Blocking of Transcription Factor E2F/DP by Dominant-Negative Mutants in a Normal Breast Epithelial Cell Line Efficiently Inhibits Apoptosis and Induces Tumor Growth in SCID Mice

By Ralf C. Bargou,*‡ Christian Wagener,* Kurt Bommert,* Wolfgang Arnold,* Peter T. Daniel,*‡ Markus Y. Mapara,*‡ Edgar Grinstein,* Hans D. Royer,* and Bernd Dörken*‡

*From the *Max Delbrück Center for Molecular Medicine, Berlin-Buch, Germany; and the ‡Humboldt University of Berlin, Universitätsklinikum Rudolf Virchow, Robert Rössle Klinik, Berlin-Buch, Germany*

Summary

The transcription factor E2F is regulated during the cell cycle through interactions with the product of the retinoblastoma susceptibility gene and related proteins. It is thought that E2F-mediated gene regulation at the G1/S boundary and during S phase may be one of the rate-limiting steps in cell proliferation. It was reported that in vivo overexpression of E2F-1 in fibroblasts induces S phase entry and leads to apoptosis. This observation suggests that E2F plays a role in both cell cycle regulation and apoptosis. To further understand the role of E2F in cell cycle progression, cell death, and tumor development, we have blocked endogenous E2F activity in HBL-100 cells, derived from nonmalignant human breast epithelium, using dominant-negative mutants under the control of a tetracycline-dependent expression system. We have shown here that induction of dominant-negative mutants led to strong downregulation of transiently transfected E2F-dependent chloramphenicol acetyl transferase reporter constructs and of endogenous c-*myc*, which has been described as a target gene of the transcription factor E2F/DP. In addition, we have shown that blocking of E2F could efficiently protect from apoptosis induced by serum starvation within a period of 10 d, whereas control cells started to die after 24 h. Surprisingly, blocking of E2F did not alter the rate of proliferation or of DNA synthesis of these cells; this finding indicates that cell-cycle progression could be driven in an E2F-independent manner. In addition, we have been able to show that blocking of endogenous E2F in HBL-100 cells led to rapid induction of tumor growth in severe combined immunodeficiency mice. No tumor growth could be observed in mice that received mock-transfected clones or tetracycline to block expression of the E2F mutant constructs in vivo. Thus, it appears that E2F has a potential tumor-suppressive function under certain circumstances. Furthermore, we provide evidence that dysregulation of apoptosis may be an important step in tumorigenesis.

Apoptosis and cell cycle are closely linked and tightly regulated processes that control tissue development, differentiation, and homeostasis (1). It is well established that deregulated expression and altered function of the genes involved in cell cycle regulation contribute to the pathogenesis of cancer (2). In the past few years, evidence accumulated that resistance toward apoptosis is another important factor for tumor development (3). This has originally been shown in the case of B cell malignancies (3–5). One important regulator of apoptosis is the *bcl-2* oncogene, which was identified at t(14;18) chromosomal translocation breakpoints in the majority of follicular B cell lymphomas. These translocations lead to juxtaposition of the *bcl-2* gene on chromosome 18, with the Ig heavy chain gene on chromosome 14. Subsequent overexpression of the *bcl-2* gene renders the lymphoma cells resistant to apoptosis. Thus dysregulation of apoptosis may be a causative event in the evolution of B cell malignancies.

The tumor suppressor gene *p53* exerts its tumor-suppressing function by regulating both cell cycle and apoptosis. Induction of apoptosis by *p53* is at least in part due to its ability to regulate transcription of members of the *bcl-2* gene family (6–8). Recently, we have shown that the death-promoting gene *bax*, another member of the *bcl-2* gene family,

The first two authors contributed equally to this work.

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is strongly downregulated in breast cancer tissue (9). Thus, dysregulation of apoptosis might be not only an important step in the development of lymphoma but also in the development of solid tumors.

Apoptosis may sustain tissue homeostasis by balancing the effects of proliferation. The regulatory coupling of proliferation and apoptosis is suggested by several recent findings. A line of investigation relevant to this hypothesis has focused on features shared by the pathways of apoptosis and proliferation. These studies have revealed that entry into S phase, an initial step in cell cycle progression, may also occur in the pathway of cell death (10). One such study of prostate epithelial cells showed that apoptosis induced by testosterone withdrawal is preceded by reentry into the cell cycle: after receiving a stimulus that induces apoptosis, quiescent prostate epithelial cells enter S phase and then die without completing the cell cycle. A second line of investigation has focused on regulatory genes that control both proliferation and apoptosis. Expression of genes important for cell cycle regulation, including *cyclin D*, c-*myc*, *Rb*, and *p53*, is associated not only with proliferation, but also with cell death (6, 11–13). E2F-1, the first member of the family of E2F transcription factors, is thought to play a critical role in G1/S progression of the cell cycle (14–16). E2F usually appears to function to control the transcription of a group of genes that encode proteins important for cell cycle progression during S phase; these proteins include dihydrofolate reductase, thymidine kinase, and DNA polymerase α (14). Transcriptional activities of E2F are modulated during the cell cycle, mainly by the formation of complexes between E2F and several key regulators of cell cycle such as the retinoblastoma protein and related proteins (17–22). E2F-1 can form a heterodimer with another E2F-like protien DP-1 and have a synergistic effect on its transcriptional activity (23, 24). Recently, it has been shown that artificial overexpression of exogenous E2F-1 in fibroblasts promotes S phase entry and subsequently leads to apoptosis (25–28).

