Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 10436–10440, October 1995
Pharmacology

Lack of functional retinoblastoma protein mediates increased resistance to antimetabolites in human sarcoma cell lines

WEIWEI LI*, JIANGUO FAN*, DANIEL HOCHHAUSER*, DEBABRATA BANERJEE*, ZBIGNIEW ZIELINSKI*, ALEX ALMASAN†, YUXIN YIN†, RUTH KELLY†, GEOFFREY M. WAHL†, AND JOSEPH R. BERTINO*‡

*Program for Molecular Pharmacology and Therapeutics, Memorial Sloan–Kettering Cancer Center, New York, NY 10021; and †Gene Expression Lab, The Salk Institute, San Diego, CA 92186

Communicated by Paul A. Marks, Memorial Sloan–Kettering Cancer Center, New York, NY, July 12, 1995 (received for review November 7, 1994)

ABSTRACT **Growth inhibition assays indicated that the IC_{50} values for methotrexate (MTX) and 5-fluorodeoxyuridine (FdUrd) in HS-18, a liposarcoma cell line lacking retinoblastoma protein (pRB), and SaOS-2, an osteosarcoma cell line with a truncated and nonfunctional pRB, were 10- to 12-fold and 4- to 11-fold higher, respectively, than for the HT-1080 (fibrosarcoma) cell line, which has wild-type pRB. These $Rb^{-/-}$ cell lines exhibited a 2- to 4-fold increase in both dihydrofolate reductase (DHFR) and thymidylate synthase (TS) enzyme activities as well as a 3- to 4-fold increase in mRNA levels for these enzymes compared to the HT-1080 ($Rb^{+/+}$) cells. This increase in expression was not due to amplification of the DHFR and TS genes. Growth inhibition by MTX and FdUrd was increased and DHFR and TS activities and expression were correspondingly decreased in Rb transfectants of SaOS-2 cells. In contrast, there was no significant difference in growth inhibition among these cell lines for the nonantimetabolites VP-16, cisplatin, and doxorubicin. A gel mobility-shift assay showed that parental SaOS-2 cells had increased levels of free E2F compared to the Rb-reconstituted SaOS-2 cells. These results indicate that pRB defective cells may have decreased sensitivity to growth inhibition by target enzymes encoded by genes whose transcription is enhanced by E2F proteins and suggest mechanisms of interaction between cytotoxic agents and genes involved in cell cycle progression.**

A wide variety of human cancers demonstrate loss or mutation in the tumor suppressor gene p53 and the retinoblastoma tumor suppressor gene (*Rb*) (reviewed in refs. 1 and 2). *Rb* encodes a nuclear phosphoprotein that has been implicated in the transition between the G_1 and S phases of the cell cycle and is phosphorylated starting at a point after mid-G_1 (3–7) by cyclin D–cdk4 (8) and/or cyclin E–cdk2 complexes (9, 10).

The viral oncoproteins E1A, simian virus 40 T antigen, and papilloma virus E7 bind to hypophosphorylated Rb protein (pRB) and displace cellular pRB binding proteins such as the E2F family of transcriptional activators (11, 12). Cells lacking pRB or sequences important for its interaction with E2F would therefore be expected to have high levels of "free" E2F, a shortened G_1 phase, and increased transcription of E2F target genes such as dihydrofolate reductase (DHFR) (13, 14), thymidylate synthase (TS) (15), and other genes necessary for entry and progression through S phase. Overexpression of E2F-1, a member of the E2F family, can reverse pRB-mediated G_1 growth arrest and allow entry into S phase (16).

Newly derived human soft tissue sarcoma cell lines (five studied in detail to date) are inherently resistant to methotrexate (MTX), when compared to HT-1080, a well-established fibrosarcoma cell line obtained from ATCC (17). Mechanisms of this inherent resistance are attributed to decreased retention of MTX in four of these cell lines due to decreased polyglutamate formation or impaired uptake (17). The fifth cell line (HS-18), although able to transport MTX and make long-chain polyglutamates comparable to the MTX-sensitive HT-1080 fibrosarcoma cell line, had an increased level of the MTX target enzyme DHFR compared to the other cell lines, without evidence of DHFR gene amplification. The subsequent finding that this cell line lacked both copies of chromosome 13, which contains the gene for pRB (18), led us to investigate further the relationship between pRB and resistance to MTX as well as FdUrd, antimetabolite drugs that inhibit enzymes involved in DNA replication.

