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Deregulated Expression of E2F Family Members Induces S-Phase Entry and Overcomes $p16^{INK4A}$-Mediated Growth Suppression

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The E2F family of transcription factors regulate genes, whose products are essential for progression through the mammalian cell cycle. The transcriptional activity of the E2Fs is inhibited through the specific binding of the retinoblastoma protein, pRB, and the pRB homologs p107 and p130 to their transactivation domains. Seven members of the E2F transcription factor family have been isolated so far, and we were interested in investigating the possible contribution of the various E2Fs to cell cycle control. By presenting the results of the generation of cell lines with tetracycline-controlled expression of E2F-1 and E2F-4 and microinjection of expression plasmids for all members of the E2F family, we demonstrate here that the pRB-associated E2Fs (E2F-1, E2F-2, and E2F-3) all induce S phase in quiescent rat fibroblasts when expressed alone. In contrast, the p107/p130-associated E2Fs require the coexpression of the heterodimeric partner DP-1 to promote S-phase entry and accelerate G_1 progression. Furthermore, the pRB-associated E2Fs were all able to overcome a G_1 arrest mediated by the $p16^{INK4}$ tumor suppressor protein, and E2F-1 was shown to override a G_1 block mediated by a neutralizing antibody to cyclin D1. The $p16^{INK4}$-induced G_1 arrest was not affected by expression of E2F-4, E2F-5, or DP-1 alone, but simultaneous expression of E2F-4 and DP-1 could overcome this block. Our results demonstrate that the generation of E2F activity is rate limiting for G_1 progression, is sufficient to induce S-phase entry, and overcomes a p16-mediated G_1 block, and since E2F-1, E2F-2, and E2F-3 are associated with pRB, they are the most likely downstream effectors in the p16-cyclin D-pRB pathway. Furthermore, our data suggest that the two subsets of E2Fs are regulated by distinct mechanisms and/or that they have distinct functions in cell cycle control. Since E2F-4 and E2F-5 cannot promote S-phase entry by themselves, our results may provide an explanation for the apparent lack of aberrations in p107 or p130 in human cancer.

Entry into and progression through the mammalian cell cycle are highly regulated processes, which at the molecular level involve a number of positively and negatively acting proteins. Many data suggest that among the negative regulators, the prototypic tumor suppressor, pRB, is crucial for proper cell cycle control (for a review, see reference 60). The gene for pRB, *RB-1*, was the first mammalian tumor suppressor gene to be cloned, and as such it has attracted much attention. Mutations in the *RB-1* gene have been found not only in retinoblastomas but also in a variety of human tumors such as osteosarcomas, small-cell lung carcinomas, prostate carcinomas, and cervical cancers (60). The identification of *RB-1* mutations in a diversity of human tumors was the first indication that pRB may have a more global regulatory role in cell proliferation. This indication has since been substantiated by a large number of experiments, and in particular it has been demonstrated that overexpression or microinjection of wild-type pRB inhibits cell cycle progression in mid- to late G_1 (15, 26, 47).

Recently it has been proposed that the type D cyclins in association with CDK4 or CDK6 are responsible for regulating the restriction point in late G_1 by initiating the phosphorylation of pRB (27, 38, 39, 57). This proposal is based on data demonstrating the pRB-specific kinase activity of cyclin D/CDK complexes, the requirement for cyclin D activity for G1 progression, the lack of such a requirement in pRB-negative cells, the timing of the cyclin D1 execution point in late G_1, and the reduced dependence on growth factors of cells overexpressing cyclin D1 or lacking pRB (27, 38, 39, 57, 58). In addition, we and others have recently demonstrated a close link between the ability of the $p16^{INK4A/CDKN2}$ (p16) tumor suppressor protein, an inhibitor of CDK4- and CDK6-associated kinase activity, to inhibit progression into S phase and the presence of a functional pRB (31, 40, 41). These data have suggested a model in which p16, cyclin D-dependent kinases, and pRB are functionally linked in a pathway that controls the passage of the restriction point.

The ability of the retinoblastoma protein (pRB) to inhibit cell cycle progression is dependent on its interaction with transcription factors belonging to the E2F family (reviewed in references 20, 33, and 44). In addition to binding to pRB, the E2F transcription factors also bind to two pRB-related proteins, p107 and p130, in association with cyclin A/CDK2 or cyclin E/CDK2 (33). The structural similarity between p107, p130, and pRB (the so-called pocket proteins) and the fact that overexpression of p107 and p130 specifically inhibit E2F-dependent transcription (33, 59) suggest that p107 and p130 have some role in regulating cell cycle progression, in part through E2F. In this regard it is interesting that the pocket proteins form complexes with E2F at different phases of the cell cycle. p130 is primarily associated with E2F in G_0 and early G_1, whereas pRB and p107 are complexed with E2F in the late G_1 and S phases (6, 34, 43, 59). Despite this apparent difference in timing, overexpression of the pocket proteins has in each case been reported to arrest certain cells at some point of the G_1 phase of the cell cycle (33, 59).

The cloning of several members of the E2F transcription

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factor family has provided us with the molecular tools for elucidating the role of these factors in cell cycle control. The prototype, E2F-1, was isolated by exploiting its direct association with pRB (23, 30, 56), and a number of related proteins (E2F-2 to E2F-5) (5, 13, 25, 28, 35, 53) as well as proteins with which it forms functional heterodimers (DP-1 and DP-2) (14, 61) have subsequently been identified. Dimerization between an E2F and a DP is essential for high-affinity DNA binding, transcriptional activity, and binding to pocket proteins (3, 5, 24, 32, 61). The isolation of several cDNAs coding for E2F activity raises the question of how different members of the family contribute to cell cycle control, and some insight has already been reported: E2F-1, E2F-2, and E2F-3 preferentially bind to pRB and not to p107 or p130; E2F-4 specifically binds to p107 and p130, and since E2F-5 was isolated by virtue of its interaction with p130 and p107, it is assumed to be specific for p107 and p130 (5, 13, 25, 35, 53, 59). DP-1 and DP-2 do not appear to contribute to the specificity of this interaction, since these proteins are found associated with pRB, p107, and p130 (59, 61). Although this difference in binding specificity between the various E2F family members suggests that each member may have a specific function, no such data have been reported.

We have initiated an analysis of the function of each member of the E2F family, and in this work we demonstrate that expression of E2F-2 and E2F-3 alone, as has previously been shown for E2F-1 (29), is sufficient to induce S phase in quiescent fibroblasts. Surprisingly, high levels of E2F-4 and E2F-5 expression are not sufficient to induce S phase. The pRB-associated E2Fs are all able to promote S-phase entry in cells that are otherwise blocked in G_1 by neutralizing antibodies to cyclin D1 or overexpression of p16 protein. In contrast to E2F-1, E2F-2, and E2F-3, the p107/p130-associated E2Fs strictly require the coexpression of DP-1 to induce S phase and overcome a p16-mediated cell cycle block. Our data suggest that in addition to being regulated by different pocket proteins, the activities of the two subsets of E2Fs are regulated by different mechanisms and/or have different functions in cell cycle control. Moreover, our data suggest that p16, type D cyclins and associated kinases, and pRB all regulate the G_1 restriction point in the mammalian cell cycle by regulating the activity of the pRB-associated E2Fs.

MATERIALS AND METHODS

Plasmids. Plasmids pUHD15-1 and pUHD10-3 for generating the tetracycline-responsive cell lines were the kind gift of M. Gossen and H. Bujard (16). Full-length hemagglutinin (HA)-tagged E2F-1 was subcloned into pUHD10-3, generating pUHDHAE2F-1 (18). A pUHD10-3 plasmid containing the full-length HA-tagged E2F-4, pTETHAE2F-4, was the kind gift of R. Bernards.

The expression plasmids pCMVE2F-1, pCMVE2F-1 (Y411C), pCMVE2F-1 (E132), and pCMVE2F-1(1-374) have been described before (21, 22). A plasmid expressing an HA-tagged version of E2F-1 was generated by cloning an *Eco*RI-*Xba*I fragment containing the full-length open reading frame of E2F-1 plus sequences containing information for the HA tag from pBSKHAE2F-1 (24) into blunt-ended pCMVneoBam (2). To generate pCMVHAE2F-2, pBSKHAE2F-2 was first constructed by a three-fragment cloning, with a *Bam*HI-*Eco*RV (nucleotides [nt] 429 to 1129) fragment from pGEXE2F-2 (28), a PCR-generated E2F-2 *Eco*RV-*Xba*I (nt 1129 to 1744) product, and *Bam*HI-*Xba*I-cut pBSKHA (24). pCMVHAE2F-2 was subsequently constructed by subcloning a *Bam*HI fragment containing full-length E2F-2 into *Bam*HI-cut pCMVHAE2F-1. pBSKHAE2F-3 and pCMVHAE2F-3 were constructed by cloning a PCR fragment of E2F-3 (nt 67 to 1488) (35) into *Bam*HI-cut pBSKHA and *Bam*HI-cut pCMVHAE2F-1, respectively. pCMVHAE2F-4 was generated by subcloning an *Eco*RI-*Bam*HI fragment containing full-length HA-tagged E2F-4 from pTETHAE2F-4 into blunt-ended pCMVneoBam.

