[CANCER RESEARCH 57, 2668–2675, July 1, 1997]

Vitamin E Succinate Inhibits Proliferation of BT-20 Human Breast Cancer Cells: Increased Binding of Cyclin A Negatively Regulates E2F Transactivation Activity

Jennifer M. Turley, Francis W. Ruscetti, Seong-Jin Kim, Tao Fu, F. Victoria Gou, and Maria C. Birchenall-Roberts[1]

Laboratory of Leukocyte Biology, Division of Basic Sciences, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702 [J. M. T., F. W. R., F. V. G.]; Laboratory of Chemoprevention, National Cancer Institute, Bethesda, Maryland 20892 [S-J. K.]; and Intramural Research and Support Program, Science Applications International Corporation-Frederick, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702 [M. C. B-R., T. F.]

ABSTRACT

Vitamin E succinate (VES) inhibited the proliferation of the estrogen receptor-negative human breast cancer cell line, BT-20, in the G_1 phase of the cell cycle. The E2F proteins are integral transcriptional components in the regulation of cell growth. Overexpression of E2F-1 blocked the ability of VES to inhibit BT-20 cell growth, suggesting that VES regulation of E2F-1 activity leads to growth arrest of BT-20 cells. VES, although having little effect on E2F-1 steady-state protein levels, decreased E2F-1 phosphorylation and transactivation activity and increased cyclin A binding to E2F-1. GAL4-E2F-1 deletion mutant studies indicated that cyclin A negatively regulates E2F function. In VES-treated BT-20 cells, the cyclin A protein exhibited reduced kinase activity, which correlated with decreased steady-state levels and binding of cyclin-dependent kinase-2 to cyclin A and increased steady-state levels and binding of $p21^{cip1}$ to cyclin A and cyclin-dependent kinase-2. The functional consequence of the negative regulatory effect of VES on E2F-1 function was shown by the ability of VES to inhibit the transcriptional activation of an E2F-1 responsive gene, c-*myc*. These studies show that VES induces growth inhibition of BT-20 cells through a mechanism that involves cyclin A-negative regulation of E2F-mediated transcription.

INTRODUCTION

The best characterized function for vitamin E is that of a fat-soluble membrane antioxidant (1). VES,[2] a derivative of vitamin E, does not possess antioxidant properties unless the succinate group is removed by a nonspecific esterase (2). VES is a potent inhibitor of neoplastic cells *in vitro* (3–10) and *in vivo* (11). The exact mechanism of action of VES in growth inhibition and differentiation is unknown. Studies have shown vitamin E and/or VES to modulate adenylate cyclase and cyclic AMP-dependent proteins (12, 13), inhibit protein kinase C activity (14–16), and regulate TGF-β protein production (4, 5). Other growth-inhibitory agents like retinoic acid and TGF-β have been reported to negatively regulate E2F activity during growth inhibition (17, 18).

Cell cycle progression is largely controlled by pathways that link the cell cycle machinery to the transcription apparatus. Consequently, transcriptional components play a central role in the regulation of the cell cycle. Members of the E2F (E2F 1–5) and DP (DP 1–3) families of transactivators form heterodimers to regulate the expression of genes that enable cell cycle progression (19, 20). E2F has been characterized as a growth-stimulatory protein *in vitro* and *in vivo* (20, 21). Recent studies in E2F knockout mice ($E2F^{-/-}$) indicate that E2F, in addition to playing an important role in cell proliferation, can also function as a tumor suppressor (22, 23). E2F, particularly E2F-1, functions to enhance cell growth by advancing quiescence cells through G_1 into S phase (20). Typically, E2F-1 activity is inhibited during G_1 by binding with the dephosphorylated RB susceptibility gene product (RB:E2F-1; Refs. 24–26). The RB-related proteins p107 and p130 also form distinct complexes with E2F proteins, although usually not E2F-1 (19, 27). The binding of cyclins and cdks to RB:E2F, p107:E2F, and E2F alone has been shown to regulate E2F transcriptional activation (28–32). In particular, E2F-1 transactivating ability can be negatively regulated by stably bound cyclin A kinase (cyclin A:cdk-2) in S phase (31, 32). It has been suggested that the cyclin:cdk components of these complexes impart kinase activity that triggers the phosphorylation of nuclear proteins like RB and E2F (33, 34).

Cyclins, their associated kinases (cdks), and cyclin-inhibitory proteins are integral components in the coordinated progression of the cell cycle (35–37). These proteins function to modify the biological activity of transcriptional factors. Normally, cyclin A-kinase regulates the G_1-S transition and S phase by participating in transcription [*e.g.*, cyclin A:cdk-2 stimulates c-*myc* transcription (38)] and DNA replication (39). Cyclin A, however, when expressed in G_1, has been demonstrated to function as a G1 cyclin (40, 41). Cyclin A activity in late G_1 depends on cyclin A association with cdk-2 and the cip, $p21^{cip1}$. $p21^{cip1}$ is a ubiquitous inhibitor of cyclin kinases and a critical regulator of cell cycle progression (35, 37, 39, 42). $p21^{cip1}$ has been shown to independently bind cdk-2, cyclin A, and the proliferating cell nuclear antigen (42). The association of $p21^{cip1}$ with cyclin A:cdk-2 inhibits kinase activity (37) and interferes with transcriptional activation events mediated by this complex (39, 43). In many breast cancer cells, cyclins A, D, and E are overexpressed (44, 45). In BT-20 cells, cyclin A mRNA is overexpressed (45). Overexpression of cyclin A may be due to the presence of mutant p53 in BT-20 and many other estrogen receptor-negative breast cancer cells (46), because wild-type p53 has been shown to suppress cyclin A expression (47). The derangement of cyclin expression in human breast cancer may be linked to tumorigenesis (45).

The E2F protein regulates c-*myc* transcription *in vitro* (48) and *in vivo* (21) and also regulates the expression of other growth-related genes like *cdc2*, DNA polymerase α, and thymidine kinase (49). E2F stimulates c-*myc* transcription by binding to E2F consensus sequences within the c-*myc* promoter (38, 50). c-*myc* is overexpressed in breast carcinomas, particularly primitive estrogen receptor-negative (anti-estrogen therapy-resistant) breast tumor cell lines (51). In addition, down-regulation of c-*myc* is associated with growth inhibition of human breast cancer cells (52, 53). Thus, we focused our investigation on VES regulation of E2F-mediated transcription using the primitive human estrogen receptor-negative cell line, BT-20, which expresses E2F, cyclin A, and c-*myc*.

MATERIALS AND METHODS

Cell Culture. BT-20 human estrogen receptor-negative breast cancer cells were derived from an advanced, infiltrating, duct-epithelial cell carcinoma

Received 12/13/96; accepted 5/5/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed. Phone: (301) 846-1442; Fax: (301) 846-6862.

[2] The abbreviations used are: VES, vitamin E succinate; TGF, transforming growth factor; RB, retinoblastoma; cdk, cyclin-dependent kinase; cip, cdk-interacting protein; CAT, chloramphenicol acetyltransferase; mIg, mouse IgG.

following mastectomy (54). BT-20 cells (American Type Culture Collection, Rockville, MD) were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 units/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine, nonessential amino acids, and HEPES buffer (Life Technologies, Inc.).

Proliferation Assays. BT-20 cells were cultured at a concentration of 5×10^4/ml in 96-well, flat-bottomed tissue culture plates (Costar, Cambridge, MA) at 37°C in a humidified atmosphere of 5% CO_2. Cells were treated in the presence or absence of VES (10 μg/ml), vehicle (0.1% ethanol and 5 μg/ml succinic acid), or antioxidants (butylated hydroxyanisol of 20, 10, and 5 μM), butylated hydroxytoluene (20, 10, and 5 μM), and *N*-acetyl cysteine (10^{-3}, 10^{-4}, and 10^{-5} M; Sigma Chemical Co., St. Louis, MO; Refs. 55 and 56) for 24 or 48 h. The vehicle, which is the proper control, was used in every experimental protocol and never varied by >5% from the untreated control. Cells were pulsed with 1 μCi of tritiated thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) during the last 8 h of culture, and harvested onto glass fiber filters; radioactivity was measured by liquid scintillation counting. Triplicate counts were averaged, and the percentage of proliferation was calculated from untreated control cells. Alternatively, cell number and viability were determined by trypan blue dye exclusion analysis. For the CMV-E2F-1 proliferation assay, BT-20 cells were transiently transfected with no plasmid (mock), 20 μg pBR322 (Life Technologies, Inc.), or 20 μg of CMV-E2F-1 (57) by electroporation. Cells were then cultured for 24 h in RPMI supplemented with 10% fetal bovine serum. Following culture, 5×10^4 viable cells were placed into 96-well tissue culture plates and treated for 24 h in the presence and absence of VES (10 μg/ml). Cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay per the manufacturer's instructions (Promega Corp., Madison, WI).

Cell Cycle Analysis. BT-20 cells were cultured for 24, 48, and 72 h in the presence and absence of VES (10 μg/ml). Following culture, cells were washed twice with PBS and fixed in 1 ml of ice-cold ethanol for 1 h at 4°C. Fixed cells were pelleted and resuspended in 0.5 ml of PBS containing 30 μg/ml propidium iodide and 1 mg/ml RNase A (Sigma), and analyzed for DNA content by flow cytometry (3).

