Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 12823–12827, December 1994
Biochemistry

Oncogenic capacity of the E2F1 gene

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Communicated by Gordon G. Hammes, August 17, 1994

ABSTRACT **Previous experiments have identified the E2F transcription factor as a potential downstream target for the action of cellular regulatory activities, such as the Rb tumor suppressor protein, that control cell growth and that, when altered, contribute to the development of human tumors. In light of these findings, we have assayed the ability of the E2F1 and DP1 genes, which encode heterodimeric partners that together create E2F activity, to act in an oncogenic fashion. We find that E2F1, particularly in combination with the DP1 product, cooperates with an activated *ras* oncogene to induce the formation of morphologically transformed foci in primary rat embryo fibroblast cultures. In addition, an E2F1 chimeric protein, in which sequences involved in Rb binding have been replaced with the herpesvirus VP16 activation domain, exhibits increased transformation activity. Cells transfected with E2F1 and DP1 or the E2F1-VP16 chimera form colonies in soft agar and induce tumor formation in nude mice. We conclude that deregulated E2F1 expression and function can have oncogenic consequences.**

It has become clear that the loss of Rb function contributes to the loss of cell growth control found in retinoblastoma as well as various other tumors (1). Considerable evidence implicates the E2F transcription factor as a critical target for Rb (2–5). The interaction of Rb with E2F correlates with the capacity of Rb to arrest cell growth in G_1 phase, as seen by the analysis of Rb mutants that have been assayed for growth suppression activity (6–9). Moreover, the ability of the viral oncoproteins E1A, simian virus 40 large tumor antigen, and human papillomavirus E7 to transform cells is dependent upon their ability to bind to Rb family members and release active E2F (2, 10, 11).

Phosphorylation of the Rb protein, likely mediated by the G1 cyclins and associated kinases (12–17), appears to inactivate Rb and allows progression through the cell cycle. Indeed, the PRAD1/Bcl1 oncogene, which is up-regulated in many parathyroid adenomas, breast carcinomas, and B-cell lymphomas, is a rearranged cyclin D1 gene (18). A consequence of Rb phosphorylation is the regulation of interaction with E2F since only the underphosphorylated form of Rb is active in E2F binding (2, 13). Thus, these G_1 cyclins may regulate E2F activity through their ability to regulate the activity of Rb and possibly other Rb family members.

Taken together, these observations suggest that the inactivation of Rb function results in the activation of E2F and is tightly associated with oncogenesis. It then follows that deregulated E2F gene activity may also have oncogenic results. We now find that the E2F1 gene (19–21) can be oncogenic in conjunction with an activated *ras* oncogene, suggesting that regulation of E2F activity by Rb may be critical in maintaining normal cellular growth control.

MATERIALS AND METHODS

Plasmids. Rous sarcoma virus (RSV) E2F1 was constructed by inserting the E2F1 cDNA (21) into the *Hin*dIII and *Xba* I sites of Rc/RSV (Invitrogen). RSV E2F1-VP16 was constructed using a *Bgl* II/*Xba* I DNA fragment encoding the transcriptional activation domain of VP16 (amino acids 413–490) (22), which was prepared from the plasmid pMSVP16 by the PCR using the oligonucleotides:

5′-GAGAGGAGATCTCCGCCCCCCGACCGATGTC-3′

5′-GCGCGCTCTAGACTACCCACCGTACTCGTCAAT-3′.

This VP16 *Bgl* II/*Xba* I fragment was then used to replace the normal activation domain of E2F1 by exchanging the VP16 fragment for a *Bgl* II/*Xba* I fragment in the pseudowild-type E2F1 construct pcDNA-E2F1$_{B358}$, which has been described (23). RSV E1A was constructed by inserting the E1A$_{12S}$ cDNA from cytomegalovirus (CMV) E1A$_{12S}$ (24) into Rc/RSV. pCMV HADP1 and the activated *ras* (T24) expression vector, HO6T1, have been described elsewhere (25, 26). The 4XE2 CAT reporter (27) and Rb and Rb(J82) expression plasmids (6) have been described elsewhere.

