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Early Gene Responses to Transforming Growth Factor-β in Cells Lacking Growth-Suppressive RB Function

ALEJANDRO ZENTELLA,[1] FRANCES M. B. WEIS,[1] DAVID A. RALPH,[2] MARIKKI LAIHO,[2] AND JOAN MASSAGUÉ[1]*

Howard Hughes Medical Institute[1] and Cell Biology and Genetics Program,[2] Memorial Sloan-Kettering Cancer Center, New York, New York 10021

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The growth-suppressive function of the retinoblastoma susceptibility gene product, RB, has been implicated in the mediation of growth inhibition and negative regulation of certain proliferation related genes by transforming growth factor-β1 (TGF-β1). Early gene responses to TGF-β1 were examined in order to determine their dependence on the cell cycle and on the growth-suppressive function of RB. TGF-β1, which rapidly elevates the steady-state level of *junB* and PAI-1 mRNAs and decreases that of c-*myc* mRNA, induces these responses in S-phase populations of Mv1Lu lung epithelial cells containing RB in a phosphorylated state. Since in this state RB is presumed to lack growth-suppressive activity, the response to TGF-β1 was also examined in DU145 human prostate carcinoma cells whose mutant RB product lacks growth-suppressive function. In these cells, TGF-β1 also decreases c-*myc* expression at the transcription initiation level. These results suggests that the c-*myc*, *junB*, and PAI-1 responses to TGF-β1 are not restricted to the G_1 phase of the cell cycle and that down-regulation of c-*myc* expression by TGF-β1 can occur through a mechanism independent from the growth-suppressive function of RB.

Transforming growth factor-β (TGF-β), a paracrine polypeptide that exists in various isoforms (36, 45), can inhibit the growth of epithelial cells by arresting their proliferative cycle in late G_1 phase (22, 27, 39, 44). Entry of Mv1Lu mink lung epithelial cells and MK mouse keratinocytes into S phase can be prevented by addition of the TGF-β isoform TGF-β1 during early or middle G_1 but not by adding TGF-β1 during the late stages of G_1 (22, 39, 44). These observations and other evidence have linked mediation of growth inhibition by TGF-β to its ability to control the activity of the *RB* gene product in middle G_1 (27). *RB* is a growth suppressor gene whose loss of function, as a result of mutation or deletion, leads to oncogenic transformation (34) that can be reverted, at least in some instances, by reintroduction of normal *RB* alleles into the cell (6, 23, 52). The gene product, RB, is a nuclear phosphoprotein that is expressed throughout the cell cycle. Its phosphorylation level, however, oscillates in a cell cycle-dependent manner (8, 12, 14a, 20, 33). Underphosphorylated RB prevails in G_0 and during G_1. RB is rapidly phosphorylated as cells approach S phase and remains phosphorylated until M phase. The transforming protein of simian virus 40, T antigen, binds unphosphorylated RB (14a, 32) and other related cellular proteins (15, 17). This interaction is thought to perturb the growth-suppressive function of RB. These observations favor the hypothesis that the form of RB actively involved in growth suppression is the unphosphorylated form (14a, 32).

In Mv1Lu mink lung epithelial cells, TGF-β interferes with the events that lead to RB phosphorylation in late G_1, thus retaining RB in its presumed growth-suppressive state (27). TGF-β seems to act by preventing the appearance of a RB kinase in middle G_1 rather than by inactivating this kinase once it has appeared or by inducing dephosphorylation of RB. This conclusion is supported by the observation that if TGF-β is added during the last 2 h of G_1 or during S, when RB is already phosphorylated, TGF-β does not cause RB dephosphorylation and does not prevent phosphorylation of RB molecules newly synthesized during that period. TGF-β1 also inhibits RB phosphorylation in Mv1Lu cells expressing T antigen (27). In the presence of T antigen, however, TGF-β no longer inhibits cell growth, presumably because T antigen interferes with the growth-suppressive activity of RB or related proteins. Taken together, these observations have suggested that inhibition of RB phosphorylation by TGF-β is not a consequence of arrest in early G_1 but rather an event in the TGF-β response that may lead to cell cycle arrest in late G_1 (27).