To express proteins containing an N-terminal epitope for the antibody 9E10 (9), pBSKMY was generated by cloning oligonucleotides coding for the epitope into *Eco*RI-*Bam*HI-digested pBSK$^-$. pBSKMYE2F-4 and pBSKMYE2F-4dl4 were constructed by cloning *Bam*HI-*Xba*I fragments from pcDNAE2F-4 and pcDNAE2F-4dl4 (kind gift of D. Ginsberg and D. Livingston [13]) into pBSKMY. pCMVMYE2F-4 and pCMVMYE2F-4dl4 were subsequently generated by subcloning *Eco*RI fragments from pBSKMYE2F-4 and pBSKMYE2F-4dl4 into blunt-ended pCMVneoBam. pCMVMYE2F-5 was constructed by subcloning an *Eco*RI-*Xba*I fragment coding for an N-terminal MYC-tagged E2F-5 from pcDNA3MYE2F-5 (kind gift of C. Sardet) into blunt-ended pCMVneoBam.

pCMVpRB, pCMVpRBΔ22, pCMVDP-1, pCMVHADP-1, pCMVp107, pCMVCD20, and pX-p16 have been described before (24, 40, 47, 63). pCMVluc was the kind gift of A. Fattaey, and pBabepuroBcl-2 was the kind gift of G. Evan (10). To construct an expression plasmid containing full-length HA-tagged p130, a 5′ fragment (nt 70 to 1180 [36]) generated by PCR was cloned into the *Bam*HI site of pBSKHA, giving rise to pBSKHAp130-5′. Subsequently, an *Xba*I-fragment (nt 1032 to 3548) isolated from pBSF-p130 (kind gift of P. Whyte) was cloned into *Xba*I-cut pBSKHAp130-5′, generating pBSKHAp130. Finally pCMVHAp130 was constructed by cloning an *Eco*RV-*Sac*II fragment containing HA-tagged full-length p130 cDNA into blunt-ended pCMVneoBam.

Fragments generated by PCR were sequenced in all cases to verify that no misincorporation had occurred during the reaction.

Tissue culture. R12 cells are Rat1 cells stably expressing the tet-VP16 transactivator, and were the kind gift of D. Resnitzky and S. Reed (51). R12 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS) and 500 μg of G-418 (Life Technologies) per ml. The human osteosarcoma cell line U-2 OS and the human cervical carcinoma cell line C-33A were grown in Dulbecco's modified Eagle medium containing 10% FCS.

Cell synchronization. Cells were synchronized in G_0/G_1 phase by culturing in medium containing 0.1% FCS (RE2F clones) or no serum (R12 cells) for 48 h. Under these conditions, less than 5% of the cells incorporated bromodeoxyuridine (BrdU) after overnight pulse-labeling. Alternatively, cells were accumulated in mitosis by incubation in the presence of 40 ng of nocodazole (Sigma) per ml for 18 h. Rounded mitotic cells were dislodged by gentle shaking and pipeting and after two washes in phosphate-buffered saline (PBS) were restimulated by replating into a fresh medium. The degree of cell cycle synchrony and the progression into S phase were monitored by incubation with BrdU (100 μg/ml; Sigma) for the indicated times.

Transfections. R12 cells were electroporated with a Genepulser apparatus (Bio-Rad) with a pulse of ~40 ms and a field of 675 V/cm. For the generation of stable cell lines, R12 cells were electroporated with 5 μg of pUHDHAE2F-1, pTETHAE2F-4, or pUHD15-1 in combination with 0.5 μg of pBabeBcl-2. Two days after electroporation, stable clones were selected in medium containing puromycin (1 μg/ml; Sigma) and tetracycline (1 μg/ml). Cell lines were generated by ring cloning, and the expression of E2F-1 or E2F-4 was determined 12 to 16 h after removal of tetracycline.

In transient transfections R12 and derivatives thereof were electroporated as described above, with 5 μg of $E2F_4CAT$ (a synthetic promoter with four E2F DNA-binding sites in front of the chloramphenicol acetyltransferase [CAT] reporter gene) and 2 μg of pCMVluc as a control for electroporation efficiency. Electroporated cells were seeded in media with or without tetracycline, and harvested 24 to 36 h after electroporation. U-2 OS or C-33A cells were transfected essentially as described previously (1). For Western blotting (immunoblotting) and preparation of cell extracts, cells were transfected with 10 to 20 μg of expression plasmid, and carrier DNA (salmon sperm DNA) was added to a total of 24 μg. For CAT assays, cells were transfected with the expression vector as indicated in the figure legends (e.g., Fig. 3) together with 2 μg of $E2F_4CAT$, 2 μg of pCMVluc, and salmon sperm DNA to a total of 24 μg per 9-cm-diameter plate. Cells were harvested 36 h after transfection.

CAT and luciferase assays. At 24 to 36 h posttransfection, cells were resuspended in 0.025 M Tris-HCl (pH 8.0). The cells were freeze-thawed three times and centrifuged at 20,000 × *g* for 5 min. Supernatants were assayed for CAT by a fluor diffusion method, essentially as described previously (46), and luciferase activity was determined with a Berthold 9501 luminometer as described by de Wet et al. (8).

Immunological reagents. Antibodies to simian virus 40 T antigen (PAb419), c-MYC (9E10), HA tag (12CA5), human E2F-1 (KH20), and cyclin D1 (DCS6) have previously been described (9, 11, 17, 24, 39). Affinity-purified rabbit peptide antibodies to pRB and p107 were the kind gift of P. Whyte.

Preparation of whole-cell extracts. Whole-cell extracts were prepared from the rat cell lines by adding two packed-cell volumes of buffer C (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.6], 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 25% glycerol), and the concentration of NaCl was adjusted to 0.42 M (final concentration) by the addition of 5 M NaCl. The cells were freeze-thawed once and left on ice for 20 min. Extracts were centrifuged at 20,000 × *g* for 20 min, and the supernatants were used for gel retardation assays.

Gel retardation assays. Gel retardation assays were performed as described previously (23). A 10-μg aliquot of cell extract was used directly in a binding buffer containing 20 mM HEPES (pH 7.6), 40 mM KCl, 1 mM $MgCl_2$, and 0.1 mM EDTA, to which 0.1 to 0.5 ng of ^{32}P-labeled probe and 1 μg of sonicated salmon sperm DNA were added. To test for the presence of specific proteins, 1 μl of tissue culture supernatant was included in the binding reaction. The reaction products were separated on 4% polyacrylamide gels in 0.25× Tris-borate-EDTA at 4°C at 200 V. Gels were then dried and exposed to films. E2F oligonucleotides for gel retardation assays contained two E2F recognition sites as in the E2 promoter (21).

Expression and purification of GST fusion proteins. Glutathione *S*-transferase (GST)-p16 protein was expressed and purified from *Escherichia coli* as described previously (40). After binding to glutathione-agarose, the proteins were washed and eluted with reduced glutathione (Sigma) and the concentration was determined by the method of Bradford (Bio-Rad). The purity and size of the eluted proteins were then evaluated by Coomassie staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Western blotting and immunoprecipitation. For Western blotting, cells were lysed directly in 1× SDS sample buffer (60 mM Tris [pH 6.8], 2% SDS, 100 mM DTT, 10% glycerol, 0.02% bromphenol blue) or whole-cell extracts were prepared as described above. Protein extracts (20 to 75 μg) were separated by SDS-polyacrylamide gel electrophoresis (8% polyacrylamide) and blotted onto nitrocellulose by semidry blotting. Nitrocellulose filters were stained with Ponceau S to confirm uniform transfer of proteins, and filters were blocked in 5% nonfat dry milk in PBS containing 0.05% Tween 20. Subsequently, the filters were probed with the antibodies indicated in the figure legends (e.g., Fig. 1) and then with horseradish peroxidase-coupled secondary antibodies and were developed by the ECL system (Amersham).

For immunoprecipitations, 300-μg aliquots of whole-cell extracts were diluted in E1A lysis buffer (250 mM NaCl, 0.1% Nonidet P-40, 50 mM HEPES [pH 7.0]) containing 5 mM EDTA, 1 mM dithiothreitol, phenylmethylsulfonyl fluoride (50 μg/ml), leupeptin (1 μg/ml), and aprotinin (1 μg/ml) and incubated with protein A-Sepharose-precoupled 12CA5 antibody. The protein A-Sepharose beads were washed three times in E1A lysis buffer, and precipitated proteins were further processed for Western blotting as described above.

