Molecular Mechanisms of Loss of β_2-Microglobulin Expression in Drug-Resistant Breast Cancer Sublines and Its Involvement in Drug Resistance[†]

Besim Ogretmen, Mary D. McCauley, and Ahmad R. Safa*

Medical University of South Carolina, Hollings Cancer Center, Department of Experimental Oncology, 171 Ashley Avenue, Charleston, South Carolina 29425

Received March 12, 1998; Revised Manuscript Received May 22, 1998

ABSTRACT: In this study, we investigated the mechanism of the loss or decreased expression of β_2-microglobulin (β_2m) in several drug-resistant sublines of MCF-7 and in a doxorubicin (DOX)-resistant variant of the T-47D breast cancer cell line. β_2m protein and RNA are not expressed in highly metastatic, multidrug-resistant MCF-7/Adr cells with high resistance to DOX. Nuclear run-on transcription and RNA stability assays demonstrate that while β_2m in MCF-7/Adr cells is transcribed, its mRNA is rapidly degraded after synthesis in these cells, indicating that it is controlled by post-transcriptional mechanisms. We also show that an MCF-7 subline (MCF-7/Adr-5) expressing a very low level of resistance to DOX has a decreased level of β_2m expression. Treatment with actinomycin D revealed that the half-life of β_2m mRNA in MCF-7 and MCF-7/Adr-5 cell lines was comparable. Nuclear run-on transcription analysis revealed a decreased rate of β_2m transcription in MCF-7/Adr-5 cells compared to that in MCF-7 cells. Moreover, β_2m mRNA remained undetectable in MCF-7/Adr cells following cycloheximide treatment. However, in MCF-7 cells, increased β_2m mRNA was observed after 12 h, and a similar level of increased mRNA expression was observed after 36 h of cycloheximide treatment in MCF-7/Adr-5 cells; these results suggest that one of the mechanisms controlling β_2m mRNA expression might be a negative regulatory protein in MCF-7/Adr-5 cells. Analysis of the β_2m status of other drug-resistant MCF-7 sublines revealed that deregulation of β_2m is not limited to DOX resistance, but can also be detected in cells selected for resistance to mAMSA and DOX-verapamil. In addition, our data show that reduced β_2m expression correlates with the decreased levels of estrogen receptor (ER) expression in the DOX-resistant MCF-7/Adr and T-47D/Adr-4 human breast cell lines. Furthermore, we provide evidence that the partial inhibition of β_2m by antisense RNA results in 2−3-fold decreased sensitivity of MCF-7 cells to DOX and mAMSA. Moreover, the addition of exogenous β_2m protein near its physiological human serum concentration can modulate the DOX sensitivity of the MCF-7 antisense β_2m and control transfectants. Therefore, these results indicate that lost or decreased β_2m expression is involved in the development of the drug-resistant phenotype and correlates with the loss of ER in human breast cancer cell lines.

The major histocompatibility complex (MHC) is a group of genes that encodes for proteins associated with the function of the immune system (*1*). There are two classes of MHC proteins: Class I, which are involved in presenting foreign antigens to cytotoxic T cells and are generally found on the surface of most cells; and Class II, which are present only in antigen-presenting cells (*2*). Class I MHC proteins consist of two polypeptide chains: a 45 kDa heavy chain (HC) consisting of a cytoplasmic region, a transmembrane region, and an extracellular domain with N-linked glycosylation sites, and a 12 kDa chain (extracellular light chain) referred to as β_2-microglobulin (β_2m) (*2*, *3*). It is reported that β_2m is a nonglycosylated protein, which has no transmembrane domain, and is associated with cells by interacting with the extracellular region of HC. β_2m makes extensive contacts with all three domains of the HC, therefore the conformation of the HC is very much dependent upon the presence or absence of β_2m; β_2m also influences antigenic peptide binding (*4*). Recently, other β_2m-associated proteins that perform nonantigen-presenting functions have been identified, including the neonatal Fc receptor (*5*) and the product of the hereditary hemochromatosis gene (*6*). Other than their immunological functions, Class I MHC proteins are reported to regulate the proliferation of normal and malignant cells (*3*). In fact, Rowley et al. (*7*) recently demonstrated that β_2m stimulates the growth of PC-3 prostatic carcinoma cells in vitro. It is also known that the expression level of Class I MHC molecules is an important factor in determining the growth and metastatic properties

[†] This work was supported by research grants from the National Cancer Institute (CA 56078), the American Cancer Society (DHP-100A), and research supports from Hollings Cancer Center to A.R.S.

* Author to whom correspondence should be addressed. Telephone: (843) 792-9192. Fax (843) 792-3200.

[1] Abbreviations: β_2m, β_2-microglobulin; DOX, doxorubicin; ER, estrogen receptor; MDR, multidrug resistant; P-gp, P-glycoprotein; CDDP, cisplatin; AdVp, DOX-verapamil; Tx, taxol; IC_{50}, concentration of drug which decreases cell survival by 50%; RT-PCR, reverse transcription-polymerase chain reaction; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; TCA, trichloroacetic acid; CHX, cycloheximide; act D, actinomycin D; PBS, phosphate-buffered saline; GSPi, glutathione S-transferase; GSPx, glutathione peroxidase; MHC, major histocompatibility complex; Vpr, virus type 1 viral protein; TAP, transporter in antigen processing.

S0006-2960(98)00573-X CCC: $15.00
Published on Web 07/21/1998

of certain human tumors, including melanoma as well as breast and lung cancers (*8*, *9*).

Multidrug resistant (MDR)-MCF-7/Adr human breast cancer cells are known to be highly metastatic, while the sensitive parental MCF-7 cell line possesses a low metastatic potential (*10*). In addition, several different mechanisms, including overexpression of P-glycoprotein (P-gp), glutathione-related detoxifying enzymes (*11*), and resistance to apoptosis induced by different cytotoxic drugs (*12*), are reported to contribute to drug resistance in MCF-7/Adr cells. In the present study, we report the complete loss of β_2m in multidrug-resistant MCF-7/Adr cells, but not in drug-sensitive MCF-7 human breast cancer cells. We further investigated whether the loss or decreased expression of β_2m is seen in several drug-resistant variants of MCF-7, including sublines expressing low and high levels of resistance to DOX, taxol, cisplatin, DOX-verapamil, and mAMSA. We also examined the β_2m status of the ER-positive T-47D and the ER-negative MDA-MB-231 breast cancer cell lines and their DOX-resistant variants, and several different human cancer cells and their drug-resistant derivatives. Moreover, we determined whether decreased β_2m is involved in the development of drug resistance by introducing an expression vector containing a cDNA fragment of the β_2m gene in the antisense orientation into MCF-7 cells and determining the decreased sensitivity of the transfectants to several cytotoxic agents.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions. The human breast cancer cell line MCF-7 and its multidrug-resistant derivative MCF-7/Adr were obtained from Dr. Kenneth H. Cowan (National Cancer Institute, Bethesda, MD). MCF-7/Adr-5 and T-47D/Adr-4 were isolated by stepwise selection of MCF-7 and T-47D cells, respectively, exposed to increasing concentrations of DOX in our laboratory. KB-3-1 human epidermoid carcinoma cells and its DOX-, colchicine-, and vinblastine-resistant derivatives designated as KB-A1, KB-C1, and KB-V1, respectively, were obtained from Dr. Igor B. Roninson (Department of Genetics, University of Illinois, Chicago, IL). The human leukemia cell line HL-60 and its DOX-resistant derivative, HL-60/Adr, were obtained from Dr. Melvin S. Center (Division of Biology, Kansas State University, Manhattan, KS). MCF-7 sublines (*13*) resistant to etoposide (VP-16), cisplatin (CDDP), DOX-verapamil (AdVp), taxol (Tx), and mAMSA were kindly provided by Dr. Susan E. Bates (National Cancer Institute, Bethesda, MD). MDA-MB-231 and its DOX-resistant derivative, MDA-MB-231A1, were obtained from Dr. Michael W. DeGregorio (Division of Hematology and Oncology, University of California, Davis, CA). Cells were maintained in RPMI-1640 medium containing 10% fetal calf serum and 100 ng/mL each of penicillin and streptomycin (Life Technologies, Grand Island, NY) at 37 °C in 5% CO_2. Drugs were removed from the resistant cell lines one week before each assay.