Transfections and Chloramphenicol Acetyltransferase (CAT) Assays. C-33A (Rb−/−) cells were maintained and transfected as described (23). CAT and β-galactosidase assays were done as described (23).

Gel Mobility Shift Assays. Gel shift assay conditions using extracts from transiently transfected SAOS-2 cells (Rb−/−) have been described (25, 28). GST-Rb and GST-Rb (J82) (where GST = glutathionine *S*-transferase) have been described (23). Gel mobility shift assays using extracts from transformed cell lines were done essentially as described (29) using 1 or 0.5 μg of whole cell extract and the adenovirus E2 promoter as a probe. The VP16 antibody is a rabbit polyclonal antiserum (AC3-1) raised against the VP16 transcriptional activation (amino acids 413–454) and was a gift of Steven Triezenberg.

Transformation Assays. Primary rat embryo cells (Bio-Whittaker) were plated at 10^6 cells per 100-mm plate in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). After 24–48 hr, cells were transfected with calcium phosphate precipitates containing 5 μg of either Rc/RSV, RSV E1A, RSV E2F1, RSV E2F1-VP16, and/or CMV HADP1, 2 μg of HO6T1 (T24 *ras*), and pGEM vector to a total of 20 μg. At 48 hr after transfections, cells were split 1:5 into DMEM supplemented with 5% FCS and containing 400 mg of G418 per ml (Geneticin). Medium was changed every 5 days and cells were incubated 2 weeks before transformed foci were counted.

Growth in Soft Agarose. Primary rat embryo cells were plated, transfected, and G418-selected as above. After 2 weeks, G418-resistant cells were trypsinized, counted, and resuspended in DMEM supplemented with 10% FCS at 10^4 cells per ml. Five thousand cells (0.5 ml) were then mixed with 1 ml of 0.5% molten agarose (Sea Plaque GTG, FMC) containing DMEM and 10% FCS for a final concentration of 0.33% agarose. This mix was then plated onto 60-mm basal agar plates containing DMEM, 10% FCS, and 0.5% agarose.

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Abbreviations: CAT, chloramphenicol acetyltransferase; GST, glutathionine *S*-transferase; RSV, Rous sarcoma virus; FCS, fetal calf serum.

