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The Transcription Factor E2F-1 Is a Downstream Target of RB Action

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Reintroduction of RB into SAOS2 ($RB^{-/-}$) cells causes a G_1 arrest and characteristic cellular swelling. Coexpression of the cellular transcription factor E2F-1 could overcome these effects. The ability of E2F-1 to bind to RB was neither necessary nor sufficient for this effect, and S-phase entry was not accompanied by RB hyperphosphorylation under these conditions. Furthermore, E2F-1 could overcome the actions of a nonphosphorylatable but otherwise intact RB mutant. These data, together with the fact that RB binds to E2F-1 in vivo, suggest that E2F-1 is a downstream target of RB action. Mutational analysis showed that the ability of E2F-1 to bind to DNA was necessary and sufficient to block the formation of large cells by RB, whereas the ability to induce S-phase entry required a functional transactivation domain as well. Thus, the induction of a G_1 arrest and the formation of large cells by RB in these cells can be genetically dissociated. Furthermore, the ability of the E2F-1 DNA-binding domain alone to block one manifestation of RB action is consistent with the notion that RB-E2F complexes actively repress transcription upon binding to certain E2F-responsive promoters. In keeping with this view, we show here that coproduction of an E2F1 mutant capable of binding to DNA, yet unable to transactivate, is sufficient to block RB-mediated transcriptional repression.

Inactivating mutations of the retinoblastoma gene (*RB-1*) exist in a variety of human tumor cells. Reintroduction of a wild-type *RB-1* gene, or its product (RB), into cells which lack RB function can suppress their ability to grow in culture and/or form tumors in nude mice (5, 34, 41, 73, 74, 84, 86, 93). The RB-mediated growth arrest of cultured cells results from a block to exit from G_1 (15, 17, 24, 37, 74, 86, 93). In certain $RB^{-/-}$ cells, restoration of pRB function produces characteristic morphological changes, including marked cellular enlargement (37, 41, 73, 74). This does not appear to be a toxic effect, as the resultant "large cells," which contain 2N DNA, are viable for many weeks (37). It is not clear whether these morphological changes, which are also seen in senescent cells (22), are due to the imposition of a G_1/S block per se or reflect additional activities of pRB related, perhaps, to exit from the cell cycle.

How RB performs its G_1 exit-blocking function is not clear. Within the protein there is an ~400-residue domain, referred to as the RB pocket, which can bind with high affinity to the three different DNA tumor virus transforming proteins: adenovirus E1A, papovavirus large T antigen (T), and human papillomavirus E7 protein (40, 44, 49). The transformation function of these proteins is linked to their RB-binding activity. In turn, all naturally occurring loss-of-function *RB-1* mutations known to be compatible with stable protein expression map to the pocket (5, 38, 39, 53, 78, 81). This finding suggested that one or more cellular proteins normally interact with the RB pocket, that such an interaction is linked to the RB growth-controlling function, and that T, E1A, and E7 act as "stalking-horses" for these proteins and prevent them from binding to RB.

RB is a differentially phosphorylated protein. It is unphosphorylated (pRB) through much of G_1. A few hours before S, it is first phosphorylated and is again phosphorylated at least twice thereafter. Late in mitosis, it is enzymatically dephosphorylated (6, 8, 12, 13, 62, 63, 65). By the time cells enter the next G_1, they contain largely pRB again (62). These findings, the preferential binding of T to pRB (61), and the fact that T stimulates S-phase entry of G_1-arrested cells suggest that pRB is responsible for the G_1-blocking effect of RB and that its phosphorylation is linked to the release of such a block. The effects of transforming growth factor beta on epithelial cells further support this view (18, 58). Indeed, RB is a substrate for certain G_1 cyclin-cdk complexes, and its G_1 exit-blocking function can be inactivated in parallel with its phosphorylation by them (17, 37, 52).

A number of specific cellular proteins can bind in vitro to the RB pocket (14, 15, 17, 19, 26, 27, 32, 43, 50, 51, 55, 66, 77, 88). One of them, the transcription factor E2F, associates with pRB in mid-late G_1 and in S (68, 71, 82). These complexes can be disrupted by T or E1A. Furthermore, intact RB, and certain pocket-containing fragments thereof, when introduced into some $RB^{-/-}$ cell lines, induce a G_1 block, and this activity correlates with the ability of these RB proteins to bind E2F (17, 24, 34, 36, 73, 74, 93). A major question, then, is whether RB-E2F complexes truly contribute to an RB-induced G_1 block and, if so, how.

E2F-binding sites exist in a number of growth-regulatory genes (4, 11, 28, 35, 64, 67, 69, 72, 87). In particular, a subset of these genes encode products which play a role in DNA synthesis. For example, the dihydrofolate reductase (DHFR), DNA polymerase α, *cdc2*, and thymidine kinase genes contain potential E2F-binding sites (4, 11, 35, 64, 69, 72). The dramatic increase in DHFR messenger at the G_1/S boundary of G_0-arrested cells stimulated to enter the cell cycle is known to depend on the integrity of E2F sites (83). On the other hand, inactivation of the E2F sites in the DHFR and *cdc2* promoters did not per se silence them or markedly inhibit their basal function (11, 35, 64). Rather, it led to a failure of timely stimulation around G_1/S (82a, 83). If silencing of certain E2F-stimulated promoters is essential to the imposition of an RB-dependent G_1 block and RB-E2F complex formation is linked

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to this process, do RB-E2F complexes effect the proper modulation of the right E2F-stimulated promoters, even though they do not depend upon free E2F function for intrinsic power?

By screening expression libraries with a recombinant form of pRB, a cDNA encoding a transcription factor with E2F-like properties was cloned recently (32, 50, 80). It encodes a 437-amino-acid protein termed E2F-1. Recent work by several laboratories indicates that there is a complex family of transcription factors capable of recognizing a canonical E2F-binding site, of which E2F-1 and a second cloned relative, DP-1, are but two members (3a, 9, 16, 19a, 20a, 21, 46, 60). E2F-1 binds to RB in vivo, prefers pRB over its phosphorylated derivatives, and contains an N-terminal DNA-binding domain and a potent C-terminal transactivation domain (20, 31, 32, 50, 80). An 18-amino-acid sequence, embedded within the latter domain, is both necessary and sufficient for pRB binding (32). Heterodimerization with DP-1 enhances the ability of E2F-1 to bind to DNA and to pRB (3, 33, 57).

In this report, we describe the results of experiments aimed at probing the relationship between pRB-E2F complex formation and the imposition of an RB-dependent G_1 exit block. The results suggest that RB-E2F complexes are necessary and active contributors to this effect and operate, at least in part, by repressing the transcription of certain E2F DNA site-containing genes.

MATERIALS AND METHODS

Plasmids. To make pSG5-E2F-1 and pSG5-E2F-1 (R.O [reverse orientation]), the E2F-1 cDNA insert from pSP72-RBAP-1(wt) (50) was excised by partial digestion with *Bam*HI and *Bgl*II and subcloned into the *Bam*HI site of pSG5 (Stratagene). pSG5-E2F-1(dl18), pSG5-E2F-1(dl53), and pSG5-E2F-1(dl18+dl53) were constructed by site-specific single-strand mutagenesis, using the Bio-Rad Muta-gene kit as directed, CCGGGGGAGAAGTCACGCGCTAGCGC CAAGAAGTCCAAGAAC and TCCCCACCCCACGAGGCCGCTAGCTGT GACTTTGGGGACCTC as primers, and pSG5-E2F-1 grown in *Escherichia coli* CJ236 as the template. To create pSP72-E2F-1(1-196), the *Hin*dIII-*Sma*I E2F-1 cDNA fragment from pSP72-RBAP-1 was ligated into the backbone DNA fragment generated by digesting a plasmid containing the E2F-1 cDNA corresponding to the basic helix-turn-helix (HLH) region [pSP72-E2F-1(bHLH) (57)] with *Hin*dIII and *Sma*I. The E2F-1(1-196) insert from the pSP72-E2F-1(1-196) was excised as a *Bam*HI-*Bgl*II fragment and then subcloned to the *Bam*HI site of the pSG5 vector. pSG5-E2F-1(1-241) was generated by ligating the *Bam*-*Bgl*II insert of pSP72-E2F-1(1-241) (a gift of W. Krek) into the pSG5 vector. pSG5-E2F-1(1-127) was generated by cutting the insert of pSP72(1-127) (a gift of W. Krek) with *Bam*HI and *Bgl*II and subcloning it into the pSG5 vector. pSG5-E2F-1(dl24) and pSG5-E2F-1(1-196; *dl*24) were generated by subcloning the *Bam*HI-*Sma*I fragment of pGST-E2F-1(1-127; dl24) (a gift of W. Krek) into the *Bam*HI-*Sma*I backbones of pSG5-E2F-1 and pSG5-E2F-1(1-196), respectively. pSG5-E2F-1(41-196) was made by replacing the *Bam*HI-*Sma*I E2F-1 cDNA insert of pSG5-E2F-1(1-196) with the *Bam*HI-*Sma*I E2F1 cDNA fragment from pGST-E2F-1(41-127) (a gift of W. Krek). To make pSG5-E2F-1(1-368), the E2F-1 cDNA was first PCR amplified by using primers GCGCTCCCCGGGGGAGAAGT CACGCTATG and GCGCCTCGAGTCAGGGAGCCCGCAGGCTGCC. The latter primer introduces a stop codon after codon 368 followed by an *Xho*I site. The PCR product was digested with *Sma*I and *Xho*I and used to replace the *Sma*I-*Xho*I E2F-1 cDNA insert of pSG5-E2F-1. To create pSG5-E2F-1(1-368) VP16, the E2F-1 cDNA from pSG5-E2F-1 was PCR amplified by using primers A (ACCACCTGATGAATATCTG) and B (GGTCGGGGGGGGCCGTCGAG GGAGCCCGCAGGCTGCC), and the VP16 cDNA from pUHD15-1 (25) was amplified by using primers C (GGCAGCCTGCGGGCTCCCTCGACGGCCCC CCCGACC) and D (GCGCCTCGAGCTACCCACCGTACTCGTC). One microliter of each 100-μl PCR mixture was then combined and PCR amplified by using primers A and D. This product was digested with *Bcl*I and *Xho*I and used to replace the internal *Bcl*I-*Xho*I E2F-1 cDNA fragment in pSG5-E2F-1. The resulting chimera encodes VP16 residues 411 to 490. Double-stranded DNA sequencing of plasmids pSG5-E2F-1(1-368) and pSG5-E2F-1(1-368)VP16 to confirm partial sequences was performed with a Sequenase kit (U.S. Biochemical) as instructed by the manufacturer.