One approach to establish causal links between the various effects of TGF-β has been to use cells that express T antigen or related proteins (27, 28, 44). Since T antigen and other DNA tumor virus transforming proteins bind RB and this is thought to perturb RB function (16, 32, 53), the ability of these proteins to inhibit specific effects of TGF-β could be interpreted as evidence that such responses lie downstream of RB in a pathway leading to growth inhibition. The growth-inhibitory effect of TGF-β is lost in Mv1Lu lung epithelial cells and MK mouse keratinocytes that express T antigen (27, 44). Certain effects of TGF-β in Mv1Lu cells, i.e., stimulation of *junB* and extracellular matrix gene expression, are not affected by the presence of T antigen, suggesting that these responses do not involve RB (28). However, down-regulation of c-*myc* transcription by TGF-β is lost in MK cells expressing T antigen, which led to the proposal that growth inhibition by TGF-β is caused by down-regulation of c-*myc* expression through a mechanism that involves the growth-suppressive function of RB or a related protein (44).

During studies on the ability of TGF-β to regulate expression of certain genes during the cell cycle, we observed that the response of c-*myc*, *junB*, and the plasminogen activator inhibitor-1 (PAI-1) gene to TGF-β in Mv1Lu cells was as intense in S phase as it was in G_1 phase. Since S-phase cell populations contained RB in the phosphorylated state,

* Corresponding author.

which is thought to be inactive as a growth suppressor, we examined TGF-β responsiveness in a human prostate carcinoma cell line that harbors a mutant *RB* gene defective in growth-suppressive function. The results show that certain early gene responses to TGF-β, including down-regulation of c-*myc* transcription, can occur through a mechanism that is independent of the growth-suppressive function of RB.

MATERIALS AND METHODS

Cell culture and transfections. The Mv1Lu mink lung cell line (CCL-64; American Type Culture Collection) was maintained in minimal essential medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 1× nonessential amino acids (GIBCO), penicillin (100 IU/ml), and streptomycin 100 mg/ml. DU145 human prostate carcinoma cells (HTB 81; American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum.

Porcine TGF-β1 (R&D Systems, Minneapolis, Minn.) was used in these studies at a final concentration of 100 pM.

mRNA assays. Total cellular RNA was isolated and, where indicated, enriched for poly(A)$^+$ RNA by chromatography over oligo(dT)-Sepharose as described previously (48). RNA was separated on 1% agarose gels containing formaldehyde and transferred to Duralon-UV membranes (Schleicher & Schuell). Specific mRNAs were detected by using as probes cDNAs corresponding to human *junB* (p465.20 [47]; obtained from D. Nathans), human c-*myc* (pcmycc41-3R/C [21]; obtained from R. Dalla-Favera), and human PAI-1 (PAI-1A1 [1]; obtained from P. Andreasen), all labeled with ^{32}P by random priming (Multiprime; Amersham). As a control, the filters were probed with ^{32}P-labeled glyceraldehydephosphate dehydrogenase (GAPDH) cDNA (pRGAPDH-13) (19). Hybridization signals were recorded on a phosphor screen for 12 h and scanned with a Phosphorimager 400-E (Molecular Dynamics, Sunnyvale, Calif.) set to detect ^{32}P radioactive emissions over a 5-order linear range of sensitivity. The hybridization signals were digitized and quantified with the Image Quant software (Molecular Dynamics). Background values were determined from equivalent surface areas near each hybridization signal and were subtracted from each value before normalization. The specific c-*myc*, *junB*, and PAI-1 mRNA values presented in the figures were normalized relative to the GAPDH signal value in the same lane.

Nuclear run-on transcription assay. DU145 cells were grown to 75% confluency in 150-mm-diameter tissue culture dishes. At various times after treatment with TGF-β1, nuclei for run-on transcriptions were prepared and frozen as described previously (3). In all experiments, the final concentration of TGF-β1 was 100 pM. Three dishes were harvested for each time point. Probes and protocols used for these experiments were similar or identical to those previously described (5, 9, 21). Briefly, a stock solution consisting of 25 mM Tris (pH 8.0), 12.5 mM $MgCl_2$, 325 mM KCl, 1.25 mM CTP, 1.25 mM ATP, 1.25 mM GTP, 2.5 mM dithiothreitol, and 200 U of RNasin per ml was made. A 60-μl sample of this stock was added to 220 μl of thawed nuclei; 20 μl of [α-^{32}P]UTP (3,000 Ci/mmol) was also added and gently mixed. The nuclei were incubated for 30 min at 30°C. This was sequentially followed by RNase-free DNase I digestion at 30°C for 10 min, proteinase K treatment in the presence of 0.5% sodium dodecyl sulfate, phenol-chloroform extraction, and precipitation with ammonium acetate and 2-propanol. The sequence of steps beginning with the DNase I digestion were repeated once. The resulting labeled RNA was hybridized to slot blots in a buffer containing 10 mM TES (pH 7.4) 0.2% SDS, 10 mM EDTA, 0.3 M NaCl, 1× Denhardt's solution, 0.5% dry milk, and 250 μg of *Escherichia coli* tRNA per ml. Hybridization was carried out at 65°C for 36 h. Blots were washed twice at room temperature, twice for 1 h each time at 65°C, and three times at 37°C for 30 to 60 min each time. All washes were with 2× SSC, and the first 37°C wash (always 30 min) also contained 2.0 μg of DNase-free RNase A per ml. All probes used in these experiments were cloned into M13 vectors and were single stranded. They corresponded to sequences from the human c-*myc* gene intron 1 and exons 1 and 2, in both orientations, to GAPDH and to vector sequences, respectively, as was previously described (21) and were the kind gift of R. Dalla-Favera (Columbia University).

