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Expression of the E2F1 transcription factor overcomes type β transforming growth factor-mediated growth suppression

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ABSTRACT **Inhibition of cell growth by type β transforming growth factor (TGF-β) occurs in mid-G_1 and is associated with decreased G_1 cyclin-dependent kinase activity and maintenance of the retinoblastoma tumor suppressor protein Rb in an underphosphorylated, growth-suppressive state. A variety of recent experiments suggest that a functional target of Rb is the E2F transcription factor. In addition, the growth-suppressive effects of TGF-β can be overcome by expression of viral oncogene products that dissociate E2F from Rb and Rb-related polypeptides. These results suggest the possibility that control of E2F may be a downstream event of TGF-β action. Consistent with that possibility is the observation that E2F1 RNA levels are drastically reduced in TGF-β-treated cells. We have also used a recombinant adenovirus containing the human E2F1 gene to overexpress the E2F1 product in mink lung epithelial cells that were growth arrested with TGF-β. We find that overexpression of E2F1 can overcome the TGF-β-mediated effect as measured by the activation of cellular DNA synthesis. These results suggest that a likely downstream target for the cyclin-dependent kinases, which are controlled by TGF-β, is the activation of E2F.**

The proliferation of eukaryotic cells is tightly regulated through a delicate balance of positive and negative regulatory proteins that exert their effects during the first gap phase (G_1) of the cell cycle (1, 2). Through a combination of genetic and biochemical approaches, a number of genes have been identified that encode these regulatory proteins. Perhaps the most critical of the positive-acting genes are those encoding the G_1 cyclins, which control rate-determining steps in G_1 progression (1, 2). When quiescent cells are stimulated to reenter the cell cycle, these G_1 cyclins are induced to accumulate, thereby activating associated kinases. This leads to the phosphorylation of downstream targets and eventual entry into S phase. The activity of these G_1 cyclin kinases is modulated by a family of proteins including p21, p16, and p27 (3–10), which define a part of the negative control of G_1. In addition, the p21 gene is regulated by the p53 tumor suppressor (11), another negative regulator of cell growth.

The initiation and control of this regulatory circuit is mediated by both positive- and negative-acting extracellular factors that interact with specific cell surface receptors. One example of a negative-acting factor is type β transforming growth factor (TGF-β), a multifunctional cytokine that inhibits the proliferation of a wide variety of cell types (12). Mink lung epithelial cells (Mv1Lu) are highly responsive to the antiproliferative effects of TGF-β and, as such, these cells have been used as a model system in which to study the mechanisms of TGF-β-dependent growth inhibition. In mink lung cells, TGF-β inhibits cellular proliferation largely through its ability to downregulate the activity of the cyclin-dependent kinases cdk2 and cdk4 (13, 14). TGF-β inactivates cdk4 directly by decreasing the level of cdk4 expression and constitutive expression of cdk4 renders Mv1Lu cells unresponsive to the effects of TGF-β (13). In contrast, TGF-β does not affect the levels of cdk2 (13, 14) but downregulates cdk2 activity (14) by recruiting p27 (10), a protein that is able to bind to and inactivate cyclin E–cdk2 complexes.

At least one of the targets for G_1 cyclin-dependent kinase activity is the retinoblastoma tumor suppressor gene product Rb (1), yet another negative regulator of cell growth. The phosphorylation of Rb by the G_1 cyclin-dependent kinases appears to abolish its growth regulatory activity. TGF-β-dependent growth inhibition of Mv1Lu cells also correlates with the accumulation of hypophosphorylated Rb (15). In addition, expression of simian virus 40 (SV40) T antigen, which binds and inactivates the hypophosphorylated form of Rb, reverses the TGF-β-mediated arrest of Mv1Lu cell growth (15).

A variety of studies have now provided strong evidence implicating the E2F transcription factor as a relevant downstream target for control by Rb and thus the overall pathway that controls G_1 progression. The interaction of Rb with E2F inhibits the transcriptional activating capacity of E2F (16–18). Since E2F appears to control the transcription of a group of S-phase genes such as dihydrofolate reductase, DNA polymerase α, and thymidine kinase, the control of E2F by Rb or Rb family members can be seen as a control of S-phase entry.