Cell Survival Assay. The concentrations of DOX, vincristine, taxol, and etoposide (VP-16) that inhibited cell growth by 50% (IC_{50}) were determined from cell survival plots obtained by treating the cells in 24-well plates containing 1 mL of the growth medium with various concentrations of the drugs for 96 h at 37 °C in 5% CO_2, as previously described (*12*). Triplicate wells were used for each treatment. The final concentration of DMSO (the drug solvent) in the growth medium was less than 0.1% (v/v) which had no effect on cell growth and survival. VP-16, taxol, and vincristine were the generous gift of Bristol Myers (Wallingford, CT), the National Cancer Institute, and Eli Lilly (Indianapolis, IN), respectively.

The effects of purified exogenous human β_2m protein (Sigma, St. Louis, MO) on cell survival was determined by MTT cytotoxicity assay as we described elsewhere (*14*). In short, the cells were plated into 96-well plates containing 100 μL of the growth medium in the absence or presence of increasing concentrations of DOX with 5 or 10 μg/mL of purified β_2m protein at 37 °C in 5% CO_2 for 96 h. They were then treated with 25 μL of 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) for 4−5 h. After lysing the cells in 100 μL of lysis buffer (*14*), the plates were read in a microplate reader (Dynatech, Chantilly, VA) at 570 nm. The IC_{50} concentrations of DOX were then determined from cell survival plots as previously described (*14*).

Cell Cycle Analysis. The cell cycle distribution of MCF-7/pcDNA3 and MCF-7/pcDNA3-αβ_2m cells was analyzed as described elsewhere (*15*). In short, 70−80% confluent cell cultures ($1-2 \times 10^6$) were washed with PBS and resuspended in 200 μL of 0.9% saline. After the cells were fixed by the addition of 3 mL of 70% ethanol, they were centrifuged at 200*g* for 5 min and dissolved in 1 mL of PBS. The cells were then stained with 5 μL of propidium iodide (1 mg/mL) at 22 °C for 30 min, and the cell cycle distribution was measured using a Coulter Epics Flow Cytometer (Coulter Corp., Miami, FL).

Determination of Growth Rate. MCF-7 and MCF-7/Adr cells were plated into 24-well plates at 1.5 and 3.0×10^4 cells/well in 1 mL of growth medium. After washing, the cells were trypsinized and then counted using a Coulter counter (Coulter Electronics, Hialeah, FL) every 24 h for 8 days. The doubling time for each cell line was then determined from its growth curves, in which each point is the average determination from triplicate wells in two independent experiments.

Western Blot Analysis. β_2m protein levels in cells were measured by Western blot analysis as we described elsewhere (*16*). In short, total proteins (80 μg/lane) were separated by 5−15 or 10−20% SDS−PAGE containing 4.5 M urea, blotted onto an Immobilon membrane, and then β_2m levels were detected using 1 μg/mL of primary rabbit polyclonal anti-human β_2m antibody (Boehringer Mannheim, Indianapolis, IN) and a 1:2500 dilution (v/v) of secondary peroxidase-conjugated anti-rabbit antibody. An amount of 1 μg/mL of mouse monoclonal C219 (*12*) antibody that recognizes P-gp (both *MDR*1 and *MDR*2 P-gp), or rabbit polyclonal anti-topoisomerase IIα (*17*), anti-Bcl-2 (C-20), anti-Bax (N-20), or anti-ER (HC-20) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) that recognize human topoisomerase IIα (Topo IIα), Bax, Bcl-2, or ER, were used in Western blots with peroxidase-conjugated secondary anti-mouse or anti-rabbit antibody (1:2500), respectively. The proteins were visualized using the ECL protein detection kit (Amersham, Arlington Heights, IL) as described by the manufacturer. The membranes were then exposed to Kodak X-Omat film for

various times. Equal loading was confirmed by Coomassie Blue staining of SDS−PAGE strips cut from gels containing 80 μg of protein/lane of each sample prior to blotting. The protein levels were quantitated by densitometry of the autoradiograms.

RT-PCR Analysis. The mRNA levels of β_2m were analyzed by RT-PCR as follows. Total RNA (1 μg), isolated using a modified SDS-phenol technique as previously described (*18*), was used in reverse transcription reactions. The resulting total cDNA was then used in PCR to measure the mRNA level of β_2m using primers spanning the full-length β_2m cDNA. The intactness of total RNA samples was checked by running them on 0.8% denaturing agarose gels followed by ethidium bromide staining (*19*) before RT-PCR analysis. The primers and PCR conditions were as follows: β_2m 1 (forward) 5′ GGCGGGCATTCCTGAAGC (847−864), β_2m 2 (reverse) 5′ CTCCATGATGCTGCTTAC (2245−2262), rRNA (forward) 5′ TTACCAAAAGTGGCCCACTA (1501−1520), rRNA (reverse) 5′ GAAAGATGGTGAACTATGCC (1826−1845), β-actin (forward) 5′ CAGAGCAAGAGAGGCATCCT (216−235), and β-actin (reverse) 5′ TTGAAGGTCTCAAACATGAT (405−424).

The reactions were performed at 94 °C for denaturation, 60 °C for annealing, and 72 °C for amplification for 30 cycles for β_2m and 22 cycles for rRNA or β-actin to achieve linear conditions. These linear amplification cycles were determined separately as described elsewhere (*20*). The amplified fragments were then separated on 2% agarose gels and visualized by ethidium bromide staining. The mRNA or rRNA levels were quantitated by densitometry. All of the primers were synthesized by Life Technologies (Grand Island, NY). The specificity of the amplified β_2m fragments was determined by direct sequencing.