Other assays. [^{125}I]iododeoxyuridine incorporation assays (27), RB protein Western immunoblot assays (27), bromodeoxyuridine (BUdR) labeling of replicating nuclei (27), TGF-β receptor affinity labeling assays (35), and metabolic labeling of proteins secreted into the culture medium (28) were done as previously described. The RB signal was quantified as described above for mRNA signals.

RESULTS

Early TGF-β1 response genes in Mv1Lu cells include *junB* (28) (Fig. 1), PAI-1 (28) (Fig. 1), and c-*myc* (Fig. 1). In exponentially growing cells, *junB* and PAI-1 expression increased, whereas c-*myc* expression decreased in response to TGF-β1. TGF-β1 elevated *junB* and PAI-1 mRNA levels at least 10-fold over the basal level, with the response being detectable by 20 to 40 min after TGF-β1 addition, maximal by 1.5 h (*junB*) or 2 h (PAI-1), and declining thereafter (Fig. 1). TGF-β1 decreased c-*myc* mRNA levels to about 50% relative to controls, with the response being maximal 1 h after TGF-β1 addition and sustained at this level for at least 4 h (Fig. 1).

To determine whether the ability of TGF-β1 to regulate expression of these genes was restricted to a particular period within G_1, as its ability to inhibit RB phosphorylation is, Mv1Lu cells were placed in G_0 by contact inhibition at a high cell density. Cells were then stimulated to enter the cell cycle by being replated at low density in the presence of 10% fetal bovine serum. Two parameters, RB phosphorylation and DNA synthesis, were measured in order to monitor the progression of these cultures through the G_1 and S phases. The relative level of phosphorylated RB was determined by Western immunoblot analysis using anti-RB antibodies, which detects the phosphorylated form(s) of RB as a band that migrates more slowly on electrophoresis gels than does underphosphorylated RB (15, 33). As previously shown (27), by 6 h after stimulation with serum, all of the RB was still in the fast, underphosphorylated form (Fig. 2). Conversion of RB to the slowly migrating form was nearly complete 18 h after stimulation with serum but did not take place if TGF-β1 was added at the time of cell release from G_0 (Fig. 2). Entry into S phase was monitored by assaying the incorporation of the nucleotide analog BUdR into replicating nuclei, using anti-BUdR indirect immunofluorescence (27). Entry into S phase lagged 3 h behind RB phosphorylation and was evident in most cells 21 h after stimulation with serum (Fig. 2).

The ability of TGF-β1 to regulate c-*myc*, *junB*, and PAI-1 mRNA levels in these cultures throughout G_1 and S phases was determined by adding TGF-β1 for 90 min at different