It was the aim of this study to examine the role of E2F/DP1 in apoptosis and cell cycle regulation. Using the tetracycline-dependent expression system, we established cell lines containing inducible dominant–negative DP-1 or E2F-2 mutants. Our major findings are that (1) downregulation of endogenous E2F/DP activity does not affect the proliferation rate in the breast epithelial cell line HBL-100 but (2) leads to downregulation of c-*myc* expression, (3) enhanced resistance to apoptosis, and (4) induction of tumorigenic growth in severe combined immunodeficiency (SCID)[1] mice.

Materials and Methods

Cells and Culture Conditions. HBL-100 cells were originally isolated from breast milk of an apparently healthy woman (9). Cells were maintained in RPMI 1640 (Seromed-Biochrom, Hamburg, Germany), 10% heat-inactivated FCS, 2 mM L-glutamine (GIBCO, Karlsruhe, Germany) and penicillin–streptomycin (Seromed-Biochrom, Hamburg, Germany).

Preparation of mRNA and Reverse Transcription–PCR (RT-PCR). RNA preparation was performed using the guanidium isothiocyanate (GTC)/CsCl method. Briefly, cells or tissue were lysed with GTC solution (4 M GTC, 20 mM sodium acetate, pH 5.2, 0.1 mM dithiothreitol, 0.5% Sarcosyl (Sigma Chemical Co., Grünwald, Germany). The resulting GTC–cell lysate was layered on top of a CsCl cushion (5.7 M, 100 mM EDTA). After ultracentrifugation at 150,000 *g* for 23 h at 18°C, the RNA pellet was resuspended in Tris-EDTA (Tris, 10 mM; EDTA, 5 mM, pH 7.4) and precipitated by 3 M sodium acetate and ethanol at −80°C for 30 min. Total RNA was extracted from cells and tissues as described above. Purification of poly(A^+) RNA from 10 μg of total RNA was performed using the Dynabead mRNA purification kit (Dynal, Oslo, Norway). Detection of mRNA by PCR was performed as described, using a Geneamp RNA PCR kit (Perkin-Elmer Cetus, Überlingen, Germany) and a thermocycler (Bachhofer, Reutlingen, Germany), according to the PCR protocol. DP-1, ΔDP-1, and ΔE2F-2 PCR products were sequenced and subsequently cloned into pUHD-3 and pKEX expression plasmids (see below). The following primers were used for amplification: ΔDP-1: 5′ primer, 5′-CCGGAATTCCGGATGGCAAAAGATGCCGGT-3′; 3′ primer, 5′-CGCGGATCCGCGCTATTACAGGTTCTTGAAGGCAAT-3′; ΔE2F-2: 5′ primer, 5′-CCGGAATTCCGGATGCTGCAAGGGCCCCGG-3′; 3′ primer, 5′-CGCGGATCCGCGCTATTACAGAGAGCAGCTCTGGAT-3′; DP-1: 5′ primer, 5′-CCGGAATTCCGGATGGCAAAAGATGCCGGT-3′; 3′ primer, 5′-CGCGGATCCGCTCAGTCGTCCTCGTC-3′.

To analyze inducible ΔE2F-2, ΔDP-1, or DP-1 expression, RT-PCR was performed using an upstream primer from the 5′ transcribed region of the expression plasmid pUHD-3 (5′-CAGATCGCCTGGAGACGCC-3′; this procedure resulted in fragment lengths of 784, 790, and 1,315 bp, respectively). β-actin was amplified using the following primers: upstream, 5′-GAGCTGCGTGTGGCTCCCGAGG-3′; downstream, 5′-CGCAGGATGGCATGGGGGAGGGCATACCCC-3′ (resulting fragment length, 246 bp).

Stable Cell Line HBL-100 Expressing Inducible ΔDp-1 or ΔE2F-2. In the first step, the plasmid pUHD 15-1 (29) containing the tetracycline repressor gene (*tet*) fused with the viral VP16 coding region was linearized with ScaI, cotransfected with the resistance plasmid pUC18 (puromycin resistance; Boehringer Mannheim, Mannheim, Germany), and selected for stable HBL-100 cell lines.

Truncated ΔDp-1 or ΔE2F-2 c-DNA contained the DNA binding and dimerization domain of DP-1 (1–235 aa) or E2F-2 (1–233 aa) but were devoid of the NH_2-terminal transactivation domain (23, 30–32). Constructs were derived from RT-PCR, sequenced, and cloned into the expression plasmid pUHD 10-3 (29). This plasmid was cotransfected with the resistance plasmid pKEX (33) (hygromycin resistance) in a second step into the stable cell lines.

Modulation of ΔDp-1 or ΔE2F-2 expression was induced by incubating the cells in medium in the presence (2 μg/ml) or absence of tetracycline. In addition, full-length DP-1 clones (1–410 aa) and mock transfectants containing no insert in pUHD 10-3 were generated by the same protocol. Furthermore, using the expression plasmid pKEX, we selected HBL-100 clones, which expressed ΔDp-1 constitutively (ΔDp-1c).

Electrophoretic Mobility Shift Assay (EMSA). Preparation of nuclear extracts and EMSAs were performed as described (34, 35). Briefly, 2.5 μg of nuclear extract was incubated with 1 μg of poly

[1] *Abbreviations used in this paper:* CAT, chloramphenicol acetyl transferase; EMSA, electrophoretic mobility shift assay; GTC, guanidium isothiocyanate; RT, reverse transcription; SCID, severe combined immunodeficiency.

(deoxyinosine–deoxycytidine) (poly [dI–dC]) for 20 min on ice and ^{32}P-labeled double-stranded oligonucleotides containing a binding site for E2F/DP (5′-ATTTAAGTTTCGCGCCCT-TTCTCAA-3′) and subsequently analyzed by native PAGE.