This study addresses the relationship between absent or abnormal pRB and sensitivity to drugs that target DHFR and TS. We have compared the growth inhibition of a human sarcoma cell line containing pRB (HT-1080) with cell lines either lacking pRB (HS-18) or having a truncated pRB incapable of binding E2F (SaOS-2). We have also obtained viable SaOS-2 sublines stably transfected with Rb after G-418 selection (see below). Cells lacking functional pRB were more resistant to growth inhibition by MTX and FdUrd compared to pRB-expressing cell lines. In cell lines lacking pRB, levels of DHFR and TS enzyme activity as well as mRNA expression were higher. Furthermore, cell lines lacking pRB had increases in free E2F as measured by gel-retardation assays.

MATERIALS AND METHODS

Chemicals. MTX was obtained from Lederle Laboratories (Pearl River, NY). FdUrd was supplied by Roche Laboratories (Nutley, NJ). Doxorubicin, cisplatin, and VP-16 were obtained from Sigma. [^{3}H]MTX and [5-^{3}H]dUMP (20 Ci/mmol; 1 Ci = 37 GBq) were purchased from Moravek Biochemicals (La Brea, CA). [α-^{32}P]dCTP and [γ-^{32}P]ATP were from DuPont/NEN. The Rb polyclonal antibody Ab-2 was obtained from Oncogene Science, E2 oligonucleotide probe (E2pro) and DHFR E2F binding oligonucleotides were synthesized with an Applied Biosystems oligonucleotide synthesizer. E2pro corresponds to the −70 to −32 region of the adenoviral E2 promoter.

Cell Lines. HT-1080, a human fibrosarcoma cell line, and SaOS-2, an osteosarcoma cell line, were obtained from the American Type Culture Collection (ATCC). HS-18, a human liposarcoma obtained from an untreated patient, was established as a cell line in this laboratory. HT-1080, SaOS-2, and HS-18 cells were maintained as monolayer cultures in RPMI 1640 medium containing 10% fetal bovine serum. The doubling times of exponentially growing HT-1080, SaOS-2, and HS-18 cells were 26, 39, and 36 h, respectively.

Cell Growth Inhibition Studies. Exponentially growing HT-1080, HS-18, and parental and Rb-transfected SaOS-2 cells (5×10^3 cells per cm^2) were exposed to various concentrations of

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

Abbreviations: pRB, retinoblastoma protein; MTX, methotrexate; DHFR, dihydrofolate reductase; TS, thymidylate synthase.

‡To whom reprint requests should be addressed.

drugs (MTX, FdUrd, doxorubicin, cisplatin, or VP-16) for 5 days. Cells then were counted with a model ZB Coulter Counter. IC_{50} values were determined from the growth inhibition data.

Western Blot Analysis of pRb. pRb was detected by immunoblotting as described with a polyclonal Rb antibody (Oncogene Science) (19).

DHFR Activity Assay. Midlogarithmic phase growing cells were harvested, washed twice with cold PBS, and resuspended in 50 mM Tris·HCl (pH 7.5) containing 10% (vol/vol) glycerol, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Cellular extract was obtained by sonication followed by centrifugation at 30,000 × *g* for 30 min at 4°C. The supernatant was used for enzyme assays. DHFR activity was determined as described (20).

TS Activity Assay. Midlogarithmic phase cells ($\approx 10^7$) were suspended in 1 ml of extraction buffer consisting of 50 mM Tris·HCl (pH 7.5), 50 mM 2-mercaptoethanol, 10% glycerol, and 0.1% Triton X-100. Cells were disrupted by freeze–thawing three times and centrifuged at 42,500 × *g* for 30 min at 4°C. The supernatant was assayed for TS activity, by the ^3H release assay, using [5-^{3}H]dUMP as described (21).

Northern Blot Analysis. Total RNA was extracted from exponentially growing cells with RNazol (Biotecx, Houston). A total of 40 μg of RNA was electrophoresed in an agarose gel containing 1.5% formaldehyde and transferred to a nylon filter and probed with ^{32}P-labeled DHFR cDNA (22). The filter was washed and quantitated with a blot analyzer (Betascope 603) and autoradiographed. After stripping, the filter was reprobed with TS cDNA (23). Hybridization of the same filter with 36B4 cDNA (24) served as an internal control for RNA loading.

Transfection of SaOS-2 Cells. SaOS-2 cells were transfected at 70% confluency with the transfection reagent DOTAP with the plasmid pLRbRNL (ATCC) containing the Rb cDNA (Boehringer Mannheim) (25). After selection with G418 (800 μg/ml) for 4 weeks, resistant clones were isolated and expanded into cell lines in medium containing G418 (200 μg/ml).

