $p21^{WAF1}$ (73). Similarly, an E2F-p107-cyclin A-CDK2 complex can be dissociated by $p21^{WAF1}$ or $p27^{KIP1}$ (87, 88). In both cases, disruption of the corresponding four-member complexes can be achieved by the induction of $p21^{WAF1}$ via the p53 pathway (73, 88). The observation of a similar effect on fibroblasts upon AAV2 infection indicates that a relatively moderate elevation of the $p21^{WAF1}$ steady-state level (Fig. 6A) is sufficient to completely arrest cells in the G_1 phase of the cell cycle.

By upregulation of $p21^{WAF1}$ gene expression, AAV activates one of the central genes involved in cell cycle control (30, 85), response to genotoxic stress (21, 22, 55), tumor suppression (23, 30, 56), differentiation (28, 29, 40, 52, 57, 65, 75), and cellular senescence (61). Interestingly, infection with AAV2 has previously been shown to give rise to potentially similar effects in different systems. For example, it has been demonstrated that AAV2 infection increases the sensitivity of transformed cells to genotoxic stress under in vitro (2, 32) and in vivo (43, 81) conditions. Furthermore, AAV infection induces the appearance of differentiation markers in human leukemia, melanoma, and immortalized keratinocyte cell lines (3, 44). It is therefore tempting to speculate that AAV-induced upregulation of the $p21^{WAF1}$ tumor suppressor gene is involved in phenomena described above. Indeed, in the adenocarcinoma cell line, A549, demonstrated to be sensitized to genotoxic stress by AAV2 (43), the $p21^{WAF1}$ gene was upregulated in a manner comparable to that of human fibroblasts (32a).

The way by which AAV2 induces $p21^{WAF1}$ gene expression is of interest. The $p21^{WAF1}$ gene promoter is regulated by different transcription factors and signal transducers (31, 45). Since stabilization of p53, which was shown to induce $p21^{WAF1}$ in response to DNA damage, is not visible in AAV2-infected cells, it is more likely that one of the p53-independent pathways is used. As such, it has been demonstrated that activation of the $p21^{WAF1}$ gene promoter can be achieved by transform-

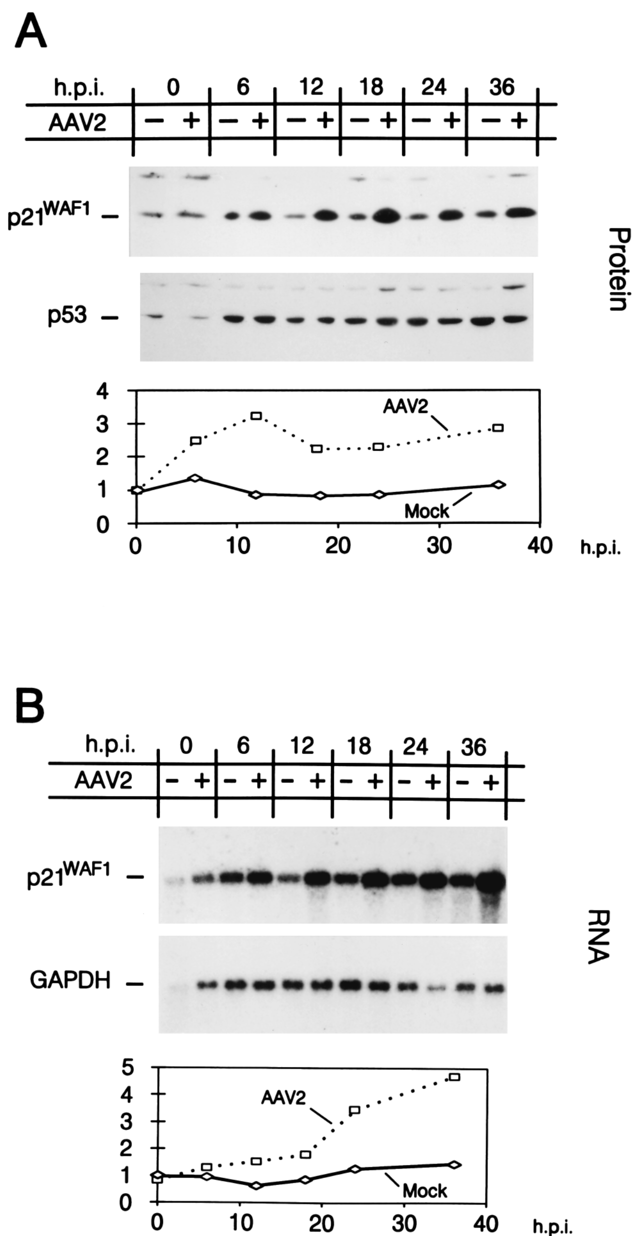

FIG. 6. $p21^{WAF1}$ upregulation is p53 independent. (A) Western blot analysis of MS28 cell extracts infected by either heat-inactivated adenovirus type 2 (−) or AAV2 (+) at an MOI of 500 for the expression of $p21^{WAF1}$ and p53. $p21^{WAF1}$ enhanced-chemiluminescent signals of three independent blots were densitometrically quantitated, normalized to actin, and plotted to give a graphical representation of the results. For each time point on the graph, the amount of $p21^{WAF1}$ protein detected in the mock control at time zero was set at 1. (B) Northern blot analysis of parallel cultures for the presence of $p21^{WAF1}$ mRNA. Northern signals were quantitated by using a phosphorimager, normalized to GAPDH mRNA levels, and plotted graphically. For each time point on the graph, the amount of $p21^{WAF1}$ mRNA detected in the mock control at time zero was set at 1. h.p.i., hours postinfection.