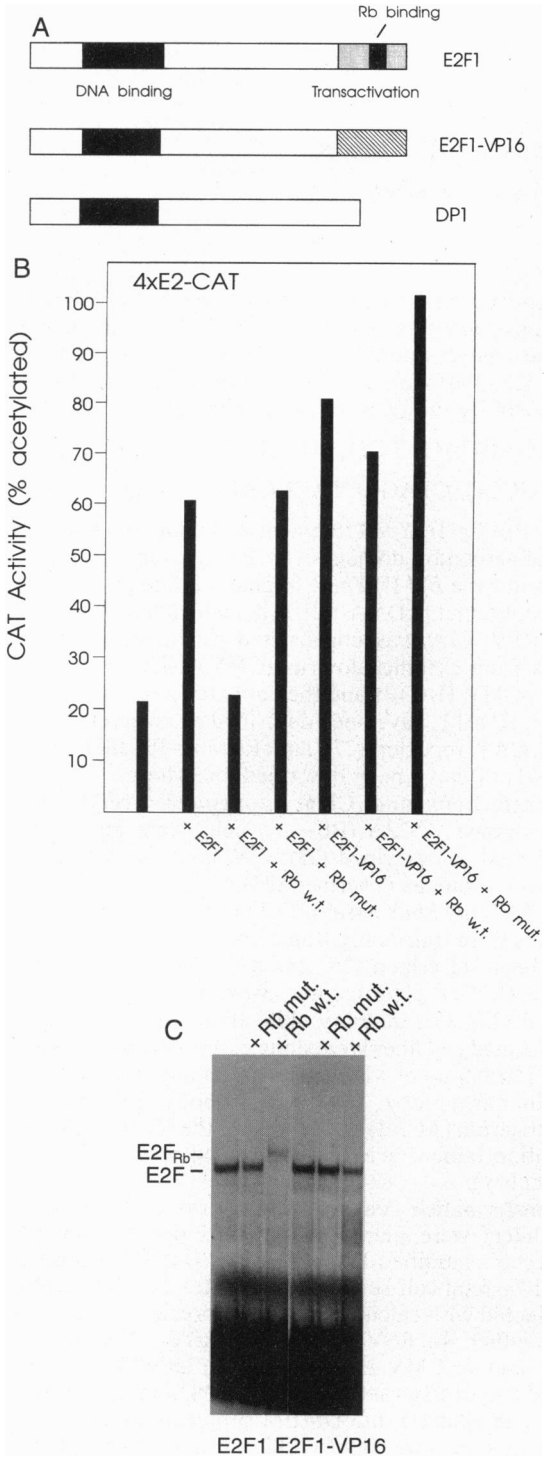

FIG. 1. Transcriptional activity of E2F1 and an E2F1-VP16 chimera. (*A*) Structure of E2F1, E2F1-VP16 chimera, and DP1. The schematic depicts the DNA binding domain and transcriptional activation domains of E2F1, E2F1-VP16, and DP1. The E2F1-VP16 chimera contains amino acids 1–358 of E2F1 fused to amino acids 413–490 of VP16 (22). (*B*) Rb repression of E2F1 and E2F1-VP16. C-33A cells were transfected with 5 μg of 4XE2 CAT, 2 μg of RSV β-galactosidase, 200 ng of RSV E2F1 or RSV E2F1-VP16, 8 μg of SV Rb, SV Rb(J82), or vector alone, and 4 μg of salmon sperm DNA as carrier. CAT assay results were normalized for β-galactosidase assay results as a control for transfection efficiency. (*C*) Interaction of Rb with E2F1 and the E2F1-VP16 chimera. SAOS-2 cells were transfected with plasmids expressing E2F1 and DP1 or E2F1-VP16 and DP1 as described in the text. Extracts (200 ng) from transfected SAOS-2 cells were used in an electrophoresis mobility shift assay with a fragment of the adenovirus E2 promoter as probe. Binding reactions also included 200 ng of GST or GST fusions with wild-type Rb or the nonbinding Rb mutant (J82) (23) as indicated.