[^{3}H]Thymidine Incorporation Assay. 5×10^4 cells/well were cultured in medium in 96-well round-bottom plates at 37°C for 72 h. The cells were pulsed with 1 μCi of [^{3}H]Thymidine (Amersham, Braunschweig, Germany) after 3 d of culture, and DNA synthesis was measured during the last 16 h of culture. The cells were harvested onto glass filters, and the incorporated radioactivity was measured using a TopCount counter (Canberra Packard, Frankfurt, Germany).

Chloramphenicol Acetyl Transferase (CAT) Assay. CAT assays were performed using a reporter plasmid E2F4CAT containing the CAT gene under the control of an E2F-dependent promoter (36). Transfection with 20 μg of superhelical reporter plasmid DNA per 10^6 cells in 9-mm culture dishes was carried out by the calcium phosphate coprecipitation technique as described previously (37). At 48 h after transfection, cellular proteins were extracted by a freeze–thaw procedure, and the CAT activity was determined as described previously (37).

Immunocytology. Immunoperoxidase staining of HBL-100 cells was performed using the Vecta stain ABC kit (Vector Laboratories, Burlingame, CA), according to the manufacturer's protocol. Anti–c-*myc* mAb was purchased from Dianova (Hamburg, Germany); anti-PCNA mouse mAb was purchased from Oncogene Science (Cambridge, MA). Polyclonal anti–Ki-67 rabbit serum was purchased from Dako (Hamburg, Germany).

Determination of Apoptotic Cells by Acridine Orange Staining. Cells were cultured as described above. Apoptosis was induced by serum depletion. After induction of apoptosis, cultures were seeded at 3×10^5 cells per 1-ml tissue culture well in RPMI 1640, stained with acridine orange (5 μg/ml), and observed by fluorescence microscopy. The number of fragmented nuclei, which reliably indicates apoptosis, was determined.

DNA Fragmentation Assay. For DNA fragmentation analysis, cell lysates were obtained by incubating a cell pellet containing 10^6 cells in 20 ml of lysis buffer (10 mM EDTA, 50 mM Tris, pH 8, 0.5% sarcosyl, 0.5 mg/ml proteinase K) for 1 h at 50°C. After addition of 5 ml of RNase (1 mg/ml) and another incubation for 1 h at 50°C, lysates were electrophoresed on a 2% agarose gel containing ethidium bromide. The gel was run in a buffer containing 26 M Na_2HPO_4, 33 mM NaH_2PO_4, 10 mM EDTA; it was photographed under UV illumination.

Xenotransplantation of Cells into SCID Mice. HBL-100 cells (10^6) were injected subcutaneously into C.B.-17 *scid/scid* mice. The mice were obtained from our own breeding colony and were kept in isolators under stringent conditions in the central animal laboratory of the Max Delbrück Center for Molecular Medicine. Microbiological controls were performed regularly by addition of sterile sentinel animals to the colony. Tumors were measured in millimeters in two dimensions, length (*a*) and width (*b*), using calipers. Tumor volume (*V*) was calculated according to $V = ab^2/2$, where *a* is the longer of the two measurements. To repress ΔDP-1 expression in vivo, the drinking water was replaced with 2.5% sucrose containing 1.0 mg/ml tetracycline hydrochloride (Sigma Chemical Co., St. Louis, MO) as described previously (38).

Results and Discussion

Truncated DP-1 and E2F-2 Constructs Block Endogenous E2F/DP Activity. To downregulate endogenous E2F/DP activity, we designed DP-1 and E2F-2 mutants (ΔDP-1 and ΔE2F-2) that may act in a dominant-negative manner to functionally inactivate endogenous E2F activities. Both constructs, ΔDP-1 and ΔE2F-2, contained DNA-binding domains but were devoid of the transactivation domain (23, 30–32). By use of a tetracycline-controlled expression system, the constructs were expressed in HBL-100 cells (a nontumorigenic permanent cell line) derived from nonmalignant human breast epithelium. Withdrawal of tetracycline led to the induction of ΔDP-1 or ΔE2F-2 mRNA expression which was shown by RT-PCR (Fig. 1 *a*). The upstream primer was chosen from the 5′ transcribed region of the expression plasmid to discriminate between exogenous and endogenous ΔDP-1 or ΔE2F-2. We monitored the induction of ΔDP-1 and ΔE2F-2 by EMSAs and found that both ΔDP-1 and ΔE2F-2 competed for DNA binding with the endogenous E2F/DP-1 heterodimer (Fig. 1 *b*). Tetracycline withdrawal led to complete disappearance of the endogenous E2F/DP binding complex, which was re-