To further explore the role of E2F as a potential downstream target of pathways that may be controlled by TGF-β, we have tested whether overexpression of E2F could reverse a TGF-β-mediated growth arrest in Mv1Lu cells. To this end, we constructed a recombinant adenovirus containing the human E2F1 gene, which encodes one of the polypeptides responsible for E2F activity. We have used this vector to infect cells treated with TGF-β and we find that E2F1 expression can indeed overcome a TGF-β-mediated inhibition of S-phase entry.

MATERIALS AND METHODS

Cell Culture. Mink lung epithelial cells (Mv1Lu) and 293 cells were obtained from the ATCC. REF-52 cells were obtained from Keith Burridge (University of North Carolina). Mink lung epithelial cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing nonessential amino acids together with 10% fetal bovine serum.

Generation of Recombinant Adenoviruses. The overlap recombination procedure was used to introduce the E2F1 gene, under the control of a cytomegalovirus (CMV) promoter, into the adenovirus genome (19). The E2F1 cDNA was

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Abbreviations: TGF-β, type β transforming growth factor; SV40, simian virus 40; CMV, cytomegalovirus.
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cloned into the *Bam*HI site of the adenovirus recombination plasmid pGEM-CMV. This places the cDNA downstream from a CMV promoter. Downstream of the cDNA are sequences containing a SV40 splice donor/acceptor and a SV40 polyadenylylation site. Flanking the inserted sequences are adenovirus sequence from genome positions 1–194 and 3325–5788, nucleotides that provide the adenovirus left terminus and sequences for overlap recombination. The plasmid contains a unique *Age* I site that is used for linearization. DNA was prepared from the adenovirus type 5 *in340* (20) and digested to completion with *Xba* I and *Cla* I, each of which cuts the viral DNA only once. This digested DNA was then transfected into 293 cells along with a linearized plasmid containing the E2F1 construct. An identical construct that did not contain the cDNA sequence was processed in parallel. Five days after transfection, lysates were prepared and potential recombinant viruses were isolated by plaque assays on 293 cells. After plaque purification, recombinants were detected by isolation of viral DNA and analyzed by restriction digestion and Southern hybridization. Viral stocks were obtained by infection of monolayer cultures of 293 cells and crude cellular lysates were prepared by repeated cycles of freezing and thawing. Titers of viral stocks were determined by plaque assay on 293 cells. After plaque purification, recombinants were detected by restriction digestion and Southern hybridization. The E2F1 recombinant virus was termed AdE2F1 and the control virus containing only the vector and no cDNA insert was termed MbAd2.

Virus Infections. All infections were performed at an input multiplicity of 100 plaque-forming units/cell. Cells were infected in medium containing reduced serum (0.2%) for 2 hr at 37°C. For infection of TGF-β-treated cells, TGF-β was maintained in the medium during the infection procedure. The $AdE1A_{12S}$ virus contains the 12S cDNA in place of E1A genomic sequences (21) and was kindly provided by E. Moran.

Western Blot Assays for E2F1. Mv1Lu cells were treated with TGF-β and infected with the MbAd2 control virus or the AdE2F1 virus. Whole cell lysates were prepared from the infected cells at various times after infection and 50 μg of protein was loaded onto a SDS/10% polyacrylamide gel. Separated proteins were transferred to nitrocellulose and probed with an E2F1-specific monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1000 dilution. Secondary antibody detection was performed by the ECL protocol (Amersham).

Northern Analysis of E2F1 mRNA. Total RNA was isolated by the RNazol method (Biotecx Laboratories, Houston) from mink lung epithelial cells, which were either treated with 100 pM TGF-β2 for 24 hr or untreated. Poly(A)-containing RNA was isolated by protocols provided by Promega. The samples were run on a gel and transferred to GeneScreen*Plus*. Methylene blue staining of the blot confirmed that equal amounts of RNA were loaded in each lane. The blot was probed with ^{32}P-labeled random-primed human E2F1 cDNA probe.