Plasmids. The basal level of c-*myc* transcription was measured in transient transfection assays using chimeric constructions of the c-*myc* promoter driving the *CAT* reporter gene. Plasmids containing sequences located 5′ upstream to the P1 transcription start site and between the P1 and P2 transcriptional start sites of the human c-*myc* gene were constructed by inserting fragments of the promoter sequence into a pGEM-CAT vector with the *Hin*dII and *Xba*I enzymes (58). The constructions were designated according to the 5′ nucleotide position relative to the c-*myc* P2 start site (+1). Deletion mutants had 3′ flanking c-*myc* gene sequences to position +48. The c-*myc*-promoter-CAT constructions used were M1 (−510 to +48; contains the P1 and P2 transcriptional start sites with their respective TATA boxes), M9 (−79 to +48; contains the P2 transcriptional start site with its respective TATA box and two E2F transcriptional control sites; −65 to −57 and −42 to −34), M9ΔE2F (M9 construction with the E2F sites mutated from GCGGGAAA and GATCGCGC to ataGGAAA and GATCGatc, respectively; mutation sites are shown as lowercase), M10 (−45 to +48), and M10ΔE2F (M10 construction with the E2F site mutated from GATCGCGC to GATCGatc; mutation sites are lowercase). As a control, c-*jun* transcription was measured in transient transfection assays using a chimeric construction of the c-*jun* promoter (position −132 to +170; containing a TATA box, and SP1, CTF, and AP1 transcriptional control sites) driving the *CAT* reporter gene (59). Full-length GAL4-E2F-1 (a generous gift of William G. Kaelin; Ref. 57) and GAL4-E2F-1 deletion mutants (M1, deletion of amino acids 1–100; and M2, deletion of amino acids 1–100 and 401–437) were constructed by inserting a PCR-generated fragment that contains murine E2F-1 sequences into the *Sal*I and *Xba*I sites in the polylinker pGEX-2TK (57). The GAL4 construction encodes 437 amino acids of the E2F-1 protein fused to the DNA-binding domain of GAL4 (amino acids 1–147). The control construction, pSG-147, encodes only the GAL4 DNA-binding domain. The reporter construction, G5B, encodes five GAL4 DNA binding sites upstream of the adenovirus E1b TATA box driving CAT and has been described previously (60). The E2F-4 cDNA was a gift of Robert A. Weinberg (19). GAL4-E2F-4 was constructed as described above for GAL4-E2F-1.

CAT Assays. BT-20 cells were transfected with the M1, M9, M9ΔE2F, M10, or M10ΔE2F human c-*myc* promoter plasmids, the human c-*jun* control plasmid, or the GAL4-E2F plasmids (full-length GAL4-E2F-4 or GAL4-E2F-1 or the M1 or M2 GAL4-E2F-1 deletion mutants) by electroporation. In the GAL4-E2F experiments, control cells were transfected with the pSG-147 control plasmid and/or the G5B reporter plasmid. Cells were then treated with medium (untreated), vehicle, or VES (10 μg/ml) and tested for CAT activity as described previously (61). Cells were then treated with VES and were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2. Following incubation, cells were washed two times in PBS, and extracts were prepared by repeated freeze-thaw. Protein concentrations were determined using the Bio-Rad protein assay, and equal amounts of protein were used to measure for the CAT enzyme. CAT enzyme activity was determined according to the method of Gorman *et al.* (62) and normalized for transfection efficiency by cotransfection with the human growth hormone expression plasmid, PSV6H. Secreted growth hormone in the medium was measured by RIA (Nichols Institute, San Juan Capistrano, CA). The percentage of conversion was determined by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

Metabolic Labeling. BT-20 cells were treated with medium (untreated), vehicle, or VES (10 μg/ml) for 24 or 48 h and then pelleted by centrifugation. An equal number of cells per treatment were washed twice in phosphate-free RPMI (Life Technologies, Inc.) and starved for 20 min in phosphate-free medium at 37°C in a humidified atmosphere of 5% CO_2. Cells were then pelleted by centrifugation, resuspended in 1 ml phosphate-free media containing 2.5% fetal bovine serum that had been dialyzed against phosphate-free RPMI (Sigma) and the respective treatment, transferred into a 24-well tissue culture plate (Costar), and labeled for 2 h at 37°C with 200 μCi of [^{32}P]P_i (3000 Ci/mmol; New England Nuclear). Following culture, labeled cells were washed three times in PBS and lysed for 30 min on ice in RIPA lysis buffer (0.15 M NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% SDS, and 50 mM Tris, pH 7.4) containing phosphatase inhibitors (1 mM sodium fluoride and 1 mM sodium orthovanadate) and proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 mM DTT). Cellular debris was removed by centrifugation (10 min at 15,000 rpm), and lysates were incubated with antibodies to normal mIg or E2F-1 and immunoprecipitated as described below. Following immunoprecipitation, samples were analyzed by SDS-10% PAGE and autoradiography. Quantification of autoradiographed bands was determined by densitometric analysis (Molecular Dynamics).

Immunoprecipitations. Five hundred μg of protein from total cellular lysates were precleared with protein G-plus agarose and then incubated overnight at 4°C with mouse anti-human E2F-1, mouse anti-human cyclin A, rabbit anti-human cyclin E, rabbit anti-human Cdk-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or normal mIg (Sigma). Antibody-protein complexes were immunoprecipitated with protein G-plus agarose (Santa Cruz Biotechnology, Inc.) for 1 h at 4°C, and protein G-agarose beads were washed three times in RIPA lysis buffer and then boiled for 5 min in sample buffer. Immunoprecipitated proteins were either analyzed by the kinase assay or resolved by SDS-PAGE. Radiolabeled proteins were visualized by autoradiography, and protein-protein interactions were determined by subsequent Western analysis as described below. Anti-E2F-1 immunoblots were probed with rabbit anti-human cyclin A (Upstate Biotechnology, Inc., Lake Placid, NY), and anti-Cyclin A immunoblots were probed with rabbit anti-human Cdk-2 (Upstate Biotechnology, Inc.) or rabbit anti-human p21 (Santa Cruz Biotechnology, Inc.).

Kinase Assay. Following immunoprecipitation, samples were washed three times in RIPA lysis buffer, once in kinase buffer [20 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 1 mM DTT], and resuspended in 20 μl of kinase buffer containing 3.75 μM ATP. Three μg of histone 1 and 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; New England Nuclear) were added to each reaction, and samples were incubated for 30 min at 37°C. The kinase reaction was terminated by the addition of sample buffer, and samples were loaded onto a 12% SDS-polyacrylamide gel. The gel was washed overnight with 1% sodium PP_i/5% trichloroacetic acid to remove free ^{32}P, dried, and analyzed by autoradiography. Quantification of autoradiographed bands was determined by densitometric analysis.

Western Analysis. Proteins from total cell lysates (200 μg) or from immunoprecipitations were transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH) by electroblotting, and membranes were blocked for 1 h in Tris-buffered saline (TBST; 0.15 M NaCl, 25 mM Tris, and 0.5% Triton X-100, pH 7.4) containing 5% nonfat dry milk (blocking buffer). Membranes were incubated with primary antibodies (mouse anti-human c-*myc*,

mouse anti-human E2F-1, and mouse anti-human cyclin A were from Santa Cruz Biotechnology, Inc.; rabbit anti-human cdk-2 was from Upstate Biotechnology, Inc., mouse anti-human p21 was from Transduction Laboratories (Lexington, KY), and mouse anti-human β-actin was from Sigma diluted in blocking buffer for 1 h, washed three times with TBST, incubated with biotin-labeled secondary antibodies (Kirkegaard and Perry Labs, Gaithersburg, MD), diluted 1:5000 in blocking buffer for 1 h, washed three times with TBST, incubated with peroxidase-labeled streptavidin tertiary antibodies (Kirkegaard and Perry Labs), diluted 1:5000 in blocking buffer for 20 min, washed six times with TBST, and developed using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL). All reactions were performed at room temperature on a rocking platform. Quantification of protein levels was determined by densitometric analysis.

RNase Protection Assay. Total RNA was isolated from 1×10^7 untreated, vehicle-treated, and VES-treated (10 μg/ml) BT-20 cells by the Totally RNA isolation kit, per the manufacturer's instructions (Ambion, Austin, TX). A linearized 251-bp cDNA fragment spanning exons 2 and 3 of the human c-*myc* proto-oncogene and a linearized 127-bp cDNA fragment spanning exons 3–4 of the human β-actin gene were used as probe templates (Ambion; Refs. 63 and 64). The c-*myc* and *β-actin* cDNA templates were transcribed to RNA using an SP6 transcription kit in the presence of [^{32}P]UTP (800 Ci/mmol; New England Nuclear), per the manufacturer's instructions (Ambion). The ^{32}P-labeled human c-*myc* and human *β-actin* RNA probes were hybridized overnight at 52°C to 30 μg of RNA using the RPA II kit, per the manufacturer's instructions (Ambion). Following incubation, samples were RNase digested for 30 min at 37°C and then precipitated in ethanol. Nondigested, double-stranded (hybridized) RNA was analyzed by 6% TBE-urea (89 μM Tris base, 89 μM boric acid, 2 μM EDTA, and 7 μM urea) acrylamide gel electrophoresis and autoradiography. Quantification of autoradiographed bands was determined by densitometric analysis. c-*myc* levels were normalized to the respective *β-actin* controls.