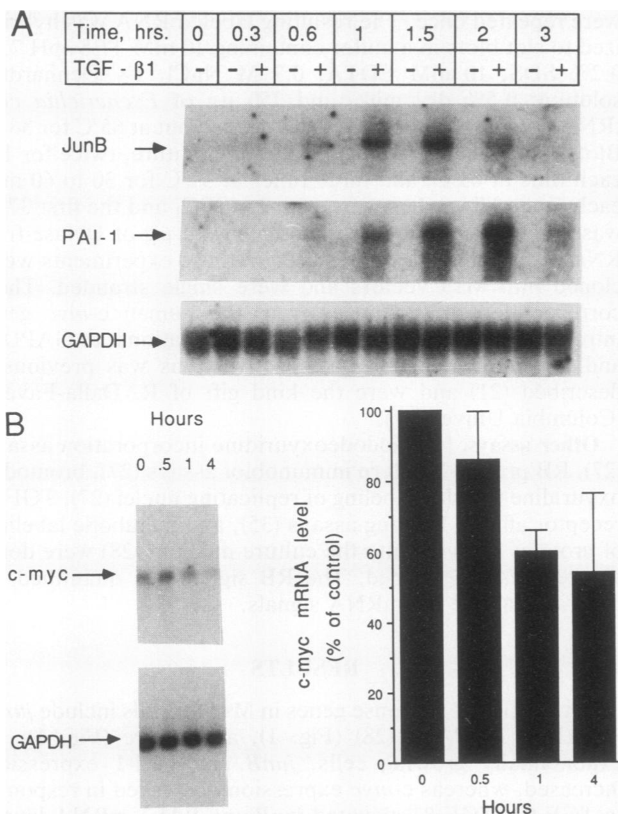

FIG. 1. Early TGF-β response genes in exponentially growing Mv1Lu cell cultures. (A) Exponentially growing cultures received 100 pM TGF-β1 (+) or no TGF-β1 (−). Total RNA was obtained at the indicated times after this addition and was assayed for *junB*, PAI-1, and GAPDH mRNA levels by Northern (RNA) blot analysis. (B) Poly(A)$^+$ RNA was obtained from exponentially growing cultures that had been exposed to 100 pM TGF-β1 for the indicated times and was used to determine the level of c-*myc* mRNA. The plot illustrates the c-*myc* mRNA level relative to the level of cells which did not receive TGF-β. The GAPDH mRNA level in each sample was used as an internal control to normalize the c-*myc* mRNA value. Data are averages ± standard deviations of two separate experiments.

times after cells were released from G_0. TGF-β1 increased *junB* and PAI-1 mRNA levels and decreased c-*myc* mRNA levels regardless of the time at which it was added to the cultures (Fig. 2). The effect of TGF-β1 on the expression of these three genes was as strong in S-phase cells as it was in G_1-phase cells. Quantitation of the c-*myc* mRNA signal after 90 min with TGF-β1 showed that the decrease in c-*myc* mRNA level in response to TGF-β was stronger during late G_1 phase and S phase that it was during early G_1 phase (Fig. 2B). During late G_1 and S phases, TGF-β1 decreased the c-*myc* mRNA level signal by approximately 70% relative to cells that did not receive TGF-β1 (Fig. 2B). The basal c-*myc* mRNA level in Mv1Lu cells was transiently elevated in response to serum as is common in quiescent cultures of many other cell types (46). Serum did not affect the basal level of *junB* or PAI-1 mRNA under these conditions (Fig. 2C).

These results indicated that TGF-β can regulate the expression of *junB*, PAI-1, and c-*myc* irrespective of the level of RB phosphorylation present in the cells. Since RB is thought to be growth suppressive only in the underphosphor-