TGF-β Growth Arrest Assay. Mink lung epithelial cells were placed into 12-well plates at a density of 2×10^4 cells per well, grown overnight, and then placed in DMEM containing 0.2% fetal bovine serum. Cells were incubated for 24 hr in 100 pM TGF-β2 and either infected with the adenovirus vectors or washed and placed into DMEM containing 10% fetal bovine serum. Infected cells were continually incubated in the presence of TGF-β2. Cultures were grown for 22, 26, or 30 hr and during the last 2 hr were labeled with [^{3}H]thymidine (4 μCi/ml; 1 Ci = 37 GBq). Cells were lysed and the amount of [^{3}H]thymidine incorporation into the DNA was quantitated with a scintillation counter. For BrdUrd assays, cells were labeled with 10 μM BrdUrd for the indicated time period, fixed, and then stained with BrdUrd-specific antibody as described (22).

CsCl Density Gradient Analysis of Viral and Cellular DNA. Adenoviral and cellular DNA were separated by buoyant density gradient centrifugation as described (23). Mv1Lu cells were treated with TGF-β for 24 hr. As in other experiments, the cells were either washed free of TGF-β and the medium was replaced with medium containing 10% serum or the cells were infected. At 20 hr postinfection, the cells were labeled with 2 μCi of [^{3}H]thymidine per ml for 4 hr. The cells were then washed and total DNA was prepared by SDS/proteinase K digestion as described. A sample of each lysate (0.5 ml) was mixed with 9.5 ml of a CsCl solution of density 1.70348 g/ml in 10 mM Tris·HCl, pH 8.5/1 mM EDTA/0.1% sarcosyl and centrifuged at 18°C for 15 hr at 55,000 rpm in a VTi 65 rotor. In addition, 5×10^5 cpm of ^{32}P-labeled adenovirus DNA (labeled by nick-translation) was added to each sample prior to centrifugation as an internal marker. Fractions of 0.2 ml were collected and 50 μl of each was trichloroacetic acid precipitated and assayed in a liquid scintillation counter.

RESULTS

E2F1 mRNA Levels Decline After TGF-β Treatment of Mink Lung Epithelial Cells. Previous experiments have shown that the E2F1 mRNA accumulates during G_1 in response to growth stimulation (24). E2F1 RNA is nearly undetectable in quiescent cells or in early G_1 and then increases as cells progress through G_1. Given the past observations of a TGF-β-mediated inhibition in G_1, together with the G_1 control of E2F1, we have assayed for an effect on E2F1 gene expression upon treatment with TGF-β as a starting point in assessing the relationship between the action of TGF-β and the control of E2F. E2F1 RNA expression was measured by Northern analysis of poly(A)-containing mRNA prepared from TGF-β-treated and untreated mink lung epithelial cells. As shown in Fig. 1, E2F1 RNA levels were markedly reduced in TGF-β-treated cells, consistent with the fact that TGF-β causes an early G_1 arrest. This is also consistent with recent data demonstrating that the E2F1 promoter can be activated by G_1 cyclin kinase activities (22) and that these kinase activities are downregulated in TGF-β-treated cells (13, 14, 25, 26).

Construction of an Adenovirus E2F1 Expression Vector. To achieve efficient expression of the E2F1 gene product in quiescent cells, we constructed an adenovirus expression vector in which the adenovirus E1 region, including the E1A and E1B genes, was replaced by the human E2F1 cDNA (27–29) under the control of the CMV immediate-early promoter (Fig. 2*A*). As shown in Fig. 2*B*, large amounts of the E2F1 protein are expressed in Mv1Lu cells after infection with the recombinant virus AdE2F1 in contrast to the infection of cells with a virus lacking the E2F1 insert, MbAd2.

Our recent experiments have shown that E2F1 overexpression can induce quiescent cells to enter S phase (22). We tested the ability of the virus to induce S phase in quiescent REF-52 cells. REF-52 cells were incubated in 0.2% serum for 48 hr and then either mock infected, infected with the E2F1 expression

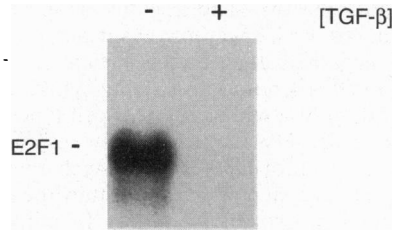

FIG. 1. Effect of TGF-β treatment on E2F1 RNA levels. Poly(A)-containing RNA was prepared from untreated (lane −) Mv1Lu cells or cells treated with 100 pM TGF-β2 for 24 hr (lane +). RNAs were analyzed by Northern blot followed by hybridization with a ^{32}P-labeled E2F1 cDNA.