E2F Binding Activity Assay. Whole cell extracts from cells were prepared as described (26). E2F binding activity was determined by gel-retardation assays (27). The reaction mixture contained, in a total of 20 μl, the binding buffer [50 mM KCl, 20 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 10% glycerol, 0.5 mM dithiothreitol, and 1% Nonidet P-40], 20 μg of extract protein, 20 μg of bovine serum albumin, 2 μg of salmon sperm DNA, and 1 ng of ^{32}P-labeled E2 oligonucleotide probe (5′-GATCAGTTTTCGCGCTTAAATTTGAGA-AAGGGCGCGAAACTAG-3′) (28). After a 30-min incubation the reaction products were separated on a 4% polyacrylamide gel in 0.25× TBE buffer at 4°C. The gel was dried and exposed to x-ray films.

Cell Cycle Analysis. Midlogarithmic phase growing cells (1×10^6 cells) were washed in ice-cold PBS, fixed in methanol, and stored at 4°C. The cells were stained with propidium iodide (100 μg/ml) and analyzed on a Becton Dickinson fluorescence-activated cell sorter according to published procedures (29, 30).

RESULTS

Relationship Between Expression of Rb Protein and Drug Sensitivity. Our initial studies established that HT-1080 cells have significant levels of pRB as determined by Western blotting. However, pRB was not detected in HS-18 and SaOS-2 cells, using a polyclonal anti-Rb antibody. An anti-pRB monoclonal antibody (clone 53-245; PharMingen) was able to detect the 95-kDa truncated pRB in parental SaOS-2 cells (31) (data not shown).

Compared with HS-18 and SaOS-2 cell lines, HT-1080 cells were more sensitive to both MTX and FdUrd (the IC_{50} values for MTX were 12- and 10-fold lower and those for FdUrd were 11- and 4-fold lower than those obtained in HS-18 and SaOS-2 cells, respectively; Table 1). The effect was specific for this class of antimetabolites since no significant difference in growth inhibition was evident among these cell lines for VP-16, cisplatin, and Adriamycin.

Target Enzyme Levels in Relation to MTX and FdUrd Resistance. Based on growth inhibition studies as described above, the HS-18 and SaOS-2 cells were more resistant to MTX and FdUrd compared to HT-1080 cells. We examined the level of the target enzymes for MTX (DHFR) and FdUrd (TS) in relation to MTX and FdUrd resistance. DHFR activities measured in HS-18 and SaOS-2 cells were 2- to 3-fold higher than those obtained in HT-1080 cells (Table 1). Higher TS activities (4- to 8-fold) were also noted in HS-18 and SaOS-2 cells compared to HT-1080 cells (Table 1). MTX uptake and polyglutamate formation were also measured in these cell lines as impaired MTX uptake and accumulation of polyglutamates may be important mechanisms of MTX resistance (17). There was no significant difference in MTX uptake or polyglutamate formation among the cell lines (data not shown). Thus, the relative resistance to MTX and FdUrd in the two Rb-lacking cell lines compared to the HT-1080 cell line is attributed to increased levels of the target enzymes in these cells.

DHFR and TS Gene Amplification and Expression. No significant difference was detected for DHFR or TS gene copy number in the cell lines (data not shown). However, Northern blot analysis showed that HS-18 and SaOS-2 cells have a 2- to 4-fold higher level of mRNA expression of DHFR and TS compared to the HT-1080 cell line (Fig. 1).

Reversal of MTX and FdUrd Resistance in the SaOS-2 Cell Line After Transfection with Rb cDNA. Based on the above results, intrinsic resistance to MTX and FdUrd appeared to be associated with absence of functional pRB and increased levels of DHFR and TS in the HS-18 and SaOS-2 cell lines. To confirm this relationship, Rb cDNA was introduced into one of these cell lines to determine whether transfection of the Rb gene decreased resistance to MTX and FdUrd. Eight stable

Table 1. DHFR and TS activity and growth inhibition by MTX, FdUrd, Adriamycin, VP-16 (etoposide), and cisplatin in human cell lines containing or lacking pRb protein