Microinjection. The cells were grown on glass coverslips with a small marked area to facilitate the location of injected cells. All microinjections were performed in HEPES-buffered tissue culture medium to compensate for pH changes during injection. Cells were injected by using the Zeiss automatic injection system connected with an Eppendorf injector. Injection capillaries (GC120TF-10; Clark Electromedical Instruments) were pulled to fine tips with the Sutter Instruments P-87 puller. Each cell was injected at a pressure between 50 and 150 hPa. The basic computer settings were as follows: angle, 45°; speed, 20; time of injection, 0.0 s. The total injection time per coverslip did not exceed 20 min. For protein microinjection, DCS-6 antibody was purified on a protein A column and subsequently dialyzed against PBS. The concentration in the needle at the time of injection was 5 mg/ml. GST-p16 fusion protein was purified as described above, dialyzed against microinjection buffer containing 10 mM HEPES (pH 7.5) and 50 mM KCl, and injected at a concentration of 2 mg/ml. Purified mouse immunoglobulin G (1 mg/ml; Sigma) was added as a microinjection marker. Proteins were injected into the perinuclear space. For DNA microinjection, purified plasmids were diluted in PBS and injected directly into cell nuclei. The concentration of each plasmid in the needle is specified for each experiment in the appropriate figure legends. In most experiments, a plasmid expressing the CD20 protein was coinjected (10 μg/ml) as a marker of cells productively expressing proteins from the injected cDNAs.

For experiments involving DNA microinjection, we prefer to present the results as a complete set of data from one typical experiment in which the same batch of cells was treated under exactly identical conditions. Despite some variation among experiments caused by different batches of cells and slight modifications in the timing of evaluation of BrdU incorporation, all the results were reproduced 2 to 5 times.

Immunofluorescence. Cells microinjected with DCS-6 or GST-p16 were fixed for 10 min in cold methanol-acetone (1:1, vol/vol), rinsed in PBS, and incubated for 30 min at room temperature with goat anti-mouse secondary antibody (Amersham; 1:100) followed by Texas red-conjugated streptavidin (Amersham; 1:100). For BrdU detection, coverslips were subsequently incubated with 1.5 N HCl for 10 min, washed extensively in PBS, and stained with fluorescein isothiocyanate-coupled anti-BrdU antibody (nondiluted; Becton Dickinson) for an additional 30 min at room temperature. Finally, nuclear DNA was costained with Hoechst 33258 (1 mg/ml, in PBS) and mounted in Mowiol (Sigma). CD20 expression on the plasma membrane was detected by incubating with anti-CD20 monoclonal antibody (Becton Dickinson) for 30 min before fixing the cells. For direct staining of expressed E2Fs or DP-1, the coverslips were incubated with specific antibodies (all of mouse origin) for 1 h at room temperature and further processed as described above.

Fluorescence-activated cell sorter analysis. The cells were trypsinized and fixed in 70% methanol for 30 min at 4°C. After washing in PBS, the cells were resuspended in a buffer containing 50 μg of propidium iodide per ml, 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 10 μg of RNase A per ml and analyzed on a Coulter Epics XL flow cytometer. Typically, 10^4 cells per sample were recorded and DNA distribution was measured with Multi-Cycle AV software (Phoenix Flow Systems).

RESULTS

Generation of cell lines with inducible expression of *E2F* genes. In order to study the specific function of the individual genes in the E2F family, two approaches were chosen. In the first approach cell lines were generated with conditional expression of E2F-1 or E2F-4, and in the other approach expression plasmids were microinjected into synchronized tissue culture cells (see below). For generating cell lines with inducible expression of E2F-1 and E2F-4, the tetracycline resistance operator-repressor system developed by Gossen and Bujard (16) was used. In this system the VP-16 transactivator is fused to the tetracycline resistance repressor, and the binding to the TetO operator is regulated by the presence of tetracycline. Stable cell lines expressing the Tet-VP16 fusion protein from a constitutive promoter are generated, and into these cell lines is introduced a plasmid with the gene of interest located downstream of a promoter containing seven TetO binding sites. Transcription of the introduced gene only occurs in the absence of tetracycline. Rat-1 fibroblasts containing the Tet-VP16 transactivator (clone R12) were kindly provided by D. Resnitzky and S. Reed (51) and have been used before to establish cell lines with conditional expression of cyclin D1, cyclin E, or cyclin B1. Previously, we have unsuccessfully tried to establish cell lines that constitutively express high protein levels of each member of the E2F family (19). The lack of success may be ascribed to the fact that high protein levels of the E2F family members are toxic for the cells (42); for E2F-1, this toxicity has been demonstrated to be partly caused by apoptosis (49, 55, 62). Since it was reported that clones established in the R12 cell line exhibited a low basal expression in the presence of tetracycline (51), we were concerned that E2F-inducible clones in the R12 cell line could not be isolated. In order to prevent a potential apoptotic effect of basal E2F expression, R12 cells were electroporated with expression plasmids containing the *E2F-1* or the *E2F-4* cDNAs downstream of the *tetO* promoter together with a plasmid containing a puromycin resistance gene or with a plasmid containing the genes for puromycin resistance and the antiapoptotic protein, Bcl-2 (10). To distinguish exogenously produced E2F-1 and E2F-4 from the endogenous proteins, *E2F-1* and *E2F-4* cDNAs were fused to a DNA sequence coding for the influenza virus HA epitope that is recognized by the 12CA5 antibody (11). Puromycin resistant cell lines were screened for inducible expression of E2F-1 and E2F-4, and cells containing the expression plasmid without insert were isolated as controls. Although there was no evidence of basal expression of the E2Fs in the presence of tetracycline, we were not able to isolate cell lines with detectable conditional expression of E2F-1 or E2F-4 in the absence of Bcl-2. However, in the presence of Bcl-2, two cell lines with conditional expression of E2F-1 or E2F-4 were generated, RE2F-1 and RE2F-4 (Fig. 1A). No detectable E2F-1 or E2F-4 was synthesized in the presence of tetracycline. The conditionally expressed E2F-1 migrated like wild-type human E2F-1, as a characteristic doublet at 60 kDa (Fig. 1A) (23, 30), and E2F-4 produced in RE2F-4 migrated like wild-type human E2F-4, as a set of heterogeneous bands at 60 kDa (Fig. 1A) (13), suggesting that the production and processing of exogenously produced proteins occurred properly. This suggestion was further supported by indirect immunofluorescence staining (with the 12CA5 antibody), which demonstrated a strong nuclear-staining pattern in the RE2F-1 cells and a nuclear and cytoplasmic staining in RE2F-4 cells after removal of tetracycline.

Several other experiments were performed to characterize conditionally expressed E2F-1 and E2F-4. To test whether the expressed E2F-1 and E2F-4 retained their abilities to bind to E2F DNA-binding sites, RE2F-1 and RE2F-4 cells grown in the absence of tetracycline were harvested, and whole-cell extracts were prepared. As shown in Fig. 1B, the addition of the 12CA5 antibody specifically supershifted an E2F DNA-binding complex in cell extracts prepared from RE2F-1 and RE2F-4