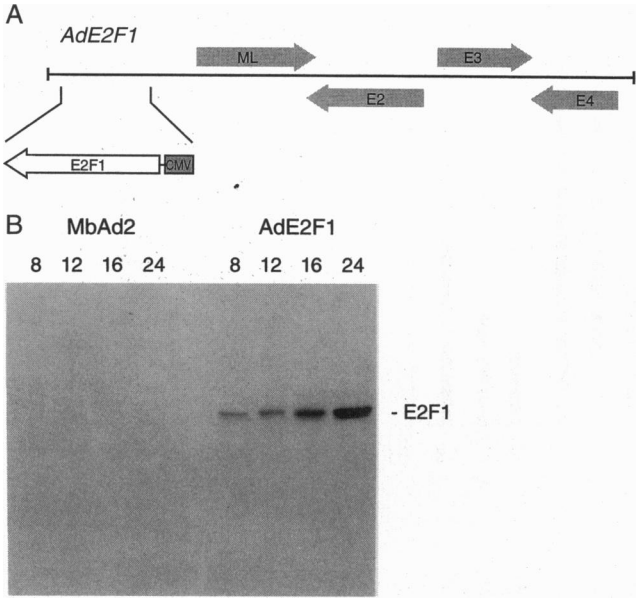

FIG. 2. Overexpression of E2F1 from an E2F1 recombinant adenovirus. (*A*) Structure of the AdE2F1 recombinant adenovirus. Adenovirus sequences from positions 191–3325 have been replaced with a CMV expression cassette containing the E2F1 cDNA. (*B*) E2F1 protein accumulation after infection with the AdE2F1 recombinant virus. Mv1Lu cells were infected with the AdE2F1 recombinant virus or the MbAd2 vector virus. Extracts were prepared at the indicated time, proteins were separated by SDS gel electrophoresis, a Western blot was prepared, and then the E2F1 polypeptide was detected with a specific antibody.

virus, or, as a control, infected with an $E1A_{12S}$-expressing virus. BrdUrd was added to the medium after infection and the cells were maintained in 0.2% serum for 30 hr. The cells were then fixed and stained for BrdUrd incorporation. As shown in Fig. 3, ≈25% of the cells infected with the AdE2F1 vector were positive for BrdUrd incorporation, whereas infection with the control virus yields 96% positive cells. This efficiency is similar to that observed for the ability of a microinjected E2F1 cDNA

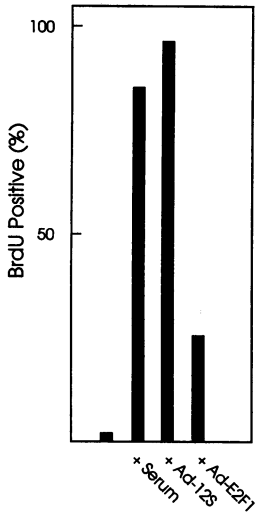

FIG. 3. Induction of DNA synthesis after infection of quiescent cells with the E2F1 recombinant virus. REF-52 cells were brought to quiescence by growth in 0.2% serum for 48 hr. The cells were then either mock infected, restimulated with serum, or infected with AdE1A_{12S} or AdE2F1 virus. After infection, BrdUrd was added to the medium and the cells were fixed and stained for BrdUrd incorporation at 30 hr after infection. Results are expressed as percentage nuclei positive for BrdUrd incorporation.

to induce S phase (22). Furthermore, mock-infected cells failed to become positive for BrdUrd incorporation. Thus, presumably as a consequence of overexpression of E2F1, this virus vector is able to induce the quiescent cells to enter S phase after infection and expression of E2F1.