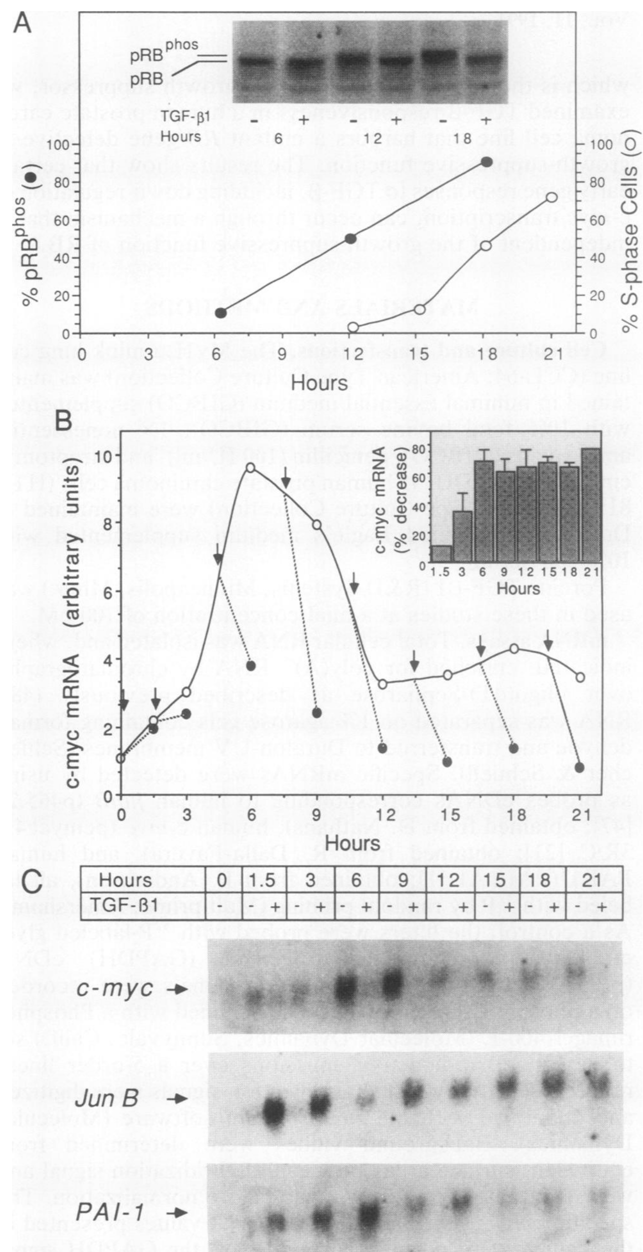

FIG. 2. Early gene responses to TGF-β1 in G_1 and S phase Mv1Lu cell cultures. Density-arrested Mv1Lu cell cultures were released from contact inhibition by replating at low density in the presence of serum-containing medium. (A) Kinetics of RB phosphorylation and entry into S phase after release of these cultures from contact inhibition, determined, respectively, by Western blot analysis, which detects phosphorylated RB (pRB^{phos}) as a slowly migrating band (inset), and by BUdR incorporation into nuclei, as described in the text. Samples of cells that received TGF-β1 at the time of release from contact inhibition were included in the RB assays to facilitate identification of the slower-migrating species. Plotted are the proportion of RB protein migrating in the slow position and the proportion of nuclei with positive BUdR immunostaining at the indicated times after release from contact inhibition. (B and C) Levels of c-*myc*, *junB*, and PAI-1 mRNAs, determined by Northern blot analysis of poly(A)$^+$ RNA (C) at the indicated times after release from contact inhibition. Some cultures (+ in panel C) received 100 pM TGF-β1 90 min prior to the assay. The relative c-*myc* mRNA level in the blots was quantified to illustrate the transient increase due to serum (B) and the effect of TGF-β1 added at the indicated times (arrows in B). The relative decrease in c-*myc* mRNA levels caused by TGF-β1 at each time point is presented in the inset. Data are the averages ± standard deviations of two separate experiments.

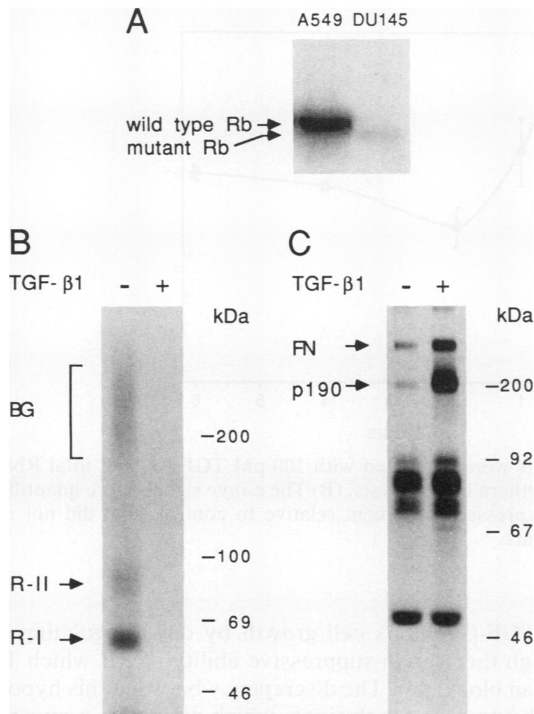

FIG. 3. RB protein and TGF-β receptors in DU145 human prostate carcinoma cells. (A) Western immunoblot of RB protein from two human cell lines, one of which (DU145 cells) expresses a faster-migrating form of RB as the result of a deletion in the *RB* gene (6). (B) Assay in which cell surface proteins in DU145 cells were affinity labeled by cross-linking with ^{125}I-TGF-β1 alone (−) or in the presence of an excess native TGF-β1 (+). Samples were electrophoresed, and the labeled products were detected by autoradiography of the gels. The specifically labeled bands correspond to TGF-β receptors I (R-I) and II (R-II), and beta-glycan (BG), respectively. (C) DU145 responsiveness to TGF-β, determined by an increase in the levels of newly synthesized fibronectin (FN) and an unknown 190-kDa secretory protein (p190). Cells were incubated for 24 h in the presence or absence of 250 pM TGF-β1 in medium that contained [^{35}S]methionine during the last 4 h of incubation. Samples from the radiolabeled medium were subjected to electrophoresis and fluorography to display newly synthesized secretory products.