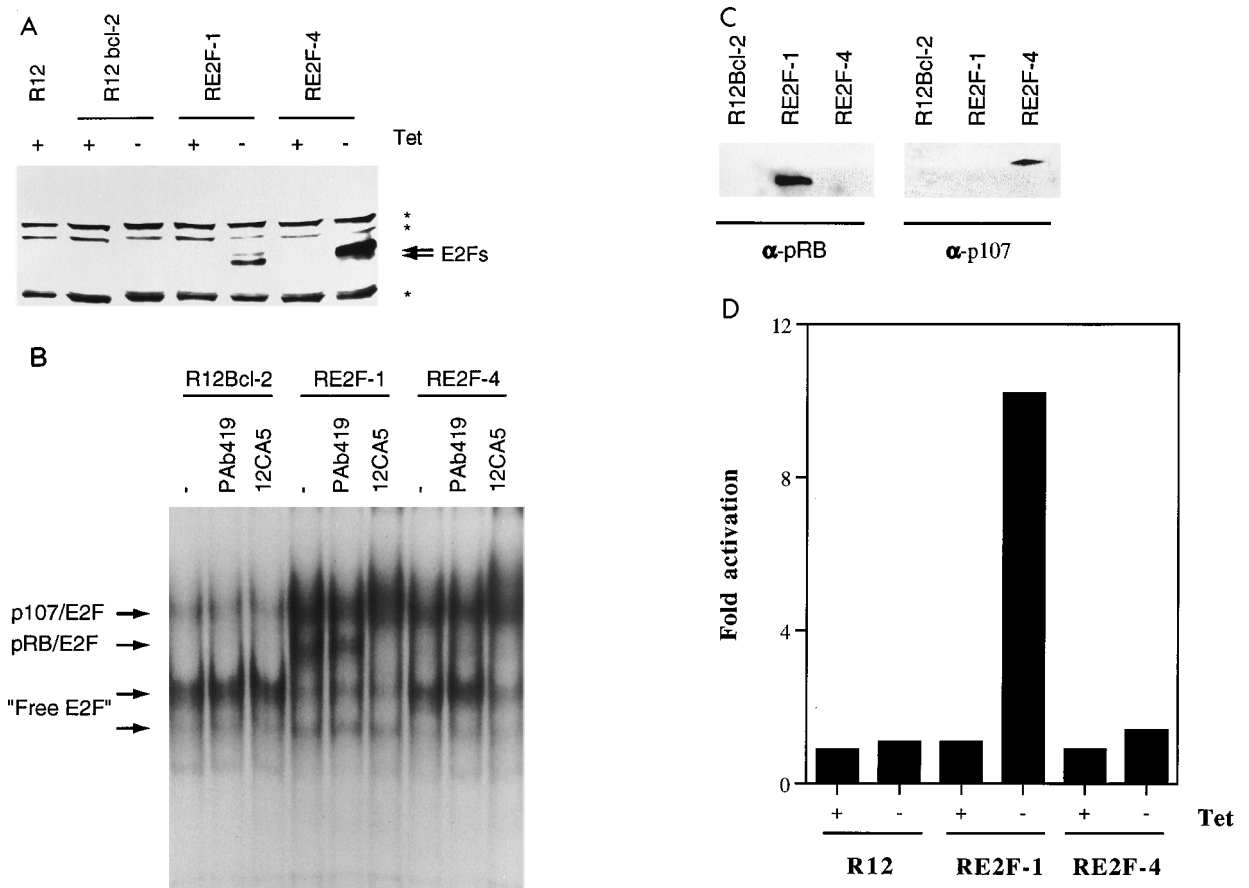


FIG. 1. RE2F-1 and RE2F-4 cell lines. (A) Conditional expression of E2F-1 and E2F-4 in rat cells. Cell lines were grown in the presence (+) or absence (−) of 1 μg of tetracycline (Tet) per ml for 20 h, and whole-cell extracts were prepared as described in Materials and Methods. Protein (75 μg) was separated on an 8% acrylamide gel, blotted onto nitrocellulose, and probed with a monoclonal antibody, 12CA5, to the HA epitope. Note that in addition to recognizing the inducible proteins, the 12CA5 antibody recognizes endogenous proteins of approximately 80, 70, and 50 kDa (indicated by asterisks). (B) Induced E2F-1 and E2F-4 bind to the consensus DNA-binding site. Whole-cell extracts were prepared from cell lines grown for 20 h in the absence of tetracycline. Cell extract (10 μg) from the indicated cell lines was incubated with a radiolabeled oligonucleotide containing two E2F DNA-binding sites. The presence of the HA-tagged E2F-1 and E2F-4 proteins was determined by the addition of the monoclonal antibody 12CA5. A monoclonal antibody to simian virus 40 large T antigen (PAb419) served as a control. p107/E2F and pRB/E2F depict E2F complexes containing the respective proteins as determined by specific antibodies. "Free E2F" denotes E2F not bound to any other protein(s). (C) Conditionally expressed E2F-1 and E2F-4 retain their in vivo binding specificities to pRB and p107, respectively. Cell extracts (300 μg) prepared from cells grown in the absence of tetracycline for 16 h were precipitated with the 12CA5 antibody as described in Materials and Methods. Immunoprecipitated proteins were separated on an 8% acrylamide gel, blotted onto nitrocellulose, and probed with a rabbit peptide antibody to pRB (α-pRB) or a rabbit peptide antibody to p107 (α-p107). (D) Transactivation of an E2F-dependent promoter. The indicated cell lines were electroporated with 5 μg of $E2F_4CAT$ and 2 μg of pCMVluc as described in Materials and Methods. Cells were grown in the presence (+) or absence (−) of tetracycline (Tet) (1 μg/ml) for 36 h before harvesting. CAT and luciferase activities were determined as described in Materials and Methods. Fold activation refers to units of CAT activity normalized to the luciferase activity for each cell extract. The basal level of $E2F_4CAT$ was set to unity. Results similar to the presented data have been obtained in four independent experiments.

cells but not from R12Bcl-2 cells, demonstrating that the E2F-1 and E2F-4 proteins expressed in these cells retain E2F DNA-binding activity.

Since E2F-1 and E2F-4 are primarily found associated with pRB and p107 or p130, respectively, we wanted to know whether the expressed proteins retained this binding specificity. As shown in Fig. 1C, 12CA5 antibodies coimmunoprecipitate p107 and not pRB in RE2F-4 cells. Similarly E2F-1 was found to be associated with pRB and not p107 in RE2F-1 cells. These results show that the conditionally expressed E2F-1 and E2F-4 bind the E2F DNA recognition site and that the proteins retain their specificities in binding to p107 and pRB.

Furthermore, we tested whether induced expression of E2F-1 or E2F-4 could transactivate a synthetic promoter, $E2F_4CAT$, containing four E2F DNA-binding sites in front of the CAT reporter gene (24). The cell lines were electroporated with the $E2F_4CAT$ reporter and grown in the presence or absence of tetracycline for 24 h before harvesting. As shown in Fig. 1D, a 10-fold increase in CAT activity was measured in RE2F-1 upon removal of tetracycline, whereas no detectable increase in CAT activity was observed in RE2F-4, R12, and R12Bcl-2 (data not shown). These results are consistent with previously published results, in which it was demonstrated that overexpression of E2F-1 alone efficiently transactivates an E2F-dependent promoter (23, 24), whereas E2F-4 alone has a very low

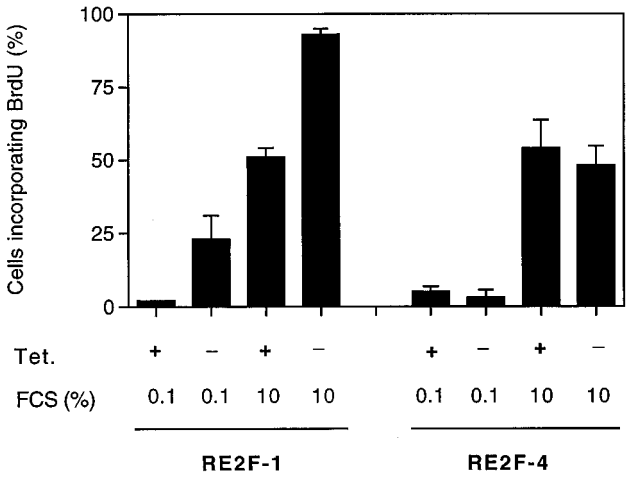


FIG. 2. Stimulation of S-phase entry by induced expression of E2F-1 but not E2F-4 in RE2F clones. Rat fibroblasts conditionally expressing E2F-1 or E2F-4 growing on glass coverslips were synchronized in G_0 by serum deprivation in the presence (+) of tetracycline (Tet.) for 48 h. Expression of E2F-1 and E2F-4 was then stimulated by removal (−) of tetracycline either with or without concomitant stimulation by 10% FCS. Simultaneously, BrdU was added to monitor DNA synthesis. After 20 h the cells were fixed, stained for BrdU incorporation, and counterstained with Hoechst for nuclear DNA. The graph summarizes the percentage of cells incorporating BrdU, calculated from several different coverslips in randomly chosen areas. Each bar represents the mean value for at least 500 counted cells (error bars, standard deviations).

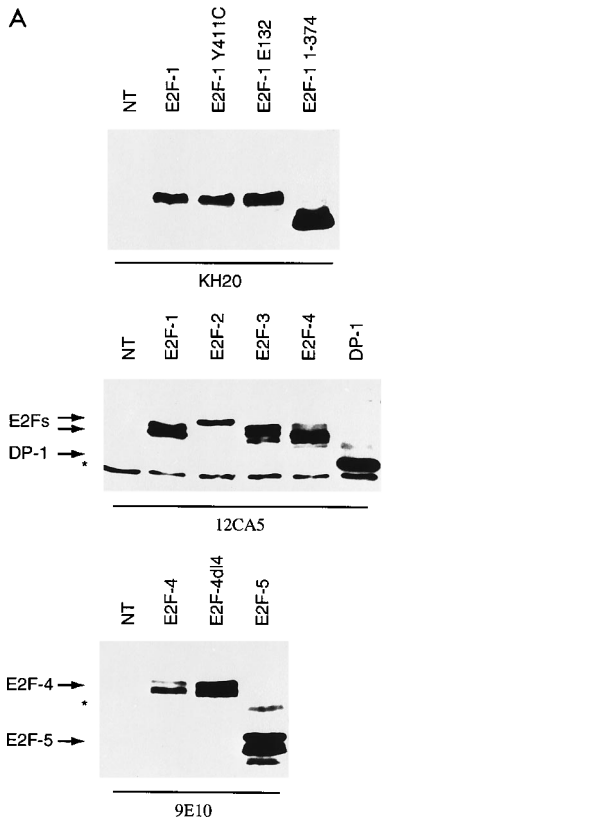
level of activity towards this promoter (5, 13). In conclusion, our data show that conditionally expressed E2F-1 and E2F-4 contain the expected characteristics of the two proteins.

Expression of E2F-1 alone, but not E2F-4, can induce S-phase entry in quiescent fibroblasts and shorten G_1 progression. Previously, it has been demonstrated that overexpression of E2F-1 in serum-starved rat fibroblasts can promote S-phase entry (29, 49, 55). To test if both E2F-1 and E2F-4 were able to stimulate S phase, we starved RE2F-1 and RE2F-4 cells in 0.1% serum in the presence of tetracycline for 48 h to induce quiescence (Fig. 2). Cells were then kept in 0.1% serum in the presence or absence of tetracycline or in medium containing 10% serum with or without tetracycline. As shown in Fig. 2, expression of E2F-1 was sufficient to induce S-phase entry in 20 to 30% of RE2F-1 cells kept in 0.1% serum within 20 h, whereas expression of E2F-4 in serum-starved RE2F-4 cells did not increase the number of cells in S phase. RE2F-4 cells were able to recover from serum starvation, since approximately 50% of the cells actively synthesized DNA within the first 20 h after serum addition (Fig. 2). Fluorescence-activated cell sorter data confirmed that approximately 20% of RE2F-1 cells kept in 0.1% serum were in S phase after removal of tetracycline within 20 h, demonstrating that BrdU incorporation is due to bona fide S-phase entry rather than DNA repair (data not shown).