To make pBS(RSV)GAL4-E2F-1(dl18), the *Bgl*II-*Eco*RI fragment of pSG5-E2F-1(dl18) was ligated, in the presence of an *Eco*RI-*Xba*I linker, into pBS (RSV)GAL4-E2F-1(285-437) (50) cut with *Bam*HI and *Xba*I. The plasmids encoding GAL4, GAL4/E2F-1(1-263), GAL4/E2F-1(1-363), and GAL4/E2F-1 (285-437), and the reporter plasmid, 3X(GAL4)BG-CAT, have been described previously (20, 50).

pcDNA-HAE2F1 was a gift of W. Krek (57). pcDNA-HAE2F1(1-368) was created by ligating the *Bam*HI-*Xho*I E2F1 cDNA fragment from pSG5-E2F-1 (1-368) into pcDNA(HA) (57) linearized with these two enzymes. The *Bam*HI-*Sal*I E2F-1 cDNA fragment from pcDNA-E2F-1(132) (10) was then used to replace the corresponding wild-type (wt) cDNA fragment in pcDNA-HAE2F1 (1-368) to create pcDNA-HAE2F1(1-368;132). The E2F-1 cDNA fragments from pSP72-E2F-1(1-127) and pSP72-E2F-1(1-196) were excised by restriction with *Bam*HI and *Eco*RI and subcloned into pcDNA linearized with these two enzymes to create pcDNA-HAE2F1(1-127) and pcDNA-HAE2F1(1-196), respectively.

Cell culture, transfections, and $^{32}P_i$ labeling. SAOS2 human osteosarcoma cells were grown in Dulbecco's modified Eagle's medium (Sigma) with 10% heat-inactivated HyClone bovine serum at 37°C. U-20S human osteosarcoma cells were grown in Dulbecco's modified Eagle's medium (GIBCO) containing 10% HyClone bovine serum. The transfection procedure was essentially as described previously (74) except that cells were transfected at 90% confluence. For growth suppression assays, SAOS2 cells were transfected with 8 μg of pCMV-RB and 24 to 48 μg of pSG5-E2F-1 or derivatives thereof. For the large-cell assays, the cell growth medium was supplemented with G418 (300 μg/ml) 48 h after transfection. Four to five days after G418 selection, 500 cells were assayed quantitatively for large cells by light microscopy.

Western blotting (immunoblotting), immunoprecipitation, and immunofluorescence. The Western blotting, immunoprecipitation, and immunofluorescence experiments were performed as described previously (74). The antibodies against E2F-1 used in this study were a mouse monoclonal antibody, SQ41 (50), and a rabbit polyclonal antibody, P98 (50). P98 (1:200 dilution) was used in the immunofluorescence staining studies. The subcellular location of wt E2F-1 and E2F-1 mutants was determined by indirect immunofluorescence staining by using a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G secondary antibody (Boehringer Mannheim) after transfection of the plasmids encoding the various proteins. For double immunofluorescence staining, cells were transfected with pCMV-RB or pCMV-RB and pSG5-E2F-1. Following fixation with 1.5% paraformaldehyde, cells were permeabilized by immersion in phosphate-buffered saline (PBS) containing 0.3% Triton X-100 for 5 min and stained for both RB and E2F-1. RB antibody was visualized by an FITC-conjugated anti-mouse secondary antibody (Boehringer), and E2F-1 antibody was visualized through the use of a rhodamine-conjugated anti-rabbit second antibody (Boehringer).

DNA binding assays. After transfection, cell extracts from SAOS2 cells were prepared by using modified whole-cell extract lysis buffer (50). E2F gel mobility shift assays were performed essentially as described by Shirodkar et al. (82). Five microliters of cell extract from each transfectant, containing approximately equal amounts of protein (~8 μg), was added to the DNA binding reaction mixture just before the addition of the probe. The ^{32}P-labeled synthetic probe was a replica of the adenovirus E2 promoter. Where indicated, competitor oligonucleotides were added as described previously (74).

McKay assays were performed essentially as described previously (54). Briefly, EBC cell extracts (74) were incubated with 1 μg of antihemagglutinin (anti-HA) antibody (12CA5; Boehringer), 1 μl of ^{32}P-labeled E2 probe prepared as described above, 20 μg of single-stranded DNA (Sigma), and 100 μl of 1:1 protein A-Sepharose in a total volume of ~800 μl for approximately 1 h at 4°C. The Sepharose was then washed five times with NETN (74) and transferred to a new Eppendorf tube. Bound probe was eluted by boiling the Sepharose for 5 min in sample buffer (50% formamide–0.1% bromophenol blue), electrophoresed in a 12% acrylamide denaturing Tris-borate-EDTA gel, and detected by autoradiography.

CAT and luciferase assays. Chloramphenicol acetyltransferase (CAT) assays were performed as previously described (20). Luciferase assays using the luciferase reporter plasmid containing the E2F-1 promoter were performed as previously described (70).

Flow cytometric analysis. SAOS2 cells were transfected with indicated plasmid combinations (pCMV-RB and pSG5 plasmids containing various E2F cDNA derivatives). Three days after transfection, the cells were subjected to RB immunofluorescence staining and propidium iodide staining for DNA. Flow cytometry analysis was then performed as described elsewhere (17, 37). When CD19 was used as a marker, 2 μg of the CD19-encoding plasmid (kindly provided by T. Tedder) and 8 μg of pCMV-RB and 24 to 48 μg of E2F-1-encoding plasmids were present in each transfection mixture. The cells were trypsinized, washed with PBS containing 0.1% bovine serum albumin and 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.3), and then reacted with an anti-CD19 antibody (CD19.15; 1:500 dilution) (kindly provided by T. Tedder) for 1 h on ice. The cells were then washed and incubated with an FITC-conjugated goat anti-mouse second antibody (15 μg/ml; Boehringer) for 40 min on ice. The cells were washed again and then fixed in 70% ethanol at 4°C overnight. Prior to sorting, the cells were washed again and then treated with an RNase A (5 μg/ml) and propidium iodide solution (69 μM propidium iodide, 38 mM sodium citrate) for 20 min at 37°C.

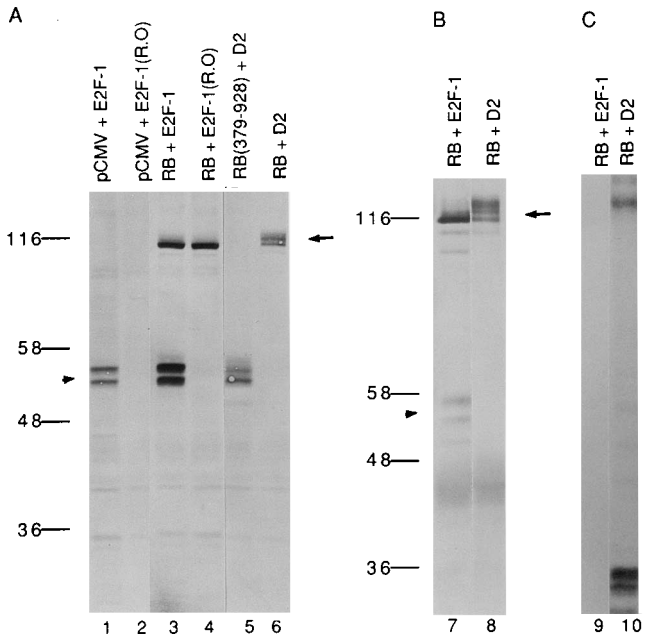

FIG. 1. Cotransfection of RB- and E2F-1-encoding plasmids. (A) Lysates (~50 μg) of SAOS2 cells, transfected with the indicated protein(s), were subjected to Western blotting with a mixture of the two anti-RB monoclonal antibodies (XZ91 and XZ56) and an anti-E2F-1 monoclonal antibody (SQ41) 2 days after transfection. Proteins were resolved in SDS–8.75% polyacrylamide gels, and bands were visualized colorimetrically. The arrow and arrowhead indicate the positions of RB and E2F-1, respectively. Molecular masses of the prestained marker protein (in kilodaltons) are indicated at the left. (B and C) SAOS2 cells were metabolically labeled with $^{32}P_i$ 36 h after transfection. After immunoprecipitation with a mixture of XZ91 and purified SQ41, protein A-Sepharose-bound proteins were resolved in an SDS–7.5% polyacrylamide gel, transferred to nitrocellulose, and detected by immunoblotting with anti-RB and anti-E2F-1 antibodies (B) or by autoradiography after blotting (C).

RESULTS

Cotransfection of RB with E2F-1 cDNAs in SAOS2 cells. The first question was whether coexpression of RB and E2F-1 alleles in an $RB^{-/-}$ cell line neutralizes one or more of the RB effects therein. To determine whether the synthesis of E2F-1 alters the expression of a simultaneously introduced *RB-1* cDNA, we constructed a mammalian expression plasmid, pSG5-E2F-1, in which the E2F-1 open reading frame was linked to the simian virus 40 early promoter. This plasmid was then transfected into SAOS2 ($RB^{-/-}$ osteosarcoma) cells along with either pCMV-RB(wt) or pCMV-RB(379-928). RB(379-928) is the smallest RB fragment that we have identified to date which retains the ability to arrest $RB^{-/-}$ cells in G_1 (17, 74). Similar results were obtained by others using an analogous RB fragment (23). It is phosphorylated during the cell cycle, in parallel with wt pRB (17, 74). The parental plasmid, pCMV-Neo-Bam (2), served as a control for the effects of transfection.