ing growth factor treatment (17), C/EBPα activation (77), or MyoD induction (29, 65). Whether infection by AAV2 activates one or more of these pathways or there is a direct effect on the $p21^{WAF1}$ promoter is an open question. Three scenarios by which AAV2 may interact with cellular factors are suggested. First, the binding of the virus to its receptor can stimulate a signal transduction cascade, which then in turn is responsible for the induction of $p21^{WAF1}$ gene expression. A precedent for this is the STAT pathway which mediates activation of the $p21^{WAF1}$ promoter by epidermal growth factor or gamma interferon (11). Second, viral proteins which either are constituents of the viral capsid or are included within it may interfere with cellular factors responsible for $p21^{WAF1}$ transcription. Both hypotheses are supported by the fact that a significant amount of infectious viral particles are needed to trigger growth arrest in fibroblasts, indicating that either the number of activated receptor molecules has to exceed a critical limit or that a considerable amount of protein has to enter the cell. Third, viral gene products, like Rep78 or Rep68, may cause the observed effects. Indeed, perturbation of the cell cycle was demonstrated in cell lines which inducibly express Rep78 (86). However, the rapid induction of the $p21^{WAF1}$ promoter which leads to visible upregulation of $p21^{WAF1}$ mRNA by 3 h after infection (data not shown) may argue against this possibility; replication of the single-stranded genome of AAV2 and transcription and translation of the *rep* genes take longer. In addition, infection with recombinant AAV2 particles in which AAV-encoded genes were replaced by a *lacZ* expression cassette still caused growth arrest of human fibroblasts, indicating that viral gene expression is not needed (32a). Further investigations to clarify which of these pathways is responsible for the observed effects are in progress.

One interesting feature of AAV2-induced upregulation of $p21^{WAF1}$ is the striking difference between mRNA and protein levels. The constant and more-or-less linear increase in the $p21^{WAF1}$ mRNA level was not reflected by the protein level. Instead, there was an increase, peaking at 12 h, followed by a plateau phase. This suggests that at this time point, either the efficiency of initiation of translation is reduced or the protein is destabilized. However, only positive regulation of the half-life of the $p21^{WAF1}$ protein has been reported to occur upon C/EBPα-induced inhibition of cell proliferation (77). Prelimi-

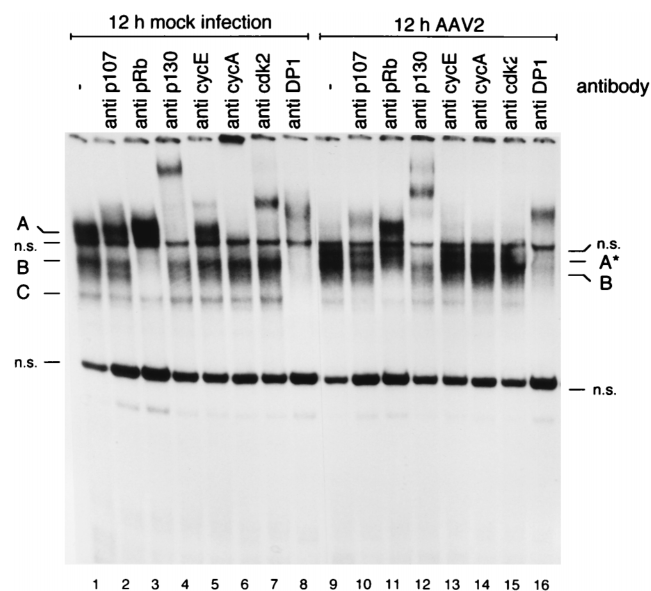

FIG. 7. Disruption of E2F complexes by AAV infection. Bandshift experiments with extracts from mock- and AAV2-infected MS28 cells, harvested 12 h after infection, were performed in the presence of specific antibodies to p107 (lanes 2 and 10), pRB (lanes 3 and 11), p130 (lanes 4 and 12), cyclin E (lanes 5 and 13), cyclin A (lanes 6 and 14), and CDK2 (lanes 7 and 15). Bands which could not be supershifted by a DP1 antibody (lanes 8 and 16) were classified as nonspecific (n.s.). −, no antibody. The positions of E2F complexes are indicated. A*, complex A disrupted and shifted to a position of higher mobility.

nary experiments indeed indicated that the rate of translational initiation of the p21^{WAF1} mRNA was reduced in AAV2-infected cells at later time points (82a). Since dephosphorylation of pRB family proteins is expected to lead to depletion of free E2F and to a shutoff of E2F-controlled genes, additional CDK-inhibitory activity would no longer be needed to maintain the growth-arrested phenotype. As a consequence, one would expect AAV2-arrested cells, like contact-inhibited cells, to reversibly remain in this state until a strong proliferative signal induces cell division. Indeed, this has been shown to be the case. After a first round of trypsinization, AAV2-infected primary human cells start to proliferate, although at a lower rate; after a second round, they are indistinguishable from mock controls (4). Altogether, it seems likely that AAV2-induced growth arrest in primary human fibroblasts is mainly mediated by p21^{WAF1}.

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