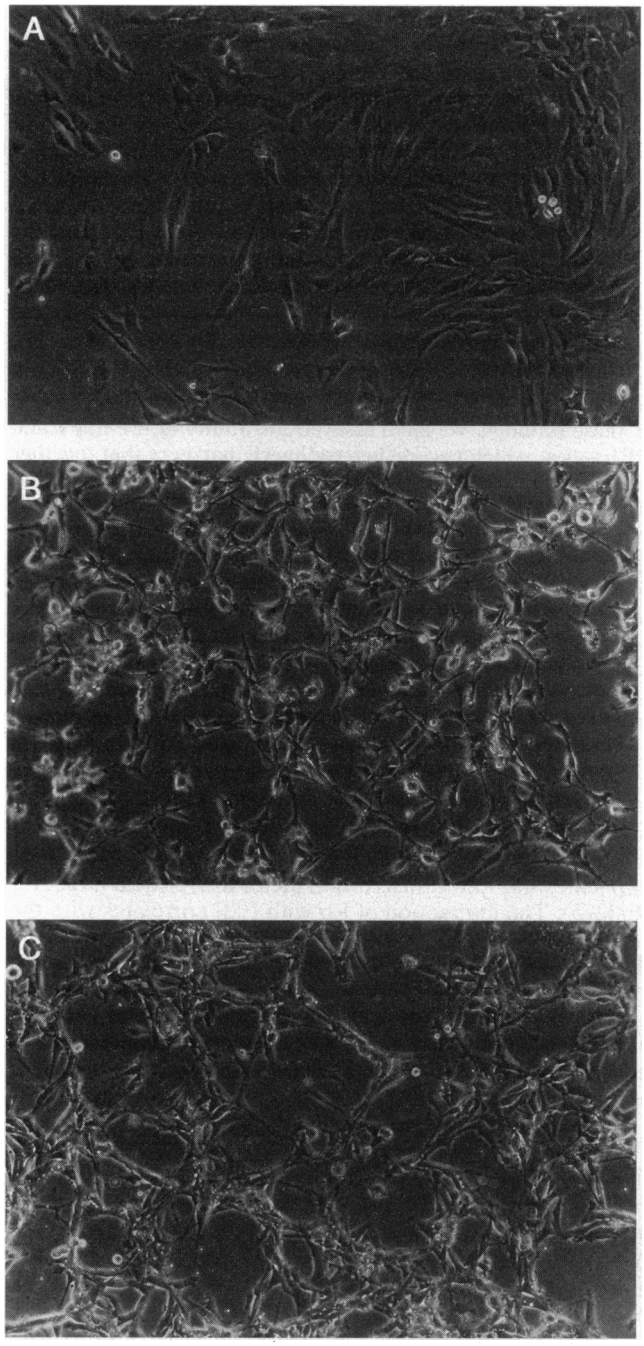

FIG. 2. Morphology of transformed foci and cell lines. (*A*) Photomicrograph of a typical morphologically transformed foci: rat embryo fibroblasts. (*B* and *C*) Photomicrographs of cell lines established by cloning transformed foci induced by transfection of the E1A and E2F1-expressing plasmids. (*B*) E2F1/DP1/*ras*. (*C*) E2F1-VP16/*ras*. (×96.)

After incubation for 2 weeks at 37°C, 5% CO_2, visible colonies were counted from triplicate plates.

Tumor Formation in Nude Mice. Primary rat embryo cells were plated, transfected, and selected in G418 as described above. G418-resistant cells were trypsinized, counted, and resuspended in DMEM at 3–4 × 10^6 cells per ml. Cells (1.5–2 × 10^6) were then injected into 3- to 4-week-old nude mice at two different injection sites as described (30). After 3 and 4

Table 1. Transformation of primary rat embryo fibroblasts by altered E2F1 expression

Plasmid	No. of foci per plate*								Average†
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7	Exp. 8	
ras	2.0	1.6	0.4	0.7	2.0	1.3	0.7	1.0	1.2 ± 0.61
E1A + *ras*	41.0	29.4	21.4	34.3	47.8	46.7	35.3	72.0	41.0 ± 15.29
E2F1 + *ras*	5.2	3.6	0.4	3.0	2.8	1.7	7.7	5.6	3.8 ± 2.33
E2F1-VP16 + *ras*	11.0	15.2	4.2	10.7	14.2	9.3	9.0	8.2	10.2 ± 3.47
DP1 + *ras*	ND	12.6	3.6	8.0	1.6	5.7	1.0	0.6	4.7 ± 4.39
E2F1 + DP1 + *ras*	18.8	14.0	11.6	10.0	4.8	7.3	20.3	9.0	12.0 ± 5.43
E2F1-VP16 + DP1 + *ras*	16.4	17.4	10.4	10.0	15.4	1.0	15.0	13.6	12.4 ± 5.32

ND, not determined.
*Average number of morphologically transformed foci per plate after selection in G418 for 2 weeks.
†Combined average ± SD of foci per plate from the eight separate experiments.

weeks, the mice were scored for the presence of tumors at the injection sites.

RESULTS

Transcriptional Activity and Rb Binding Properties of E2F1 and E2F1-VP16. If the growth-suppressing activity of Rb reflects the control of E2F, then overexpression of E2F1 might be expected to create a phenotype similar to the loss of Rb function. Likewise, production of an E2F1 protein that could no longer interact with Rb, but which still retained the ability to activate transcription, might also be expected to bypass the normal function of Rb in growth suppression. As an approach to this latter possibility, we constructed an E2F1-VP16 chimera in which the transcriptional activation domain of E2F1, including the 18-amino acid region known to be responsible for Rb binding, was substituted with the transcriptional activation domain of the herpesvirus protein VP16 (Fig. 1*A*). As shown in Fig. 1*B*, cotransfection with a plasmid expressing Rb inhibited the ability of E2F1 to activate the test promoter, whereas an Rb mutant that is deficient in E2F binding was unable to inhibit E2F1-mediated transactivation. In sharp contrast, expression of the Rb protein had little effect on the transactivation capacity of the E2F1-VP16 chimeric protein.