ylated state (14a, 32), the preceding results raised the possibility that negative regulation of c-*myc* and activation of *junB* and PAI-1 by TGF-β1 might be independent from the growth-suppressive function of RB. To further examine this possibility, we studied the TGF-β1 response in a human tumor cell line, DU145, that lacks growth-suppressive RB function as the result of a defect in the *RB* gene. This human prostate carcinoma cell line has a full complement of TGF-β receptors, as determined by affinity labeling using ^{125}I-TGF-β1 and the cross-linking agent disuccinimidyl suberate. The experiment readily detected TGF-β receptors I and II and beta-glycan in DU145 cells (Fig. 3B). Receptors I and II are TGF-β-binding membrane glycoproteins of 53 and 75 kDa, respectively. As determined with a panel of TGF-β receptor-defective Mv1Lu cell clones, these receptors are required to mediate TGF-β responses, including inhibition of RB phosphorylation, inhibition of cell growth, and elevation of extracellular matrix protein expression (7, 29, 30). Beta-glycan is a membrane proteoglycan that binds TGF-β via its 110-kDa core protein and may function as a regulator of TGF-β availability to the cell (2, 11, 49).

We determined that the TGF-β receptors in DU145 cells were functional by examining the expression of major secretory components of the extracellular matrix which TGF-β elevates in many other cells (36). When exposed to TGF-β1, DU145 cells increased the production of secretory proteins of 190 and 210 kDa (Fig. 3C). The 210-kDa protein is fibronectin, as determined by immunoprecipitation with an anti-human fibronectin antibody (data not shown). The identity of the 190-kDa protein is unknown, but it might correspond to thrombospondin, which is a 190-kDa secretory protein whose expression is strongly increased by TGF-β in various cell lines (28, 41). In contrast to previous observations (54), DU145 cells were not growth inhibited by up to 0.5 nM TGF-β1 or TGF-β2 (data not shown).

DU145 cells contain a deletion of exon 21 in the *RB* gene (6, 24). This defective *RB* allele directs the synthesis of a 100-kDa protein smaller than the product of the normal *RB* allele (6) (Fig. 3A) and unable to bind T antigen or undergo cell cycle-dependent phosphorylation (6). Transfection of a normal *RB* allele into DU145 cells either restores a nontumorigenic phenotype (23) or causes growth arrest (14). Therefore, DU145 cells provided an appropriate system to determine whether TGF-β1 could down-regulate c-*myc* expression in the absence of growth-suppressive RB function.

Addition of TGF-β1 to exponentially growing DU145 cells decreased the steady-state level of c-*myc* mRNA (Fig. 4). After 2 h with TGF-β1, this level was approximately 50% of the level in cells that did not receive TGF-β1 and remained low for at least 6 h after TGF-β1 addition. This effect was similar to the effect on c-*myc* mRNA levels in Mv1Lu cells (Fig. 1B). Furthermore, nuclear run-on transcription assays indicated that TGF-β1 decreased c-*myc* expression in DU145 cells by repressing c-*myc* transcription (Fig. 5). cDNA probes complementary to sequences from exons 1 and 2 and intron 1 of the human c-*myc* gene (21) were used in these assays to determine whether TGF-β1 affected c-*myc* transcript initiation or elongation. Using this assay system, maximal inhibition of c-*myc* transcription was observed in cells exposed to TGF-β1 for 2 h. The run-on transcription signals obtained from cells at this time point were less than 20% of the signal obtained from untreated cells with each of the c-*myc* probes (Fig. 5). The effect of TGF-β1 on c-*myc* transcription slowly declined after 2 h, closely matching the kinetics of decrease of c-*myc* mRNA levels in response to TGF-β1. Taken together, these results indicate that DU145 cells lacking growth-suppressive RB function can respond to TGF-β with a decrease in steady-state c-*myc* mRNA level due to a lower c-*myc* transcription initiation rate.