Statistical Analysis. The cell proliferation data were converted to logarithms to achieve variance stabilization and analyzed by ANOVA followed by *t* test and/or Duncan's multiple comparison test.

RESULTS

VES Inhibits the Proliferation of BT-20 Cells. VES significantly inhibited the growth of estrogen receptor-negative BT-20 human breast cancer cells in a dose-dependent manner while maintaining greater than 90% viability. Tritiated thymidine incorporation studies showed VES at 10 μg/ml to optimally inhibit the proliferation of BT-20 cells by 42 and 82%, following 24 and 48 h of culture, respectively (Fig. 1*A*). The appropriate vehicle control (0.1% ethanol and 5 μg/ml succinic acid), as well as fat-soluble antioxidants butylated hydroxyanisol and butylated hydroxytoluene and water-soluble antioxidant *N*-acetyl cysteine, did not affect BT-20 cell proliferation (data not shown). This suggests that VES possesses nonantioxidant-related growth-inhibitory properties. Enumeration of cell number in VES-treated BT-20 cell cultures showed decreases in cell proliferation similar to those obtained with the tritiated thymidine incorporation assay (Fig. 1*B*). Cell cycle analysis of BT-20 cells showed VES to induce G_1 cell cycle arrest following 24, 48, and 72 h of culture (Table 1).

Overexpression of E2F-1 Blocks VES-induced Growth Inhibition of BT-20 Cells. E2F activity is tightly regulated and known to be inhibited in G_1 as an early response to growth-inhibitory signals (17, 18). Because VES treatment inhibited BT-20 cell growth in G_1, we determined whether VES functioned through the E2F pathway. To demonstrate a role for E2F-1 in BT-20 cell proliferation, BT-20 cells were transiently transfected with CMV-E2F-1, pBR322 (control plasmid), or no plasmid (mock). VES significantly ($P < 0.0001$) inhibited the growth of mock- and pBR322-transfected cells by 59 and 50%, respectively (Fig. 2). CMV-E2F-1-transfected cells were found to possess slightly increased proliferative capacity (7–10%; Fig. 2). VES treatment of CMV-E2F-1-transfected cells did not significantly inhibit cell growth (16%; Fig. 2). In addition, we used an E2F-binding element decoy [described recently by Morishita *et al.* (21)] to further implicate a role for E2F in VES-induced growth arrest of BT-20 cells. The presence of a wild-type but not mutant E2F decoy prevented growth arrest of BT-20 cells by VES treatment (data not shown). These results provide evidence that in BT-20 cells, VES regulation of E2F-1 activity can lead to growth arrest.

Table 1 *VES induces a G_1 shift in BT-20 cells*

Treatment	G_1	G_2	G_2-M
Untreated	45%	30%	25%
VES 24 h	61%	22%	17%
VES 48 h	66%	16%	18%
VES 72 h	75%	9%	16%

BT-20 cells were untreated or VES-treated (10 μg/ml) for 24, 48, or 72 hours. Following culture, cell cycle analysis was performed by flow cytometry. The data are from a representative experiment ($n = 3$).

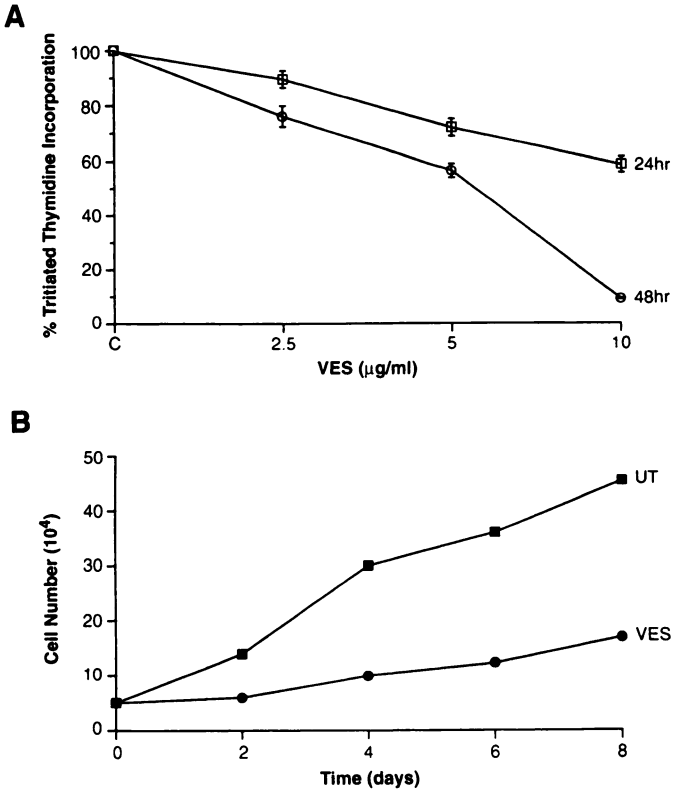

Fig. 1. VES inhibits proliferation of BT-20 cells. *A*, tritiated thymidine incorporation study of cells cultured for 24 or 48 h in the presence or absence of increasing concentrations of VES. The data are expressed as the percentage of maximal proliferation from a representative experiment ($n = 3$) as compared to untreated control cells. ANOVA and regression analysis showed VES to inhibit cell growth in a dose-dependent (linear) fashion ($P < 0.001$). The Duncan's multiple comparison test showed 10 μg of VES to significantly inhibit cell proliferation ($P < 0.01$). *Bars*, SE. *B*, growth of BT-20 cells in the presence of medium (*UT*, untreated) or VES (10 μg/ml) for 2, 4, 6, and 8 days. The data are the cell number $\times 10^4$ from a single representative experiment ($n = 3$).

VES Regulates the E2F-1 Protein. Changes in the level of protein expression and phosphorylation can alter the functional activity of transcription factors. To study the regulation of E2F-1 steady-state protein and phosphorylation levels by VES, Western analysis and *in vivo* phosphorylation studies were performed. VES reduced E2F-1 protein levels 30% following 24 h of culture (Fig. 3*A*). *In vivo* ^{32}P cell labeling, followed by immunoprecipitation, with E2F-1 antibodies indicated an 82% decrease in E2F-1 phosphorylation following 24 h of VES treatment (Fig. 3*B*). Control immunoprecipitation with normal mIg demonstrated the specificity of the E2F-1 protein immunoprecipitation and inhibition of phosphorylation by VES (Fig. 3*B*). Results

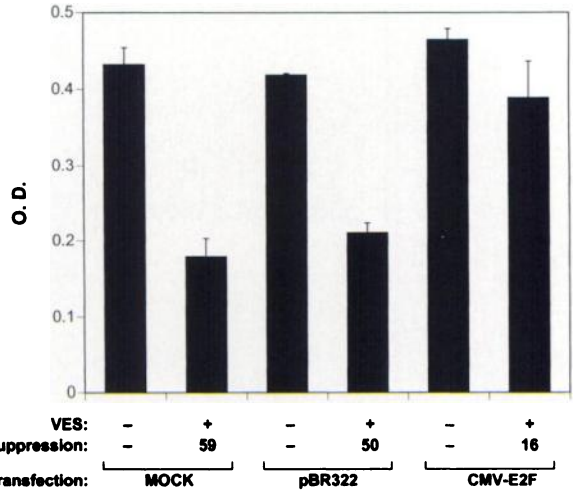

Fig. 2. Overexpression of E2F-1 blocks VES-induced growth suppression of BT-20 cells. BT-20 cells were transiently transfected with no plasmid (*MOCK*), pBR322 (control plasmid), or CMV-E2F-1 and treated in the presence and absence of VES (10 μg/ml) for 24 h. Following culture, proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are the mean absorbance (*O.D.*) from a representative experiment (n = 4); *bars*, SE. ANOVA followed by t test showed VES to significantly inhibit the proliferation of mock- and pBR322-transfected cells ($P < 0.0001$) but not CMV-E2F-1-transfected cells ($P = 0.052$). The VES-induced growth suppression of CMV-E2F-1-transfected cells was found to be significantly different from that of mock- and pBR322-transfected cells ($P < 0.01$).

obtained from vehicle-treated cells were similar to those of untreated cells, and results at 48 h were similar to those at 24 h (data not shown).