Two days after transfection, cell extracts were prepared, resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and transferred to nitrocellulose. RB and E2F-1 were visualized by Western blot analysis using a mixture of monoclonal antibodies against RB (XZ91 and XZ56) and E2F-1 (SQ41). The RB-encoding plasmids, when introduced into SAOS2 cells in the presence of a plasmid carrying E2F-1 in the antisense orientation, led to the appearance of un(der) phosphorylated RB (pRB) only (Fig. 1, lane 4). Coexpression of E2F-1 and RB cDNAs led to the appearance of RB (at ~110 kDa; arrow in Fig. 1A) and of the characteristic E2F-1 doublet at ~58 kDa (arrowhead) without a significant change in the abundance or migration of the coproduced RB species (Fig. 1; compare lanes 3 and 4). Thus, coexpression of E2F-1 did not interfere with the synthesis of RB or its state of phosphorylation in this assay.

That the wt RB and RB(379-928) cDNAs used in these studies could direct the synthesis of more slowly migrating, overtly phosphorylated RB species (pRBphos) was shown earlier, by transfecting the corresponding expression plasmids into the $RB^{+/+}$ cell line U-20S (17, 74). Similarly, the wt RB- and RB(379-928)-encoding cDNAs led to the production of pRB phos in SAOS2 cells when cotransfected with cyclin D2, as originally shown by Ewen et al. (17) (Fig. 1, lanes 6, 8, and 10). Cyclin D2 cotransfection into SAOS2 with an RB-encoding plasmid also overrode the RB-induced G_1 block in these cells (17). Thus, these two RB alleles encoded proteins which could be phosphorylated in G_1, as expected, when confronted with the appropriate enzyme(s).

Phenotypic alteration of SAOS2 cells resulting from cotransfection of RB and E2F-1. SAOS2 cells exhibit distinctive morphological changes, most notably cell flattening and enlargement, following RB synthesis. The effect is linked, in part, to the function of the RB pocket (37, 41, 73, 74, 86). These large cells began to appear within 2 days after RB transfection and correlated with the RB growth suppression phenotype, since various mutant forms of RB unable to induce this phenotype were also unable to arrest the growth of these cells. In keeping with earlier findings (37, 74), the large-cell phenotype developed after either pCMV-RB or pCMV-RB(379-928) was transfected. The same effect was observed when either of these RB alleles was cotransfected with a control plasmid, pSG5-E2F-1(R.O), which contains a reverse-orientation E2F-1 insert linked to the simian virus 40 early promoter (Fig. 2). pSG5-E2F-1(R.O), when transfected alone, had no discernible effect. In contrast, cotransfection of pSG5-E2F-1 with either pCMV-RB or pCMV-RB(379-928) led to a major reduction in the number of large cells (Fig. 2 and Table 1). The suppression of the large-cell phenotype by E2F-1 was similar to that observed following cointroduction of E1A and RB into SAOS2 cells (Fig. 2).

To test whether both RB and exogenous E2F-1 were synthesized in the same cells and to determine the relationship, if any, between the simultaneous synthesis of the two proteins and their subcellular distribution, double-label immunofluorescence staining with anti-RB and anti-E2F-1 antibodies was performed on cotransfected SAOS2 cells. Under the staining conditions used, the endogenous E2F-1 signal was not detected, and no RB signal was present in untransfected cells. An E2F-1 signal in singly transfected cells was predominantly nuclear (data not shown). Upon cotransfection with RB, both the newly synthesized RB and E2F-1 could be detected in the same cells, and both proteins appeared to be nuclear when cotransfected (Fig. 3E and F). Using an excess of pSG5-E2F-1 to pCMV-RB, we found that the majority of the RB^+ cells stained positively for E2F-1, confirming the coexpression of the two alleles in the relevant cells. These data, in conjunction with the Western blot results cited above (Fig. 1), suggest that the ability of E2F-1 to override the RB-induced large-cell phenotype was not due to an alteration of the abundance or subcellular distribution of RB within cells which overproduce E2F-1.

The vast majority of large cells stained positively for RB, but ~10 to 20% did not. Moreover, ~5 to 10% of the total RB^+ cells in either an RB-transfected or an RB–E2F-1-cotransfected culture stained positively for RB but revealed a normal

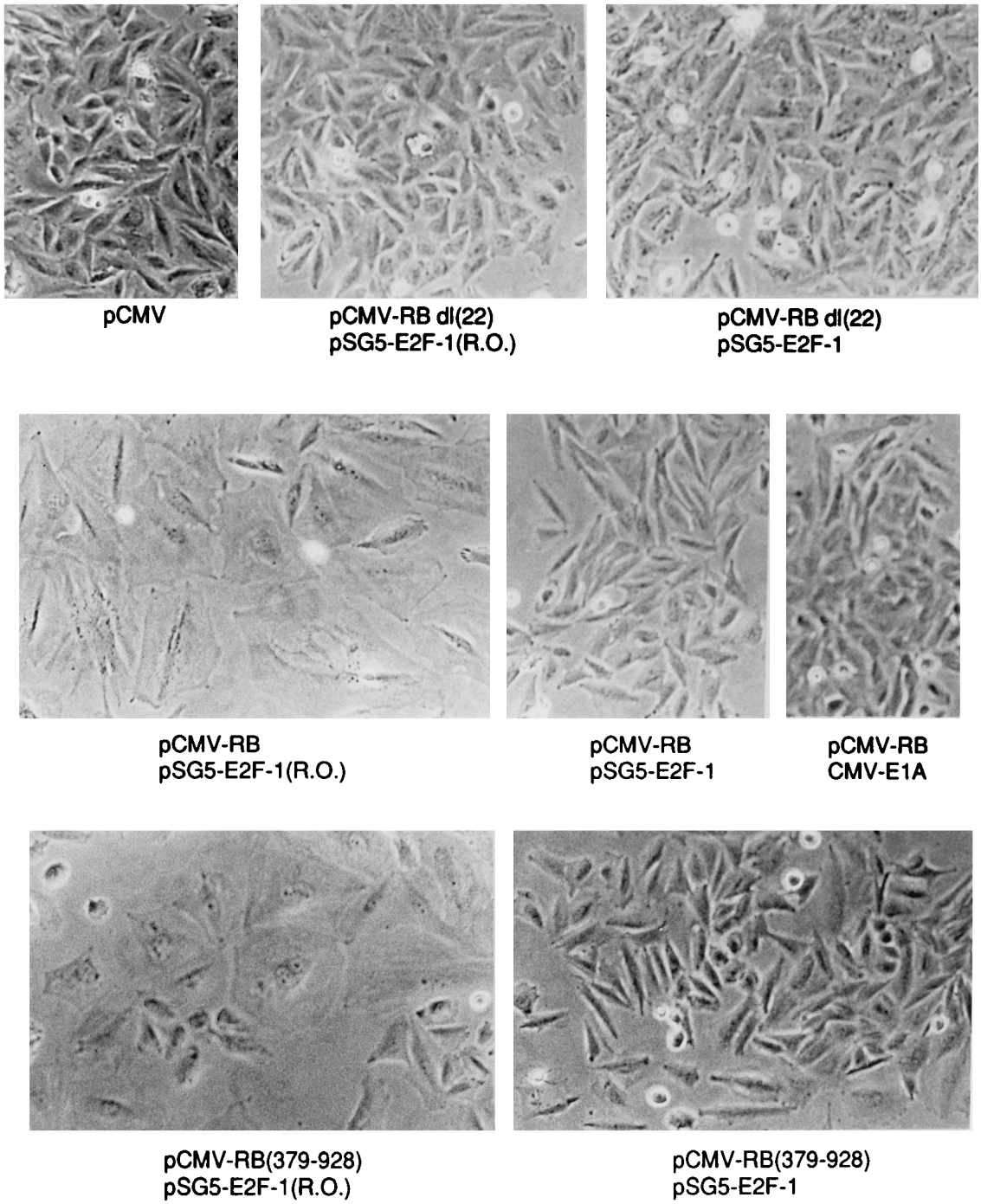

FIG. 2. Phase-contrast micrographs (magnification, ×250) of SAOS2 cells, transfected with the indicated plasmids, after 4 days of cultivation in G418-containing medium.

morphology. Indeed, in the latter case, these cells did not stain positively for E2F-1. Among several possibilities, these findings suggest that RB and E2F-1 can have biological effects at concentrations below those required to score positively by immunofluorescence. In support of the view that E2F-1 can serve its function(s) without being detected, the endogenous E2F-1 signal was undetectable in nontransfected cells by immunofluorescence under these assay conditions, although it is present in these cells, as shown by immunoblotting of concentrated cell extracts (12a). It is also possible that RB-transfected SAOS2 cells secrete paracrine factors which promote enlargement of cells which either were untransfected or synthesized insufficient RB to enlarge on their own.

Design of E2F-1 mutants. Initial structure-function studies of E2F-1 identified a transcription activation domain at its C terminus (residues 368 to 437) and an RB-binding domain (residues 409 to 426) embedded within it (20, 32, 50, 80). Within the N-terminal region, there is a putative HLH domain

TABLE 1. Large-cell production

Plasmids	No. of large cells/500 cells		Avg %	% Reduction
	Expt 1	Expt 2		
RB + control	39	28	6.7	
RB + E2F-1	7	8	1.5	78
RB + E2F-1(dl18)	5	6	1.1	84
RB + E2F-1(dl24)	6	10	1.6	76
RB + E2F-1(dl53)	29	31	6.0	10
RB + E2F-1(dl53+dl18)	32	33	6.5	3
RB + E2F-1(1-241)	8	10	1.8	73
RB + E2F-1(1-196)	8	11	1.9	72
RB + E2F-1(1-196; dl24)	9	7	1.6	76
RB + E2F-1(1-127)	31	29	6.0	10
RB + E2F-1(41-196)	11	17	2.8	58
pCMV + control	3	2	0.5	93

(residues 128 to 181) which, when synthesized as a bacterial glutathione *S*-transferase fusion protein, can bind specifically to a canonical E2F DNA-binding site (32, 45, 50, 80). Immediately upstream of this HLH domain is a basic cluster, and more toward the C terminus, there is a hydrophobic heptad repeat (putative zipper region), which serves to support heterodimer formation with the E2F-1 partner, DP-1 (3, 33, 57). Another domain (residues 76 to 99), located upstream of the basic cluster, which can bind to the cyclin A protein both in vitro and in vivo, has also been recently defined (56). On the basis of these observations, a series of E2F-1 deletion mutations affecting these various regions was generated and subcloned into the pSG5-E2F-1 vector (Fig. 4). The question at hand was whether one or more of these functional regions contribute to the ability of E2F-1 to neutralize the RB large-cell effect.