TGF-β-Induced Cell Cycle Arrest Is Overcome by E2F1 Overexpression. Given the data that suggest that E2F activation is an important event of late G_1 progression such that E2F1 overexpression can induce S phase, as well as the data of Fig. 1 showing control of E2F1 RNA levels by TGF-β, we have determined whether E2F1 overexpression can overcome a TGF-β-induced cell cycle arrest. Two approaches to this general question were taken. First, we asked if E2F1 overexpression could prevent cells from leaving the cell cycle as a result of TGF-β treatment. In this experiment, mink lung epithelial cells were infected with AdE2F1 or the control virus MbAd2 and then treated with TGF-β for 36 hr. At this time, BrdUrd was added, incubation continued for another 12 hr, and then BrdUrd incorporation was measured by indirect immunofluorescence. As shown in Fig. 4*A*, TGF-β treatment resulted in a 10-fold reduction in BrdUrd-positive cells. Infection with the MbAd2 control virus did not alter this inhibition. In contrast, infection with the AdE2F1 virus, or an adenovirus that expresses the $E1A_{12S}$ product, prevented the inhibition.

The second approach asked whether E2F1 overexpression could overcome a TGF-β-mediated block of cell growth that had already taken place. Mink lung epithelial cells were arrested with TGF-β for 24 hr and then infected with AdE2F1 or MbAd2. In addition, cells were washed free of TGF-β and the cells were stimulated by adding 10% serum. In this experiment, induction of DNA synthesis was measured by [^{3}H]thymidine incorporation so as to use short pulse labels to obtain a kinetic measure of DNA synthesis. Thymidine incorporation was measured in 2-hr labeling periods beginning at 20, 24, and 28 hr after infection. As shown in Fig. 4*B*, which is the average for three independent experiments, cells that were infected with the control virus remained arrested with no evidence of DNA synthesis, whereas cells infected with the AdE2F1 virus exhibited a significant incorporation of [^{3}H]thymidine, based on the comparison to the level achieved by the addition of serum. Moreover, analysis of BrdUrd incorporation in a separate experiment revealed that >50% of the E2F1-expressing cells were replicating DNA as compared to 30% of the serum-stimulated cells (Fig. 4*C*).

A possible complication of using adenovirus vectors in these experiments is that viral DNA replication may be occurring, which would be measured as thymidine incorporation or BrdUrd incorporation. This is not likely given that wild-type adenoviruses replicate inefficiently in rodent cells and the fact that the vectors used lack E1A and E1B genes. Nevertheless, to investigate this possibility, [^{3}H]thymidine-labeled DNA was analyzed by CsCl density gradient centrifugation to separate viral DNA from cellular DNA. As shown in Fig. 5, only one peak of labeled DNA was observed with either the serum-stimulated sample or the sample from an infection with the AdE2F1 virus, and this peak is clearly separate from that of viral DNA that was added as an internal marker. We conclude that the overexpression of E2F1 encoded by the recombinant adenovirus does lead to the induction of cellular DNA synthesis, thus overcoming the TGF-β-mediated inhibition.

DISCUSSION

A variety of experiments suggest an important role for the E2F transcription factor in cell growth control and regulation of G_1 progression. These include the fact that the capacity of Rb to act as a growth suppressor is dependent on the ability to interact with and inhibit the function of E2F (30, 31). In addition, a Rb-mediated arrest of cells in G_1 can be overcome by coexpression of the E2F1 cDNA product (32). Finally, recent experiments have shown that expression of the E2F1