Nuclear Run-On Transcription Assay. The nuclear run-on transcription assay was performed using nuclei isolated from MCF-7, MCF-7/Adr, and MCF-7/Adr-5 cells as described by Groudine et al. (*21*) and Greenberg and Ziff (*22*). In summary, nuclei were prepared by lysing $3-4 \times 10^6$ cells in 1 mL of lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% IGEPAL CA-630 detergent) for 5 min on ice. After treatment with 50 μg/mL DNase-free RNase A on ice for 30 min, the nuclei were washed twice with the lysis buffer and resuspended in 100 μL of freezing buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA) and then immediately frozen. The clean nuclei were then thawed and the nuclear run-on transcription assay was performed at 30 °C for 30 min by adding 100 μL of reaction buffer containing 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 0.5 mM each of ATP, CTP, GTP, and 100 μCi of [α-^{32}P]UTP. Reactions were stopped by RNase-free DNase I treatment (100 units) at 30 °C for 10 min. After deproteinization by proteinase K (25 μg/mL) for 30 min at 42 °C, the labeled mRNA was extracted by phenol/chloroform (49:1) and then precipitated using 500 μL of 100% ethanol and 30 μL of 3 M sodium acetate, pH 5.2. Equal counts of TCA-precipitated transcripts from each cell line were used for hybridizations of slot blots, prepared by slot blot apparatus (Schleicher & Schuell, Keene, NH) as described by the manufacturer, containing the denatured DNA fragments. All PCR products were isolated from agarose gels using the Qiaex II Gel Extraction Kit (Qiagen, Chatsworth, CA). Hybridizations of the slot blots were performed in hybridization buffer as previously described (*19*), containing 50% formamide, 10% dextran sulfate, 0.5% nonfat dried milk, 1% SDS, and 250 μg/mL of denatured salmon sperm DNA, at 50 °C for 72 h in a personal hybridization oven (Stratagene, Menasha, WI), and then the membranes were washed three times each with 2× SSC-1% SDS and 0.1× SSC-1% SDS at 62 °C for 20 min. The membranes were then exposed to Kodak X-Omat film at −80 °C for 7−12 days. High-stringency washes were performed using 1× SSC-1% SDS in 50% formamide at 42 °C.

Determination of mRNA Stability. The optimal actinomycin D (act D) concentration that inhibits >95% of RNA synthesis in living cells was determined as follows: MCF-7 and MCF-7/Adr cells (1×10^5) were plated into 24-well plates and grown in 1 mL of RPMI-1640 medium for 24 h and then treated with serial dilutions of act D in the same medium for 2 h. The stock solution of act D was prepared as 10 mg/mL in DMSO; the final concentration of DMSO in both treated and untreated cells was less than 0.1%. The drug was then removed, and the cells were pulsed with 4 μCi/mL [^{3}H]uridine for 2 h. Following washes with PBS, micropreparation of total RNA was performed using the modified SDS/phenol method as we previously described (*18*). After isolation, total RNA samples were resuspended in 25 μL of nuclease-free water (Promega, Madison, WI) and then 1 μL of the samples were spotted onto Gene Screen nylon membranes (DuPont, Boston, MA). The membranes were air-dried, extensively washed in 5% trichloroacetic acid (TCA) in 20 mM sodium pyrophosphate, and then soaked in 70% ethanol. Air-dried membranes were then placed into 5 mL of scintillation fluid (Packard, Meriden, CT) and quantitated by scintillation counting.

After the optimal act D concentrations for each cell line were determined, the mRNA stability of β_2m was studied. Cells were plated into 6-well plates containing 5 mL RPMI-1640 medium and grown until they reached 70−80% confluence. They were then treated with the optimal act D concentration for 0, 8, and 16 h, at which times total RNAs were isolated by the modified SDS/phenol method. After RNA concentrations were determined, the levels of β_2m mRNA and rRNA were measured by RT-PCR using 1 μg of total RNA for each sample as described.

Inhibition of Protein Synthesis Using Cycloheximide. The effects of protein synthesis inhibition using cycloheximide (CHX) on β_2m gene expression in MCF-7, MCF-7/Adr, and MCF-7/Adr-5 cells were investigated as described (*23*). In short, the optimal CHX concentrations that inhibit 95% of the protein synthesis in each cell line (grown in 24-well plates) were determined by measuring the total incorporation of [^{35}S]methionine (4 μCi/mL) into TCA precipitable macromolecules by scintillation counting. The results were normalized to protein concentrations and expressed relative to untreated controls. The cells were then treated with CHX at its optimal concentration for various times, and β_2m mRNA levels were analyzed by RT-PCR as described above. The levels of β-actin mRNA detected by RT-PCR were used as internal controls.

Cloning the β_2m cDNA Fragment in Antisense Orientation. The expression vector containing a 357 bp β_2m cDNA fragment in the antisense orientation, designated as pcDNA3-$\alpha\beta_2$m, was prepared as follows. The cDNA fragment was