VES Inhibits GAL4-E2F-1 Transactivation Activity. To more directly characterize the effect of VES on E2F-1 protein activity, we investigated the ability of VES to regulate the functional activity of the GAL4-E2F-1 fusion protein using transient transfection assays. The constructions used were GAL4-E2F containing full-length E2F-1, E2F-4, or deleted E2F-1 (Fig. 4*A*) and a G5B-CAT reporter. VES treatment inhibited GAL4-E2F-1 transactivation activity 81% (Fig. 4*B*). Deletion of amino acids 1–100 (E2F-1 M1), a region containing the cyclin A-binding site (19, 31), resulted in high levels of CAT activity (80% acetylation compared to 25% acetylation of full-length GAL4-E2F-1), which were not significantly regulated by VES treatment (Fig. 4*B*). Deletion of a region containing the cyclin A-binding domain (amino acids 1–100) and a region containing the pocket protein-binding domain (amino acids 401–437; E2F-1 M2; Ref. 19), almost completely eliminated CAT activity (<2% acetylation; Fig. 4*B*). In addition, VES had little effect on the transcriptional activity of GAL4-E2F-4, which lacks a cyclin A-binding site (Fig. 4*B*). The CAT activity of control cells transfected with pSG-147 (encodes the GAL4 DNA-binding domain only) was less than 1% (data not shown). These results suggest that: (*a*) the cyclin A-binding site in E2F-1 confers a negative regulatory effect on E2F-1 transactivation activity; (*b*) negative regulation of E2F-1 activity by VES requires the region containing the cyclin A-binding site; and (*c*) although the presence of a pocket protein is required for optimal E2F-1 activity, VES-mediated regulation of E2F-1 does not occur through the pocket protein region.

VES Regulates Cyclin A Function. Because the decrease in GAL4-E2F-1 activity suggested that VES signals the inhibition of E2F-1 transactivation activity via cyclin A and because BT-20 cells are known to overexpress cyclin A (45), the effect of VES on cyclin A and the cyclin A regulatory proteins, cdk-2 and $p21^{cip1}$, was

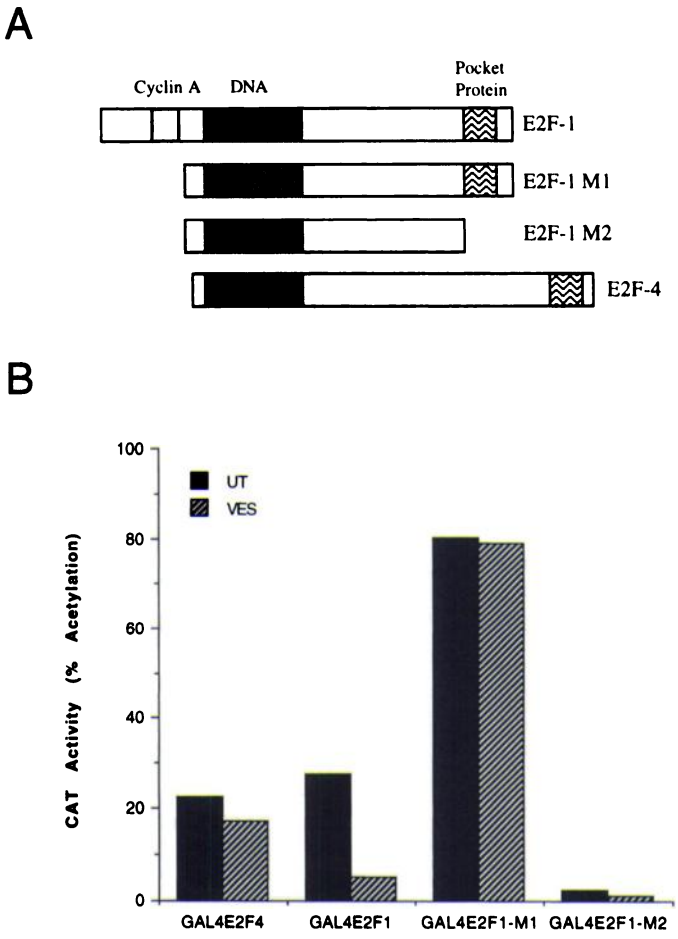

Fig. 4. VES inhibits GAL4-E2F transactivation activity. *A*, diagrammatic representation of the full-length E2F-1 and E2F-4 proteins and two deletion mutants, M1 (deletion of amino acids 1–100) and M2 (deletion of amino acids 1–100 and 401–437), of E2F-1. The cyclin A, DNA, and pocket protein (RB, p107, or p130) binding domains of the proteins are indicated. *B*, GAL4-E2F CAT assay. BT-20 cells were transiently transfected with the GAL4-E2F constructions (full-length GAL4-E2F-4 or GAL4-E2F-1 or the GAL4-E2F-1 deletion mutants M1 or M2) and the G5B reporter plasmid. Cells were treated with medium (*UT*, untreated) or VES. CAT activity was measured 24 h later, and the percentage of acetylation was determined by PhosphorImager analysis. The values were normalized as described in "Materials and Methods." The data are from a representative experiment (n = 3). The SE of the separate experiments was <8%.

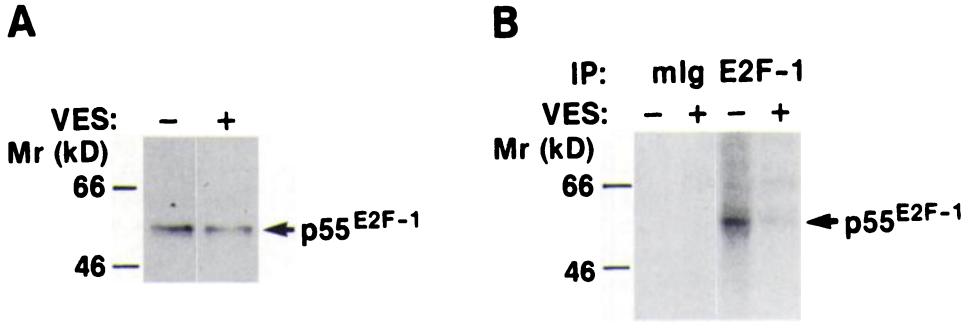

Fig. 3. Effect of VES on E2F-1 protein expression levels and phosphorylation in BT-20 cells. *A*, Western analysis of the E2F-1 protein from total cell lysates of 24-h untreated and VES-treated cells. *B*, immunoprecipitation (*IP*) of *in vivo* ^{32}P-labeled E2F-1 proteins from 24-h untreated and VES-treated cells using specific antibodies to the E2F-1 protein or normal mIg. The data are from a representative experiment (n = 3).