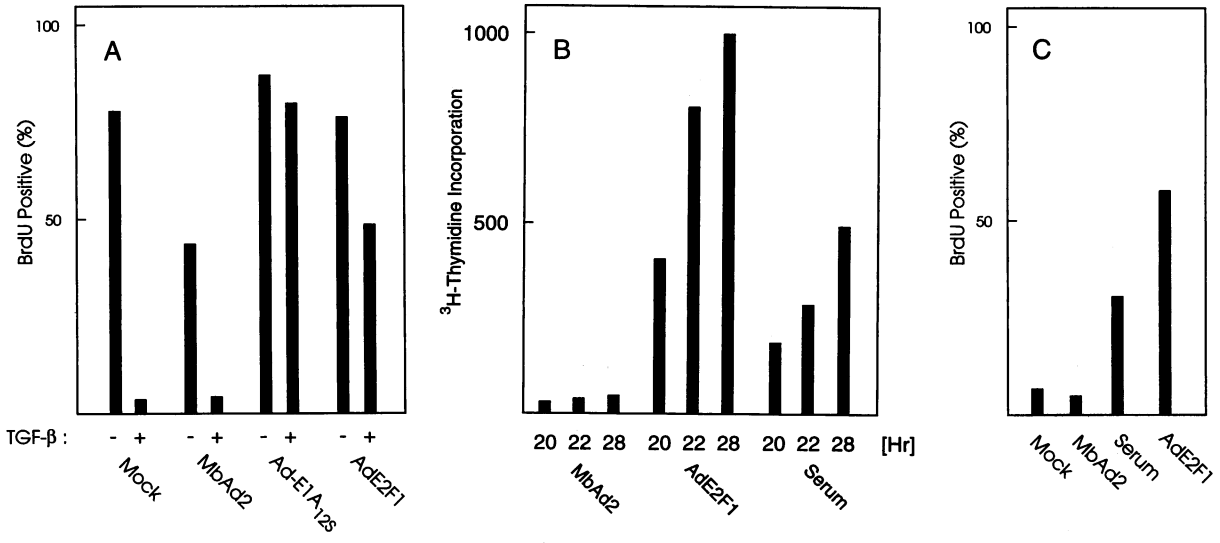

FIG. 4. Effect of E2F1 overexpression on TGF-β-mediated inhibition of cell growth. (*A*) E2F1 overexpression blocks TGF-β-mediated inhibition of cell growth. Asynchronously growing Mv1Lu cells were either uninfected or infected with the AdE2F1 virus, the Ad-E1A$_{12S}$ virus, or the MbAd2 control virus and then placed in medium containing 0.2% serum. Six hours after infection, cells were treated with TGF-β2 for 36 hr and then incubated with BrdUrd for 12 hr in the continued presence of TGF-β. BrdUrd incorporation was measured by indirect immunofluorescence and is expressed as percentage of nuclei positive for BrdUrd incorporation. (*B*) E2F1 overexpression overcomes a TGF-β-mediated growth arrest. Mv1Lu cells were treated with TGF-β2 for 24 hr. At this time there is a >90% growth inhibition as measured by [^{3}H]thymidine incorporation. Cells were then either washed and placed in medium containing 10% serum or infected with virus in the continued presence of TGF-β. Incorporation of [^{3}H]thymidine into DNA was assayed at the indicated times. (*C*) BrdUrd assays. Mv1Lu cells were treated as in *B*. BrdUrd was added to the medium at 16 hr postinfection and cells were harvested at 24 hr. BrdUrd incorporation was measured by indirect immunofluorescence.

cDNA product prevents quiescence upon serum deprivation and expression of E2F1 in quiescent cells stimulates S phase (22). These findings, taken together with additional recent studies of G_1 regulatory activities, implicate E2F as a critical downstream target of G_1 control events that lead to the induction of cellular DNA replication. In particular, previous work has shown that the Rb protein is regulated by phosphorylation and that this is likely mediated by various G_1 cyclin–cdk kinase complexes (33–36). Since the phosphorylation of Rb, and perhaps other Rb family members, affects its ability to bind to and regulate E2F (34, 37, 38), the control of E2F activity could be viewed as an event that is downstream from the action of G_1 cyclin-dependent kinases. The growth-suppressing effects of TGF-β coincide with the downregulation of G_1 kinase activities and the concomitant conversion of Rb to the underphosphorylated, growth-suppressive form. Therefore, the control of E2F activity may be an important target of the TGF-β growth-inhibitory signal. The results we present here support this model since the TGF-β-mediated growth arrest can be overcome by expression of the E2F1 product.