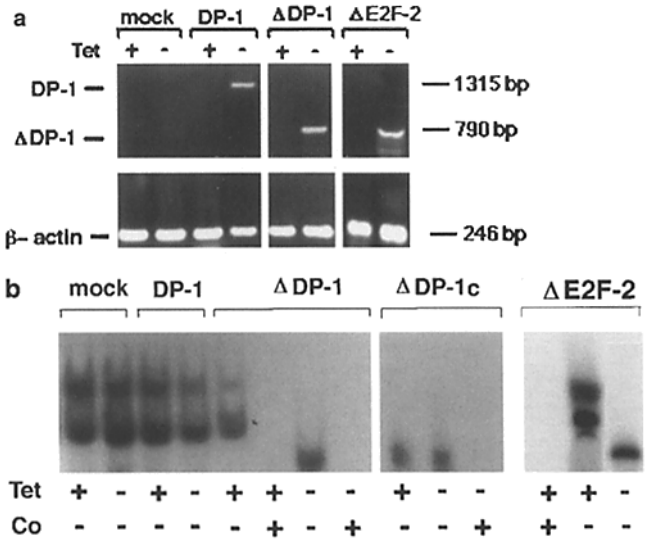
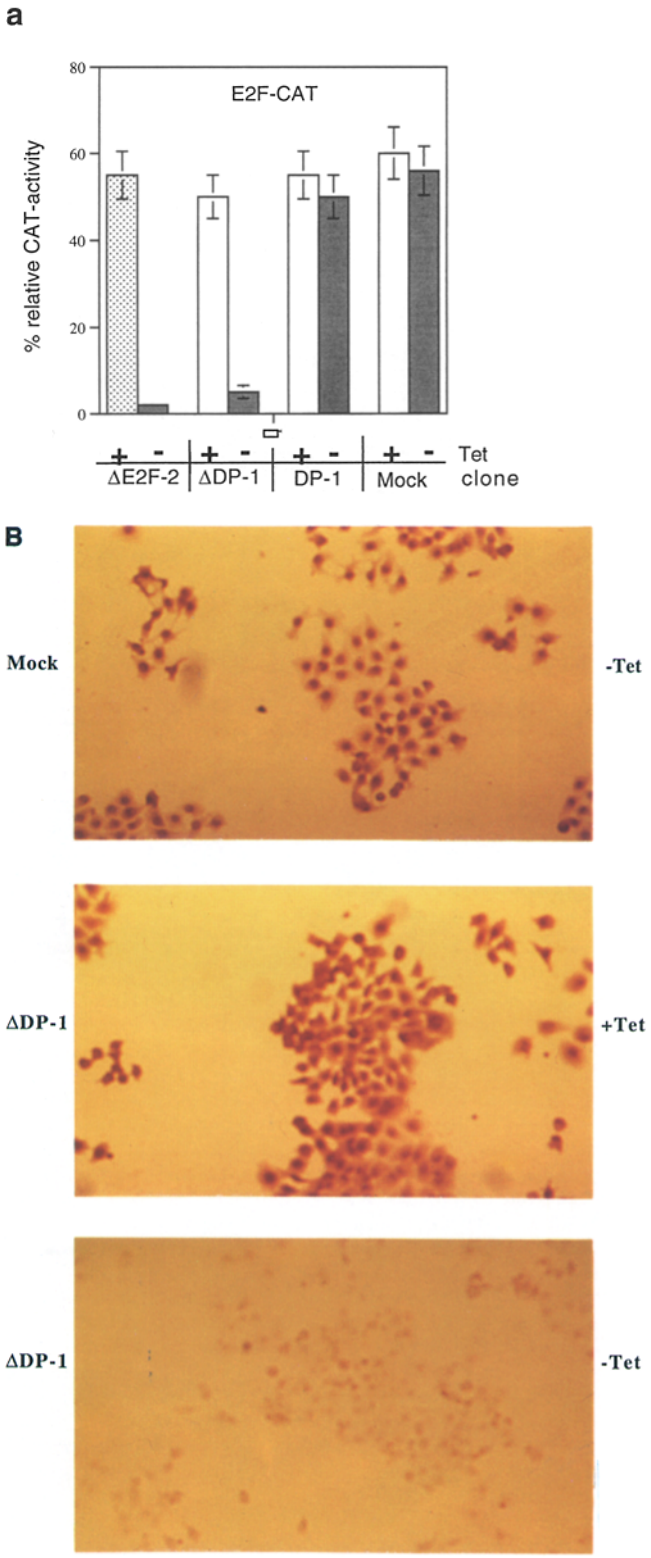


Figure 1. Inducible expression of ΔDP-1 or ΔE2F-2 in HBL-100 cells. (*a*) Stable clones, which are under transcriptional control of a tetracycline (tet)-dependent transactivator, were generated, as described in Materials and Methods. HBL-100 clones transfected with either truncated DP-1 (*ΔDP-1*), truncated E2F-2 (*ΔE2F-2*), or full-length DP-1 (*DP-1*) were analyzed by RT-PCR. The upstream primer was chosen from the 5′ transcribed region of the expression plasmid to discriminate between exogenous and endogenous DP-1. The resulting fragment lengths are indicated. Cells were cultured in the presence (+) or absence (−) of tetracycline. RNA was extracted 24 h after tetracycline withdrawal. Induction of ΔDP-1, ΔE2F-2, and DP-1 expression can be detected after tetracycline withdrawal, whereas in the presence of tetracycline, expression is completely repressed. β-Actin and mock transfectants served as controls. (*b*) Nuclear E2F/DP–binding complexes of inducible and constitutive HBL-100 clones. The same clones as in *a* and one constitutive ΔDP-1 clone (*ΔDP-1c*) were analyzed by EMSA. Where indicated, a 50-M excess of unlabeled binding oligonucleotide was added to show specificity of DNA-binding complexes. Cells were cultured in the presence (+) or absence (−) of tetracycline. Nuclear protein was extracted 24 h after tetracycline withdrawal. Induction of ΔDP-1 or ΔE2F-2 expression leads to complete disappearance of the endogenous E2F/DP binding complex, which is replaced by a smaller complex. Full-length DP-1 and mock transfectants served as controls.

placed by a faster-migrating complex that most likely contained the truncated mutants. To test whether truncated DP-1 or truncated E2F-2 inhibits E2F-dependent transcriptional activity, we transfected reporter genes under the control of an E2F-dependent promoter into ΔDP-1 and ΔE2F-2 clones of HBL-100. As depicted in Fig. 2 *a*, strongly decreased CAT activity could be observed after induction of either ΔDP-1 or ΔE2F-2.