Direct assays for Rb–E2F1 interaction are shown in Fig. 1*C*. A GST-Rb protein was added to extracts of cells that were transfected with E2F1 and DP1. A slowly migrating E2F complex was generated with the wild-type Rb protein, whereas the addition of an Rb mutant protein, known not to bind to E2F, had no effect (Fig. 1*C*). Consistent with the transactivation data, addition of the GST-Rb protein to the E2F1-VP16/DP1 extract did not result in the formation of the Rb-containing E2F complex.

Transforming Activity of E2F1 and E2F1-VP16 in Primary Rat Embryo Fibroblasts. Plasmids expressing the E2F1, E2F1-VP16, and DP1 proteins were transfected into primary rat embryo fibroblasts together with a plasmid expressing an activated Ha-*ras* oncogene. In each case, the cDNA sequences were under the control of the RSV promoter, a relatively strong promoter not subject to cell growth regulatory control. After selection in G418 for 2 weeks, plates were scored for the presence of morphologically transformed foci (Fig. 2*A* and Table 1).

The activated *ras* gene alone gave rise to an average of 1 or 2 foci per plate. In the absence of *ras*, neither E1A nor any of the E2F1 constructs gave rise to morphologically transformed foci. Addition of the E1A gene together with *ras* gave rise to a substantially increased number of morphologically transformed foci, ranging from 21 to 72 foci per plate. Cotransfection of E2F1 and *ras* as well as DP1 and *ras* gave rise to an increase in the number of transformed foci over *ras* alone in most experiments. The E2F1-VP16 product consistently gave rise to an increase in foci over that with *ras* alone, an average of an 8.5-fold increase per experiment. Cotransfecting E2F1 and DP1 together with *ras* gave an enhancement of transforming ability over either E2F1 or DP1 alone, while cotransfecting E2F1-VP16 with DP1 only slightly increased the average number of foci compared with E2F1-VP16 alone.

E1A/*ras* transfected cells consistently grew out to establish cell lines that maintained their morphologically transformed phenotype (Fig. 2*B*). In contrast, E2F1/*ras* transfected cells did not always clone well, often reverting to a normal morphology. Cells from E2F1/DP1/*ras* foci and the E2F1-VP16/*ras* foci were found to clone more efficiently than E2F1/*ras* and many of these cell lines clearly exhibited a transformed morphology (Fig. 2*B*). Moreover, the E2F1-VP16/*ras* cells were more efficient in maintaining the transformed morphology when placed into culture (≈75% of the foci retained a transformed morphology).

The ability of E2F1 or the E2F1-VP16 transfected cells to form colonies in soft agar was also assayed. Following selection in G418 for 2 weeks, aliquots of 5×10^3 cells were plated into three 60-mm plates in 0.3% agarose, and colonies were counted after 2 weeks (Table 2). Cells transfected with *ras* alone did not give rise to colonies in any of the experiments, whereas transfection of E1A together with *ras* gave rise to >300 colonies per plate, consistent with previous assays (31–33). Cells transfected with E2F1 and *ras* did not form colonies in soft agar, but cells transfected with E2F1-VP16 and *ras* did give rise to colonies. DP1 together with *ras* did yield a few small colonies in some experiments, but E2F1 together with DP1 and *ras* gave rise to larger colonies, similar in number to that seen for E2F1-VP16 and *ras*. Surprisingly, cotransfection of DP1 with E2F1-VP16 decreased the efficiency of colony formation from that seen with E2F1-VP16 alone. Although we have no clear explanation for this result, it is possible that the high level of E2F activity that would result from the interaction of DP1 with the chimeric protein could be detrimental for cell survival.