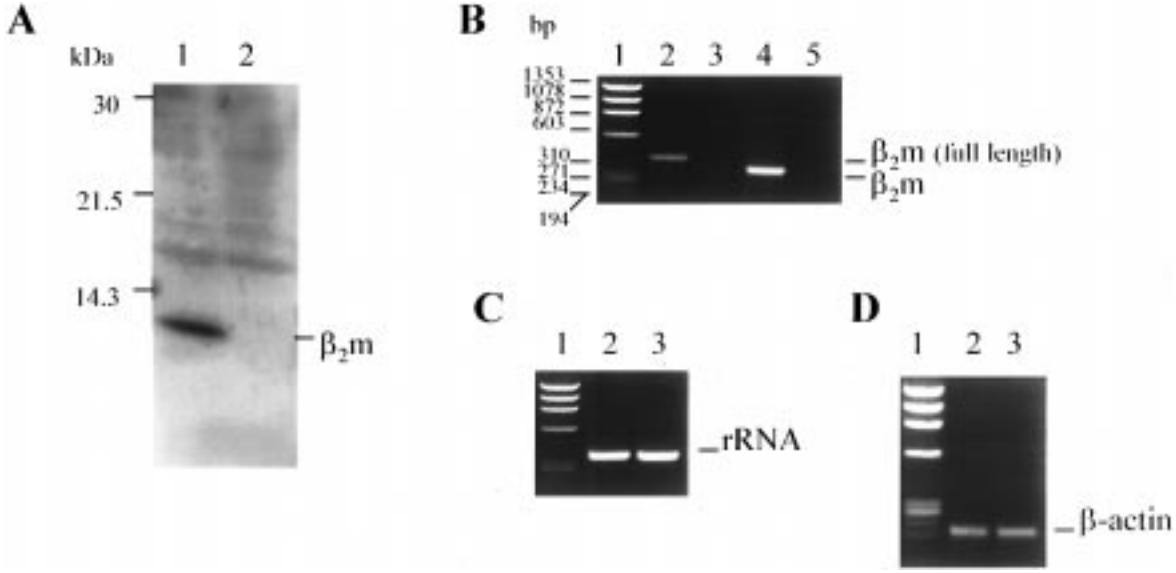

FIGURE 1: Determination of β_2m expression in MCF-7 and MCF-7/Adr cells. (A) The levels of β_2m protein (M_r 12 000) in MCF-7 (lane 1) and MCF-7/Adr (lane 2) cells were analyzed by Western blot analysis. The protein samples (80 μg/lane) were separated on 10−20% SDS−polyacrylamide gels, and β_2m protein was detected using the rabbit polyclonal anti-β_2m antibody following the transfer of the proteins from the gels to Immobilon membranes. Molecular weight markers are indicated on the left. The specificity of the β_2m antibody was confirmed using 20 ng of the purified human β_2m protein (Sigma, St. Louis, MO) in Western blot analysis as described above (data not shown). (B) The mRNA levels of β_2m in MCF-7 (lanes 2 and 4) and MCF-7/Adr (lanes 3 and 5) were detected by RT-PCR. Their rRNA or β-actin levels (Figure 1C, D, lanes 2 and 3, respectively) were used as internal controls. The 413 bp and 357 bp β_2m cDNA fragments, generated using primers 1−2 and 3−4, respectively. The RT-PCR products were run on 2% agarose gels and visualized by ethidium bromide (EtBr) staining. Lane 1 in Figure 1B−D contains *Hae*III-cut ΦX174 DNA fragments used as molecular weight markers. The results shown are representative of at least three independent experiments.

amplified with 1 μg of total RNA from MCF-7 cells by RT-PCR as described above. The primers used for the amplification of the β_2m fragment were as follows: β_2m 3 (forward) 5′P-CGGGATCCCGCTCCGTGGCCTTAGC (889−913) and β_2m 4 (reverse) 5′P-CCAAGCTTCTATCTTGGGCTGTGAC (1595−1619).

The first eight nucleotides of the primers are not complementary to the β_2m mRNA, and they contain sequences to generate *Bam*HI and *Hind*III sites at the 5′ and 3′ ends, respectively, of the amplified fragment. These restriction sites allowed cloning of the 357 bp β_2m fragment in the antisense orientation into the pcDNA3 expression vector (Invitrogen, San Diego, CA) which contains a *Hind*III site at the 5′ end and a *Bam*HI site at the 3′ end. The amplified fragments and the vector were digested with the restriction enzymes Hind III and Bam HI, ligated using T4 DNA ligase (Promega, Madison, WI) as described by the manufacturer, then transferred into *Escherichia coli* JM109 cells by $CaCl_2$ transformation (*14*).

Transfection of MCF-7 Cells with Antisense pcDNA3-αβ_2m. The expression vectors pcDNA3 and pcDNA3-αβ_2m were isolated and purified from transformed *E. coli* JM109 cells by the alkaline lysis method using Qiagen's miniplasmid isolation kit before transfections. MCF-7 cells were grown into 6-well plates containing 5 mL of Richter's improved MEM zinc option (Life Technologies) for 24 h before transfection. Stable transfections of MCF-7 cells with the above plasmids (2.5−5 μg of DNA/well) were performed by the calcium phosphate-mediated transfection method using Stratagene's modified bovine serum transfection kit as described by the manufacturer. The transfectants were initially selected in the presence of 1 mg/mL Geneticin (Life Technologies) for 48 h, then the cells were trypsinized and replated into fresh media with Geneticin to obtain individual colonies. The concentration of Geneticin was subsequently increased to 2 mg/mL in the second through fourth weeks of selection. Subsequently, the individual colonies (10 colonies/transfection) were picked from the wells using a sterile pipet tip and expanded in 10 mL of RPMI-1640 containing 2 mg/mL of Geneticin. The stable clones were then identified for the inhibition of β_2m in MCF-7 cells transfected with the pcDNA3-αβ_2m, compared to MCF-7 cells transfected with pcDNA3 (controls), by Western blotting as described above.

RESULTS

Expression of β_2m in MCF-7 and MCF-7/Adr Cells. Protein levels of β_2m in MCF-7 and MCF-7/Adr cells were detected by Western blotting using anti-human β_2m rabbit polyclonal antibody as described in Experimental Procedures. Figure 1A shows that β_2m is completely lost in MCF-7/Adr (lane 2) but not in MCF-7 (lane 1) cells. To determine whether the loss of β_2m protein in MCF-7/Adr cells is due to inhibition of its transcription, we performed RT-PCR. Total cellular RNA samples were used in RT-PCR to measure the β_2m mRNA levels by amplification of β_2m specific primers producing either full-length (413 bp) or partial (357 bp) β_2m cDNA fragments in both cell lines. As seen in Figure 1B, lanes 2 and 4, β_2m mRNA is produced in MCF-7 cells while it is not detectable in MCF-7/Adr cells (lanes 3 and 5). The rRNA and β-actin cDNA levels of MCF-7 and MCF-7/Adr cells were used as internal controls (Figure 1C,D, lanes 2 and 3, respectively). These results reveal that the loss of β_2m expression in MCF-7/Adr cells is controlled at the RNA level.

Analysis of β_2m Transcriptional Activity by Nuclear Run-On Assay. To investigate whether the loss of β_2m expression

is regulated at the transcriptional level, we performed a nuclear run-on transcription assay using the 413 bp cDNA fragment, spanning the full length of the β_2m gene. A 345 bp cDNA fragment which is specific for human rRNA generated by RT-PCR was used as an internal control probe for both cell lines. Interestingly, as seen in Figure 2A, [α-^{32}P]UTP-labeled transcripts from MCF-7 (panel 1) and MCF-7/Adr cells (panel 2) detected the full-length β_2m fragment (lower bands) as well as the rRNA fragment (upper bands). The same results were obtained in at least three independent nuclear run-on transcription assays after higher stringency washes of the membranes following hybridizations. These results show that the transcriptional signal of β_2m is specific (not due to a nonspecific hybridization of the probes) and detectable at similar levels in both cell lines.

Stability of β_2m mRNA in MCF-7 and MCF-7/Adr Cells. To investigate whether the transcriptional alteration of β_2m is due to rapid degradation of its mRNA, we examined the stability of β_2m mRNA using total cellular RNA from MCF-7 and MCF-7/Adr cells treated with the RNA polymerase inhibitor actinomycin D (act D) for 0, 8, and 16 h by RT-PCR. First, we determined the optimal act D concentration that inhibits 95% of RNA synthesis, defined as 95% reduction in [^{3}H]uridine incorporation in the cells due to act D treatment as compared to untreated controls. A 95% inhibition of RNA synthesis in MCF-7 cells was obtained at 20 μg/mL of act D and at 240 μg/mL of act D in MCF-7/Adr cells (Figure 2B). These data confirm the previous data (*24*) that act D is a substrate for P-gp, therefore MCF-7/Adr cells are more resistant to act D compared to MCF-7 cells. After treating the cells with act D at its optimal concentration, we found that the half-life of β_2m mRNA in MCF-7 cells was about 10 h (Figure 2C, lower panel, lanes 2−4). However, no signal was detected even at zero time controls in MCF-7/Adr cells (Figure 2C, lower panel, lanes 5−7). These results together with the results of the nuclear run-on assay suggest that β_2m mRNA is rapidly degraded after its synthesis in these cells. The rRNA, which is reported to have a half-life of >72 h (*24*), was used as an internal control for both cell lines, and no degradation of rRNA was observed in MCF-7 and MCF-7/Adr cells (Figure 2C, upper panel, lanes 2−4 and 5−7, respectively).