Using immunocytological staining, we were also able to show that induction of ΔDp1 led to strong downregulation of endogenous c-*myc* (Fig. 2 *b*), which contains E2F-binding sites in its promoter and has been described as a target gene of E2F/DP (39). The same result was observed using the E2F-2 mutant (not shown). Expression of Ki-67, a marker indicating that the cycling state of cells, was not affected by the induction of ΔDp-1 (Fig. 2 *b*). Thus, truncated DP-1 or E2F-2 blocked E2F/DP–dependent transcriptional activity, which was consistent with the complete disappearance of endogenous E2F7DP binding complex in EMSAs (see Fig. 1). From these results, we conclude that our truncated constructs are dominant-negative mutants that are able to block E2F/DP.

Blocking of Endogenous E2F/DP Activity Does Not Alter the Proliferation Rate of HBL-100 Cells. E2F is thought to act



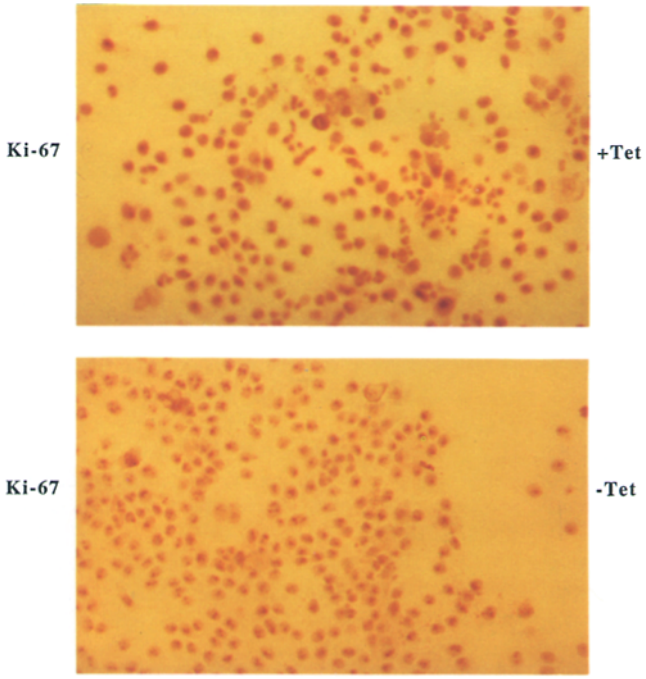


Figure 2. Downregulation of E2F-dependent promoter activity in ΔDP-1 or ΔE2F-2 expressing HBL-100 cells. (*a*) Repression of CAT activity. Transcriptional activity of endogenous E2F was analyzed in inducible HBL-100 clones, using a reporter construct under the control of an E2F-dependent promoter. Cells were cultured in the presence (+) or absence (−) of tetracycline. CAT assays were performed 24 h after tetracycline withdrawal, as described in Materials and Methods. Induction of ΔDP-1 or ΔE2F-2 expression leads to strong downregulation of E2F-dependent promoter activity. Induction of full-length DP-1 does not alter CAT activity. Mock transfectants served as controls. (*b*) Downregulation of endogenous c-*myc* expression. Expression of endogenous c-*myc* protein was analyzed in inducible ΔDP-1 clones by immunocytology, as described in Materials and Methods. Where indicated, cells were cultured in the presence (+) or absence (−) of tetracycline. Cells were fixed and stained with an indirect immunoperoxidase method 24 h after tetracycline withdrawal. Induction of ΔDP-1 expression leads to strong downregulation of c-*myc* expression in HBL-100 cells, whereas expression of Ki-67 remained unaffected in these cells. Mock transfectants served as control.

at the G1/S boundary and during S phase of the cell cycle. E2F-mediated transcriptional activation includes genes that are important for S phase progression; therefore, E2F may be one of the rate-limiting steps in cell proliferation. For this reason, we were interested to find out whether expression of the dominant-negative E2F/DP mutants would influence proliferation and DNA synthesis. Surprisingly, blocking of E2F did not alter the proliferation rate or the DNA synthesis rate of HBL-100 cells (Fig. 3). These results are in contrast to the biological effect of similar dominant-negative E2F mutants described in quiescent 3T3 fibroblasts (40). In these cells, a ΔE2F mutant blocked cell cycle progression induced by the adenovirus protein E1A. E1A acts via the E2F pathway by liberating E2F from multicomponent complexes that contain the product of the retinoblastoma gene, *pRb*, and related proteins. In the fibroblasts, cell cycle progression is E2F-dependent and could therefore be blocked by E2F dominant-negative mutants. Although we can not exclude the possibility that unaffected cell cycle progression in HBL-100 cells expressing either ΔDP-1 or ΔE2F-2 could be due to incomplete suppression of endogenous E2F activity, we propose that cell cycle progression and transcription of important S phase genes could be driven in an E2F-independent manner. This hypothesis is supported by our observation that PCNA, an important cell cycle regulating S phase protein, which is another potential target gene of E2F, was not affected by our dominant-negative mutants (not shown). Therefore, it seems that some (naturally occurring) E2F-responsive promoters can be downregulated (c-*myc*) by such mutants but others cannot be (PCNA).