Previous experiments have shown that induced expression of cyclin D1 or cyclin E leads to faster entry into S-phase of serum-starved cells in the presence of 10% serum, whereas expression of cyclin D1 or cyclin E was not sufficient to induce S phase in cells kept in 0.1% serum (45, 50, 51). Although E2F-4 expression did not lead to S-phase entry in serum-starved cells, it was therefore still a possibility that E2F-4 could confer some growth advantage to cells in 10% serum when expressed alone. In order to test this possibility, RE2F-4 and RE2F-1 cells were grown in 10% serum in the presence or absence of tetracycline, and as can be seen in Fig. 2 no detectable effect on BrdU incorporation was seen in the RE2F-4 cell line upon removal of tetracycline. In contrast, 93% of the RE2F-1 cells progressed into S phase upon E2F-1 expression, compared with 51% in the absence of E2F-1 expression (Fig. 2). These results suggest that induced expression of E2F-1 alone is sufficient to target genes whose products are involved in regulating S-phase entry, whereas expression of E2F-4 is not.

The pRB-associated E2Fs can all induce S-phase entry. In order to rule out clonal effects and to be able to study the effect of expressing E2F mutants and combinations of E2Fs and heterodimeric partners, we turned to microinjection of expression plasmids into R12 cells. A series of E2F expression plasmids were constructed and tested for their ability to synthesize high levels of protein. As shown in Fig. 3A, the expression plasmids directed high-level expression of proteins migrating with the expected molecular masses after transfection of U-2 OS cells. A very similar pattern of protein synthesis was seen after transfection of C-33A cells and Rat-12 cells (data not shown). Furthermore, the ability of the synthesized proteins to transactivate an E2F-dependent promoter was tested (Fig. 3B). In agreement with published results, E2F-1, E2F-2, and E2F-3 (23, 24, 35) were all shown to transactivate the E2F-dependent promoter when expressed alone, whereas a moderate or no effect was seen on this promoter when E2F-4 or E2F-5 was expressed (5, 25). Similar results were obtained when another E2F-dependent promoter, E2CAT (37), was used (data not shown). Consistent with published results (3, 5, 24, 32, 61), expression of DP-1 alone led to a low level of transactivation; however, DP-1 was shown to cooperate with E2F-1, E2F-4, and E2F-5 in transactivating an E2F-dependent promoter (Fig. 3B). Finally, also in accordance with published results, mutants of E2F-1 lacking the ability to bind DNA (E2F-1 E132) or the transactivating domain (E2F-1 1-374) were unable to transactivate the E2F reporter construct (Fig. 3B) and an E2F-1 protein (E2F-1 Y411C) and an E2F-4 protein (E2F-4dl4) that have lost their abilities to interact with pocket proteins retained approximately the same transactivating potentials as their wild-type counterparts.

To determine the specificity of E2F-1-induced S-phase entry, expression plasmids containing distinct mutations in the *E2F-1* gene were microinjected into serum-starved R12 cells (Fig. 4 and 5). As a marker for productively injected cells, a plasmid containing the CD20 gene under the control of the same promoter as the E2F cDNAs was coinjected. By using CD20 as a marker for productively injected cells, differences in antibody affinity to the various proteins were avoided and a comparison of the E2F-mediated effects was made possible. As demonstrated in Fig. 4C and E, productively injected cells were easily detected with an anti-CD20 antibody. S-phase induction was measured by indirect immunofluorescence with an antibody to BrdU, and cells were counterstained with Hoechst for DNA content. In agreement with published results (29, 49, 55) and the data obtained for the RE2F-1 clone, expression of wild-type E2F-1 induced S phase in serum-starved cells. This induction was dependent on the ability of E2F-1 to bind to DNA and to transactivate E2F-dependent promoters, since the DNA-binding-deficient mutant (E2F-1 E132) (7, 21) and the transactivating-deficient mutant (E2F-1 1-374 [Fig. 3B]) did not induce S phase (Fig. 5A). The ability of E2F-1 to induce S phase was not caused by preventing other proteins from binding to pRB, since an E2F-1 mutant (E2F-1 Y411C) that cannot bind pRB but retains its transcriptional activity (22) still induced S phase. Interestingly, when evaluated within the same experiment, the mutant deficient in pRB binding was more efficient in inducing S-phase progression than was the wild type



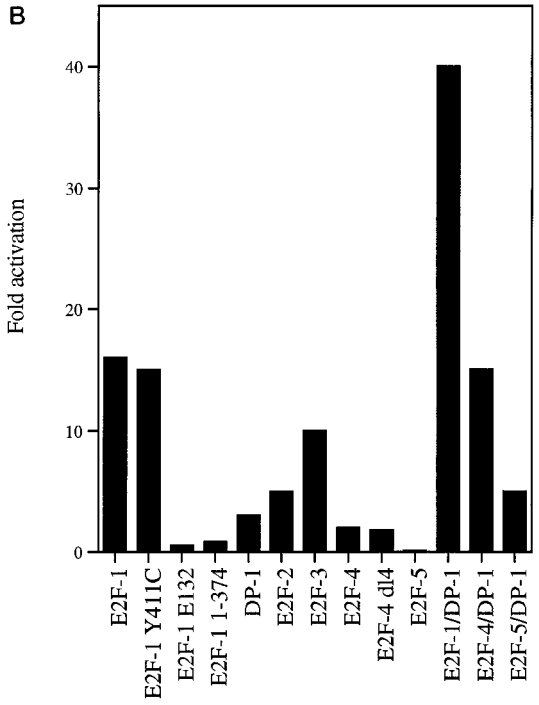


FIG. 3. Expression levels and activities of various E2F proteins. (A) U-2 OS cells were transiently transfected with expression plasmids (10 μg) containing cDNAs for the indicated proteins that were either nontagged (upper gel), HA tagged (middle gel), or MYC tagged (lower gel). Transfected cells were lysed, and 20 to 25 μg of protein was separated on 8% acrylamide gels. The gels were probed with a monoclonal antibody to E2F-1 (KH20), a monoclonal antibody to the HA epitope (12CA5), or a monoclonal antibody to c-MYC (9E10). Note the endogenous proteins (marked with asterisks) recognized by the 12CA5 antibody (~50 kDa) and the 9E10 antibody (~56 kDa). The ~56-kDa protein is seen only in the last lane of the exposed gel shown at the bottom but is present in all lanes with 9E10 on longer exposures. The exposure time for the upper picture was too short to detect endogenous E2F-1. (B) U-2 OS cells were transfected in duplicate with plasmids expressing the indicated proteins. Expression plasmids (50 ng) were cotransfected with 2 μg of reporter construct containing four E2F DNA-binding sites in front of the CAT gene ($E2F_4CAT$), 2 μg of pCMVluc, and carrier DNA to a total of 24 μg per 9-cm-diameter dish. Fold activation refers to units of CAT activity normalized to the luciferase activity for each cell extract. The basal level of $E2F_4CAT$ was set to unity. Results similar to the presented data have been obtained in both U-2 OS and C-33A cells in a minimum of four independent experiments. The E2F-1 mutants are as follows: Y411C, pRB binding deficient; E132, DNA binding deficient; and 1-374, transactivation and pRB binding deficient. The E2F-4 dl4 mutant is deficient in p107 binding.

(Fig. 5A). This result indicates that endogenous pRB inhibits the activity of wild-type E2F-1 and may provide an explanation as to why only 50 to 60% of the cells progress into S phase in the presence of wild-type E2F-1. The observed effects of the wild-type E2F-1 and the mutants were not due to different levels of expression or subcellular localization, since cells transfected with the expression constructs used for microinjection expressed similar levels of the E2F-1 proteins (Fig. 3A) and all the proteins were localized to the nucleus after microinjection as assayed with specific monoclonal antibodies (Fig. 4A and B and data not shown).

Similar to expression of the RE2F-4 clone, E2F-4 expression after microinjecting serum-starved cells was not sufficient to promote S-phase entry (Fig. 4E and F and 5B). Interestingly, it was recently demonstrated that an E2F-4 molecule with a deletion of four amino acids (E2F-4dl4) in the p107/p130-binding region could transform NIH 3T3 cells, whereas wild-type E2F-4 could not (13). This difference was ascribed to the mutant's lesser susceptibility to inhibition by p107 (and probably also by p130). The inhibitory effect of p107 or p130 may also prevent E2F-4 from stimulating S phase in serum-starved cells, and this possibility was tested by microinjecting an expression plasmid containing the cDNA for E2F-4dl4. As shown in Fig. 5B, S-phase entry was not promoted by E2F-4dl4 either, although both this mutant and the wild-type E2F-4 proteins were expressed at high levels (Fig. 3A) and could weakly transactivate an E2F-dependent promoter (Fig. 3B).