Cell line	Rb status	DHFR, $\mu mol \cdot h^{-1} \cdot mg^{-1}$	TS, $nmol \cdot h^{-1} \cdot mg^{-1}$	IC_{50}, nM		IC_{50}, μM		
				MTX	FdUrd	Adriamycin	VP-16	Cisplatin
HT-1080	+	0.06 ± 0.01	0.80 ± 0.20	7.20 ± 2.80	28.9 ± 8.10	0.21 ± 0.09	2.2 ± 0.3	2.5 ± 0.1
HS-18	−	0.13 ± 0.02	6.20 ± 0.50	84.1 ± 25.1	315 ± 9.10	0.35 ± 0.1	2.1 ± 0.3	3.1 ± 0.4
SaOS-2	−	0.14 ± 0.01	3.90 ± 0.60	79.2 ± 29.3	107 ± 12.1	0.18 ± 0.07	1.7 ± 0.3	2.8 ± 0.3
SaOS-2 31	−	0.12 ± 0.01	4.00 ± 0.10	94.4 ± 41.9	137 ± 53.4	0.29 ± 0.05	2.8 ± 0.7	4.3 ± 1.0
SaOS-2 9	+	0.07 ± 0.01	2.10 ± 0.10	29.2 ± 17.1	67.2 ± 13.2	0.24 ± 0.04	2.1 ± 0.2	3.2 ± 0.6
SaOS-2 10	+	0.06 ± 0.01	1.10 ± 0.20	41.7 ± 20.8	27.9 ± 13.1	0.24 ± 0.05	2.7 ± 0.1	3.4 ± 1.7

DHFR and TS activities were determined by a spectrophotometric assay and a [^{3}H]dUMP assay, respectively, with cell extracts obtained from exponentially grown cells. IC_{50} values were obtained by counting, using a model ZB Coulter Counter, the surviving cells after a 5-day exposure of exponentially grown cells to various concentrations of the indicated drug. Results are means ± SE from three experiments. −, Rb is either absent or nonfunctional; +, functional Rb.

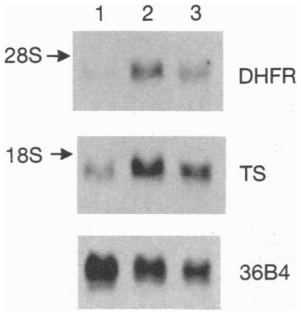

FIG. 1. Northern blot analysis of mRNA in three human cell lines. The probes were a DHFR cDNA (*Top*) and a TS cDNA (*Middle*). (*Bottom*) Control for total RNA loaded using a 36B4 probe. Lanes: 1, HT-1080 cells (pRB^+); 2, HS-18 cells (pRB^-); 3, SaOS-2 cells (pRB^-).

clones from SaOS-2 cells transfected with Rb were obtained with G418 selection and were expanded into cell lines. Seven of the eight sublines showed pRB expression by Western blotting (105–110 kDa) (data not shown). Some of these sublines had changes in morphology and were larger and flatter in appearance, as has been described (32–38). Five of the eight sublines grew slowly, with a longer doubling time than that of parental SaOS-2 cells. The three sublines that had a similar doubling time compared to the parental line (38–39 h) were selected for determination of the effect of pRB expression on cell growth inhibition by MTX and FdUrd. Two of these three sublines (S-9 and S-10) expressed full-length pRB, while subline S-31 did not (Fig. 2). As shown in Table 1, pRB^+ sublines S-9 and S-10 were more sensitive to growth inhibition by MTX and FdUrd than parental cells (IC_{50} values for MTX were 2.6- to 6.2-fold lower and those for FdUrd were 2.3- to 4.1-fold lower, respectively). In contrast, the G418 selected but pRB^- subline S-31 showed no change in sensitivity to MTX and FdUrd compared to parental cells. This pRB-mediated drug sensitivity was specific to antimetabolites since no significant change in growth inhibition to the nonantimetabolites VP-16, Adriamycin, and cisplatin was observed when parental SaOS-2 cells and the pRB-expressing sublines of SaOS-2 cells were compared (Table 1). DHFR and TS activities in Rb-transfected cells were determined to ascertain whether the increase in sensitivity to MTX and FdUrd was related to a decrease in DHFR and TS activities. As predicted, DHFR and TS activities in the S-9 and S-10 cell lines were 2- to 3-fold lower than in SaOS-2 cells and were similar to those of HT-1080 cell lines (Table 1). DHFR and TS activities in the S-31 subline were similar to the parental SaOS-2 cell line (Table 1).

The Level of Free E2F Is a Critical Factor in Determination of Drug Sensitivity. E2F-like binding sites have been found in the promoters of several genes involved in DNA replication, including DHFR (13, 14), TS (15), thymidine kinase (39, 40), human DNA polymerase α (41), and the *CDC2* gene (42). We therefore measured E2F and its complexes in these cell lines by a gel-retardation assay in order to determine free and bound forms of this family of transcription factors (Fig. 3). E2F complexes (Fig. 3, band A) were observed in HT-1080 cells

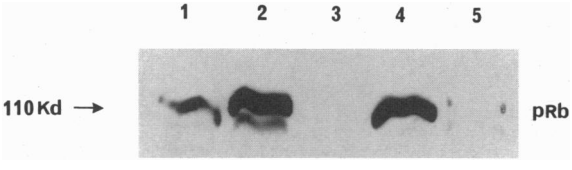

FIG. 2. Expression of pRB in Rb-transfected SaOS-2 cells. pRB was detected by immunoblotting as described. Lanes: 1, HT-1080 cells; 2, Rb-transfected SaOS-2, clone 10; 3, parental SaOS-2 cells; 4, Rb-transfected SaOS-2, clone 9; 5, Rb-transfected SaOS-2, clone 31.