Synthesis and subcellular localization of E2F-1 mutants. Each of the E2F-1 mutant expression plasmids gave rise to the synthesis of a grossly stable protein, as defined by total steady-state accumulation in E2F-1 Western blots and immunofluorescence studies (Fig. 5 and data not shown). Moreover, the abundance of each appeared to be unaffected by coexpression of an RB-encoding plasmid (data not shown). The subcellular location of wt E2F-1 and E2F-1 mutants was determined by indirect immunofluorescence staining after transfection of plasmids encoding the various proteins (Fig. 5 and data not shown). Cells producing exogenous wt E2F-1 and each of the mutants tested exhibited strong nuclear fluorescence, suggesting that the protein was properly localized. Weaker nuclear staining was observed in cells transfected with E2F-1(1-241), E2F-1(1-196), and E2F-1(41-196) than in cells transfected with the other E2F-1 mutants tested. Cells transfected with E2F-1(1-127) revealed both nuclear and cytoplasmic staining.

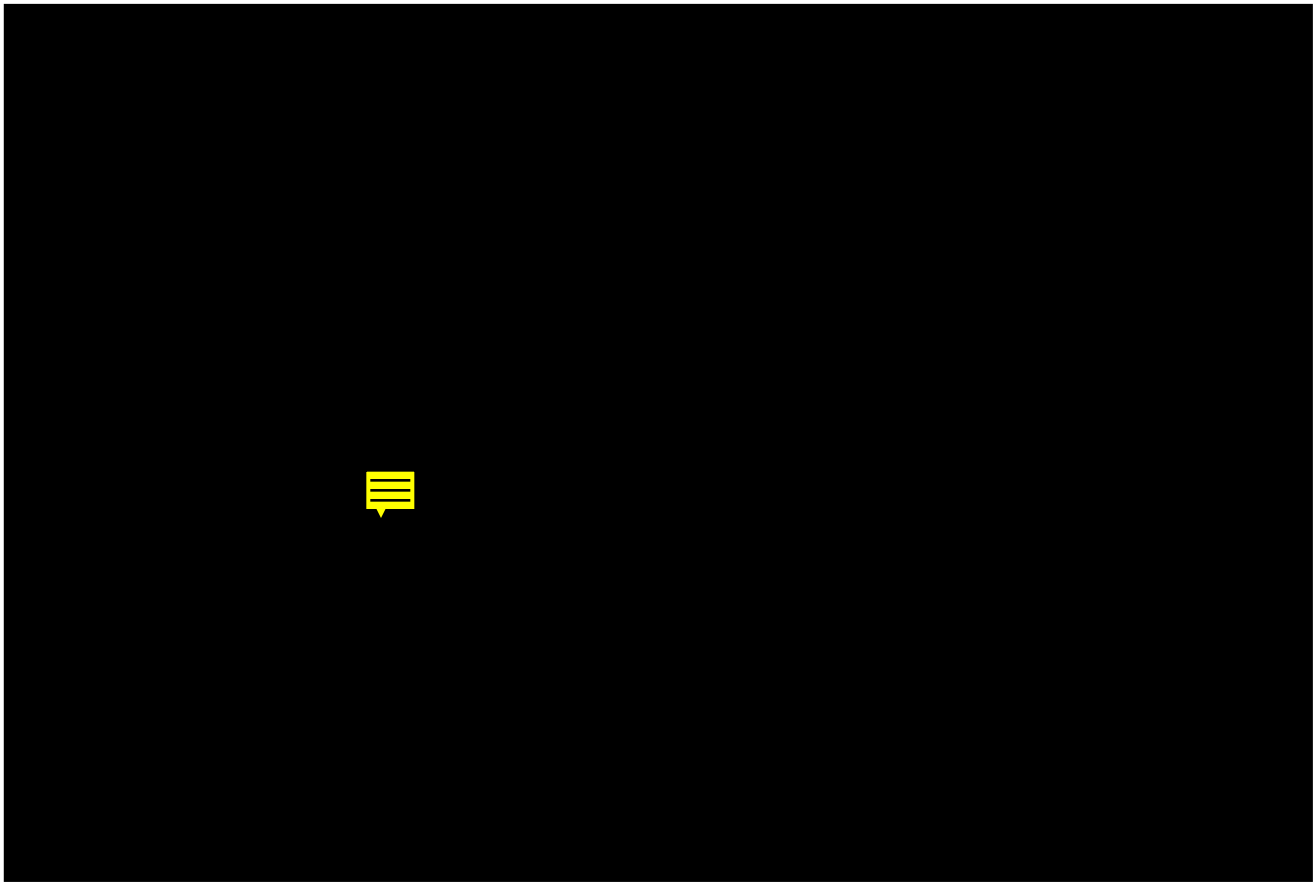

FIG. 3. Colocalization of RB and E2F-1 in transfected SAOS2 cells (magnification, ×368). Cells were transfected with pCMV-RB (A to C) or pCMV-RB and pSG5-E2F-1 (D to F); 24 to 36 h later, RB was visualized by indirect immunofluorescence staining using an FITC-conjugated secondary antibody (B and E) and E2F-1 was visualized by indirect immunofluorescence staining using a rhodamine-conjugated secondary antibody (C and F). DNA was stained with 4′,6-diamidino-2-phenylindole hydrochloride (DAPI) (A and D).

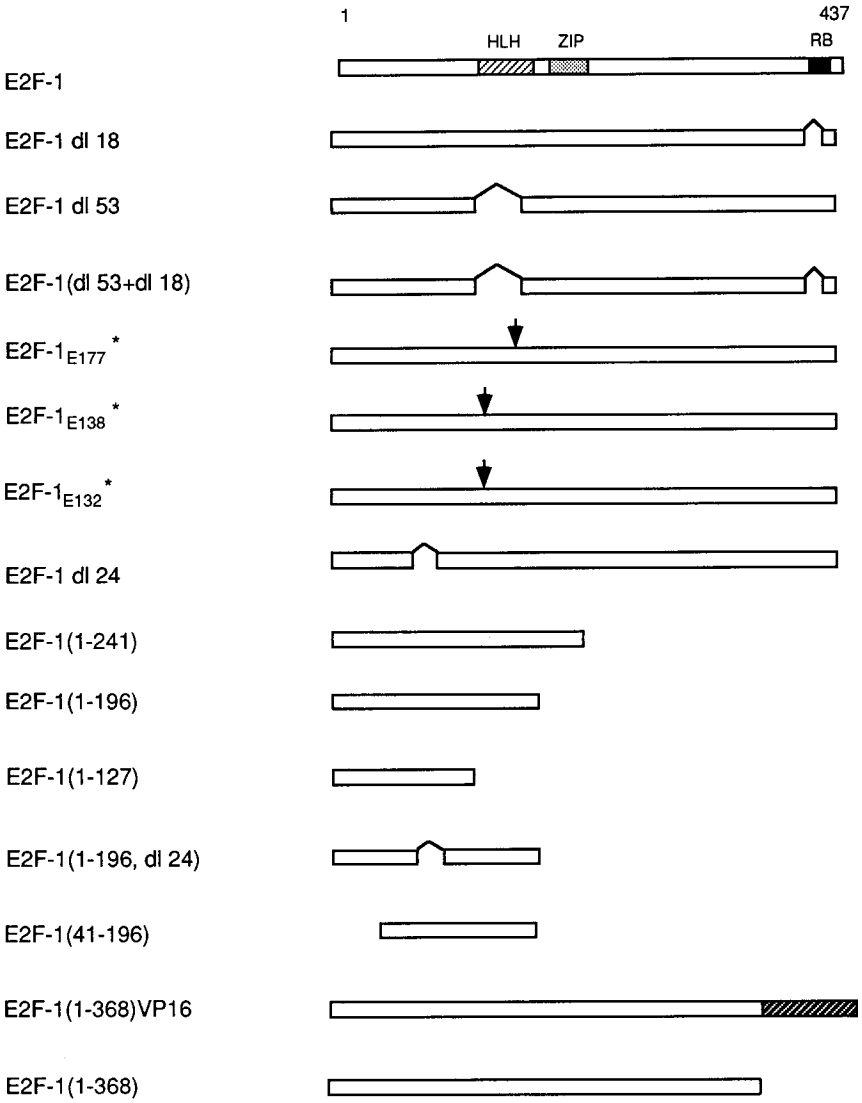

FIG. 4. Structural maps of the various E2F-1 derivatives analyzed. *, see reference 10.

The ability of E2F-1 to override the RB large-cell phenotype requires an intact DNA-binding domain but not an intact transactivation or RB-binding domain. Next, we examined whether RB binding was required for E2F-1 to neutralize the large-cell phenotype. To this end, a plasmid encoding a mutant version of E2F-1 which lacks the 18-amino-acid RB-binding sequence was introduced into SAOS2 cells. E2F-1(dl18), like wt E2F-1, prevented the large-cell phenotype, suggesting that RB binding is not required for the E2F-1 effect on cell morphology (Table 1). Another E2F-1 mutant, E2F-1(1-241), lacking both the RB-binding and transactivation domains, was also active in this assay, as was E2F-1(dl24), which lacks cyclin A-binding function.