Mechanism of Reduced Expression of β_2m in MCF-7 Cells with Low DOX Resistance. MCF-7/Adr cells express a high degree of resistance to various anticancer agents (Table 1). To determine whether the loss of β_2m is an early event in the development of the DOX-resistant phenotype, we developed an MCF-7 subline resistant to DOX by passaging the cells stepwise in increasing DOX concentrations. The DOX-resistant clone of MCF-7 cells, designated as MCF-7/Adr-5, displays about 5-fold resistance to DOX and expresses cross-resistance to taxol, VP-16 (both about 2-fold), vincristine (about 4-fold), and mAMSA (about 1.5-fold) (Table 1). The protein and mRNA levels of β_2m in this cell line were detected by Western blotting and RT-PCR, respectively, as described in Experimental Procedures. As seen in Figure 3A, β_2m is decreased about 68% in MCF-7/Adr-5 cells (lane 2), compared to MCF-7 cells (lane 1). The level of the apoptosis-related protein Bax was used as an internal control (Figure 3B, lanes 1 and 2). We have previously shown that the Bax levels are identical in both

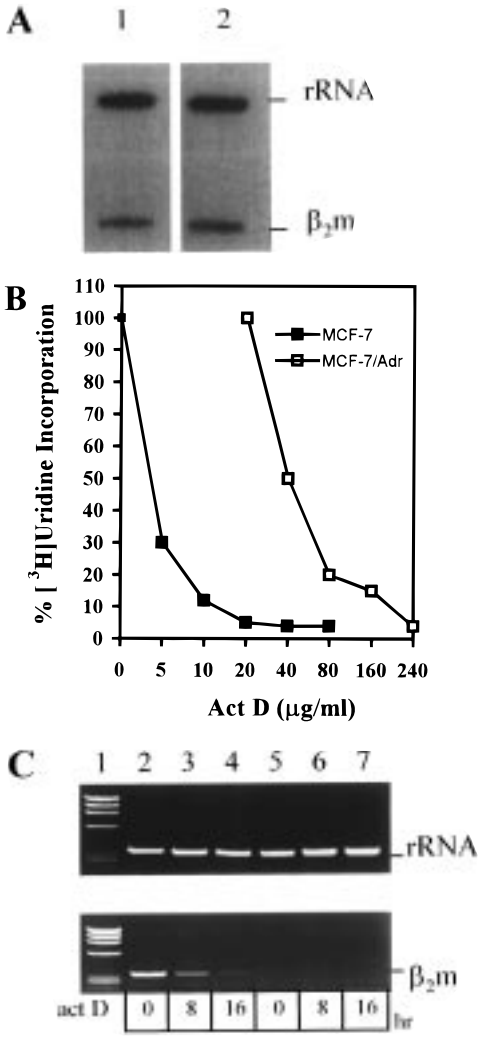

FIGURE 2: Analysis of the transcriptional activity of the β_2m gene and stability of its mRNA in MCF-7 and MCF-7/Adr cells. (A) Nuclei were isolated from MCF-7 (panel 1) and MCF-7/Adr (panel 2) cells and used in nuclear run-on transcription assays as described in Experimental Procedures. Equal counts of [α-^{32}P]UTP-labeled nuclear run-on transcripts were hybridized to Gene Screen membranes containing 413 bp full-length β_2m cDNA (lower bands) and 345 bp rRNA (upper bands) fragments immobilized using a slot blot apparatus. The autoradiograms above are the representative of three independent nuclear run-on assays in which similar results were obtained. (B) The optimal act D concentration that inhibits 95% of the [^{3}H]uridine incorporation in MCF-7 and MCF-7/Adr cells was determined as described in Experimental Procedures. Cells were grown in 24-well plates in the absence or presence of increasing concentrations of act D for 2 h, and then pulsed with 4 μCi/mL [^{3}H]uridine for 2 h at 37 °C in 5% CO_2. The TCA-precipitated [^{3}H]uridine, incorporated into RNA, was then determined by scintillation counting. Untreated cells were used as controls. Duplicate samples were used for each point. (C) The stability of β_2m mRNA in MCF-7 and MCF-7/Adr cells, grown in the presence of the act D concentration that inhibited 95% of RNA synthesis, was determined by RT-PCR as described. At various times (0−16 h), total RNA samples were extracted from cells and 1 μg of RNA was used to amplify the 357 bp β_2m cDNA (lower panel) and 345 bp rRNA (upper panel) fragments by RT-PCR in MCF-7 (lanes 2−4) and MCF-7/Adr (lanes 5−7). The amplified fragments were run on 2% agarose gels and visualized by EtBr staining. Lane 1 contains *Hae*III-cut ΦX174 DNA fragments used as molecular weight markers. Incubation time is indicated on the bottom of the gels. The results shown are representative of two independent experiments.

Table 1: Cell Survival Analysis Following Doxorubicin, Taxol, Vincristine, VP-16, and mAMSA Treatments

	IC_{50} (mM) [fold resistance][a]				
cell line	DOX	Taxol	Vincristine	VP-16	mAMSA
MCF-7	0.0034	0.0032	0.0007	0.00086	0.00031
MCF-7/Adr	20.0 [5882]	3.4 [1063]	5.6 [8095]	1.4 [1628]	0.0775 [250]
MCF-7/Adr-5	0.0163 [4.8]	0.0058 [1.8]	0.0026 [3.7]	0.0015 [1.8]	0.00043 [1.4]
MCF-7/pcDNA3	0.0035 [1.03]	0.0034 [1.06]	0.0007 [1.0]	0.0009 [1.05]	0.0003 [1.0]
MCF-7/pcDNA3-αβ_2m	0.011 [3.2]	0.0032 [1.0]	0.0007 [1.0]	0.0018 [2.1]	0.0005 [1.6]

[a] Fold resistance is described as the ratio of the IC_{50} values for MCF-7/Adr, MCF-7/Adr-3, MCF-7/pcDNA3, and MCF-7/pcDNA3-αβ_2m to that of MCF-7 cells. The cells were treated with or without various concentrations of drugs at 37 °C in 5% CO_2 for 96 h in 24-well plates. After trypsinization and counting the viable cells after each treatment, the concentrations of drugs that reduced cell survival by 50% (IC_{50}) were calculated from cell survival plots. The average of triplicate determinants in three independent experiments was used for the analysis. Standard deviations for the IC_{50} values in each treatment were between 0.00005 and 0.0001.