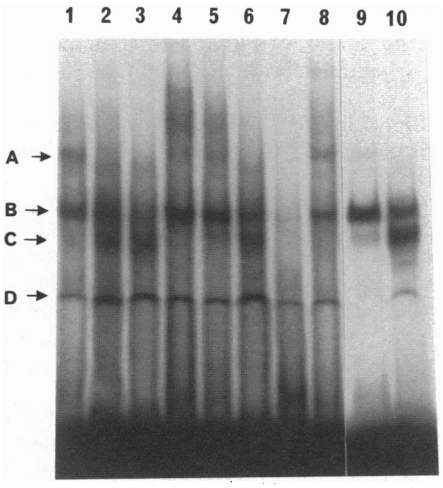

FIG. 3. E2F binding assay. Cell extracts (10 μg) were incubated with a ^{32}P-labeled E2 oligonucleotide probe. Lanes: 1, HT-1080 extract; 2, HS-18 extract; 3, SaOS-2 extract; 4, extract from Rb-transfected SaOS-2 cells (clone 9); 5, extract from Rb-transfected SaOS-2 cells (clone 10); 6, extract from Rb-transfected SaOS-2 cells (clone 31); 7, HT-1080 extract plus 100-fold excess unlabeled E2 probe; 8, HT-1080 extract plus 2 μg of monoclonal antibody to pRB; 9, HT-1080 extract plus 0.5% sodium deoxycholate; 10, SaOS-2 extract plus 0.5% sodium deoxycholate. Positions of free E2F and E2F complexes are indicated by arrows: A, E2F complexed with pRB; B and C, free E2F, of which C is the pRB-regulated free E2F; D, a nonspecific band.

(containing pRB) (lane 1) or in pRB-reconstituted SaOS-2 sublines (lanes 4 and 5) but these complexes were absent in HS-18 cells (lane 2), in parental SaOS-2 cells (lane 3), and in the S-31 subline of SaOS-2 (lane 6), cell lines lacking functional pRB. Addition of Rb antibody (lane 8) reduced the intensity of this complex and the band appeared to have supershifted. Furthermore, when the assay mixtures containing HT-1080 and SaOS-2 were treated with sodium deoxycholate (lanes 9 and 10, respectively), known to be capable of disrupting E2F complexes (43), the E2F complex (band A) was not detected, while bands B and C were not affected. Therefore, uncomplexed or free E2F appeared as a doublet (bands B and C). This is consistent with recent reports (44, 45) and may represent migration of different family members of E2F. The faster-migrating component (band C), but not the slower migrating band (band B), appeared to be regulated by pRB (compare lanes 2 and 3 and lanes 3 and 4). There was significantly more pRB-regulated free E2F in HS-18 (lane 2) and parental SaOS-2 cells (lane 3) when compared with HT-1080 cells (lane 1). However, in Rb-transfected SaOS-2 sublines [S-9 (lane 5) and S-10 (lane 6)], the level of free E2F was markedly reduced.

Cell Cycle Changes in Cells Containing and Lacking pRB. Cell cycle distribution was assessed in cells containing or lacking pRB. As shown in Table 2, HS-18 cells, which lack pRB, contained more cells in S phase than HT-1080 cells containing pRB. Compared to SaOS-2 parental cells, Rb-transfected SaOS-2 cells had a higher percentage of cells in G_1.

DISCUSSION

We recently reported that mouse embryonic fibroblasts from homozygous Rb "knockout" mice accumulated nuclear p53 under a variety of growth challenges and underwent cell death by apoptosis (46). Selective pRB inactivation by targeted expression of E7 or of a mutated T antigen, which cannot bind p53, also resulted in apoptosis *in vivo* (47). In contrast, inactivation of p53 in cells lacking functional pRB led to hyperproliferation and an increase in frequency of tumorigen-

Table 2. Cell cycle distribution of several sarcoma cell lines and Rb-transfected SaOS-2 subline