In addition, E2F-1(1-196), an N-terminal fragment which terminates before the putative zipper domain, blocked large-cell formation, whereas E2F-1(1-127), which contains the intact basic region and terminates before the HLH, failed to suppress it (Table 1). This result cannot be ascribed to insufficient E2F-1(1-127) synthesis, because E2F-1(1-127) appeared to accumulate to a higher level than E2F-1(1-196), as determined by Western blotting (data not shown). Further deletion of either the extreme N-terminal 41 amino acids or the cyclin A-binding region failed to inactivate the large-cell-suppressing effect of E2F-1(1-196), since both E2F-1(41-196) and E2F-1(1-196; dl24) reduced the number of large cells produced upon cotransfection with pCMV-RB, although the effect of E2F-1(41-196) was slightly less prominent than that of E2F-1(1-196) in the same experiments (Table 1).

Since a region of the protein containing largely its DNA-binding apparatus (HLH, the minimal unit needed for E2F DNA binding in vitro [32, 45, 50]) functioned in this assay, we examined whether the wt E2F-1 effect was dependent on the integrity of its HLH motif. Using oligonucleotide-directed mutagenesis, we constructed a deletion mutant in which the HLH was internally deleted from an otherwise intact protein to create E2F-1(dl53). Transfection of pSG5-E2F-1(dl53) gave rise to a stable protein with nuclear location (Fig. 5 and data not shown). As shown in Table 1, significant numbers of large cells developed after cotransfection of E2F-1(dl53) with RB. Consistent with this finding, E2F-1(dl53+dl18), which carries mutations in both the RB-binding and HLH regions, also failed to suppress the large-cell phenotype. These results indicate that

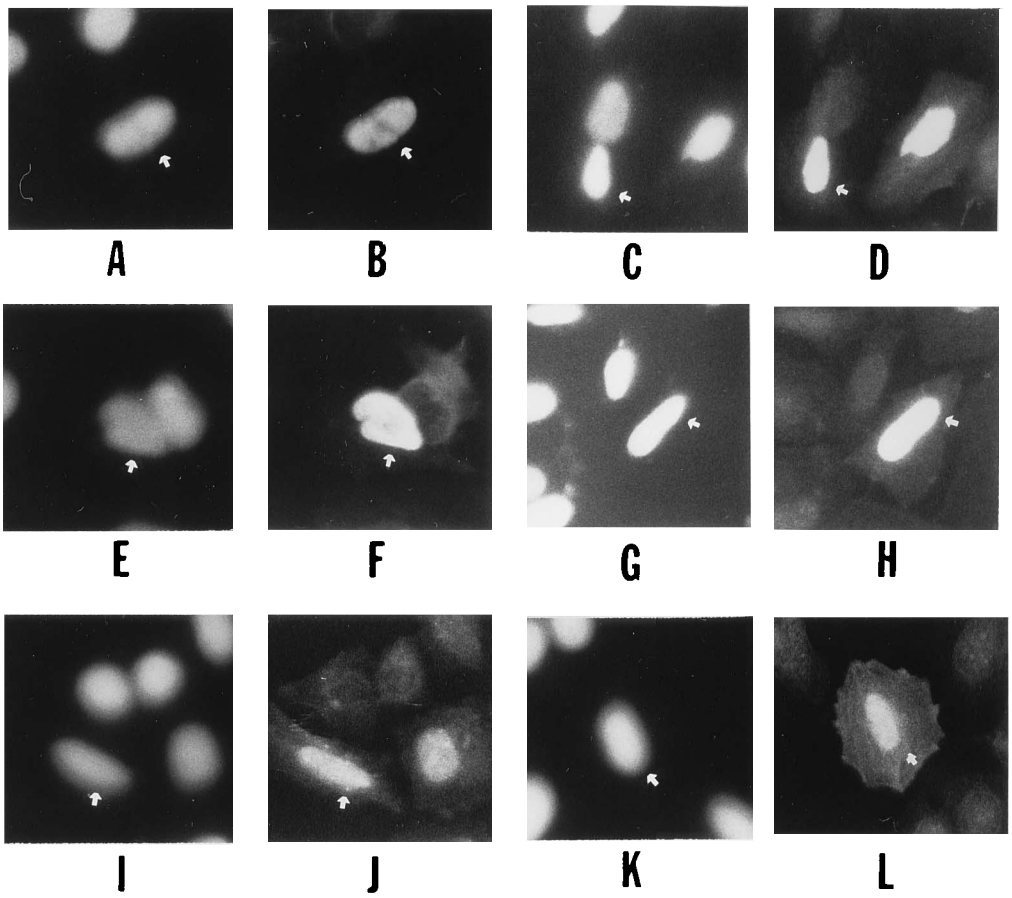

FIG. 5. Nuclear immunolocalization of various E2F-1 mutant derivatives in SAOS2 cells. Cells were doubly stained with 4′,6-diamidino-2-phenylindole hydrochloride (DAPI) for DNA (A, C, E, G, I, and K) and with a rabbit antibody raised against E2F-1 for E2F-1 and its derivatives (B, D, F, H, J, and L). A typical E2F-1-positive cell in each experiment is indicated by the arrows. SAOS2 cells were transfected with pSG5-E2F1 (A and B), pSG5-E2F1(dl18) (C and D), pSG5-E2F1(dl53) (E and F), pSG5-E2F1(dl53+dl18) (G and H), pSG5-E2F1(1-196) (I and J), and pSG5-E2F1(1-127) (K and L).

the DNA-binding unit is necessary for the E2F-1 large-cell suppression effect. Since the product of a plasmid encoding only the E2F-1 HLH was undetectable when produced in these cells, we do not know whether the E2F-1 HLH is sufficient to block the appearance of large cells.

The region of E2F-1 from residues 41 to 196 contains largely its specific DNA-binding domain, and E2F-1(dl53) has lost this region only. To further test the hypothesis that DNA binding is essential for the RB-mediated large-cell phenotype, SAOS2 cells were transfected with RB alone or cotransfected with RB and either wt E2F-1 or, separately, three E2F-1 derivatives, each of which carries two single amino acid substitutions and is unable to bind to DNA (10). Note that the residues altered in these three mutants are not required for stable binding to DP-1 in vitro (10, 33). Furthermore, the E2F-1(E132) mutant can bind to DP-1 in vivo (30). As shown in Table 2, wt E2F-1 once again suppressed large-cell formation. By contrast, none of the mutant species were active in this regard.

The N-terminal fragment E2F-1(1-196), which carries an intact HLH but not the zipper region, binds E2F sites in vivo. From the data described above, one might conclude that the DNA-binding function constitutes a key element in the ability of E2F-1 to override the RB-induced size effect. To test whether a truncated E2F-1 fragment predicted, from in vitro studies, to be competent for DNA binding actually manifests that activity, E2F-1(1-196) and E2F-1(1-127) were tested for in vivo DNA binding function. Gel mobility assays, performed on extracts of suitably transfected SAOS2 cells, showed that the former, but not the latter, retained DNA-binding function in vivo (Fig. 6A). DNA binding by E2F-1(1-196) appeared to be specific, as determined by competition experiments performed with wt or altered versions of canonical E2F DNA-binding sites (Fig. 6A). The data shown in Fig. 6A left open the possibility, however, that E2F1(1-127) DNA complexes were not detected because of their comigration with one or more nonspecific DNA complexes. Therefore, we next transfected cells

TABLE 2. Effects of mutating the E2F-1 DNA-binding domain

Plasmids[a]	No. of large cells/500 cells	Cell cycle analysis	
		% in S, G_2, M	Increase in % of cells in G_1
pCMV + control	4	42	0
RB + control	27	20	22
RB + pCMV-E2F-1	10	31	11
RB + pCMV-E2F-1(E177)	31	15	27
RB + pCMV-E2F-1(E138)	29	19	23
RB + pCMV-E2F-1(E132)	28	19	23

[a] The wt E2F-1 and derivatives used were ligated into the pcDNA I/amp vector (10).

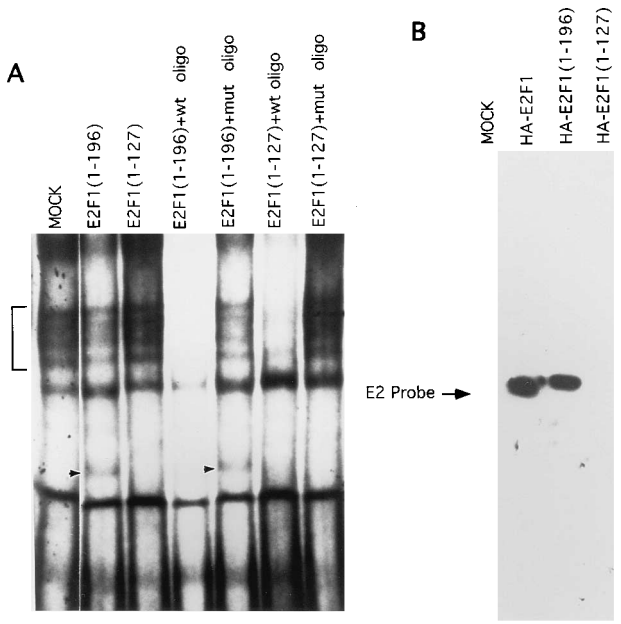

FIG. 6. DNA binding studies. (A) Gel shift experiments. Whole-cell extracts prepared from SAOS2 cells transfected with plasmids encoding the indicated E2F-1 species were incubated with a ^{32}P-labeled E2 promoter-containing oligonucleotide. Where indicated, the specificity of complex formation was demonstrated by the addition of a 100-fold molar excess of unlabeled competitor oligonucleotide DNA representing either the wt or mutant (mut) E2F-binding site. Protein-DNA complexes were resolved by electrophoresis in a 4% polyacrylamide gel. A novel band seen when E2F-1(1-196) was introduced into cells is indicated by the arrowheads. The presence of E2F-1(1-196) in this complex was confirmed by antibody supershift experiments (data not shown). Bands corresponding to endogenous E2F-containing complexes are indicated by the bracket. (B) McKay assays. Extracts prepared from SAOS2 cells transfected with plasmids encoding the indicated HA-tagged E2F species were immunoprecipitated with an anti-HA antibody in the presence of a ^{32}P-labeled E2 probe and competitor DNA. Bound probe was eluted by boiling in sample buffer, electrophoresed in a 12% acrylamide denaturing Tris-borate-EDTA gel, and detected by autoradiography.

with expression plasmids directing the synthesis of HA epitope-tagged wt E2F1, E2F1(1-196), or E2F(1-127). As a negative control, cells were transfected in parallel with the backbone vector; 48 h later, cell lysates were prepared and McKay assays were performed, using an anti-HA antibody and a ^{32}P end-labeled E2F-binding site from the E2 promoter. As can be seen from Fig. 6B, E2F1(1-196), but not E2F(1-127), retained the ability to recognize this site under these assay conditions. Taken together, these data suggest that the E2F-1 DNA-binding domain, previously identified by in vitro studies, namely, the N-terminal fragment consisting of an intact HLH and terminating before the putative zipper (32, 45, 50), can bind DNA in vivo.