DISCUSSION

This study was conducted, in part, to determine whether early gene responses to TGF-β1 are restricted to the G_1 phase of the cell cycle. Mv1Lu cells and other cell types are susceptible to growth inhibition by TGF-β1 only during G_1 phase (22, 27). Therefore, it was of interest to determine whether the ability of TGF-β1 to regulate expression of growth-related genes was also restricted to the G_1 phase. The present results show that it is not. The genes examined include the nuclear factors *junB* and c-*myc* as well as PAI-1, a secretory inhibitor of extracellular matrix proteolysis. TGF-β1 rapidly elevates *junB* and PAI-1 expression and down-regulates c-*myc* expression in Mv1Lu cells. These effects have been observed in other cell lines (13, 18, 28, 31, 42, 43, 51) and do not always correlate with growth-inhibi-

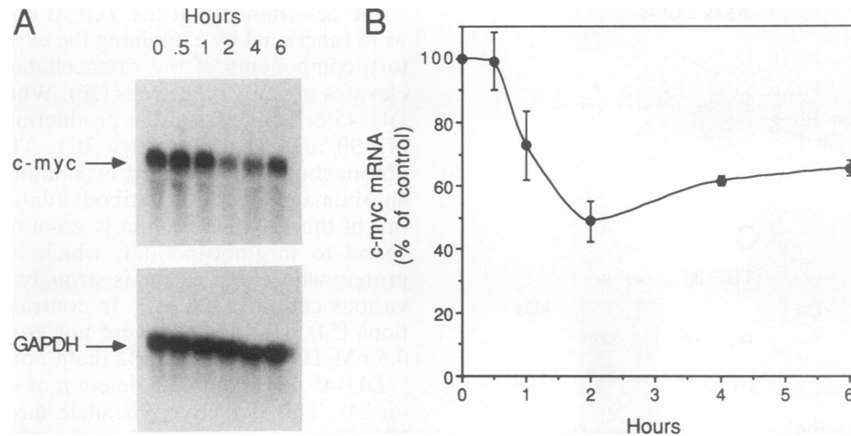

FIG. 4. Effect of TGF-β1 on c-*myc* mRNA levels in DU145 cells. (A) Cells were incubated with 100 pM TGF-β1, and total RNA was obtained at the indicated times. The c-*myc* mRNA level was determined by Northern blot analysis. (B) The c-*myc* signals were quantified and normalized against GAPDH mRNA signals from the same blot. They are expressed as percent relative to controls that did not receive TGF-β1. Data are averages ± standard deviations of three separate experiments.

tory response to TGF-β1 (10, 42, 50). Acute changes in the expression of these genes in Mv1Lu cells occur irrespective of whether TGF-β1 is added to cells during G_1 or S phase.

An important objective of this study was to determine whether the growth-suppressive function of RB was required for mediation of early gene responses to TGF-β1. Evidence provided by previous studies suggests that the mechanism through which TGF-β arrests cell cycle may involve RB (27, 44). Specifically, TGF-β treatment precludes phosphorylation of RB during late G_1 phase in Mv1Lu cells, thus retaining RB in a presumed growth-suppressive state (27). RB has also been implicated in the rapid down-regulation of c-*myc* expression caused by TGF-β1 in mouse keratinocytes (44). The latter hypothesis is based on the observation that expression of viral oncoproteins that bind RB interferes with down-regulation of c-*myc* expression by TGF-β1 (44). If these oncoproteins interfered with c-*myc* regulation by inactivating the growth-suppressive function of RB, it might be expected that inactivation of this RB function by other means should also lead to a loss of c-*myc* response to TGF-β1. The results of experiments with S-phase Mv1Lu cells discussed above are relevant here too. S-phase Mv1Lu cells contain essentially all of their RB protein in a highly phosphorylated state. Since evidence suggests that RB is inactive as a growth suppressor in this highly phosphorylated state (14a, 32, 52), these results raised the possibility that the presumptive growth-suppressive form of RB might not be required for negative regulation of c-*myc* expression by TGF-β1.

This possibility was tested with DU145 human prostate carcinoma cells which lack growth-suppressive RB function due to a deletion of exon 21 in the *RB* gene (6, 22). DU145 cells respond with down-regulation of c-*myc* expression. Furthermore, this effect is due to inhibition of c-*myc* transcription initiation by TGF-β1. Thus, the growth-suppressive function of RB, which appears to mediate cell cycle arrest by TGF-β1 in certain cells, is not required for TGF-β1 inhibition of c-*myc* expression in DU145 cells.