Blocking of Endogenous E2F/DP Activity Inhibits Induction of Apoptosis. Next we wanted to know whether blocking of endogenous E2F alters sensitivity toward apoptosis. We therefore induced apoptosis by serum depletion and asked whether the expression of either ΔDP-1 or ΔE2F-2 would affect apoptosis. Our results show that after induction of ΔDP-1 or ΔE2F-2, apoptosis could be blocked for 10 d (Fig. 4). In contrast, in the presence of tetracycline, our

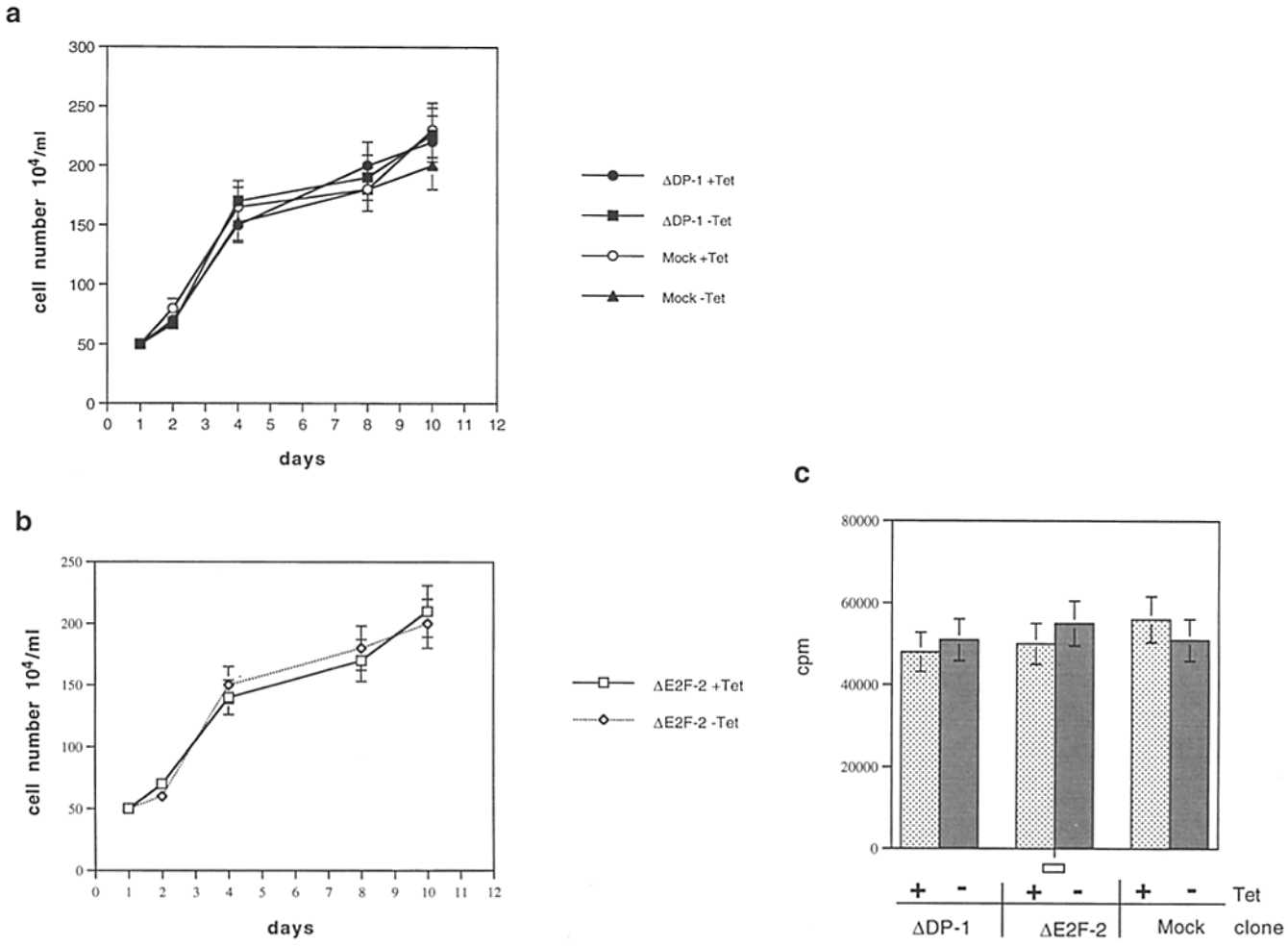


Figure 3. Proliferation rate of ΔDP-1– or ΔE2F-2–expressing HBL-100 cells. Cells were cultured in the presence (+) or absence (−) of tetracycline. (*a* and *b*) Number of viable cells was determined on days 1, 2, 4, 8, and 10 after withdrawal of tetracycline. Viability was determined by trypan blue staining. Mean values and SD of four independently performed experiments are indicated. (*c*) [^{3}H]Thymidine incorporation was measured 3 d after withdrawal of tetracycline. Again, mean values and SD of four independently performed experiments are indicated. Mock transfectants served as the control. Induction of ΔDP-1 or ΔE2F-2 expression in HBL-100 cells has no effect on the rate of DNA synthesis or cell proliferation.

mock-transfected cell clones started to die via apoptosis after 1 d of serum starvation. Therefore, it appears that induction of apoptosis by growth factor depletion in cycling cells was at least in part mediated by an E2F-dependent pathway. This concept is in accord with previous findings that in vivo overexpression of exogenous E2F-1 induced apoptosis in fibroblasts (25–28). It has been shown that constitutive c-*myc* expression causes apoptosis in serum-starved cells (11). We have shown that blocking of endogenous E2F led to downregulation of endogenous c-*myc* expression and inhibition of apoptosis. Therefore, one might speculate that E2F-dependent apoptosis could be regulated at least in part by transcription of the c-*myc* gene. Our findings are strengthened by the fact that the retinoblastoma gene product, an important regulator of E2F, also inhibits apoptosis (13).

Thus, E2F/DP is a transcription factor involved in the regulation of two different biological processes and therefore provides another example of the close linkage between apoptosis and control of the cell cycle.