E2F1 Expression in Transformed Cells. As shown in Fig. 3*A*, the majority of morphologically transformed cell lines derived from transfection with E2F1/*ras* or E2F1/DP1/*ras* were found to have increased E2F activity, as measured by DNA binding assays, whereas all morphologically normal cell lines

Table 2. Growth in soft agarose

Plasmid	No. of colonies per plate		
	Exp. 1	Exp. 2	Exp. 3
ras	0	0	0
E1A + *ras*	>300	>300	>300
E2F1 + *ras*	0	0	0
E2F1-VP16 + *ras*	26	38	>300
DP1 + *ras*	0	1	30
E2F1 + DP1 + *ras*	23	27	>300
E2F1-VP16 + DP1 + *ras*	9	0	174

Primary rat embryo fibroblasts were transfected with the indicated plasmids, selected in G418 for 2 weeks, and then plated in soft agarose. Visible colonies were then counted following a 2-week incubation.

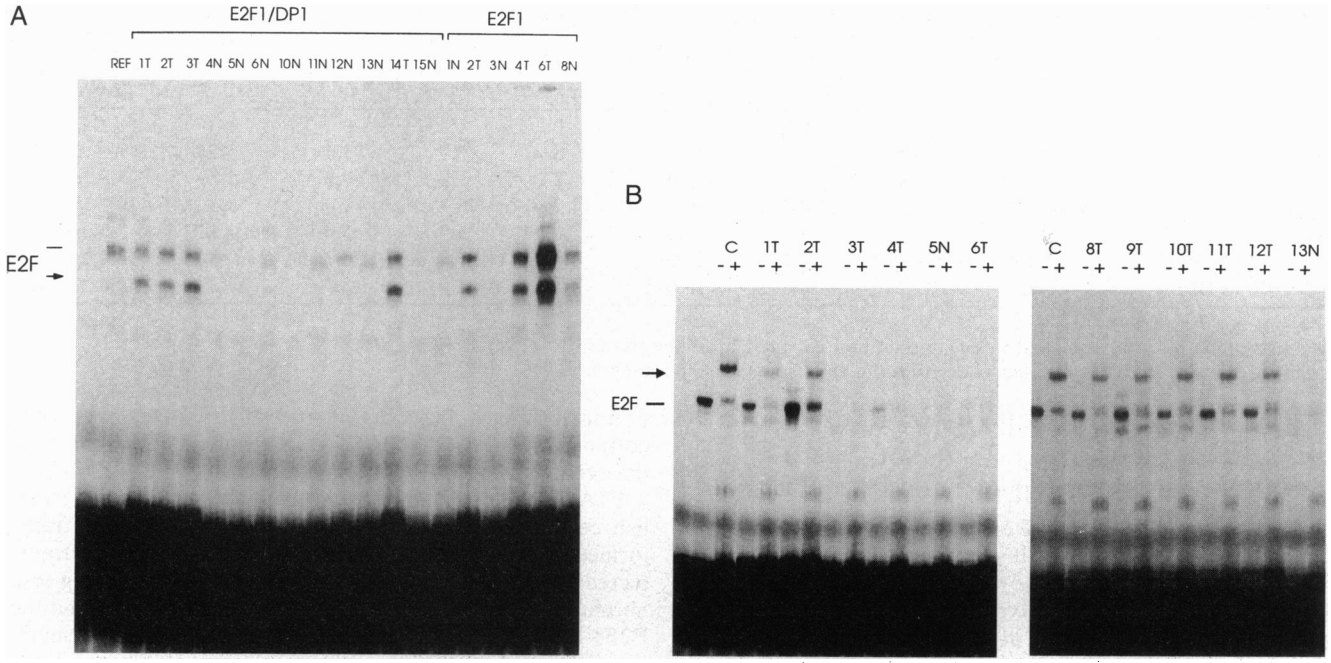

FIG. 3. E2F DNA binding activity in transformed cells. (*A*) Twelve cell lines derived from E2F1/DP1/*ras* transformed foci and six cell lines derived from E2F1/*ras* transformed foci were assayed for E2F-specific DNA binding activity. One microgram of total protein was used in each assay. Lane 1, probe only; lane 2, assay with extract from the parental primary rat embryo fibroblasts (REF) prior to transfection and selection. A "T" indicates the cell lines that maintained a transformed morphology and an "N" indicates those that reverted to a normal morphology. A fast migrating E2F–DNA complex that correlates with transformed morphology is indicated by the arrow. (*B*) Extracts from 12 cell lines derived from E2F1-VP16/*ras* foci were assayed for total E2F-specific DNA binding activity and for expression of the E2F1-VP16 chimeric protein through the addition of a VP16-specific antibody. (*Left*) Lane 1, probe alone; lanes 2 and 3 (C), extract from SAOS-2 cells (200 ng) overexpressing E2F1-VP16 and DP1 as described in the legend to Fig. 2. In the assay of lane 3, a VP16-specific antiserum was added, which resulted in the generation of a super-shifted complex as indicated by the arrow. Lanes 4–15 received 500 ng of extract from six independent E2F1-VP16/*ras* transformed cell lines and alternating lanes received the VP16 antiserum. (*Right*) Lanes 1 and 2 (C), repeat of the SAOS-2 controls shown in *A*. Lanes 3–12 received 500 ng of extract from six E2F1-VP16/*ras* transformed cell lines with alternating lanes receiving the VP16 antiserum. In each case, 2 μl of a 1:5 dilution of the antiserum was added.

were found to have lower activity than the early passage parental cells. The increased E2F binding activity was found primarily in a faster migrating species compared to the major species in the parental primary rat embryo fibroblasts. Although Western blot analysis confirmed that the E2F1 protein was expressed in the majority of these cell lines (data not shown), the faster migrating species does not appear to derive from the exogenous E2F1 protein since antibody specific for human E2F1 had little or no effect on this complex (data not shown). We thus presume that this represents an endogenous activity that is induced in these cells.