Cell line	Rb status	Fraction, %		
		G_1	S	G_2/M
HT-1080	+	54.5	20.1	25.4
HS-18	−	50.5	33.0	16.5
SaOS-2 (parental)	−	46.4	37.1	16.5
SaOS-2 9	+	61.8	24.2	14.0
SaOS-2 10	+	74.3	17.4	8.3

Cell cycle analysis was performed by staining midlogarithmic phase cells of each cell line with propidium iodide followed by flow cytometric analysis.

esis (48, 49). In this circumstance, unlike cells that contain wild-type p53, overexpression of E2F (50), c-myc (51), or E1A (52) did not commit these cells to apoptosis. The tumor cell lines used in this study, the HS-18 sarcoma cell line and SaOS-2 osteosarcoma line, lacking both functional p53 and pRB, are likely, for this reason, to be relatively resistant to growth challenges that include anticancer drugs compared to tumor cells that contain wild-type p53.

Introduction of Rb cDNA into cells lacking pRB or containing a nonfunctional form of this protein into different cell lines including SaOS-2 cells has been reported to lead to morphologic changes, slowing of growth and cell death (32–34). We obtained several clonally derived cell lines, using G418 selection that, although containing functional Rb, were able to grow with a generation time similar to that of parental cells. As expected, these transfectants had an increase in the G_1 phase of the cell cycle, consistent with the function of pRB in regulating S-phase cell entry (3). It is likely that the level of expression of pRB is one factor that may determine the viability of transfectants. High levels of expression of Rb may result in such profound G_1 block as to not allow cells to enter S phase, resulting in cessation of growth.

The HS-18 and SaOS-2 cell lines, lacking pRB or functional pRB, respectively, both express elevated levels of mRNAs encoded by DHFR and TS, two of several genes related to DNA replication that are regulated in part by E2F/DP heterodimeric transcription factors that can bind hypophosphorylated pRB (43). These data are in accord with our recent findings that $Rb^{-/-}$ mouse embryonic fibroblasts derived from knockout mice have elevated levels of DHFR and TS mRNA, as compared to syngeneic fibroblasts with the $Rb^{+/+}$ genotype (46). Compared to HT-1080 cells containing normal pRB, the HS-18 and SaOS-2 cell lines were severalfold resistant to growth inhibition by MTX and FdUrd. Furthermore, Rb expression correlates with restored sensitivity to these antimetabolites and reduced DHFR and TS mRNA synthesis in SaOS-2 cell transfectants. Studies with other cell lines selected by chronic exposure to MTX that have low level gene amplification (2- to 4-fold) and an increase in DHFR mRNA and protein clearly show that a significant (10- to 20-fold) degree of resistance to MTX results (53). As intracellular MTX levels are regulated by a saturable carrier-mediated transport process, the intracellular level of DHFR becomes a critical factor in the inhibition of tetrahydrofolate biosynthesis by this drug and consequent growth inhibition (54). In contrast, the Rb transfectants were equally sensitive to other anticancer agents that do not target enzymes whose transcription is known to be increased by free E2F.

The data presented show that there is a relationship between the level of at least one species of "free" E2F and the presence or absence of functional pRB. Five members of the E2F family have been described to date (55), and at least three E2F proteins can bind the pRB-related proteins, p107 and p130 (56, 57). A large number of genes may be regulated during different phases of the cell cycle by these distinct E2F–DP_1 heterodimers (55). As the oligomer used to detect E2F proteins in the gel-retardation assay does not distinguish between the E2F species involved, further investigation is required to determine which E2F heterodimer(s) transcriptionally activates the DHFR and the TS promoters. If transcriptional activity is demonstrated for these and other promoters containing an E2F binding site—e.g., ribonucleotide reductase (58) and DNA α polymerase (41)—it may be predicted that these pRB-deficient cells may also be resistant to inhibitors of these enzymes (e.g., hydroxyurea and aphidicolin).

These results may explain why human tumors lacking pRB or having functional abnormalities of this protein are relatively resistant to treatment with drugs that target DHFR or TS. For example, retinoblastoma cell lines are intrinsically resistant to MTX, not alkylating agents (59). In addition, small cell lung cancer cell lines that commonly lack pRB are relatively resistant to MTX (60), and MTX is not used in the treatment of this disease as patients with this tumor are usually refractory to treatment with MTX (61).

This study was supported by U.S. Public Health Service Grants PO1-CA-47179 and 5R01CA-61449 to J.R.B. and G.M.W., respectively, and by a grant from the G. Harold and Leila Y. Mathers Charitable Foundation to G.M.W. D.H. was supported by an award from the V Foundation.