Blocking of Endogenous E2F/DP Activity in Normal Breast Epithelial Cell Line HBL-100 Induces Tumor Growth in SCID Mice. We recently provided evidence that dysregulation of apoptosis might be an important step in the pathogenesis of breast cancer (9). Therefore, we were interested in determining whether enhanced resistance to apoptosis of

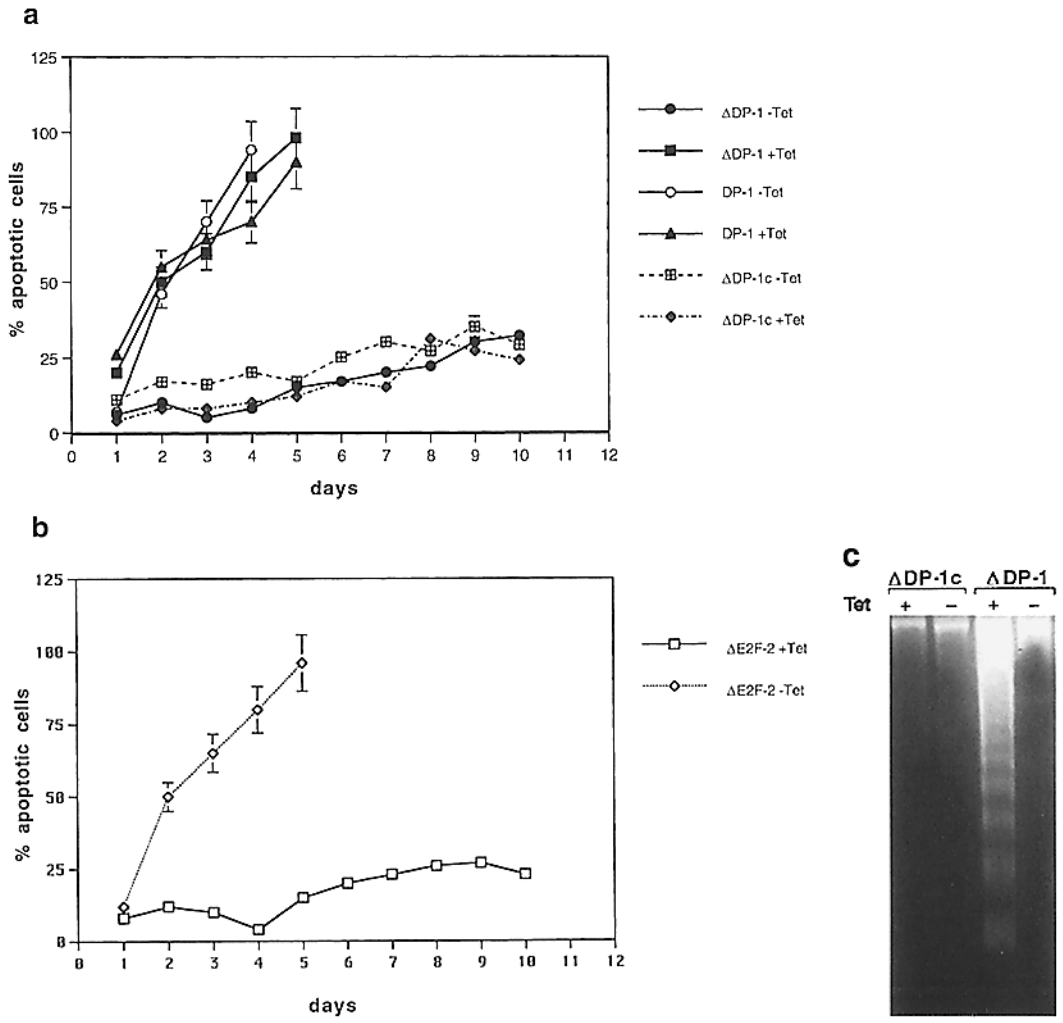


Figure 4. Inhibition of serum starvation–induced apoptosis in ΔDP-1 or ΔE2F-2 expressing HBL-100 cells. Cells of soluble ΔDP-1 or ΔE2F-2 clones or constitutive ΔDP-1 clones (ΔDP-1c) were cultured in the presence or absence of tetracycline (+/− Tet) for prolonged time periods after serum starvation. (*a* and *b*) Apoptosis was assayed morphologically by staining the nuclei with acridine orange on days 1–10. The number of fragmented nuclei (indicating apoptosis) from 100 cells was determined and is expressed as a percentage of apoptotic cells. The mean SD of four independent experiments is indicated. Induction of ΔDP-1 or ΔE2F-2 efficiently protects from apoptosis in HBL-100 cells within the observed period of 10 d. In contrast, repression of either ΔDP-1 or ΔE2F-2 expression led to induction of apoptosis after serum starvation for 2 d. In constitutively expressing cells, tetracycline has no effect on apoptosis resistance. Inducible full-length DP-1–expressing cells (DP-1) served as control. (*c*) Detection of apoptosis by DNA fragmentation in ΔDP-1–expressing HBL-100 cells. Agarose gel electrophoresis of DNA extracted from constitutive or inducible ΔDP-1 clones 2 d after serum depletion. DNA fragmentation can be observed only when ΔDP-1 expression is repressed. No DNA fragmentation can be observed in ΔDP-1–expressing cells.