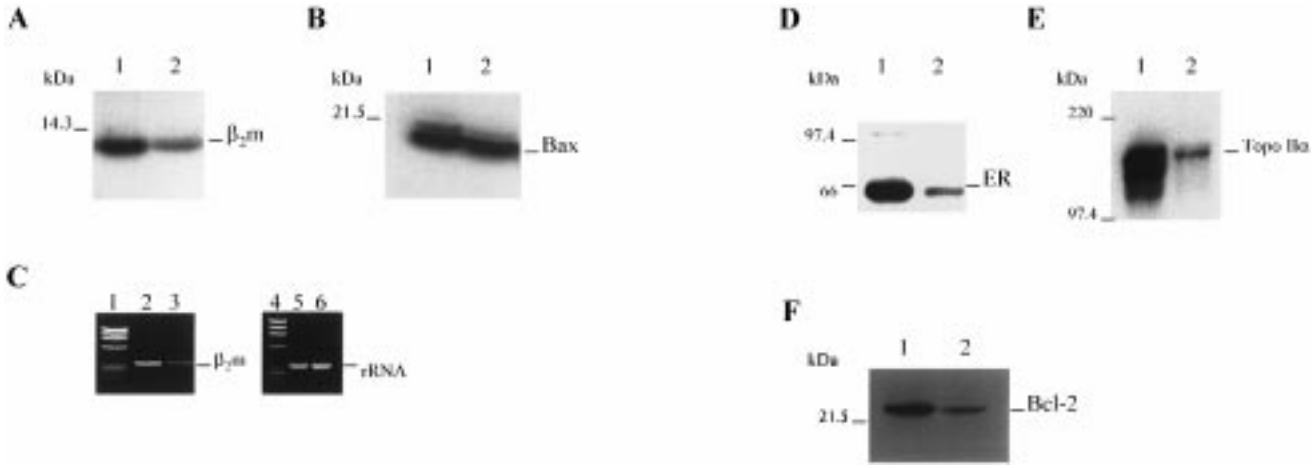

FIGURE 3: Analysis of β_2m, Bcl-2, Topo IIα, and ER expression in MCF-7 and MCF-7/Adr-5 cells. (A) β_2m protein levels in MCF-7 (lane 1) and MCF-7/Adr-5 (lane 2) cells were determined by Western blot analysis using the rabbit polyclonal anti-β_2m antibody. Protein samples (80 μg/lane) were resolved on 12−17% SDS−polyacrylamide gels and then transferred to Immobilon membranes prior to Western blotting. Molecular weight markers are indicated on the left. (B) Bax protein levels (M_r 21 000) were used as internal controls (lanes 1 and 2). (C) β_2m mRNA levels in MCF-7 (lane 2) and MCF-7/Adr-5 (lane 3) were measured by RT-PCR, and normalized against their rRNA levels (lanes 5 and 6, respectively). The 357 bp β_2m cDNA and 345 bp rRNA fragments were run on 2% agarose gels and visualized by EtBr staining. The *Hae*III-cut ΦX174 DNA fragments were used as molecular weight markers (lanes 1 and 4). (D) ER (M_r 65 000), (E) Topo IIα (M_r 170 000), (F), Bcl-2 (M_r 26 000) in MCF-7 (lane 1) and MCF-7/Adr-5 (lane 2) were determined by Western blotting. The molecular weight markers are indicated on the left.

MCF-7 and MCF-7/Adr cells (*12*). Moreover, mRNA levels of β_2m as measured by RT-PCR were about 35% lower in these cells compared to MCF-7 cells (Figure 3C, lanes 3 and 2, respectively). The β_2m mRNA levels of the cells were normalized to their rRNA levels (Figure 3C, lanes 6 and 5, respectively). Additionally, we examined the levels of P-gp, estrogen receptor (ER), Topo IIα, and Bcl-2 in MCF-7/Adr-5 cells by Western blotting as described in Experimental Procedures. Our results using C219 anti-P-gp monoclonal antibody and RT-PCR of the *MDR*1 gene showed that neither P-gp nor its mRNA were detectable in these cells (data not shown), while the expression of ER decreased about 65% in MCF-7/Adr-5 cells compared to MCF-7 cells (Figure 3D, lanes 2 and 1, respectively). Moreover, the Topo IIα and Bcl-2 levels were decreased about 70 and 40%, respectively, in MCF-7/Adr-5 compared to MCF-7 cells (Figure 3E and F, lanes 2 and 1, respectively).

To determine the molecular mechanisms which cause reduced expression of β_2m mRNA in MCF-7/Adr-5 cells, we determined the half-life of β_2m mRNA of MCF-7/Adr-5 cells using act D treatments as described in Experimental Procedures. First, the optimal concentration of act D that inhibits 95% of RNA synthesis was determined as described at 60 μg/mL (Figure 4A). The cells were then treated with act D (60 μg/mL) for 0, 8, and 16 h, and β_2m mRNA (Figure 4B, lanes 2−4) or rRNA (Figure 4B, lanes 5−7) levels were measured by RT-PCR. These results show that the half-life of β_2m mRNA in MCF-7/Adr-5 cells is about 10 h (Figure 4B), similar to that of MCF-7 cells (Figure 2C). To investigate whether the differences in the β_2m mRNA levels in MCF-7 and MCF-7/Adr-5 cells is due to transcriptional or post-transcriptional control, we performed nuclear run-on analysis as described. As seen in Figure 4C, the rate of β_2m transcription in MCF-7/Adr-5 cells is decreased about 30% compared to that in MCF-7 (panels 2 and 1, respectively).

To explore the possible involvement of a negative regulatory protein in β_2m gene expression, we inhibited protein synthesis using CHX as described in Experimental Procedures. First, the optimal concentrations of CHX that inhibit 95% of protein synthesis in MCF-7, MCF-7/Adr, and MCF-7/Adr-5 cells were determined by measuring [^{35}S]methionine incorporation into TCA precipitable macromolecules, and calculated at 200, 800, and 400 μg/mL, respectively (Figure 5A). Then, following 12 h of CHX treatment at its optimal concentration, induction of β_2m gene expression (about 50%) was detected in MCF-7 cells by RT-PCR (Figure 5B, lanes 1−4). β_2m mRNA was undetectable following protein