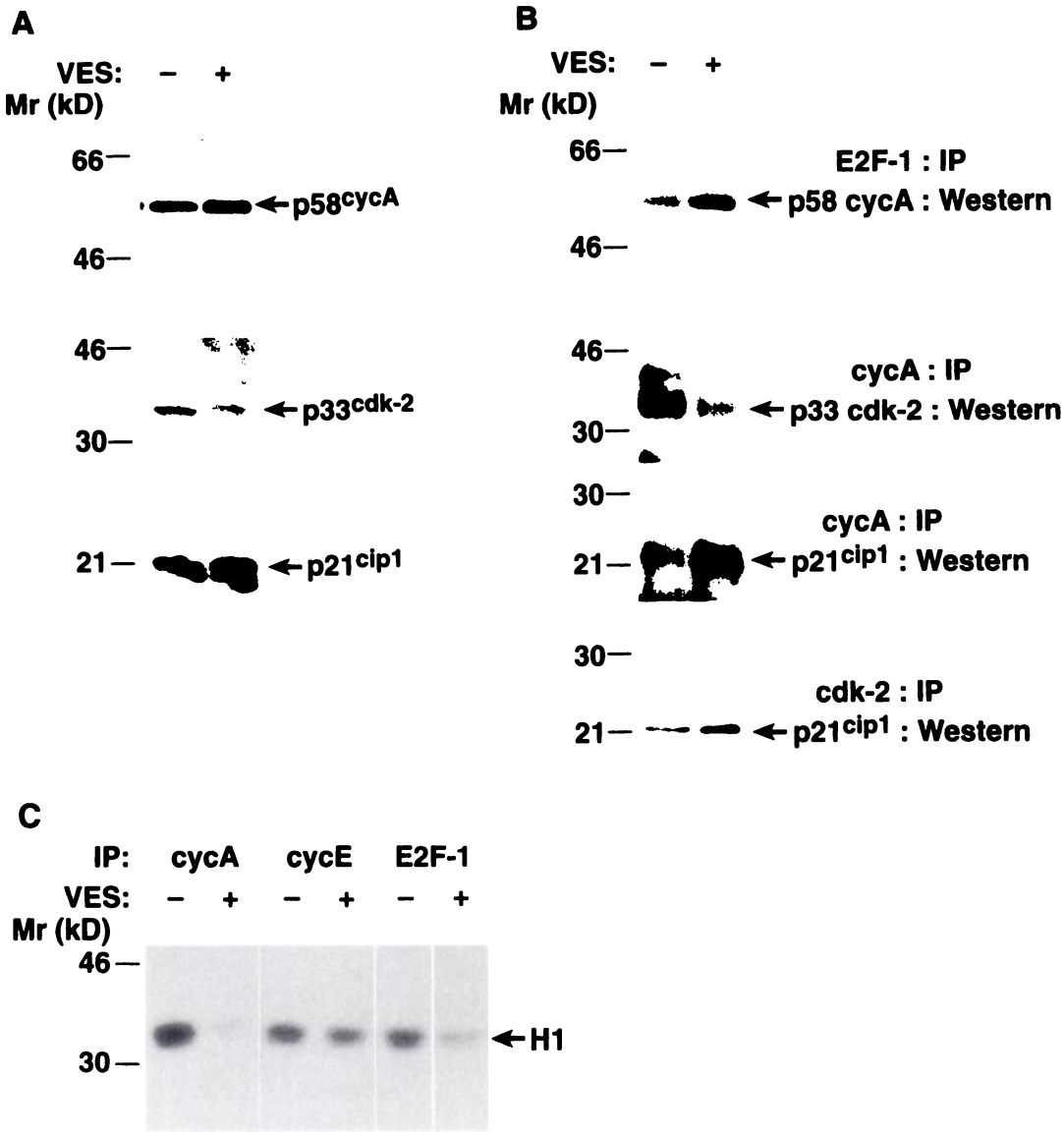

Fig. 5. Effect of VES on the cyclin A, cdk-2, and p21 proteins in BT-20 cells. *A*, Western analysis of cyclin A, cdk-2, and p21 proteins in untreated and VES-treated (10 μg/ml) BT-20 cells. *B*, immunoprecipitation (*IP*) and Western analysis of cyclin A- and cdk-2-associated proteins in untreated and VES-treated BT-20 cells. Anti-E2F-1 immunoprecipitates were probed with cyclin A antibodies, anti-cyclin A immunoprecipitates were probed with cdk-2 or $p21^{cip1}$ antibodies, and anti-cdk-2 immunoprecipitates were probed with $p21^{cip1}$ antibodies. *C, in vitro* histone 1 (*H1*) kinase assay to determine the effect of VES on cyclin A, cyclin E (control), and E2F-1-associated kinase activity in BT-20 cells. The data are from a representative experiment (n = 2).

studied. Western analysis was performed to determine the steady-state protein levels of cyclin A, cdk-2, and $p21^{cip1}$ in exponentially growing and VES-treated BT-20 cells. VES treatment had no effect on steady-state cyclin A protein levels, decreased cdk-2 protein levels 30%, and increased $p21^{cip1}$ protein levels 68% (Fig. 5*A*).

E2F-1 and cyclin A-associated kinase activity has been reported to be regulated by protein-protein interactions during cell cycle progression (31, 32, 34, 37). Immunoprecipitation with E2F-1 followed by anti-cyclin A Western blotting showed increased association of cyclin A with E2F-1 (57%) in VES-treated BT-20 cells as compared to untreated BT-20 cells (Fig. 5*B*). Because cyclin A kinase activity is increased by the association of cyclin A with cdk-2 and is inhibited by the binding of $p21^{cip1}$ to the components of the complex, the effect of VES on cyclin A protein complexes was investigated. Immunoprecipitation with cyclin A, followed by anti-cdk-2 or anti-$p21^{cip1}$ Western blotting, showed a 76% decrease in the binding of cdk-2 to cyclin A and an 81% increase in the binding of $p21^{cip1}$ to cyclin A in VES-treated BT-20 cells, as compared to untreated BT-20 cells (Fig. 5*B*). Immunoprecipitation with cdk-2, followed by anti-$p21^{cip1}$ Western blotting, showed a 40% increase in the binding of $p21^{cip1}$ to cdk-2 in VES-treated cells as compared to untreated cells.

Histone 1 kinase assays were performed with anti-cyclin A, anti-cyclin E, and anti-E2F-1 immunoprecipitates to determine the associated kinase activity of these proteins in VES-treated cells. Cyclin A:cdk-2 is known to phosphorylate and regulate the activity of many proteins including E2F-1 (65–67). VES decreased cyclin A-associated kinase activity (*i.e.*, cyclin A:cdk-2) 98%, while having little or no effect on cyclin E-associated kinase activity (*i.e.*, cyclin E:cdk-2; Fig. 5*C*). In addition, the kinase activity of E2F-1-associated proteins was decreased 70% following VES treatment (Fig. 5*C*). Results obtained from vehicle-treated cells were similar to those of untreated cells, and results following 24 h of culture were similar to those obtained following 48 h of culture (data not shown).

VES Inhibits c-*myc* Transcription, mRNA, and Protein Levels in BT-20 Cells through an E2F-dependent Pathway. E2F-1 is known to positively transactivate the expression of many genes in-

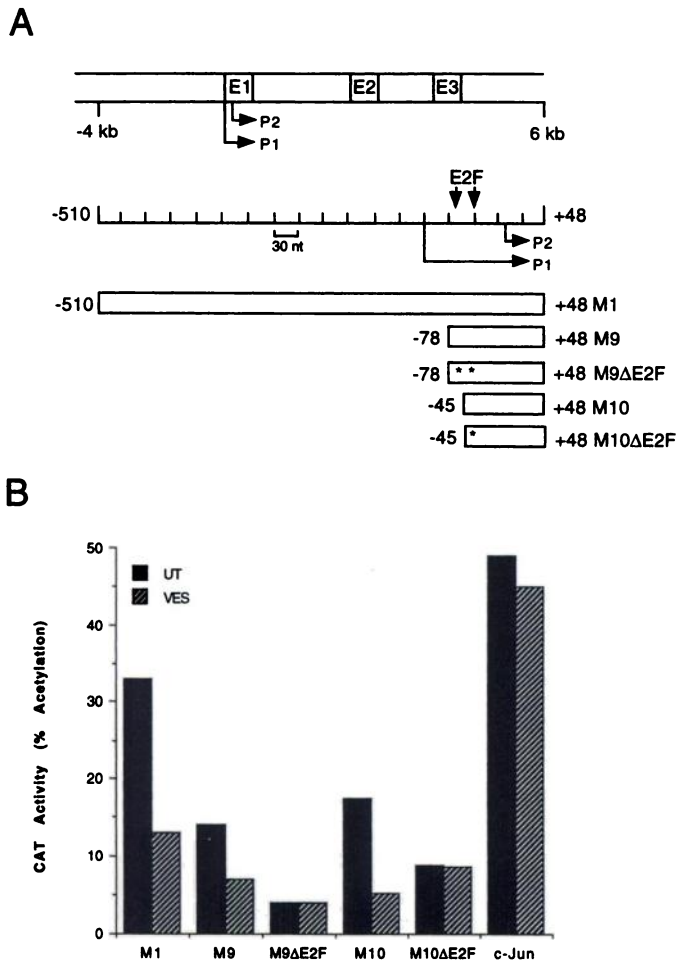

Fig. 6. VES inhibits c-*myc* transcription in BT-20 cells. *A*, diagrammatic representation of chimeric constructions of the c-*myc* promoter driving the *CAT* reporter gene. The P1 and P2 transcriptional start sites and the E2F transcriptional control sites are indicated. *B*, transient transfection CAT assay. BT-20 cells were transfected with the M1, M9, M9ΔE2F, M10, M10ΔE2F, or c-*jun* CAT plasmids by electroporation, treated with medium (*UT*, untreated) or 10 μg/ml VES for 48 h, and tested for CAT activity. The percentage of acetylation was determined by PhosphorImager analysis. The values were normalized as described in "Materials and Methods." The data are from a representative experiment (n = 3). The SE of the separate experiments was <8%.

cluding c-*myc* (38, 48, 50). To study the functional relevance of VES regulation of E2F-1 activity, we examined the effect of VES on c-*myc* transcription using c-*myc* promoter-CAT constructions (Fig. 6*A*; described in "Materials and Methods"). Transient transfection assays showed the high basal level of c-*myc* transcription in BT-20 cells (Fig. 6*B*), which was unaffected by vehicle treatment (data not shown). VES inhibited the transcription of the M1, M9, and M10 constructions 61, 43, and 70%, respectively, following 48 h of culture [Fig. 6*B*; similar results were observed following 24 h of culture (data not shown)]. VES decreased c-*myc*-CAT activity whether there were one or two E2F regulatory sites present. The M9 construction contains a known inhibitory element (68), which could explain why the level of M9-CAT activity is lower than that for the M10 construction. VES had no effect on the transcription of the M9ΔE2F and M10ΔE2F constructions (Fig. 6*B*). In addition, VES did not affect transcriptional expression of a human c-*jun*-promoter-CAT construction, which lacks E2F binding sites (Fig. 6*B*). These results indicate that VES treatment of BT-20 cells results in inhibition of E2F-mediated c-*myc* promoter expression. Because VES negatively regulated c-*myc* transcription through an E2F-dependent mechanism, we studied the effect of VES on c-*myc* steady-state mRNA and protein levels. VES decreased the levels of c-*myc* mRNA transcripts, as determined by RNase protection assay. Kinetic analysis showed VES to markedly decrease c-*myc* mRNA levels following 4 h of culture (Fig. 7*A*). c-*myc* mRNA levels remained suppressed at least through 48 h of culture (Fig. 7*A*). Western analysis indicated that VES also decreased c-*Myc* protein levels 88% following 24 of culture (Fig. 7*B*). c-*myc* protein levels remained suppressed at least through 48 h of culture (Fig. 7*B*). Vehicle treatment had no effect on basal c-*myc* expression levels (data not shown). Together, these results show a correlation between VES-mediated inhibition of E2F-driven c-*myc*-promoter-CAT expression, down-regulation of c-*myc* mRNA and protein levels, and cyclin A-negative regulation of E2F activity in VES growth-suppressed BT-20 cells.