The majority of the morphologically transformed cells derived from transfection with the E2F1-VP16 chimera with ras were found to have an increased E2F-specific DNA binding activity (Fig. 3*B*). This increased binding activity is found primarily in a complex involving the chimeric protein as demonstrated by the ability of a VP16-specific antiserum to interact with and retard the mobility of the complex ("+" lanes). Although the majority of the transformed cell lines derived from E2F1-VP16 transfection exhibited evidence of the E2F1-VP16 DNA protein complex, two cell lines (3T and 6T) did not. We do note, however, that upon longer exposure it is apparent that there was an increase in the faster migrating species in the extracts of these cells that was typical of the cells transformed by E2F1 or E2F1/DP1 (data not shown).

E2F1 Transfected Cells Induce Tumors in Nude Mice. Finally, the oncogenic potential of the E2F1-transfected cells was tested by assaying their ability to form tumors in nude mice. Aliquots of 1.5–2 × 10^6 transfected and selected cells were injected subcutaneously into nude mice and tumors were scored after 2–3 weeks. In four separate experiments, no tumors were observed following injection of cells that were transfected with *ras* alone (Table 3). Cells transfected with E1A together with *ras* gave rise to visible tumors in every experiment. Tumor formation with cells transfected with *ras* and E2F1 or DP1 alone was less efficient, generating tumors that were usually smaller and slower developing than E1A plus *ras*, particularly in the case of DP1/*ras*. In contrast, cells transfected with E2F1-VP16, E2F1/DP1, or E2F1-VP16/DP1 together with *ras* gave rise to tumors of a similar size, or often larger, than those derived from the E1A and *ras* transfected cells.

DISCUSSION

It is now evident that various genes encoding regulatory activities that govern the mammalian cell cycle, particularly the progression of quiescent cells through G_1 and into S phase, are targets for alterations that underlie the develop-

Table 3. Oncogenic potential of E2F1-transformed cells in nude mice

Plasmid	Experiment 1	2	3	4
ras	−	−	−	−
E1A + *ras*	+	+	+	+
E2F1 + *ras*	+	+/−	+	+
E2F1-VP16 + *ras*	+	+	+	+
DP1 + *ras*	+	+/−	+/−	−
E2F1 + DP1 + *ras*	+	+	ND	ND
E2F1-VP16 + DP1 + *ras*	+	+	+	ND

Primary rat embryo fibroblasts were transfected with the indicated plasmids, selected in G418 for 2 weeks, and then injected into nude mice. −, No tumors observed after 4 weeks; +/−, small tumors observed after 4 weeks; +, large tumors observed after 3 weeks; ND, not determined.