Release of RB-induced G_1 arrest by transactivation-competent E2F-1. To determine whether cotransfection of E2F-1 releases an RB-dependent G_1 block and, if so, what elements within the E2F-1 molecule were required, flow cytometry analysis of cotransfected SAOS2 cells was performed.

RB and E2F-1 expression plasmids were introduced along with a third plasmid encoding the cell surface protein CD19 (85). The CD19 marker was used as a means of selectively trapping transfected cells carrying a specific tag after the cells were coated with a suitable anti-CD19 monoclonal antibody. The generic goal of this experiment was to determine which phase(s) of the cell cycle the transfected cells were in. The synthesis of CD19 alone did not affect the normal distribution of unsynchronized SAOS2 cells in the cell cycle (data not shown). Therefore, it was possible to transfect them with RB and CD19, with and without a wt or mutant allele of E2F-1, and search for dissolution, by the transcription factor, of the RB cell cycle block.

TABLE 3. Cell cycle analysis of E2F-1 mutants by flow cytometry

Plasmids	Expt 1[a]		Expt 2	
	% in S, G_2, M	Increase in % of cells in G_1	% in S, G_2, M	Increase in % of cells in G_1
pCMV + control	50	0	52	0
RB + control	26	24	15	37
RB + E2F-1	34	16	36	16
RB + E2F-1(dl18)	37	13	46	6
RB + E2F-1(dl24)	31	19		
RB + E2F-1(dl53)	14	36	18	34
RB + E2F-1(dl53+dl18)	17	33		
RB + E2F-1(1-241)	21	29	15	37
RB + E2F-1(1-196)	18	32		
RB + E2F-1(1-196; dl24)	22	28		
RB + E2F-1(1-127)	20	30		
RB + E2F-1(41-196)	19	31		

[a] The numbers were calculated among FITC-positive cells; a total of 15,000 cells were counted for each experiment. DNA content was measured by propidium iodide fluorescence intensity.

The results of these experiments are shown in Tables 2 to 4. In keeping with earlier reports, introduction of RB alone led to G_1 arrest, manifested by a ~25 to 30% net increase of $CD19^+$ G_1 cells over the number observed in the vector-transfected control population. Cotransfection of RB and E2F-1 led to a significant decrease in the percentage of RB-positive cells in G_1 compared with what was observed in a culture transfected with RB alone. E2F-1 alone exhibited no significant effect on cell cycling, and a known RB pocket mutant unable to block cell growth was also inactive in this assay, as predicted (17, 34, 73, 74, 86) (data not shown). Consistent with the observations of Ewen et al. (17), cotransfection of cyclin D2 and RB reproducibly led to RB overt phosphorylation (Fig. 1) and movement of a fraction of the RB-blocked population into $S/G_2/M$ (data not shown).

Next, we examined the effect of cotransfecting various E2F-1 mutants on an RB-induced G_1 block. As shown in Table 3, synthesis of RB alone led to a G_1 block, and cotransfection of E2F-1 with RB promoted S-phase entry by a significant fraction of the otherwise blocked cells. In keeping with the large-cell phenotype suppression results, E2F-1(dl18) also overrode the block, resulting in a significant decrease in the number of $CD19^+$ G_1 cells, whereas E2F-1(dl53) and E2F-1(dl53+dl18) were inert. In keeping with the results on the large-cell phenotype, none of the three more subtly mutated, DNA-binding-defective E2F-1 derivatives [E2F-1(E177), E2F-1(E138), and E2F-1(E132) (10)] were active in overcoming an RB-induced G_1 block (Table 2). Each of these proteins appeared to be as stable as wt E2F-1, as determined by steady-state Western blot analysis, and was nuclear in location, as determined by immunofluorescence (data not shown). This finding suggests strongly that RB-E2F complexes interact specifically with one or more promoter-linked E2F sites to block G_1 exit. The fact that these mutants retained RB-binding activity, when tested (10), suggests that RB binding is not sufficient for overriding the G_1 block established in these experiments. This observation, along with the ability of the E2F-1(dl18) to overcome an RB block, suggests that the E2F-1 effect observed here is not simply due

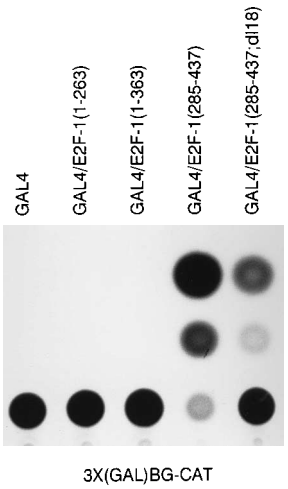

FIG. 7. E2F-1(dl18) retains transactivating activity. SAOS2 cells were transfected with plasmids encoding the indicated GAL4/E2F-1 chimeras along with a CAT reporter plasmid containing three tandem GAL4 DNA-binding sites. CAT assays were performed as described previously (20).

to a T-like sequestration or pocket-emptying effect on the cosynthesized RB protein. Hence, E2F-1 likely plays an active role in promoting G_1 exit in these cells.

Since the cyclin A-binding mutant was active in these assays, this function does not contribute to the E2F-1 override of the RB G_1 block. E2F-1(1-241), E2F-1(1-196), E2F-1(1-196; dl24), and E2F-1(41-196), all of which suppressed the large-cell phenotype, were, however, inactive in this assay (Table 3 and data not shown). The results with the deletion mutants of E2F-1 were further confirmed in experiments in which a CD19 plasmid was not included in the transfection mixtures and transfected cells were selected after staining with an anti-RB monoclonal antibody (PMG3-245; PharMingen) (data not shown). Hence, the method of selecting and scoring the phenotype of transfected cells did not contribute to the observed results.

Of note, the E2F-1 mutants which failed to release an RB G_1 block lack either an E2F-1 transactivation domain or a DNA-binding domain. This observation suggests that both the specific DNA-binding and transactivation functions of E2F-1 are required for this protein to overcome a G_1 block. To test this hypothesis further, we assayed E2F-1(dl18) for transactivation function by fusing it to the GAL4 DNA-binding domain and testing its ability to transactivate a GAL4 CAT reporter plasmid. GAL4/E2F-1(dl18) clearly functioned as a transactivator in this assay, although it was less active than the wild-type GAL4/E2F-1 protein tested in parallel (Fig. 7). In addition, we constructed expression plasmids encoding either E2F-1(1-368), an E2F-1 fragment which terminates immediately before its transactivation domain, or E2F-1(1-368) fused to the herpesvirus VP16 C-terminal acidic activation domain, E2F-1(1-368)VP16. As shown in Table 4, E2F-1(1-368)VP16, like the wt protein, can override an RB-induced G_1 block and, in fact, did so more effectively than the latter when tested in parallel. In contrast, E2F-1(1-368) was inactive in these assays. These data again suggest that for E2F-1 to override an RB-induced G_1 block, it must contain functional transactivation and DNA-binding domains.

An E2F-1 transactivation-defective mutant which retains DNA-binding function can block RB-mediated transcriptional repression. Our results, taken together with earlier studies which suggested that E2F-binding sites could serve as repressor elements in certain promoter contexts (11, 47, 59, 70, 89), raised the possibility that the induction of large cells by RB was linked to the ability of RB-E2F complexes to bind to, and repress, certain E2F-responsive promoters. To test this possibility, we examined whether coproduction of an E2F-1 transactivation-defective mutant which retained DNA-binding function could block the ability of RB to repress a model E2F-responsive promoter. To this end, SAOS2 cells were transfected with the following: (i) a reporter plasmid containing the E2F-1 promoter fused to luciferase (70); (ii) expression plasmids encoding either wt or mutant (deleted of exon 22) RB; and (iii) expression plasmids encoding E2F-1(1-368) (which retains the ability to bind to DNA but lacks the E2F-1 transactivation domain) (20, 50) or E2F-1(1-368;132) (a DNA-binding-defective mutant derivative thereof) (10). Both of these E2F-1 expression plasmids gave rise to the production of stable proteins, as determined by Western blot analysis (data not shown). The E2F-1 promoter contains four potential E2F sites which appear to mediate the repression of this promoter under low-serum conditions (47, 70). Following transfection, cell extracts were prepared and luciferase assays were performed. As can be seen from Fig. 8, wt (but not mutant) RB repressed the E2F-1 promoter in these assays. Coproduction of E2F-1(1-368), but not E2F-1(1-368;132), reproducibly inhibited the ability of RB to repress this promoter. Thus, the coproduction of an E2F-1 mutant which lacks its transactivation domain but retains the ability to bind to DNA can block RB-mediated transcriptional repression.