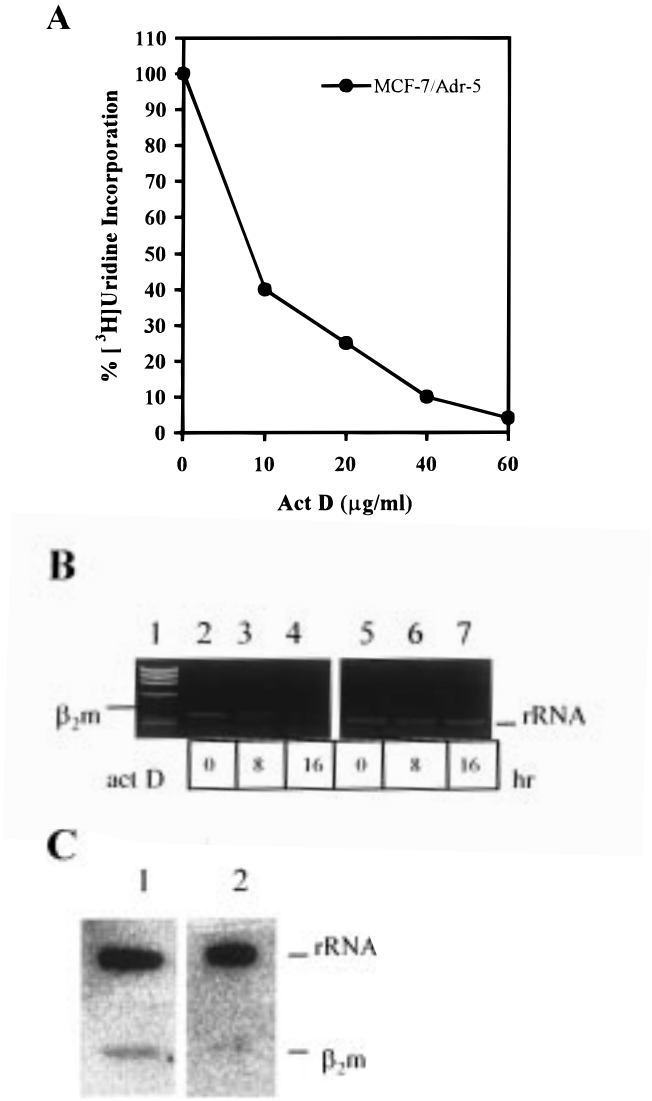

FIGURE 4: Analysis of the transcriptional activity of the β_2m gene in MCF-7/Adr-5 cells. (A) The act D concentration that inhibits 95% of the RNA synthesis in MCF-7/Adr-5 cells were determined as described. Cells were grown in the absence or presence of increasing concentrations of act D for 2 h and then pulsed with 4 μCi/mL [^{3}H]uridine for 2 h. The TCA-precipitated [^{3}H]uridine was measured by scintillation counting. Untreated cells were used as controls. Duplicate determinants were used for each treatment. (B) The stability of β_2m mRNA (lanes 2−4) and rRNA (lanes 5−7) in MCF-7/Adr-5 cells, grown in the presence of 60 μg/mL of act D for 0, 8, and 16 h were determined by RT-PCR as described. The incubation time is indicated on the bottom of the gel. (C) The transcriptional activity of β_2m in MCF-7 (panel 1) and MCF-7/Adr-5 (panel 2) cells was determined by nuclear run-on assay as described. The membranes containing the 413 bp β_2m (lower bands) and 345 bp rRNA (upper bands) fragments were hybridized with the equal counts of labeled transcripts for 72 h at 50 °C as described. The results shown are representative of two independent experiments.

synthesis inhibition for 0−32 h in MCF-7/Adr cells (Figure 5C, lanes 1−4). However, in MCF-7/Adr-5 cells, a 50% increase in β_2m mRNA expression was detected after 32 h of CHX treatment (Figure 5D, lanes 1−4). β-actin mRNA levels remained unaffected by the inhibition of protein synthesis in these cells for 0−32 h (Figure 5B-D, lanes 5−8). Taken together, these results indicate that transcriptional regulation by a labile protein might be responsible for the alteration of β_2m mRNA expression in MCF-7/Adr-5 cells.

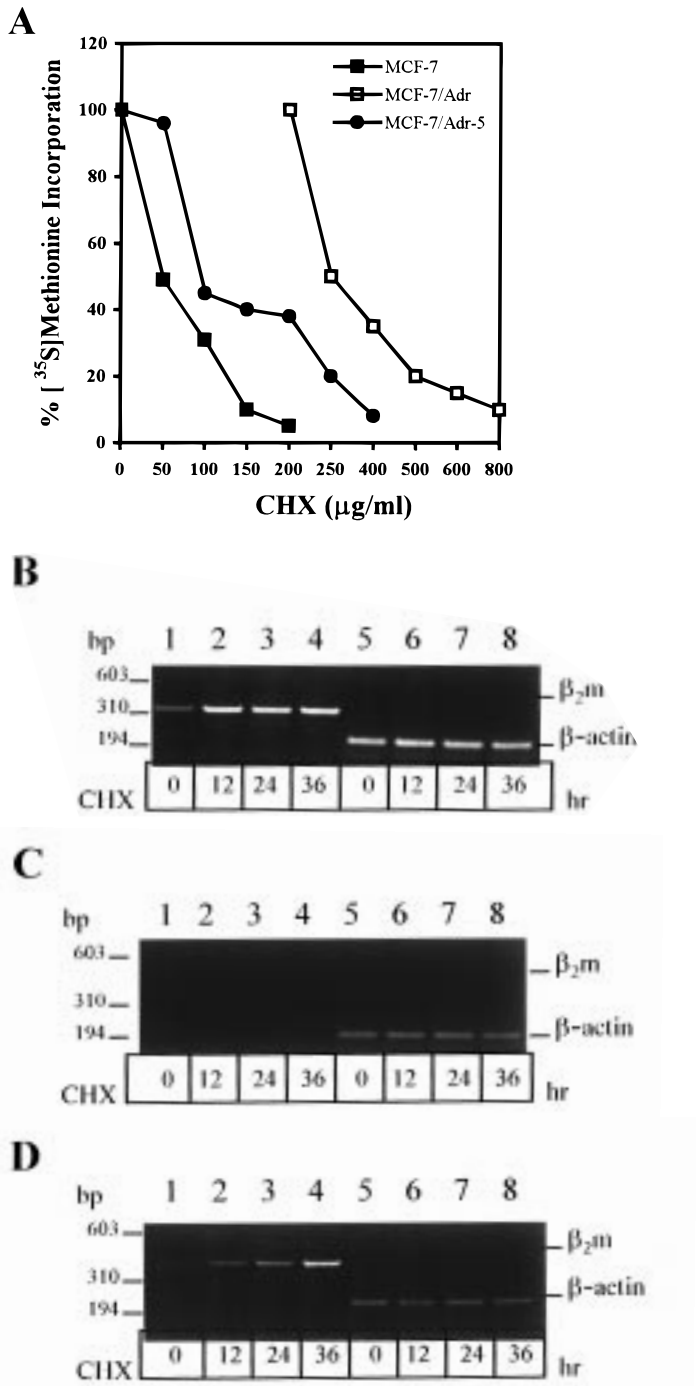

FIGURE 5: The analysis of β_2m mRNA levels after CHX treatments. (A) The CHX concentrations that inhibit 95% of protein synthesis in MCF-7, MCF-7/Adr, and MCF-7/Adr-5 were determined as described in Experimental Procedures. Cells were grown in the absence or presence of increasing concentration of CHX for 2 h and then pulsed with 4 μCi/mL [^{35}S]methionine. The TCA-precipitated labeled macromolecules were then measured by scintillation counting. Untreated cells were used as controls. Duplicate determinants were used for each treatment. The β_2m mRNA levels (lanes 1−4) and β-actin levels (lanes 5−8) following treatment with the optimal CHX concentrations were determined in MCF-7 (B), MCF-7/Adr (C), and MCF-7/Adr-5 (D) cells at various time points (0−32 h) by RT-PCR. The incubation time is indicated on the bottom of the gels. Molecular weight markers are indicated on the left. The results shown are representative of two independent experiments.