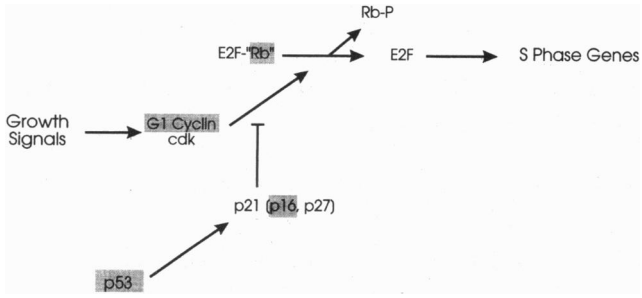

FIG. 4. Regulatory events governing cell growth control. Genes involved in G_1 regulatory events. Gene products that have been implicated in human tumorigenesis are indicated by the shading. "Rb" refers not only to Rb but also to other Rb family members (p130, p107) that are known to interact with and regulate E2F.

ment of human neoplasms (Fig. 4). The recent identification of the p21 gene as a target for control by the p53 tumor suppressor has provided a link between the action of p53 as a growth suppressor and the control of G_1 cyclin/kinase activity that regulates the cell growth cycle (34–37). The realization that the p16 gene product, a functional relative of p21, may be altered in many types of tumors provides further evidence for the interconnection (38, 39). Other studies have revealed that the *PRAD1/Bcl1* oncogene is a rearranged version of cyclin D1 (18, 40). Finally, a likely target for the action of D-type cyclins, in conjunction with the cdk4 protein kinase, is the retinoblastoma protein (Rb), the paradigm for tumor suppressors (12, 13, 15). It seems possible that any activity participating in the G_1 regulatory chain of events leading to cell proliferation is a potential oncogene.

Many of these activities that govern the progression of cells through G_1, and that are also involved in human cancers, can be seen as upstream regulators of E2F. Certainly, the data implicating E2F as a target for the action of the Rb tumor suppressor protein is the most compelling form of indirect evidence linking E2F regulation with oncogenesis. The data we present here now provide direct evidence to demonstrate that the E2F1 gene can exhibit oncogenic activity. E2F1 or DP1 alone has low and variable transformation ability but, when combined, E2F1/DP1 consistently gives rise to an increase in transformation capability in cooperation with an activated *ras* oncogene. The finding that the Rb-resistant E2F1-VP16 chimera has increased oncogenic activity supports the idea that regulation of E2F1 activity by Rb or other Rb family members is critical in maintaining normal cellular growth control. The increased oncogenic potential of E2F1-VP16 also suggests that E2F1 is not simply sequestering Rb but rather that the transcriptional activation of E2F target genes is likely involved in the ability of E2F1 or E2F1-VP16 to mediate cellular transformation. The apparently low level of E2F1 in the transformed cells suggests that only a modest increase in E2F1 activity, perhaps at inappropriate times during the cell cycle, is sufficient for altering cell growth control.

Finally, although these experiments clearly demonstrate an oncogenic potential for E2F, it remains to be shown if deregulation of E2F gene products is an actual event of human tumorigenesis. Given the participation of many of the other genes suspected to be upstream of the control of E2F1, together with the results we present here, it seems likely that alterations in E2F1 will be found to be associated with the development of human tumors.

We thank Mike Ostrowski for the mutant Ha-*ras* plasmid, Kristian Helin and Ed Harlow for the DP1 expression plasmid, and Steve Triezenberg for the VP16 antiserum. We also thank Masa Ikeda and Tim Kowalik for helpful suggestions and comments and Kaye Culler and Teresa Jenkins for help in the preparation of the manuscript. D.G.J. is supported by a fellowship from the American Cancer Society and W.D.C. is supported by a fellowship from the Damon Runyon–Walter Winchell Foundation.

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