Resistance of c-*myc* expression to down-regulation by TGF-β1 in MK cells that express T antigen or other viral oncoproteins requires the integrity of the RB-binding site in these oncoproteins (44). This observation led to the proposal that TGF-β inhibits cell growth by down-regulating c-*myc* through the growth-suppressive ability of RB which T antigen can block (44). The discrepancy between this hypothesis and the present conclusions, which dissociate c-*myc* regulation from growth-suppressive RB function, might be resolved by the possibility that mutant RB in DU145 cells can still down-regulate c-*myc* expression even though it lacks growth-suppressive activity. So far, there is no evidence in support of this possibility. In a recent report (25), a transfected RB expression vector caused an increase in the transcriptional activity of a cotransfected c-*myc* promoter in Mv1Lu cells and a decrease in other cell lines, which points to important cell-specific differences and the complex nature of any mechanism that involves RB as a mediator of c-*myc* down-regulation by TGF-β1. Alternatively, other T-binding cellular proteins (15, 17, 38) might be involved in mediating the c-*myc* response to TGF-β1. It is also possible that loss of c-*myc* response to TGF-β1 in certain T-transfected cell lines is secondary to cell transformation by this oncogene.

An additional point made by the present studies is that *RB*-defective human tumor cells can express TGF-β1 receptors. Various human retinoblastoma cell lines lack detectable TGF-β receptors I and II (26), absence of these receptors being very rare in other normal or tumor cells (37). This observation suggested that loss of growth-inhibitory response to TGF-β1 due to loss of TGF-β receptors might contribute to the escape of cells from normal growth control and lead to a progressively more tumorigenic phenotype (26). The presence of TGF-β receptors in DU145 cells shows that normal RB function is not required for expression of these receptors. Independent loss of TGF-β receptors in retinoblastoma cells might confer a further growth advantage to these cells via loss of the ability to be affected by TGF-β1 through other growth inhibitory mechanisms. Although RB appears important in the growth-inhibitory mechanism of TGF-β, TGF-β could also inhibit cell proliferation by controlling the activity of other growth-suppressive products (15, 17, 38) as well as by down-regulating mitogen receptors (4) or modulating cell adhesion receptors and extracellular matrix production in ways that constrain cell proliferation (40).

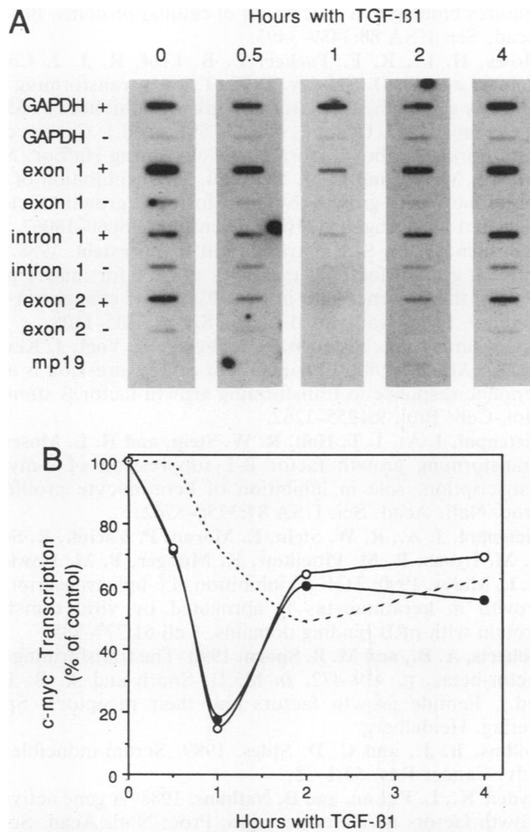

FIG. 5. Effect of TGF-β1 on c-*myc* transcription in DU145 cells. (A) Cells were incubated with 100 pM TGF-β1 for the indicated times, and nuclei from these cells were subjected to run-on transcription assays using single-stranded segments of intron 1 and exons 1 and 2 of the human c-*myc* gene in sense (+) and antisense (−) orientations (21), as described in the text. Single strands corresponding to the GAPDH gene were included in the assays for normalization purposes. The single-stranded cloning vector (mp19) was included to control for background signal. (B) The run-on transcription signal values obtained with the c-*myc* exon 1 (●) and exon 2 (○) sense strands were quantified and normalized against the GAPDH sense-strand signal and are plotted as percent relative to controls that did not receive TGF-β1. The effect of TGF-β1 on c-*myc* mRNA levels in the same batch of cells (from Fig. 4) is included in this plot (– – –) to facilitate comparison between the kinetics of both effects.

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