DISCUSSION

BT-20 cells were growth suppressed and arrested in G_1 following VES treatment. These events correlated with VES-mediated negative regulation of E2F-1 transcriptional activity and were overcome by ectopic expression of E2F-1. During growth inhibition of BT-20 cells, VES but not the appropriate vehicle control (0.1% ethanol and 5 μg/ml succinic acid) was found to: (*a*) increase the levels of cyclin A bound to E2F-1; and (*b*) inhibit E2F-1 transactivation activity through the amino terminal region of E2F, which contains the cyclin A-bind-

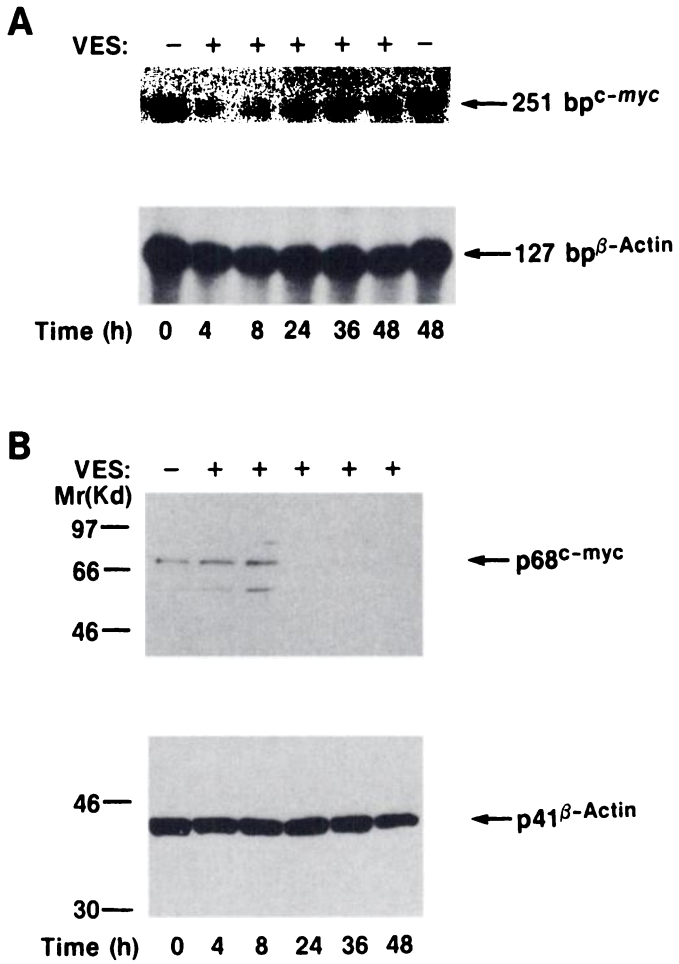

Fig. 7. VES decreases c-*myc* mRNA and protein levels in BT-20 cells. *A*, RNase protection assay for c-*myc* and β-*actin* (control) mRNA in 0, 4, 8, 24, 36, and 48 h VES-treated (10 μg/ml) and untreated BT-20 cells. *B*, Western analysis of c-Myc (*upper panel*) and β-Actin (control; *lower panel*) in 0 (untreated), 4, 8, 24, 36, and 48 h VES-treated (10 μg/ml) BT-20 cells. The data are from a representative experiment (n =2).

ing domain. VES negatively regulated cyclin A-associated kinase activity as a result of increased cyclin A:$p21^{cip1}$ and cdk-2:$p21^{cip1}$ binding and decreased cyclin A:cdk-2 binding. Our studies showed that expression of an E2F-responsive gene, c-*myc,* was decreased in response to VES treatment. Therefore, the VES growth-inhibitory effect on BT-20 cells may occur, at least in part, through cyclin A-negative regulation of E2F-1-mediated transcription.

The cyclin A protein has been shown to be expressed and to function as a G1 cyclin (40, 41). This could be the case in BT-20 cells, which overexpress cyclin A (45), particularly in VES-treated cells that growth arrest in G_1. In the G_1-S transition, phosphorylation of RB inactivates RB to liberate active E2F (41, 69). In S-phase progression, the complex between cyclin A, cdk-2, and E2F-1 negatively regulates E2F function (31). Cyclin A:cdk-2 is known to phosphorylate RB (41, 69) and E2F-1 (65–67). The functional consequences of E2F-1 phosphorylation, however, are controversial. Fagan *et al.* (26) recently demonstrated that phosphorylation of E2F-1 prevents RB:E2F binding and, therefore, maintains E2F-1 in a transcriptionally active state. On the other hand, others have demonstrated that in S phase following E2F-mediated transactivation events, the phosphorylation of E2F-1 (65–67) and DP-1 (31) by cyclin A:cdk-2 leads to decreased E2F-1:DP-1 DNA binding. It is possible that the phosphorylation and activation state of E2F-1 or DP-1 are dependent on cell cycle status, tissue type, or altered in neoplastic cells. Our data indicate that in VES-treated G_1 growth-arrested BT-20 cells, inhibition of E2F-1 transactivation activity correlates with reduced cyclin A-associated kinase activity and reduced E2F-1 protein phosphorylation.

Analysis of protein-protein interactions in VES-treated G_1 arrested BT-20 cells demonstrated an increase in cyclin A:E2F binding and a decrease in cyclin A:cdk-2 binding. As a result, the cyclin A and E2F complexes had low levels of associated kinase activity, whereas the kinase activity of cyclin E was unaffected by VES treatment. These data suggest that cyclin A binds to and negatively regulates E2F in the absence of cdk-2. In support of this, previous studies using *in vitro*-translated cyclin A was shown to bind GST-E2F-1 in the absence of cdk-2 (31, 34). Because RB has been shown to negatively regulate E2F-1 function (24–26), we investigated the presence of RB in E2F protein complexes. Gel shift assays (using several different antibodies to RB or the RB-related proteins p107 and p130) suggested that under the conditions used, RB or RB-related proteins were not associated with cyclin A:E2F (data not shown) in BT-20 cells. Furthermore, our GAL4-E2F studies suggest that the RB-binding site (pocket protein domain) in E2F-1 is required for optimal transactivation activity but not for VES-mediated regulation of E2F-1 activity. Why was RB not seen in association with cyclin A:E2F-1 in VES-treated cells? Other than technical reasons, perhaps cdk-2 is required in the complex to recruit RB. Alternatively, the overexpression of cyclin A in BT-20 cells (45) may dysregulate RB function through several mechanisms.

The mechanism by which VES treatment results in the inactivation of cyclin A-associated kinase activity in BT-20 cells involved the regulation of the $p21^{cip1}$ inhibitory protein. The subunit stoichiometry of cyclin A:cdk-2:$p21^{cip1}$ complexes varies to dictate the kinase activity of the complex. Increasing numbers of $p21^{cip1}$ molecules associated with either cyclin A alone and cdk-2 alone or with the complex cyclin A:cdk-2 gives rise to inactive protein complexes (37). In VES-treated BT-20 cells, the cyclin A protein showed decreased association with cdk-2 and increased association with $p21^{cip1}$, as compared to untreated cells. In proliferating rat liver cells, cyclin A protein levels have been shown to increase in G_1, whereas cdk-2 protein levels did not increase until late G_1 (70). Thus, VES negative regulation of cyclin A:cdk-2 kinase activity in G_1-arrested BT-20 cells may contribute to the inactivation of the E2F-1 protein and the inhibition of transcription through the E2F element.

The exact mechanism(s) by which VES inhibits tumor cell growth has not been identified. These studies indicate that VES targets the regulation of transcriptional regulatory proteins such as E2F-1 and c-*Myc* similar to other growth suppressors, such as TGF-β and retinoic acid (17, 18). Although the best characterized function of vitamin E is that of a fat-soluble antioxidant (1), these and other studies (3, 4, 8) suggest that antioxidant activity alone cannot explain the antiproliferative activity of VES.

These findings indicate a potential therapeutic role for VES in breast cancer. However, the E2F-1 knock-out mice ($E2F^{-/-}$) have again shown the difficulty in extrapolating *in vitro* established observations to the organismal level (22, 23). It has been found with E2F-$1^{-/-}$ mice that E2F-1 functions to regulate apoptosis and to suppress cell proliferation, whereas most of the *in vitro* overexpression studies support a role for E2F in the stimulation of cell proliferation. Thus, a single gene (like *E2F-1*) can function as a stimulator or inhibitor of cellular functions, depending on either the cellular environment or intrinsic differences related to its concentration and/or the concentration of its cofactors. If VES can be used *in vivo,* it has advantages over other fat-soluble vitamin tumor inhibitors because VES is relatively nontoxic (71). VES, through its growth-inhibitory properties, may function as a cancer therapeutic agent, and through its potential antioxidant property, may protect against tumor development (72, 73). Clearly, more studies are needed to determine the effects of VES *in vivo* and its mechanisms of action in normal and neoplastic cells.