Since our data suggested a functional difference between the pRB-associated E2F-1 and the p107/p130-associated E2F-4, we wanted to extend our observations to include other members of the E2F family. Serum-starved R12 cells were therefore microinjected with expression plasmids containing the cDNAs for the pRB-associated E2F-2 or E2F-3 or the p107/p130-associated E2F-5. Similar to E2F-1, the pRB-associated E2F-2 and E2F-3 alone were able to stimulate S-phase entry in R12 cells, whereas no effect was seen after microinjection of the E2F-5 expression plasmid (Fig. 5B).

E2F-4 and E2F-5 can induce S phase when coexpressed with DP-1. Since E2F-4 and E2F-5 did not have any detectable effect on S-phase entry in serum-starved cells, one of the heterodimeric partners for the E2Fs was included in our experiments. DP-1 has been demonstrated to bind the pRB-associated E2Fs and E2F-4 in vivo (59, 61), and it is present throughout the cell cycle (4). DP-1 or a DP-like protein may be an obligatory partner for the E2F proteins, and dimerization of DP-1 with one of the E2Fs leads to increased DNA-binding and transcriptional activity of the heterodimer (3, 5, 13, 24, 32). When a DP-1 plasmid shown to direct expression of high levels of DP-1 protein (Fig. 3A) was injected into serum-starved cells, only a very limited effect on cell cycle progression was seen (Fig. 5C). However, when DP-1 was coexpressed with E2F-4 or

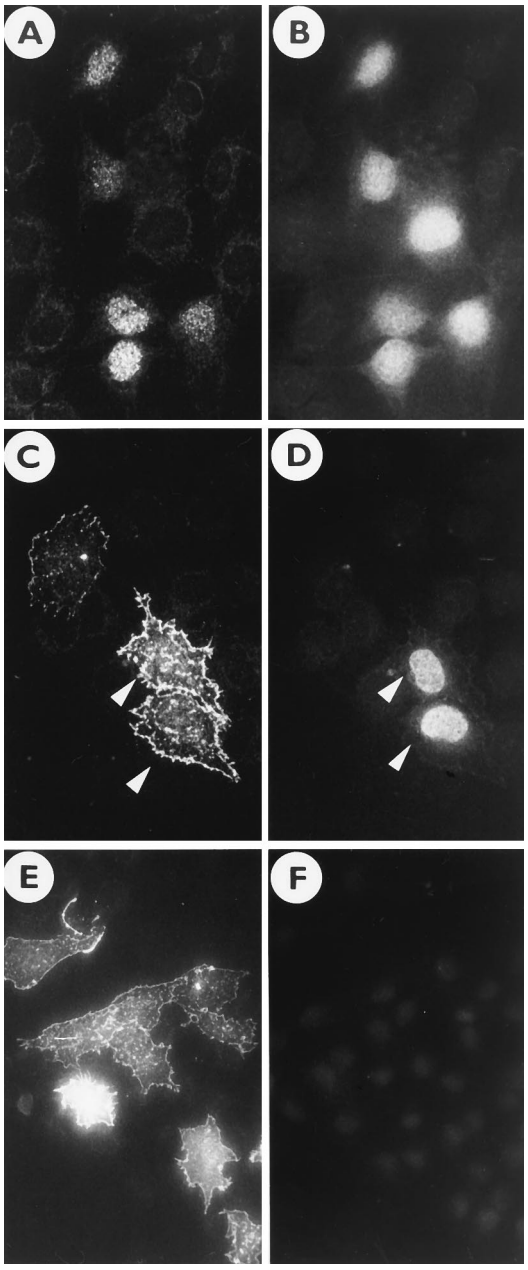


FIG. 4. Microinjection of E2F-1 but not E2F-4 stimulates DNA synthesis in rat fibroblasts. Serum-starved R12 cells were microinjected directly into the nucleus with expression plasmids (10 μg/ml) containing wild-type E2F-1 or E2F-4 together with CD20 expression vector as a marker for productively injected cells. Immediately after injection, fresh serum-free medium containing BrdU was added. After an additional 20 h, the cells were fixed and processed for indirect immunofluorescence detecting E2F-, CD20-, and BrdU-positive cells, respectively (see Materials and Methods). Injected cells expressing E2F-1, as detected by specific monoclonal antibody (KH20), are shown (A). The corresponding induction of BrdU incorporation is also shown (B). Cells microinjected with E2F-1 (C) and E2F-4 (E) plasmids together with CD20 expression vector revealed by anti-CD20 staining are shown. Two out of three E2F-1-injected cells (marked by arrowheads) (C) but none of the E2F-4-injected cells (E) stimulate BrdU incorporation (D and F).

E2F-5, approximately 30% of R12 cells entered S phase (Fig. 5C). The S-phase entry was not due to an anti-inhibitory effect of E2F-4, since the p107/p130-binding-deficient mutant, E2F-4dl4, could induce S phase in 35% of R12 cells when coexpressed with DP-1. The degree of S-phase induction mediated by E2F-4 or E2F-5 in conjunction with DP-1 could not be increased by microinjecting greater amounts of the expression plasmids (data not shown). For comparison, coexpression of E2F-1 and DP-1 was twofold more efficient than E2F-4 or E2F-5 coexpressed with DP-1 in promoting S-phase entry (Fig. 5C).

To determine the specificity of the E2F-1 or E2F-4–DP-1 effect on cell cycle progression, serum-starved cells were microinjected with expression plasmids containing the cDNAs for the pocket proteins together with the various E2F expression plasmids (Fig. 5D). E2F-1-induced S-phase progression was shown to be inhibited three- to fourfold by pRB and less than twofold by p107. These results are consistent with previously published results demonstrating that E2F-1 induced transactivation can be inhibited by pRB (22) and to a lesser extent by p107 (7). S-phase induction by E2F-4 and DP-1 was totally abolished by coexpressing p107 or p130, whereas pRB caused only a twofold reduction in BrdU incorporation. The reduction observed with wild-type pRB was specific, since a naturally occurring mutant of pRB(Δ22) did not reduce the activity of E2F-4 or DP-1.

E2F-1 overcomes G_1 blocks mediated by p16 and a cyclin D1 antibody. Recent data have suggested a close connection between the presence of a functional pRB and the ability of p16 and neutralizing antibodies to cyclin D1 to block cells in the G_1 phase of the cell cycle (see above). These data have defined a pathway in which the CDK4/CDK6-inhibitor, p16, type D cyclins, and pRB control the passage through the restriction point. Since the pRB-associated E2Fs are some of the downstream targets for pRB, we wanted to know if the block exerted by p16 or by a neutralizing antibody to cyclin D1 could be rescued by overexpression of the E2Fs.

In the first set of experiments, a previously characterized neutralizing antibody to cyclin D1, DCS-6 (38, 39), was microinjected into RE2F-1 or RE2F-4 cells (Fig. 6A). In the presence of tetracycline and 10% serum, DCS-6 caused a significant delay in S-phase entry by approximately 60 to 65% compared with those of noninjected cells in the RE2F-1 and RE2F-4 cell lines. In contrast, DCS-6 was able to inhibit S-phase entry by only 10% when RE2F-1 was grown in the absence of tetracycline and in 10% serum, and the inhibition was only 24% in serum-starved RE2F-1 cells that had been grown in the absence of tetracycline for 24 h (Fig. 6A). These data demonstrate that E2F-1 can relieve the cyclin D1 requirement for G_1 progression and suggest that E2F-1 is downstream of cyclin D1 in the same pathway as pRB. Interestingly, expression of E2F-4 alone was not able to overcome a block mediated by DCS-6.

In the second set of experiments, we evaluated the ability of the E2F-1 and E2F-4 to overcome a G_1 block mediated by p16. RE2F-1 and RE2F-4 cells were microinjected with a fusion protein, GST-p16, which previously has been demonstrated to block human and rodent cells in G_1 (40). S-phase progression was inhibited by 80 to 90% in both RE2F-1 and RE2F-4 cells microinjected with GST-p16 in the presence of tetracycline and 10% serum (Fig. 6A). However, upon removal of tetracycline in low or high concentrations of serum, S-phase progression was only inhibited by approximately 20% in the RE2F-1 cell line. In contrast, S-phase progression was still inhibited by 90% in RE2F-4 cells induced to express E2F-4.