ΔDP-1 or ΔE2F-2 expressing cells would induce tumorgenicity. We transplanted ΔDP-1 and mock-transfected HBL-100 cells into SCID mice. To suppress ΔDP-1 or ΔE2F-2 expression in vivo, tetracycline was added to the animals' drinking water. As depicted in Fig. 5, tumor growth was observed only in animals that carried either ΔDP-1– or ΔE2F-2–expressing cells and obtained no tetracycline from their drinking water. No tumor growth was observed in animals that were challenged with HBL-100 cells but that did not express truncated mutants. Therefore, we conclude that blocking of endogenous E2F activity can induce tumor growth in SCID mice and that this induction is most likely due to an enhanced resistance to apoptosis. It appears that, in cells proliferating in an E2F-independent manner, E2F has a potential tumor-suppressive function. Recently, it has been reported that induction of apoptosis in E2F-overexpressing cells depends on the expression of *p53* (27, 28). Therefore, it will be interesting to look for loss of function mutations of the E2F/DP family, particularly in *p53*-expressing tumors. Thus, we provide further evidence that dysregulation of apoptosis is an important step in tumorigenesis.

In conclusion, we found that downregulation of endogenous E2F/DP activity did not affect the proliferation rate of the breast epithelial cell line HBL-100 but led to downregulation of c-*myc* expression, enhanced resistance toward apoptosis, and induction of tumorigenic growth in SCID mice. Therefore, we propose the following three hypotheses. First, cell cycle progression can be driven in an E2F independent manner. Second, endogenous E2F is involved in the regulation of apoptosis (possibly via transcriptional regulation of c-*myc* expression). Third, under certain circumstances, E2F has a potential tumor-suppressive function.

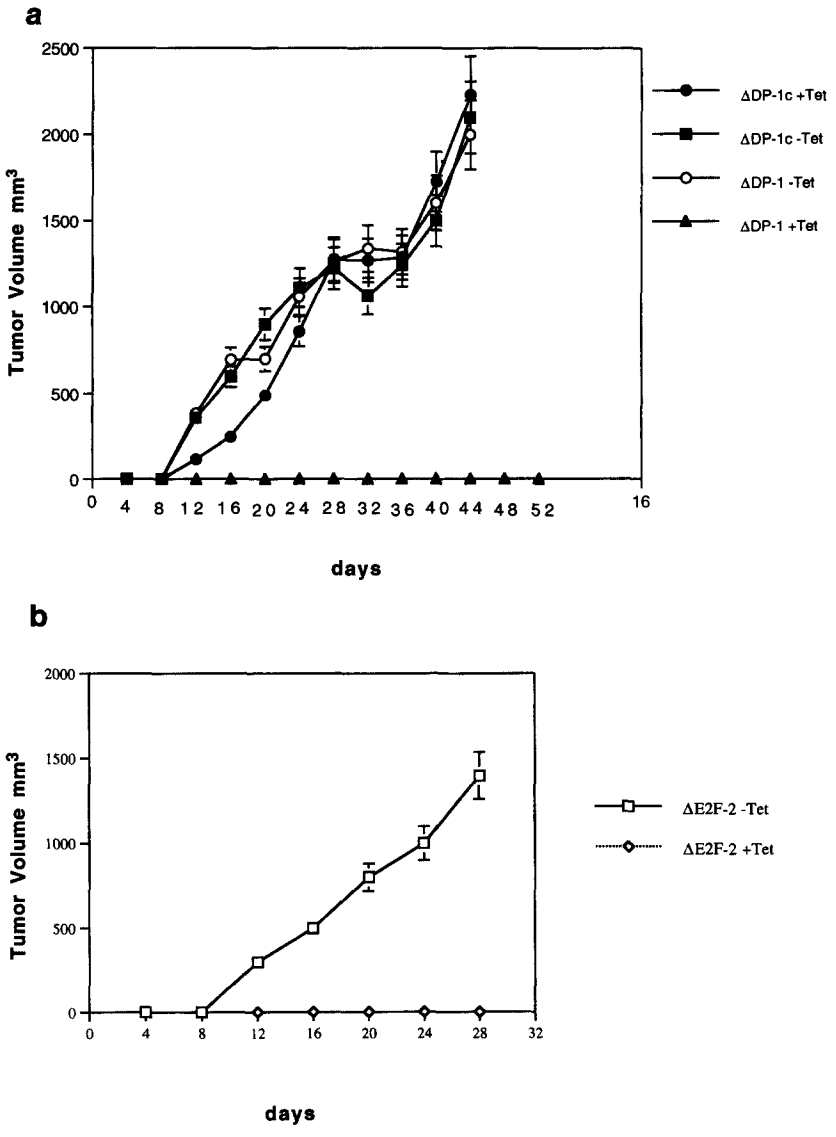


Figure 5. Tumor growth of ΔDP-1– or ΔE2F-2–expressing HLB-100 cells in SCID mice. Shown is tumor growth of inducible (*a*) ΔDP-1, (*b*) ΔE2F-2, or constitutive ΔDP-1–expressing (*a*) ΔDP-1c HBL-100 clones in SCID mice. Animals were challenged with the same cell number (10^6); where indicated, tetracycline (1 mg/ml) was added to the drinking water to suppress ΔDP-1 expression in vivo. Mean values and SD of the tumor size of 10 animals are indicated. Expression of ΔDP-1 or ΔE2F-2 leads to rapid induction of tumor growth, whereas tetracycline repression of the mutants prevents tumor growth. Since tetracycline does not affect tumor growth of constitutively expressing clones, suppression of tumor growth of inducible clones is not due to a toxic effect of tetracycline. Animals challenged with mock-transfected or nontransfected clones did not show tumor growth (not shown).

Address correspondence to Dr. Ralf C. Bargou, Max Delbrück Center for Molecular Medicine, Robert Rössle Str. 10, 13122 Berlin-Buch, Germany.

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