ACKNOWLEDGMENTS

We thank Charles Riggs for performing the statistical analysis, Louise Finch for performing the cell cycle analysis, and Rick Gontarek for assistance with the RNase protection assays.

REFERENCES

1. Niki, E., Yamamoto, Y., Komour, M., and Miyana, Y. Inhibition of oxidation of biomembranes by tocopherol. *In:* A. T. Diplock, L. J. Machlin, L. Packer, and W. A. Pryor (eds.), Vitamin E: Biochemistry and Health Implications, pp. 23–25. New York: Annals of the New York Academy of Science, 1989.
2. Prasad, K. N., and Edwards-Prasad, J. Vitamin E and cancer prevention: recent advances and future potentials. J. Am. Coll. Nutr., *11:* 487–500, 1992.
3. Turley, J. M., Sanders, B. G., and Kline, K. RRR-α-tocopheryl succinate modulation of human promyelocytic leukemia (HL-60) cell proliferation and differentiation. Nutr. Cancer, *18:* 201–213, 1992.
4. Turley, J. M., Funakoshi, S., Ruscetti, F. W., Kasper, J., Murphy, W. J., Longo, D. L., and Birchenall-Roberts, M. C. Growth inhibition and apoptosis of RL human B lymphoma cells by vitamin E succinate and retinoic acid: role for transforming growth factor β. Cell Growth & Differ., *6:* 655–663, 1995.
5. Charpentier, A., Groves, S., Simmons-Menchaca, M., Turley, J., Zhao, B., Sanders, B. G., and Kline, K. RRR-α-tocopheryl succinate inhibits proliferation and enhances secretion of transforming growth factor-β (TGF-β) by human breast cancer cells. Nutr. Cancer, *19:* 225–239, 1993.
6. Slack, R., and Proulx, P. Studies on the effects of vitamin E on neuroblastoma NIE 115 cells. Nutr. Cancer, *12:* 75–82, 1989.
7. Prasad, K. N., and Edwards-Prasad, J. Effects of tocopherol (vitamin E) acid succinate on morphological alterations and growth inhibition in melanoma cells in culture. Cancer Res., *42:* 550–555, 1982.
8. Fariss, M. W., Fortuna, M. B., Everett, C. K., Smith, J. D., Trent, D. F., and Djuric, Z. The selective antiproliferative effects of α-tocopheryl hemisuccinate and cholesteryl hemisuccinate on murine leukemia cells results from the action of the intact compounds. Cancer Res., *54:* 3346–3351, 1994.
9. Prasad, K. N., Cohrs, R. J., and Sharma, O. K. Decreased expression of c-*myc* and H-*ras* oncogenes in vitamin E succinate induced morphologically differentiated murine B-16 melanoma cells in culture. Biochem. Cell Biol., *68:* 1250–1255, 1990.
10. Cohrs, R. J., Torelli, S., Prasad, K. N., Edwards-Prasad, J., and Sharma, O. K. Effect of vitamin E succinate and a cAMP stimulating agent on the expression of c-myc, N-myc and H-ras in murine neuroblastoma cells. Int. J. Devl. Neurosci., *9:* 187–194, 1991.
11. Kelloff, G. J., Crowell, J. A., Boone, C. W., Steele, V. E., Lubet, R. A., Greenwald, P., Alberts, D. S., Covey, J. M., Doody, L. A., Knapp, G. G., Nayfield, S., Parkinson, D. R., Prasad, V. K., Prorok, P. C., Sausville, E. A., and Sigman, C. C. Clinical development plans for cancer chemopreventive agents. J. Cell. Biochem. Suppl., *20:* 282–294, 1994.
12. Sahu, S. N., Edwards-Prasad, J., and Prasad, K. N. Effect of α-tocopheryl succinate on adenylate cyclase activity in murine neuroblastoma cells in culture. J. Am. Coll. Nutr., *7:* 285–293, 1988.