The mechanism by which E2F1 overexpression overcomes TGF-β suppression of cell growth may be complex. In principle, the overproduction of E2F activity could simply overwhelm the ability of Rb and Rb family members to control E2F, regardless of the state of phosphorylation of Rb. It is also possible that the TGF-β-mediated inhibition of G_1 cyclin-dependent kinase activity, and thus inhibition of Rb phos-

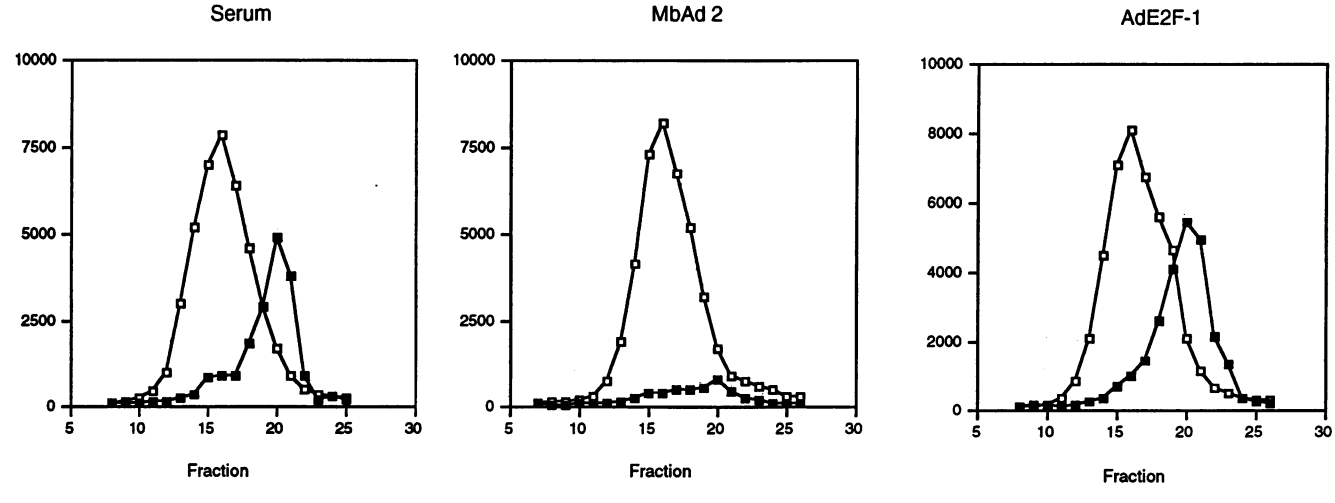

FIG. 5. CsCl density gradient analysis of labeled DNA. Conditions of the experiment were the same as described in Fig. 4*B*. Cells were blocked with TGF-β for 24 hr and then either washed free of the TGF-β or infected. At 20 hr postinfection, the cells were pulsed with [^{3}H]thymidine for 4 hr. Total DNA was prepared from the cells and analyzed by CsCl density gradient sedimentation. Adenovirus DNA, labeled *in vitro* with ^{32}P, was added to each sample as an internal marker for viral DNA. Fractions were collected from the gradient, trichloroacetic acid precipitated, and assayed in a liquid scintillation counter. For each gradient, solid squares indicate ^{3}H cpm and open squares indicate ^{32}P cpm.

phorylation, may be partly overcome in the cells expressing E2F1 since we do observe an increase in Rb phosphorylation in E2F1-expressing cells (data not shown). Although this may simply reflect the movement of these cells toward S phase, it is also possible that this may be the consequence of an E2F1-mediated activation of transcription of G_1 cyclin genes since recent experiments indicate the presence of functional E2F sites in both the cyclin D1 and cyclin E promoters (K. Ohtani, D. G. Johnson, and J.R.N., unpublished data). If there is an activation of synthesis of endogenous G_1 cyclins, it is possible that this accumulation would overcome the action of inhibitors induced by TGF-β, thus resulting in an increase in kinase activity. Finally, it is also likely that expression of the E2F1 product from the adenovirus recombinant leads to increased synthesis of the endogenous E2F1 product since recent experiments also indicate that the E2F1 promoter is autoregulated (22). Thus, the consequence of expression of the E2F1 gene from the adenovirus vector may be complex, initiating a series of events ultimately leading to increased overall levels of E2F and activation of S phase.

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