1. Greenblatt, M. S., Bennett, W. P., Hollstein, M. & Harris, C. C. (1994) *Cancer Res.* **54,** 4855–4878.
2. Schubert, E. L., Hansen, M. F. & Strong, L. C. (1994) *Ann. Med.* **26,** 177–184.
3. Goodrich, D. N., Wang, N. P., Qian, Y. W., Lee, E. Y.-H. P. & Lee, W. H. (1991) *Cell* **67,** 293–302.
4. Chen, P. L., Scully, P., Shew, J. Y., Wang, J. Y. & Lee, W. H. (1989) *Cell* **58,** 1193–1198.
5. DiCaprio, J. A., Furukawa, Y., Ajchenbaum, F., Griffin, J. D. & Livingston, D. M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1795–1798.
6. Lees, J. A., Buchkovich, K. J., Marshak, D. R., Anderson, C. W. & Harlow, E. (1991) *EMBO J.* **10,** 4279–4290.
7. Lin, B. T., Gruenwald, S., Morla, A. O., Lee, W. H. & Wang, J. Y. (1991) *EMBO J.* **10,** 857–864.
8. Ewen, M. E., Sluss, H. K., Sherr, C. J., Matsushime, H., Kato, J. & Livingston, D. M. (1993) *Cell* **73,** 487–497.
9. Hinds, P. W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S. I. & Weinberg, R. A. (1992) *Cell* **70,** 993–1006.
10. Hatakeyama, M. & Weinberg, R. A. (1994) *Genes Dev.* **8,** 1759–1771.
11. Wang, J. Y. J., Knudsen, E. S. & Welch, P. J. (1994) *Adv. Cancer Res.* **64,** 25–85.
12. Nevins, J. R. (1992) *Science* **258,** 424–429.
13. Blake, M. C. & Azizkhan, J. C. (1989) *Mol. Cell. Biol.* **9,** 4994–5002.
14. Slansky, J. E., Li, Y., Kaelin, W. G. & Farnham, P. J. (1993) *Mol. Cell. Biol.* **13,** 1610–1618.
15. Johnson, L. F. (1994) *J. Cell. Biochem.* **54,** 387–392.
16. Johnson, D. G., Schwartz, J. K., Cress, W. D. & Nevins, J. R. (1993) *Nature (London)* **365,** 349–352.
17. Li, W. W., Lin, J. T., Schweitzer, B. I., Tong, W. P., Niedzwiecki, D. & Bertino, J. R. (1992) *Cancer Res.* **52,** 3908–3913.
18. Li, W. W., Tong, W. P. & Bertino, J. R. (1995) *Clin. Cancer Res.* **1,** 631–636.
19. Sambrook, J., Fritsch, E. F. & Maniatis, J. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed., pp. 1819–1885.
20. Bertino, J. R., Perkins, J. P. & Johns, D. G. (1965) *Biochemistry* **4,** 839–846.
21. Roberts, D. (1966) *Biochemistry* **5,** 3546–3548.
22. Srimatkandada, S., Medina, W. D., Cashmore, A. R., Whyte, W., Engle, D., Moroson, B. A., Franco, C. T., Dube, S. & Bertino, J. R. (1983) *Biochemistry* **22,** 5774–5781.
23. Deng, T. L., Li, D. W., Jenh, C. H. & Johnson, L. F. (1986) *J. Biol. Chem.* **261,** 16000–16005.
24. Laborda, J. (1991) *Nucleic Acids Res.* **19,** 3998.