13. Torelli, S., Masoudi, F., and Prasad, K. N. Effect of tocopheryl succinate activity on cyclic AMP-dependent protein kinase activity in B16 melanoma cells in culture. Cancer Lett., *39:* 129–136, 1988.
14. Boscoboinik, D., Szewezyk, A., Hensey, C., and Azzi, A. Inhibition of cell proliferation by α-tocopherol: role of protein kinase C. J. Biol. Chem., *266:* 6188–6194, 1991.
15. Boscoboinik, D., Szewezyk, A., and Azzi, A. α-Tocopherol (vitamin E) regulates vascular smooth muscle cell proliferation and protein kinase C activity. Arch. Biochem. Biophys., *286:* 264–269, 1991.
16. Chatelain, E., Boscoboinik, D. O., Bartoli, G. M., Kagan, V. E., Gey, F. K., Packer, L., and Azzi, A. Inhibition of smooth muscle cell proliferation and protein kinase C activity by tocopherols and tocotrienols. Biochim. Biophys. Acta, *1176:* 83–89, 1993.
17. Ishida, S., Shudo, K., Takada, S., and Koike, K. Transcription from the P2 promoter of human protooncogene *myc* is suppressed by retinoic acid through an interaction between the E2F element and its binding proteins. Cell Growth & Differ., *5:* 287–294, 1994.
18. Schwarz, J. K., Bassing, C. H., Kovesdi, I., Datto, M. B., Blazing, M., George, S., Wang, X., and Nevins, J. R. Expression of the E2F1 transcription factor overcomes type β transforming growth factor-mediated growth suppression. Proc. Natl. Acad. Sci. USA, *92:* 483–487, 1995.
19. Sardet, C., Vidal, M., Corbrinik, D., Geng, Y., Onufryk, C., Chen, A., and Weinberg, R. A. E2F-4 and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle. Proc. Natl. Acad. Sci. USA, *92:* 2403–2407, 1995.
20. Weinberg, R. A. E2F and cell proliferation: a world turned upside down. Cell, *85:* 457–459, 1996.
21. Morishita, R., Gibbons, G. H., Horiuchi, M., Ellison, K. E., Nakajima, M., Zhang, L., Kaneda, Y., Ogihara, T., and Dzau, V. J. A gene therapy strategy using a transcription factor decoy of the E2F binding site inhibits smooth muscle proliferation *in vivo*. Proc. Natl. Acad. Sci. USA, *92:* 5855–5859, 1995.
22. Field, S. J., Tsai, F., Kuo, F., Zubiaga, A. M., Kaelin, W. G., Jr., Livingston, D. M., Orkin, S. H., and Greenberg, M. E. E2F-1 functions in mice to promote apoptosis and suppress proliferation. Cell, *85:* 549–561, 1996.
23. Yamasaki, L., Jacks, T., Bronson, R., Goillot, E., Harlow, E., and Dyson, N. J. Tumor induction and tissue atrophy in mice lacking E2F-1. Cell, *85:* 537–548, 1996.
24. Weintraub, S. J., Prater, C. A., and Dean, D. C. Retinoblastoma protein switches the E2F site from positive to negative element. Nature (Lond.), *358:* 259–261, 1992.
25. Sherr, C. J. The ins and outs of RB: coupling gene expression to the cell cycle clock. Trends Biochem. Sci., *4:* 15–18, 1994.
26. Fagan, R., Flint, K. J., and Jones, N. Phosphorylation of E2F-1 modulates its interaction with the retinoblastoma gene product and the adenoviral E4 19 kDa protein. Cell, *78*: 799–811, 1994.
27. Chittenden, T., Livingston, D. M., and DeCaprio, J. A. Cell cycle analysis of E2F in primary human T cells reveals novel E2F complexes and biochemically distinct forms of free E2F. Mol. Cell. Biol., *13:* 3975–3983, 1993.
28. Devoto, S. H., Mudryj, M., Pines, J., Hunter, T., and Nevins, J. R. A cyclin A-protein kinase complex possesses sequence-specific DNA binding activity: p33cdk2 is a component of the E2F-cyclin A complex. Cell, *68:* 167–176, 1992.
29. Shirodkar, S., Ewen, M., DeCaprio, J. A., Morgan, J., Livingston, D. M., and Chittenden, T. The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-regulated manner. Cell, *68:* 157–166, 1992.
30. Kato, J., Matsushime, H., Hiebert, S. W., Ewen, M. E., and Sherr, C. J. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev., *7:* 331–342, 1993.
31. Krek, W., Ewen, M. E., Shirodkar, S., Arany, Z., Kaelin, W. G. J., and Livingston, D. M. Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. Cell, *78:* 161–172, 1994.
32. Mudryj, M., Devoto, S. H., Pines, J., and Nevins, J. R. Cell cycle regulation of the E2F transcription factor involves an interaction with cyclin A. Cell, *65:* 1243–1253, 1991.
33. Hinds, P. W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S. I., and Weinberg, R. A. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell, *70:* 993–1006, 1992.
34. Bandara, L. R., Adamczewski, J. P., Zamanian, M., Poon, R. C., Hunt, T., and La Thangue, N. B. Cyclin A recruits $p33^{cdk-2}$ to the cellular transcription factor DRTF1. J. Cell Sci. Suppl., *16:* 77–85, 1992.
35. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. The p21 cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell, *75:* 805–816, 1993.
36. Stein, G. S., Stein, J. L., van Wijnen, A. J., and Lian, J. B. Histone gene transcription: a model for responsiveness to an integrated series of regulatory signals mediating cell cycle control and proliferation/differentiation interrelationships. J. Cell. Biochem., *54:* 393–404, 1994.
37. Zhang, H., Hannon, G. J., and Beach, D. p21-containing cyclin kinases exist in both active and inactive states. Genes Dev., *8:* 1750–1758, 1994.
38. Oswald, F., Lovec, H., Moroy, T., and Lipp, M. E2F-dependent regulation of human MYC: trans-activation by cyclins D1 and A overrides tumour suppressor protein functions. Oncogene, *9:* 2029–2036, 1994.
39. Heichman, K. A., and Roberts, J. M. Rules to replicate by. Cell, *79:* 557–562, 1994.
40. Carbonaro-Hall, D., Williams, R., Wu, L., Warburton, D., Zeichner-David, M., MacDougall, M., Tolo, V., and Hall, F. G1 expression and multistage dynamics of cyclin A in human osteosarcoma cells. Oncogene, *8:* 1649–1659, 1993.
41. Resnitzky, D., Hengst, L., and Reed, S. I. Cyclin-A associated kinase activity is rate limiting for entrance into S phase and is negatively regulated in G_1 by $p27^{Kip\ 1}$. Mol. Cell. Biol., *15:* 4347–4352, 1995.
42. Goubin, F., and Ducommum, B. Identification of binding domains on the $p21^{cip1}$ cyclin-dependent kinase inhibitor. Oncogene, *10:* 2281–2287, 1995.
43. Dimri, G. P., Nakanishi, M., Desprez, P., Smith, J. R., and Campisi, J. Inhibition of E2F activity by the cyclin-dependent protein kinase inhibitor p21 in cells expressing or lacking a functional retinoblastoma protein. Mol. Cell. Biol., *16:* 2987–2997, 1996.
44. Weistat-Saslow, D., Merino, M. J., Manrow, R. E., Lawrence, J. A., Bluth, R. F., Wittenbel, K. D., Simpson, J. F., Page, D. L., and Steeg, P. S. Overexpression of cyclin D mRNA distinguishes invasive and *in situ* breast carcinomas from non-malignant lesions. Nature Med., *1:* 1257–1260, 1995.
45. Keyomarsi, K., and Pardee, A. B. Redundant cyclin overexpression and gene amplification in breast cancer cells. Proc. Natl. Acad. Sci. USA, *90:* 1112–1116, 1993.
46. Haldar, S., Negrini, M., Monne, M., Sabbioni, S., and Croce, C. M. Down-regulation of *bcl-2* by *p53* in breast cancer cells. Cancer Res., *54:* 2095–2097, 1994.
47. Yamamoto, M., Yoshida, M., Ono, K., Fujita, T., Ohtani-Fujita, N., Sakai, T., and Nikaido, T. Effect of tumor suppressors on cell cycle-regulatory genes: RB suppresses $p34^{cdc2}$ expression and normal p53 suppresses cyclin A expression. Exp. Cell Res., *210:* 94–101, 1994.
48. Thalmeier, K., Synovzik, H., Mertz, R., Winnacker, E. L., and Lipp, M. Nuclear factor E2F mediates basic transcription and trans-activation by E1a of the human MYC promoter. Genes Dev., *3:* 527–536, 1989.
49. Nevins, J. R. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science (Washington DC), *258:* 424–429, 1992.
50. Hiebert, S. W., Lipp, M., and Nevins, J. R. E1A-dependent trans-activation of the human *MYC* promoter is mediated by the E2F factor. Proc. Natl. Acad. Sci. USA, *86:* 3594–3598, 1989.
51. Miller, T. L., Huzel, N. J., Davie, J. R., and Murphy, L. C. c-*myc* gene chromatin of estrogen receptor positive and negative breast cancer cells. Mol. Cell. Endocrinol., *91:* 83–89, 1993.
52. Watson, P. H., Pon, R. T., and Shiu, R. P. C. Inhibition of c-myc expression by phosphorothioate antisense oligonucleotide identifies a critical role for c-myc in the growth of human breast cancer. Cancer Res., *51:* 3996–4000, 1991.
53. Ray, R. B., Steele, R., Seftor, E., and Hendrix, M. Human breast carcinoma cells transfected with the gene encoding a c-myc promoter-binding protein (MBP-1) inhibits tumors in nude mice. Cancer Res., *55:* 3747–3751, 1995.
54. Lasfargues, E. Y., and Ozzello, L. Cultivation of human breast carcinomas. J. Natl. Cancer Inst., *21:* 1131–1147, 1958.
55. Hoffeld, J. T. Agents which block membrane lipid peroxidation enhance mouse spleen cell immune activities *in vitro*: relationship to the enhancing activity of 2-mercaptoethanol. Eur. J. Immunol., *11:* 371–376, 1981.
56. Schreck, R., Rieber, P., and Baeuerle, R. A. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kB transcription factor and HIV-1. EMBO J., *10:* 2247–2258, 1991.
57. Kaelin, W. G., Krek, W., Seller, W. R., DeCaprio, J. A., Ajchenbaum, F., Fuchs, C. S., Chittenden, T., Li, Y., Farnham, P. J., Blanar, M. A., Livingston, D. M., and Flemington, E. K. Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell, *70:* 351–364, 1992.
58. Siebenlist, U., Henninghausen, L., Battey, J., and Leder, P. Chromatin structure and protein binding in the putative regulatory region of the *c-myc* gene in Burkitt lymphoma. Cell, *37:* 381–391, 1984.
59. Angel, P., Hattori, K., Smeal, T., and Karin, M. The *jun* proto-oncogene is positively autoregulated by its product, jun/AP-1. Cell, *55:* 875–885, 1988.
60. Kim, S. J., Onwuta, U. S., Lee, Y. I., Li, R., Botchan, M. R., and Robbins, P. D. The retinoblastoma gene product regulates Sp1-mediated transcription. Mol. Cell. Biol., *12:* 2455–2463, 1992.
61. Birchenall-Roberts, M. C., Ruscetti, F. W., Kasper, J., Lee, H., Friedman, R., Geiser, A., Sporn, M. B., Roberts, A. B., and Kim, S. J. Transcriptional regulation of the transforming growth factor B1 promotor by v-*src* gene products is mediated through the AP-1 complex. Mol. Cell. Biol., *10:* 4978–4983, 1990.
62. Gorman, C. M., Moffat, L. F., and Howard, B. H. Recombinant genomes which express chloramphenicol acetyl-transferase. Mol. Cell. Biol., *2:* 1044–1051, 1982.
63. Nakajima-Iijima, S., Hamada, H., Reddy, P., and Kakunaga, T. Molecular structure of the human cytoplasmic β-actin gene: interspecies homology of sequences in the introns. Proc. Natl. Acad. Sci. USA, *82:* 6133–6137, 1985.
64. Gazin, C., Dupont de Dinechin, S., Hampe, A., Masson, J. M., Martin, P., Stehelin, D., and Galibert, F. Nucleotide sequence of the human c-*myc* locus: provocative open reading frame within the first exon. EMBO J., *3:* 383–387, 1984.
65. Kitagawa, M., Higashi, H., Suzuki-Takahashi, I., Segawa, K., Hanks, S. K., Nishimura, S., and Okuyama, A. Phosphorylation of E2F-1 by cyclin A-cdk2. Oncogene, *10:* 229–236, 1995.
66. Xu, M., Sheppard, K., Peng, C., Yee, A. S., and Piwnica-Worms, H. Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. Mol. Cell. Biol., *14:* 8420–8431, 1994.
67. Dynlacht, B. D., Flores, O., Lees, J. A., and Harlow, E. Differential regulation of E2F *trans*-activation by cyclin/cdk2 complexes. Genes Dev., *8:* 1772–1786, 1994.
68. Kerr, L. D., Miller, D. B., and Matrisian, L. M. TGF-β1 inhibition of transin/stromelysin gene expression is mediated through a Fos binding sequence. Cell, *61:* 267–278, 1990.
69. Horton, L. E., Qian, Y., and Templeton, D. J. G1 cyclins control the retinoblastoma gene product growth regulation activity via upstream mechanisms. Cell Growth & Differ., *6:* 395–407, 1995.
70. Castro, A., Jaumot, M., Verges, M., Agell, N., and Bachs, O. Microsomal localization of cyclin A and cdk2 in proliferating rat liver cells. Biochem. Biophys. Res. Commun., *201:* 1072–1078, 1994.
71. Bendich, A., and Machlin, L. J. Safety of oral intake of vitamin E. Am. J. Clin. Nutr., *48:* 612–619, 1988.
72. Knekt, P., Aromaa, A., Maatela, J., Aaran, R., Nikkari, T., Hakama, M., Hakulinen, T., Peto, R., and Teppo, L. Vitamin E and cancer prevention. Am. J. Clin. Nutr., *53:* 283S–286S, 1991.
73. Floyd, R. A. Role of oxygen free radicals in carcinogenesis and brain ischemia. FASEB J., *4:* 2587–2597, 1990.