Lack of effect of cotransfected E2F-1 on the state of RB phosphorylation. Cotransfection of RB-encoding plasmids with cyclin D2 into SAOS2 cells led to the appearance of more

TABLE 4. Cell cycle analysis of E2F1-VP16 chimera and nonphosphorylatable RB mutant by flow cytometry

Plasmids	Expt 1[a]		Expt 2		Expt 3	
	% in S, G_2, M	Increase in % of cells in G_1	% in S, G_2, M	Increase in % of cells in G_1	% in S, G_2, M	Increase in % of cells in G_1
pCMV + control	45	0	47	0	47	0
RB + control	14	31	20	27	18	29
RB + E2F-1	21	24	31	16	37	10
RB + E2F-1(1-368)VP16	35	10	35	12	41	6
RB + E2F-1(1-368)	14	31	22	25		
RB + E2F-1(dl53)	16	29				
RB + E2F-1(1-196)	15	30				
RBΔp34-HA + control	19	26	23	24		
RBΔp34-HA + E2F-1	26	19	39	8		

[a] The numbers were calculated among FITC-positive cells; a total of 15,000 cells were counted for each experiment. DNA content was measured by propidium iodide fluorescence intensity.

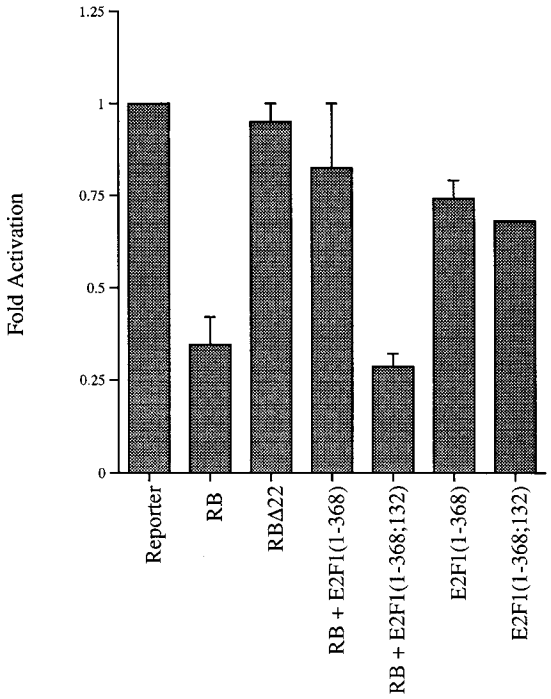

FIG. 8. A transactivation-defective E2F-1 mutant which retains DNA-binding capability can block RB-mediated transcriptional repression. SAOS2 cells grown in 100-mm-diameter dishes were transfected, in duplicate, with 5 μg of an E2F-1 promoter-luciferase reporter plasmid, 2 μg of pCMV-βgal, and the indicated pCMV-RB expression plasmids (500 ng) and pcDNA-E2F-1 expression plasmids (2 μg). The total amount of pCMV and pcDNA plasmid in each transfection was normalized by the addition of the backbone plasmid(s) (total DNA = 20 μg); 48 h later, cell extracts were prepared and luciferase and β-galactosidase assays were performed. Luciferase values were normalized for β-galactosidase and expressed relative to the activity observed with the E2F-1 promoter-luciferase reporter plasmid in the absence of the RB and E2F expression plasmids.

slowly migrating, overtly phosphorylated RB bands, as previously described by Ewen et al. (17) (Fig. 1, lane 6, 8, and 10). These bands comigrated with the pRBphos species generated after transfection of RB plasmids into U-20S cells ($RB^{+/+}$) (17, 74) and represent overtly phosphorylated RB species, as previously demonstrated by both ^{32}P labeling and exogenous phosphatase treatment experiments (74). Similar to cyclin D2, E2F appears to bind efficiently to RB(379-928), the minimal region of RB necessary for G_1 growth suppression (17, 74). However, when the state of RB phosphorylation and the release of the G_1 block in the E2F-1–RB-transfected cells were monitored in parallel, we found that E2F-1 could override an RB-induced G_1 block without causing the hyperphosphorylation of RB, as determined either by P_i labeling (Fig. 1, lanes 7 and 9) or by the migration of the RB species in SDS-polyacrylamide gels (Fig. 1; compare lanes 3 and 4). In the same experiments, cyclin D2 also overrode the block and did lead to overt RB phosphorylation (Fig. 1; compare lane 6 with lane 4 and see lanes 7 to 10).

To address this issue further, we analyzed RBΔp34-HA, an RB mutant in which the known phosphorylation sites have been mutated (29). This protein was found not to be overtly phosphorylated in vivo and to form complexes with E2F (29). As shown in Table 4, overproduction of RBΔp34-HA in SAOS2 cells led to a G_1 block. This effect could be overridden by the cotransfection of an E2F-1 expression plasmid, further supporting the notion that E2F-1 can overcome an RB-induced G_1 block without inducing overt RB phosphorylation.

Thus, two different RB-binding proteins, E2F-1 and cyclin D2 (17, 32, 50, 80), overrode the RB block, albeit with different consequences for the state of RB phosphorylation.

DISCUSSION

Introduction of wt RB into certain $RB^{-/-}$ cells induces G_1 arrest. Here we have demonstrated that the ability of RB to induce G_1 arrest can be overcome by coproduction of the cellular transcription factor E2F-1. This observation has also been made by Zhu et al. (93). In their study, E2F-1 mutants were not examined, leaving open the possibility that E2F-1, by virtue of overproduction, was inactivating RB in a T- or E1A-like manner. We found, however, that the ability of E2F-1 to bind to RB was neither necessary nor sufficient for this effect. Thus, E2F-1 was not merely acting to displace other RB-binding proteins from the RB pocket. In contrast, the ability of E2F-1 to bind to DNA and to transactivate was necessary, consistent with the notion that E2F-1 was acting as a sequence-specific, DNA-binding transcription factor in these assays. In keeping with this view, we showed that fusion of a heterologous transactivation domain to the E2F-1 DNA-binding domain restored the ability of the latter to overcome an RB-induced G_1 block. We further showed that the ability of E2F-1 to overcome an RB-induced block was not dependent upon its ability to induce overt phosphorylation of RB, such as has been shown for certain cyclins (17, 37). Indeed, we showed that E2F-1, but not cyclin D2, could overcome the action of a nonphosphorylatable RB mutant which retains pocket function. These observations, coupled with earlier biochemical studies which showed that RB normally interacts with E2F-1 in vivo, strongly suggest that E2F-1 is a downstream target of RB action.

We cannot conclude, however, that it is the only target of RB action. A number of putative RB-binding proteins (14, 15, 17, 19, 26, 27, 43, 51, 77, 88, 90), including additional E2F family members (3a, 19a, 20a, 21, 46, 60), have recently been identified, and it is possible that the ability of RB to induce a G_1 block is linked to the regulation of all or a subset of these proteins. Thus, proper regulation of E2F-1 appears to be necessary, but may not be sufficient, for RB to induce a G_1 block.

That coexpression of E2F-1 allowed SAOS2 cells to progress out of G_1 into later phases of the cell cycle, in the absence of overtly phosphorylated RB, suggests three additional conclusions. First, although un(der)phosphorylated RB (pRB) blocks replication, it does so during G_1, and not S, at least under these experimental conditions, in keeping with the results of Goodrich et al. (24). That cells can pass a G_1 block containing largely unphosphorylated or underphosphorylated wt RB is consistent with results of Dowdy et al. (15), who made a similar observation following transfection of SAOS2 cells with RB and cyclin D1. Second, given the results presented here, the presence of overtly phosphorylated RB is not an absolute requirement for S-phase entry. Third, RB–E2F-1-cotransfected cells passed through intervals in the cell cycle wherein cyclins E and A are normally synthesized and complexed with cdk2 (reference 37 and references therein). Both of these proteins, when transfected into SAOS2 in the presence of RB, led to RB hyperphosphorylation, consistent with the view that each plays a role in the normal RB phosphorylation process (37). Why, after introduction of E2F-1, RB was not overtly phosphorylated during G_1 exit and S-phase entry is a relevant question. It is possible that RB phosphorylation is a processive event. For example, it is possible that in the absence of a primary phos-

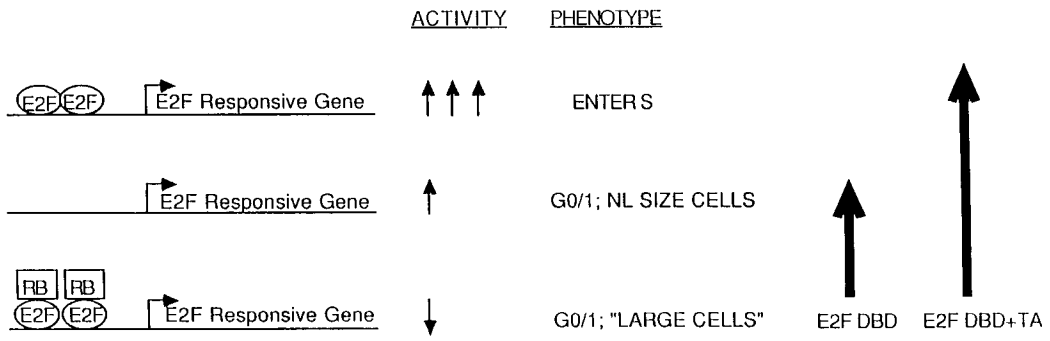

FIG. 9. Model for effects of E2F-1 expression upon RB-induced G_1 block. The effect of cointroducing the E2F-1 DNA-binding domain (DBD) alone or fused to a transactivation domain (TA) is thought to alter the transcriptional activity of certain E2F-responsive genes, which in turn affects cell size and ability to enter S phase. For simplicity, "E2F" is used to indicate heterodimeric complexes between E2F and DP family members regulated by RB.

phorylation event (e.g., catalyzed by a cyclin D2/cdk kinase complex [17, 52]), subsequent phosphorylation events mediated by cyclin E/cdk2 and cyclin A/cdk2 complexes, which arise later in G_1 and early in S in cycling cells, respectively, are not readily detectable. One theoretical caveat associated with this hypothesis is that these kinases may not be sufficiently activated under the experimental conditions used here.