Since serum-starved cells enter the G_1 phase of the cell cycle in a different way than chemically arrested cells, we determined if E2F-1 could rescue the growth-suppressing effect of p16 in nocodazole-synchronized cells. As shown in Fig. 6B, cells released from nocodazole were even more efficiently rescued

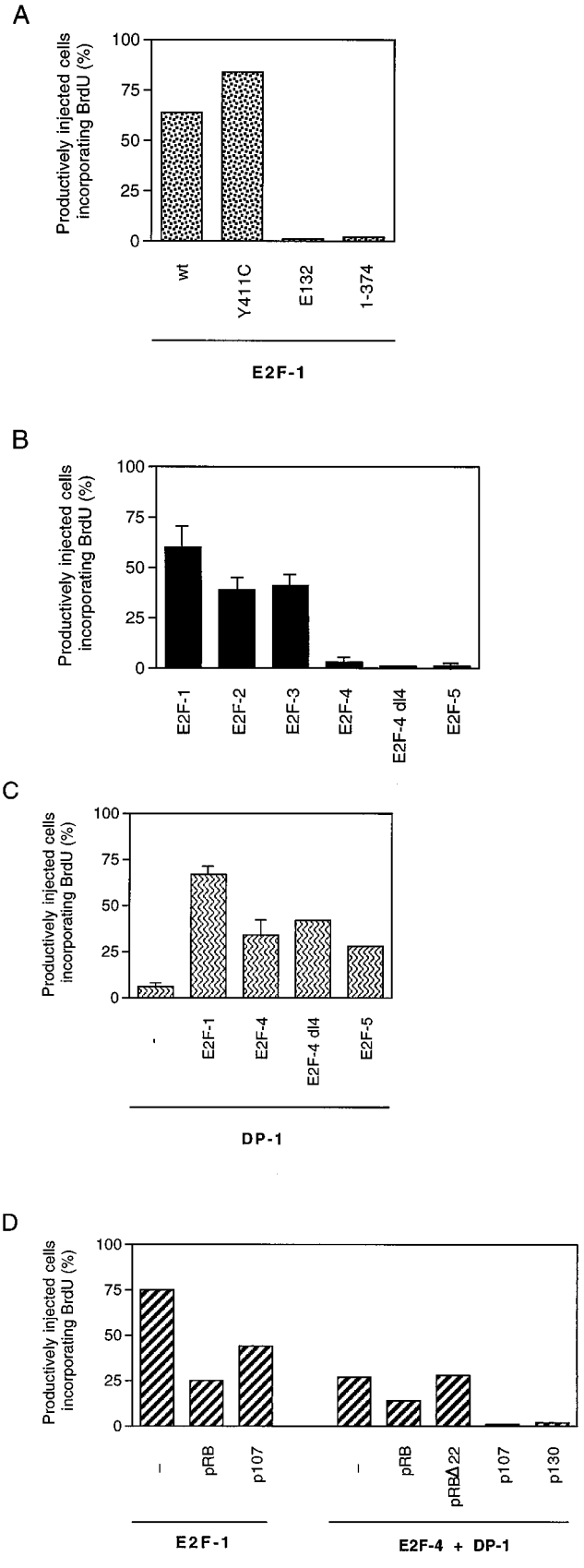


FIG. 5. E2F-induced BrdU incorporation in rat fibroblasts. (A) Ability of wild-type (wt) and mutant forms of E2F-1 to induce DNA synthesis. Quiescent R12 cells were microinjected with E2F expression plasmids containing the indicated cDNAs as described in the legend to Fig. 4. At 20 h after injection, the productively injected cells expressing CD20 were evaluated for BrdU incorporation. The diagram summarizes one complete set of data derived from one microinjection experiment performed under exactly identical conditions, thereby allowing a direct comparison between Y411C and wild-type E2F-1. Similar results were obtained in repeated experiments. (B) S-phase induction by the E2F transcription factor family. Quiescent R12 cells were microinjected and evaluated for BrdU incorporation as described above. Each bar represents the mean value for at least 500 counted cells (error bars indicate standard deviations). (C) E2F-4 and E2F-5 can induce S phase when coexpressed with DP-1. Quiescent R12 cells were microinjected and evaluated for BrdU incorporation as described above. Each bar represents the mean value for at least 500 counted cells (error bars, standard deviations). (D) Specificities of E2F-1- and E2F-4–DP-1-promoted S-phase entry. Quiescent R12 cells were microinjected with E2F-1 or with E2F-4 and DP-1 expression plasmids (10 μg/ml) together with expression plasmids (50 μg/ml) containing no insert (−), containing the genes pRB, p107, or p130, or containing a naturally occurring mutant of pRB. BrdU incorporation of productively injected cells was evaluated 20 h later. The E2F-1 and E2F-4 mutants used were as described in the legend to Fig. 3.

from a p16-mediated G_1 block by E2F-1 than were cells released from serum starvation.

Generation of E2F activity abolishes p16-mediated growth suppression. To rule out clonal effects and to determine if the other E2Fs were able to overcome a p16-mediated cell cycle block, serum-starved R12 cells were microinjected with E2F expression plasmids with or without a p16 expression plasmid. As shown in Fig. 7, p16 was not able to arrest cells that were induced into S phase by the pRB-associated E2Fs or E2F-4 in conjunction with DP-1. Furthermore, the ability of E2F-1 to override a G_1 block induced by p16 is achieved by generation of E2F activity and not by functional inactivation of pRB, since the pRB-binding deficient mutant of E2F-1 (Y411C) very efficiently induced S phase in the presence of high levels of p16 (Fig. 7). As a control for the inhibitory effect of p16, we demonstrated that R12 cells kept in 10% serum did not undergo DNA synthesis after microinjection of the p16 expression plasmid (Fig. 7). In conclusion, our data demonstrate that the generation of E2F activity by expression of the pRB-associated E2Fs or E2F-4 together with DP-1 is sufficient to overcome the G_1 block mediated by p16.

DISCUSSION

Using two experimental approaches, we have demonstrated that the pRB-associated E2Fs (E2F-1, E2F-2, and E2F-3) all can induce S-phase entry in serum-starved cells and that the p107/p130-associated E2Fs (E2F-4 and E2F-5) cannot when expressed alone. In the first approach, cell lines were generated with the inducible expression of E2F-1 and E2F-4. The proteins synthesized were shown to exhibit the expected characteristics, including protein size, DNA-binding activity, and specificity in binding to pRB and p107. Furthermore, induced expression of E2F-1 could transactivate an E2F-dependent promoter about 10-fold, whereas no detectable transactivation of this promoter by induced expression of E2F-4 was observed. Since E2F-4 expressed in the RE2F-4 cells was shown to exhibit the expected properties of wild-type E2F-4 and since E2F-4 was expressed at higher levels than E2F-1 (Fig. 1A), our results suggest that the inability of E2F-4 to transactivate the E2F-dependent promoter is not due to mutations in the transfected E2F-4 cDNA but more likely due to an intrinsic property of wild-type E2F-4. In fact, when a DP-1 expression plasmid was coelectroporated into RE2F-4 cells with the reporter construct, a specific transactivation of this reporter was mea-

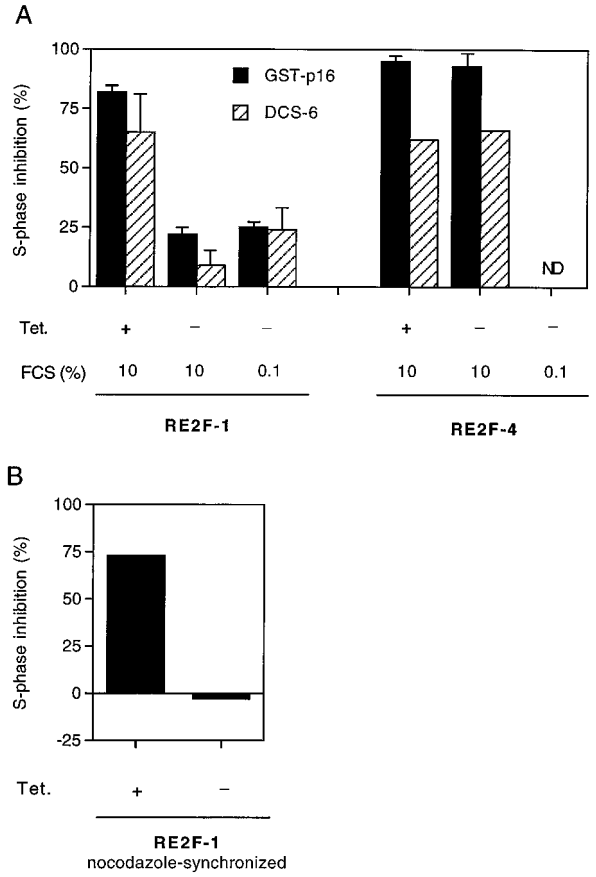


FIG. 6. Expression of E2F-1 alone but not E2F-4 can rescue G_1 blocks mediated by a neutralizing antibody to cyclin D1 and p16. (A) RE2F clones were synchronized in G_0 by incubation for 48 h in medium containing 0.1% FCS and tetracycline (Tet.). Purified DCS-6 antibody (5 mg/ml) was then microinjected perinuclearly into at least 100 cells, and cells were incubated in medium containing 10% FCS with (+) or without (−) tetracycline or an 0.1% FCS without tetracycline. BrdU was added to the media simultaneously. After an additional 18 h the cells were fixed and stained for immunoglobulin-containing cells and BrdU incorporation. Alternatively, RE2F clones were microinjected with purified GST-p16 fusion protein (2 mg/ml) together with purified nonspecific mouse immunoglobulin (1 mg/ml) as an injection marker. After incubation for 18 h in the indicated medium with or without tetracycline, the cells were fixed and stained for both immunoglobulin (injected cells) and incorporation of BrdU. Specific S-phase inhibition was calculated as $[(N - I)/N] \times 100$, where N is the percentage of BrdU incorporation in noninjected cells and I represents BrdU incorporation of cells injected with the antibody. The bars represent the mean values for three experiments (error bars, standard deviations). ND, not determined.) (B) RE2F-1 cells were synchronized by incubation in medium containing tetracycline and nocodazole (see Materials and Methods). Mitotic cells were collected and replated in fresh medium with or without tetracycline. At 4 h after replating, the cells were microinjected with GST-p16 together with mouse immunoglobulin, and after an additional 20 h, S-phase inhibition was evaluated essentially as described for panel A. From left to right, the bars summarize results obtained from 116 and 161 injected cells.