25. Stamatatos, L., Leventis, R., Zuckermann, M. J. & Silvius, J. R. (1988) *Biochemistry* **27,** 3917–3925.
26. Raychaudhuri, P., Rooney, R. & Nevins, J. R. (1987) *EMBO J.* **6,** 4073–4081.
27. Yee, A. S., Reichel, R., Kovesdi, I. & Nevins, J. R. (1987) *EMBO J.* **6,** 2061–2068.
28. Shirodkar, S., Ewen, M., DeCaprio, J. A., Morgan, J., Livingston, D. M. & Chittenden, T. (1992) *Cell* **70,** 157–164.
29. Goodrich, D. W., Ping-Wang, N., Qian, V. W., Lee, E. & Lee, W. H. (1991) *Genes Dev.* **5,** 293–302.
30. DiLeonardo, A., Linke, S., Clarkin, K. & Wahl, G. M. (1994) *Genes Dev.* **8,** 2540–2551.
31. Shew, J. Y., Lin, B. T., Chen, P. L., Tseng, B. Y., Yang, F. T. & Lee, W. H. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 6–10.
32. Huang, H.-J. S., Yee, J.-K., Shew, J.-Y., Chen, P.-L., Bookstein, R., Friedmann, T., Lee, E. Y.-H. P. & Lee, W.-H. (1988) *Science* **242,** 1563–1566.
33. Bookstein, R., Shew, J. Y., Chen, P. L., Scully, P. & Lee, W.-H. (1990) *Science* **247,** 712–715.
34. Templeton, D. J., Park, S. H., Lanier, L. & Weinberg, R. A. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3033–3037.
35. Takahashi, R., Hashimoto, T., Xu, H. J., Matsui, T., Miki, T., Bigo-Marshall, H., Aaronson, S. A. & Benedict, W. F. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5257–5261.
36. Qin, X. Q., Chittenden, T., Livingston, D. M. & Kaelin, W. G. (1992) *Genes Dev.* **6,** 953–964.
37. Wang, N. P., To, H., Lee, W.-H. & Lee, Y.-H. P. (1993) *Oncogene* **8,** 279–288.
38. Haas-Kogan, D. A., Kogan, S. C., Levi, D., Dazin, P., T'Ang, A., Fung, Y.-K. T. & Israel, M. A. (1995) *EMBO J.* **14,** 461–472.
39. Farnham, P. J., Slansky, J. E. & Kollmar, R. (1993) *Biochem. Biophys. Acta Rev. Cancer* **1155,** 125–131.
40. Don, Q. P., Zhao, S., Levin, A. H., Wang, J., Helin, K. & Pardee, A. B. (1994) *J. Biol. Chem.* **269,** 1306–1313.
41. Pearson, B. E., Nasheuer, H. P. & Wang, T. S. F. (1991) *Mol. Cell. Biol.* **11,** 2081–2095.
42. Dalton, S. (1992) *EMBO J.* **11,** 1797–1804.
43. Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M. & Nevins, J. R. (1991) *Cell* **65,** 1053–1061.
44. Johnson, D. G., Cress, W. D., Jakoi, L. & Nevins, J. R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 12823–12827.
45. Chittenden, T., Livingston, D. M. & DeCaprio, J. A. (1993) *Mol. Cell. Biol.* **13,** 3975–3983.
46. Almasan, A., Yin, Y., Keely, R., Lee, E. Y.-H. P., Bradley, A., Li, W. W., Bertino, J. R. & Wahl, G. M. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 5436–5440.
47. Howes, K. A., Ranson, H., Papermaster, D. S., Lasudry, J. G. H., Albert, D. M. & Windle, J. J. (1994) *Genes Dev.* **8,** 1300–1310.
48. Pan, H. & Griep, A. E. (1994) *Genes Dev.* **8,** 1285–1299.
49. Morgenbesser, S. D., Williams, B. O., Jacks, T. & Depinho, R. A. (1994) *Nature (London)* **371,** 72–74.
50. Wu, X. & Levine, A. J. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 3602–3606.
51. Wagner, A. J., Kokontis, J. M. & Hay, N. (1994) *Genes Dev.* **8,** 2817–2830.
52. Lowe, S. W., Schmidt, E. M., Smith, S. W., Osborne, B. A. & Jacks, T. (1993) *Nature (London)* **362,** 847–849.
53. Rosowsky, A., Wright, J. E., Cucchi, C. A., Lippke, J. A., Tantravahi, R., Ervin, T. J. & Frei, E., III (1985) *Cancer Res.* **45,** 6205–6212.
54. Goldman, I. D. & Matherly, L. H. (1985) *Pharmacol. Ther.* **28,** 77–102.
55. Sardet, C., Vidal, M., Cobrinik, D., Geng, Y., Onufryk, C., Chen, A. & Weinberg, R. A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 2403–2407.
56. Cao, L., Faha, B., Dembski, M., Tsai, L. H., Harlow, E. & Dyson, N. (1992) *Nature (London)* **355,** 176–179.
57. Cobrinik, D., Whyte, P., Pepper, D. S., Jacks, T. & Weinberg, R. A. (1993) *Genes Dev.* **7,** 2392–2402.
58. Björklund, S., Skogman, E. & Thelander, L. (1992) *EMBO J.* **11,** 4953–4959.
59. Chan, H. S. L., Canton, M. D. & Gallie, B. L. (1989) *Anticancer Res.* **9,** 469–474.
60. Hensel, C. H., Hsieh, C. I., Gadzar, A. F., Johnson, B. E., Saguchi, A. Y., Naylor, S. L. & Lee, E. Y. (1990) *Cancer Res.* **50,** 3067–3072.
61. Hande, K. R., Oldham, R. K., Fer, M. F., Richardson, R. L. & Greco, F. A. (1982) *Am. J. Med.* **73,** 413–420.