Reintroduction of wt RB into SAOS2 cells leads to three phenotypes, namely, alteration in cellular morphology (large cells), induction of a G_1 arrest, and suppression of macroscopic colony formation by cells coexpressing a selectable marker (34, 37, 41, 73, 74). Cotransfection of E2F-1 with RB overcame the first two effects but not the third (74a). The inability of cells cotransfected with E2F-1 and RB to form macroscopic colonies may be due to the ability of deregulated E2F-1 production to cause apoptosis (75, 91). Surprisingly, we found that the ability of RB to induce large cells and to induce a G_1 arrest could be genetically dissociated. In particular, we found that the E2F-1 DNA-binding function was both necessary and sufficient to block the induction of large cells, whereas the ability to overcome a G_1 block required a functional DNA-binding domain and a functional transactivation domain. For the latter, either a homologous or a heterologous transactivation domain was sufficient, given the results with E2F1(1-368)VP16, in which a foreign transactivation domain successfully replaced the natural one. Earlier studies have suggested that RB-E2F complexes may actively repress transcription from certain promoters containing E2F sites (11, 47, 59, 70, 89). Thus, it is possible that the ability of the minimal E2F-1 DNA-binding domain to block the induction of large cells by RB is due to its ability to compete with RB-E2F transcriptional repressor complexes for binding to selected E2F-responsive promoters whose products, directly or indirectly, govern cell size. Our finding that an E2F-1 transactivation-defective mutant which retained the ability to bind to DNA could block RB-mediated transcriptional repression in a transient assay is wholly consistent with this view. Given the nature of the mutants studied here, one might speculate that this possible mechanism, without the attendant activation of certain promoters by the transfected factor, is sufficient to block large-cell formation but not to move a cell out of G_1. To overcome a G_1 block, both alleviation of transcriptional repression and delivery of a stimulatory transcriptional signal would appear to be required (Fig. 9). Clearly, other models exist.

Thus, RB-E2F complexes appear to have two biological effects: G_1 blockade and production of large cells. Alleviation of the latter can be achieved without cancellation of the former. This observation raises the possibility that in SAOS2 cells, RB-E2F complexes operate at two (or more) points in the cell cycle prior to S-phase entry. In this model, the first block (the transition from 2N DNA containing large cells to 2N DNA containing normal-appearing cells) was overcome by an exogenous E2F-1 DNA-binding domain competing with one or more E2F species, presumably bound to RB, for binding to a subset of E2F-responsive genes. This effect, surprisingly, was not dependent upon the competitor stimulating a promoter per se. Nor was it dependent upon displacing endogenous E2F bound to the RB pocket, since successful elimination of cell swelling was achieved with E2F species which were unable to bind to RB at all. Overcoming the second block (between 2N normal-size cells and G_1 exit), would, in this model, also depend on the ability of E2F-1 to compete with RB-E2F complexes for binding to selected promoters as well as its ability, once bound to DNA, to deliver a transactivation signal to appropriate S-phase genes (see above).

Clearly, a more detailed biochemical understanding of why SAOS2 cells become large upon reintroduction of RB will be required before we speculate further. Among the central questions to be answered are the following. Have RB-induced large cells exited the cell cycle? Being large, has their state of differentiation changed, or are they senescent? To what extent, if any, is the repertoire of E2F-responsive genes responsible for the observed changes in cell morphology the same as those which govern S-phase entry?

Why did the DNA-binding-defective E2F-1 mutants which retained RB binding capability not disrupt the binding of RB to E2F-1 in a T- or E1A-like manner? Should they have not also scored positive if RB action requires binding to E2F-1? An uninteresting possibility is that these E2F-1 mutants bind to pRB with lower affinity than wild-type E2F. This seems unlikely given that (i) the C terminus of E2F-1 is sufficient for recognition by pRB in vivo (20, 31) and (ii) at least one of the DNA-binding-defective point mutants tested here retains the ability to bind to DP-1 in vivo (30). DP-1 enhances the ability of E2F-1 to bind to pRB (33, 57). It is also worth bearing in mind, however, that T and E1A have apparently evolved to displace proteins such as E2F from the RB pocket. It follows that they are more potent pocket emptiers than wt E2F-1. In this regard, we know that these viral proteins share a sequence (L-X-C-X-E), not found in the E2F proteins cloned to date, which enables them to bind with high affinity to the RB pocket, and that less of the RB protein is required for stable binding to the viral proteins than is required for E2F binding (1, 7, 34, 36, 42, 73, 74, 76). It is also possible that RB binds preferentially to DNA-bound E2F. If so, one would predict that a DNA-binding-defective E2F-1 protein (and hence a protein not

bound to DNA) might not efficiently disrupt RB-E2F-DNA complexes.

p107, like RB, contains a pocket domain, can bind to E2F, and, when overproduced, can repress reporter genes bearing E2F sites (79, 92, 93). Zhu et al. have shown that overproduction of p107 can inhibit the growth of certain cells, including SAOS2 cells, in G_1 (93). They further demonstrated that the ability of p107 to induce growth arrest, unlike that of RB, could not be neutralized by coexpression of E2F-1. This finding, taken together with the recent identification of multiple E2F species (3a, 19a, 20a, 21, 46, 60), and the failure, thus far, to detect stable p107–E2F-1 complexes in vivo, might suggest that pocket proteins differ with respect to their downstream E2F targets. A caveat here, however, is that the ability of p107 to suppress cell growth in the experiments described by Zhu et al. (93) did not depend upon the integrity of its pocket domain, raising the possibility that E2F regulation may not be responsible for this effect.

Given that E2F-1 can dominate a major aspect of the growth suppression function of RB (G_1 exit block), it is possible that E2F-1, under certain circumstances, behaves as an oncogene. Recent experiments that suggest that E2F-1 can induce quiescent cells to enter S phase are consistent with this view (48, 75). One might also predict that deregulated expression of E2F-1, and/or synthesis of an E2F-1 species bearing a subtle, inactivating mutation in the RB-binding domain, has the same functional consequences as loss of RB. Experiments aimed at testing this possibility are in progress.

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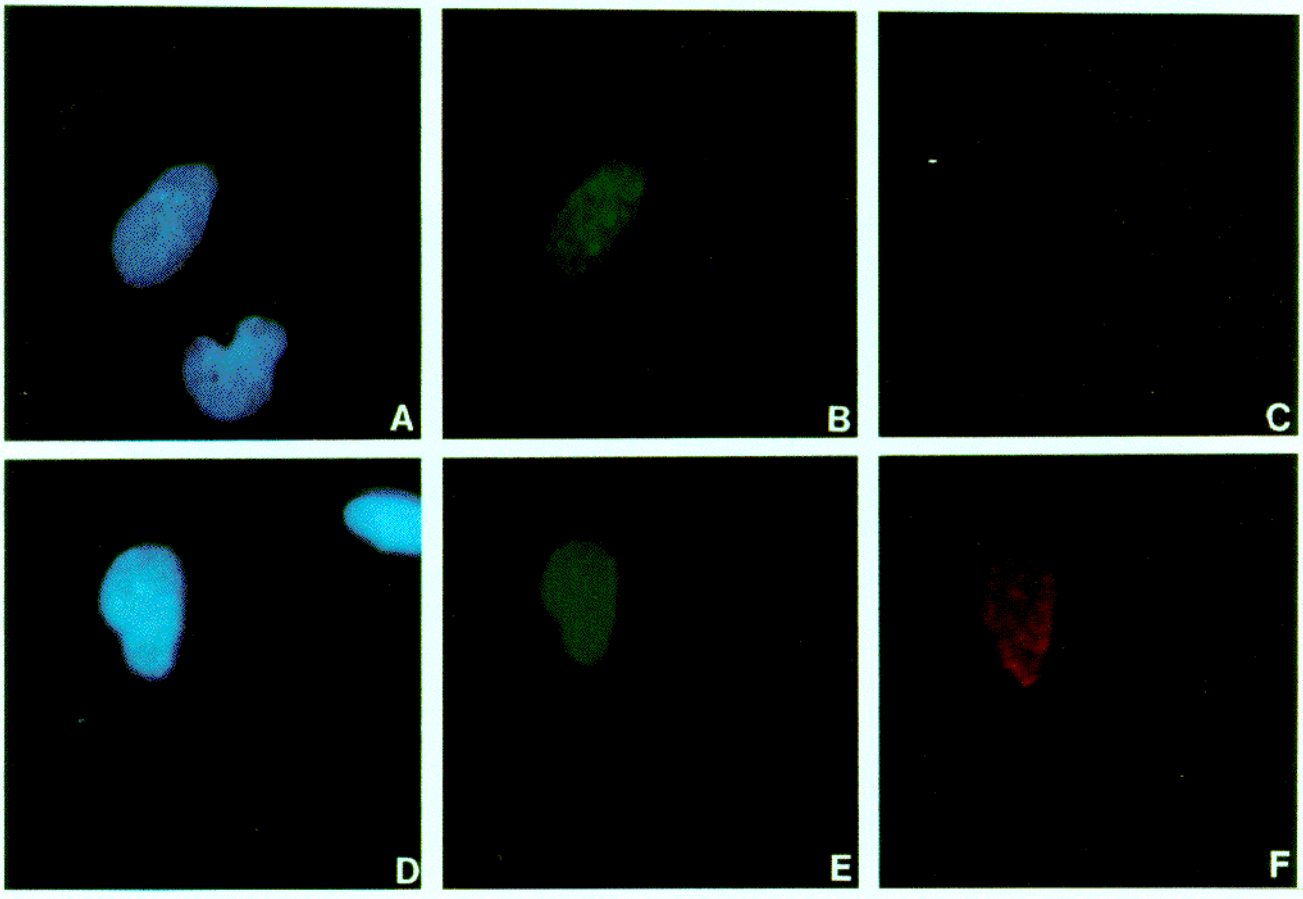
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