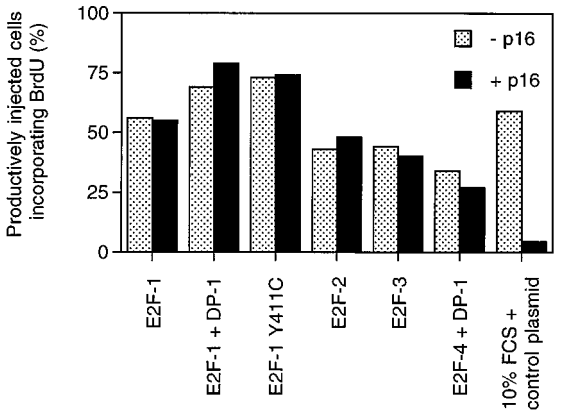


FIG. 7. Expression of pRB-associated E2Fs or E2F-4 in combination with DP-1 can overcome a p16-mediated cell cycle block. R12 cells were synchronized in G_0 by incubation for 48 h in serum-free medium and microinjected with p16 expression plasmid together with plasmids containing E2F-1, E2F-2, or E2F-3 genes or E2F-4 in conjunction with DP-1. CD20 expression plasmid was included in all samples as a marker of productively injected cells (the concentration of all plasmids was 10 μg/ml). After an additional 20-h incubation in serum-free medium containing BrdU, the cells were fixed and immunostained for CD20 and BrdU. As a control for the ability of the given concentration of p16 expression plasmid to mediate cell cycle block, the cells were injected with p16 plasmid or the parental plasmid without insert and stimulated with 10% FCS for 24 h.

sured, but only under conditions under which E2F-4 was expressed (data not shown).

In the second approach, expression plasmids containing the cDNAs of the E2Fs and various mutants thereof were microinjected into serum-starved rat fibroblasts. Using this approach, we could more readily analyze the effect on S-phase entry by expressing the E2F family members and in addition rule out possible clonal effects. Consistent with the data obtained for the inducible cell lines, E2F-1 in this experimental setting was able to induce S phase after microinjection into serum-starved rat fibroblasts kept in low concentrations of serum and E2F-4 was not. Furthermore, the ability of E2F-1 to induce S phase was dependent on the integrity of its DNA-binding domain and its transactivation domain but not its pRB-binding domain. These results are in agreement with data reported by Qin et al. (48) and suggest that the S-phase entry induced by E2F-1 is dependent on transactivation of specific promoters and not on inactivating the normal function of pRB.

Interestingly, E2F-2 and E2F-3 were also able to induce S-phase entry in serum-starved fibroblasts and E2F-5 was not. The inability of the p107/p130-associated E2Fs to induce S-phase progression was not due to differences in expression levels among the E2F family members, since similar amounts of protein were synthesized after transfection (Fig. 3A). Furthermore, microinjection of E2F-4 and E2F-5 expression plasmids at 10-fold (100 μg/ml) the standardly used concentration was not sufficient to induce S-phase progression whereas E2F-1 was even effective in inducing S-phase at a 10-fold lower concentration (1 μg/ml) (data not shown). The inability of E2F-4 and E2F-5 to stimulate entry into S phase was most likely not due to the inhibitory effect of p107 and p130 on these transcription factors, since a deletion mutant of E2F-4 that does not bind to p107 or p130 and retains its transactivating activity could not induce S phase either. In transient transfections, E2F-4 and E2F-5 only poorly activated an E2F-dependent reporter construct (Fig. 3B), as has been published by others (5, 25). This result is consistent with the data obtained for the RE2F-4 clone; however, this result could appear rather surprising, since E2F-4 and E2F-5 contain as strong a transactivating domain as does E2F-1, when expressed as GAL-4 fusion proteins (25). Taken together, these data may suggest that the E2F-dependent reporter construct used in these studies does not contain the optimal DNA structure for E2F-4 and E2F-5 binding and/or that the activity of these E2Fs are regulated in a different manner than the pRB-binding E2Fs. Data from another laboratory (12) have suggested that E2F-4 transactivates some E2F-dependent reporter constructs to a higher degree than does E2F-1. Thus, the inability of the p107/p130-associated E2Fs to induce S phase in quiescent fibroblasts may be due to the transactivation by these E2Fs of a subset of

E2F-dependent promoters that differ from those activated by the pRB-associated E2Fs.

However, when E2F-4 or E2F-5 was coexpressed with DP-1 in serum-starved cells, 30% of the cells entered the S phase of the cell cycle. The S-phase induction mediated by E2F-4 and DP-1 was specifically inhibited by p107 and p130 but not by pRB, suggesting that the activation of the pRB-associated E2Fs by DP-1 is not required for E2F-4–DP-1-promoted S-phase entry. The coexpression of p107/p130-associated E2Fs with DP-1 leads to a large increase in E2F DNA-binding activity of transfected cells (data not shown and references 5, 13, and 25), and the ability to transactivate the E2F-dependent reporter construct (Fig. 3B) (5, 25), thereby suggesting that the generation of E2F DNA-binding activity drives the cells into the S phase. In contrast, a large increase in DNA-binding activity is not required for the pRB-associated E2Fs to promote S-phase entry (Fig. 1B) (24, 61; also, data not shown). In fact, the coexpression of DP-1 with E2F-1 only marginally increases the ability of E2F-1 to promote S-phase entry (Fig. 5C). Our experiments therefore suggest that the p107/p130-associated E2Fs are able to induce S-phase entry to a certain extent under conditions under which an artificially large amount of E2F DNA-binding activity is created. E2F-dependent promoters that otherwise would not be activated by these E2Fs may therefore be activated. As a complementary explanation, one could imagine that expression of the p107/p130-associated E2Fs and not the pRB-associated E2Fs may change the subcellular localization of DP-1, or that coexpression of DP-1 with the E2Fs would localize the proteins in the nucleus. Using a monoclonal antibody to DP-1 in indirect immunofluorescence assays, we have not seen any change in subcellular localization of endogenous DP-1 after tetracycline removal in the RE2F-1 and RE2F-4 cell lines (data not shown). However, future studies are required to understand the structural differences between the p107/p130-associated and the pRB-associated E2Fs that allow the pRB-associated E2Fs to induce S-phase entry when expressed alone. In preliminary results we have identified a small domain in E2F-1 that, when substituted for a similar-sized domain in E2F-4, allows the E2F-4 chimeric protein to induce S phase in the absence of DP-1 coexpression (43a). We are currently analyzing the influence of this domain on the E2F-4 protein.

The p16-cyclin D-pRB-E2F pathway. Since E2F is a downstream target for pRB, one would predict that overexpression of any E2F transcription factor with E2F DNA-binding activity could overcome a G_1 block mediated by pRB. However, this should be a fact only if inhibition of E2F-dependent transcription is causing the G_1 arrest mediated by pRB. That E2F-1 can overcome such a block has previously been shown (48, 63). In this paper we have extended the linearity of the pathway by demonstrating that the pRB-associated E2Fs alone can abolish a p16-elicited G_1 block and that E2F-1 overrides a G_1 arrest mediated by a neutralizing antibody to cyclin D1. In recent experiments, it was demonstrated that transforming growth factor β-mediated growth suppression was overcome by E2F-1 (54). Although the molecular mechanisms of transforming growth factor β-mediated growth suppression are not clear, it has been suggested that transforming growth factor β regulates the action of G_1 cyclin-dependent kinases and thereby the phosphorylation of pRB. Our results therefore extend and corroborate the findings of Schwarz et al. (54).

In conclusion, our data suggest that E2F-1, E2F-2, and E2F-3 are immediately downstream of pRB in the p16-cyclin D-pRB pathway, since they all bind to pRB, and that they can overcome G_1 arrests mediated by p16, antibodies to cyclin D1, and pRB. Furthermore, since E2F-4 and E2F-5 cannot overcome G_1 arrests imposed by p16 or antibodies to cyclin D1 when expressed alone, our results further strengthen the possible existence of multiple pathways regulating the mammalian cell cycle (52, 60). In the p16-cyclin D-CDK4-pRB-E2F-1, -2, and -3 pathways, many alterations have been associated with human cancers, whereas no tumor-associated alterations have so far been characterized in the less-defined p107/p130-E2F-4 and -5) pathway. By demonstrating that E2F-4 and E2F-5 cannot induce S phase in quiescent cells when expressed alone, our data may suggest that there is no genetic pressure that selects for alterations in p107, p130, E2F-4, or E2F-5, thereby providing a possible explanation as to why such mutations have not been identified. In contrast, our data provide further evidence for the importance of the p16-cyclin D-pRB-E2F pathway in regulating the G_1 phase of the cell cycle. The demonstration that E2F-1, E2F-2, and E2F-3 all lead to S-phase progression may also initiate a more extensive search for alterations of the genes encoding these proteins in various types of tumors.

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