Protein and mRNA Levels of β_2m in Drug-Resistant MCF-7 Sublines. To investigate whether the inhibition of

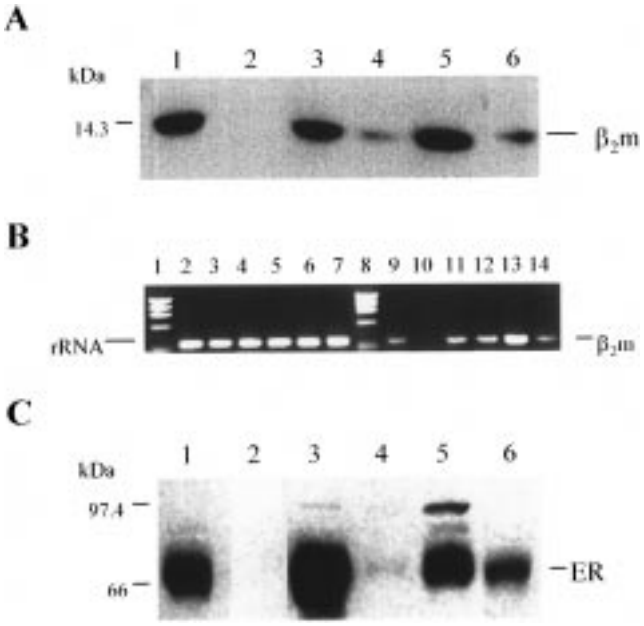

FIGURE 6: Analysis of β_2m and ER expression in drug-resistant MCF-7 sublines. (A) β_2m protein levels in MCF-7 (lane 1) and its drug-resistant sublines MCF-7/Adr, MCF-7/CDDP, MCF-7/AdVp, MCF-7/Tx200, and MCF-7/mAMSA (lanes 2−6, respectively) were determined by Western blotting using the rabbit polyclonal anti-β_2m antibody. Protein samples (80 μg/lane) were resolved on 12−17% SDS−polyacrylamide gels and then transferred to Immobilon membranes prior to Western blotting. Molecular weight markers are indicated on the left. (B) β_2m mRNA levels in MCF-7, MCF-7/Adr, CDDP, AdVp, Tx200, and mAMSA were detected by RT-PCR (lanes 9−14, respectively) and normalized to their rRNA levels (lanes 2−7, respectively). The amplified fragments (357 bp β_2m cDNA and 345 bp rRNA fragments) were run on 2% agarose gels and visualized by EtBr staining. The *Hae*III-cut ΦX174 DNA fragments were used as molecular weight markers (lanes 1 and 8). (C) ER protein levels in MCF-7, MCF-7/Adr, CDDP, AdVp, Tx200, and mAMSA cells (lanes 1−6, respectively) were determined by Western blotting using the rabbit polyclonal anti-ER (HC-20) antibody as described above. Molecular weight markers are indicated on the left. The results shown are representative of two independent experiments.

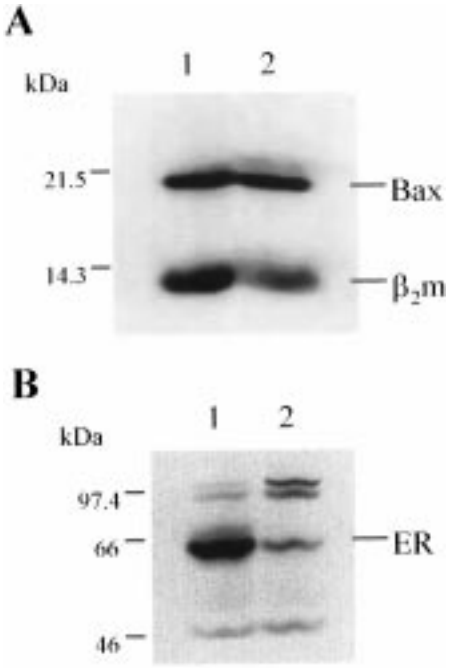

FIGURE 7: β_2m levels in T-47D and T-47D/Adr-4 cells. The protein levels of β_2m (A) and ER (B) in T-47D and T-47D/Adr-4 cells (lanes 1 and 2, respectively) were determined by Western blotting as described. Bax protein levels were used as internal controls (Figure 6A, lanes 1−2, upper band). Molecular weight markers are indicated on the left. The results shown are representative of two independent experiments.

β_2m is also associated with the resistance to other chemotherapeutic agents in MCF-7 cells, we examined several MCF-7 drug-resistant sublines. Western blot analysis of β_2m in MCF-7 and its sublines resistant to cisplatin (CDDP), doxorubicin-verapamil (AdVp), taxol (Tx), and mAMSA is shown in Figure 6A. As seen in Figure 6A, MCF-7 cells which are selected for resistance to mAMSA and AdVp had 75 and 85% decreased β_2m levels (lanes 6 and 4), respectively, compared to sensitive MCF-7 cells (lane 1). However, β_2m protein levels did not change in MCF-7/Tx200 and MCF-7/CDDP cells (lanes 5 and 3). β_2m was not detectable in MCF-7/Adr cells (lane 2). To correlate these data with the transcriptional levels of β_2m in these cells, we performed RT-PCR as described in Experimental Procedures. Figure 6B shows the RT-PCR analysis of β_2m in the cells above. The mRNA levels of β_2m decreased about 20% in MCF-7/mAMSA cells (lane 14) compared to controls (lane 9). β_2m mRNA levels in MCF-7/CDDP and MCF-7/AdVp cells were comparable to that of MCF-7 cells (lanes 11, 12, and 9, respectively). However, β_2m mRNA was not detectable in MCF-7/Adr cells (lane 10). Interestingly, its mRNA levels increased slightly, about 2-fold, in MCF-7/Tx200 cells (Figure 6B, lane 13). The β_2m mRNA levels in these cells were normalized against their rRNA levels as measured by RT-PCR (Figure 6B, lanes 2−7).

Our results showed decreased levels of β_2m and ER in MCF-7/Adr-5 compared to MCF-7 cells. Therefore, we compared the levels of ER and β_2m in several drug-resistant MCF-7 sublines. Interestingly, the ER levels in MCF-7/mAMSA and MCF-7/AdVp were 44 and 90% lower (Figure 5C, lanes 6 and 4, respectively) than that of MCF-7 cells (lane 1). However, the ER levels in MCF-7/Tx200 and MCF-7 were comparable (Figure 6C, lanes 5 and 1, respectively), and the expression of the ER level is increased about 1.7-fold in MCF-7/CDDP, compared to MCF-7 cells (Figure 6C, lanes 3 and 1, respectively). The MCF-7/Adr cell line, which is reported to be ER-negative (*12*), was used as a negative control (Figure 5C, lane 2). Taken together, these results suggest that the loss or decreased expression of β_2m during the development of drug resistance in MCF-7 cells can be initiated by drugs such as DOX, mAMSA, or AdVp, but not by taxol or cisplatin. The decreased level of ER in these cells seems to correlate with lost or decreased β_2m levels in MCF-7/Adr, MCF-7/AdVp, and MCF-7/mAMSA.

To determine whether β_2m inhibition is detectable in other drug-resistant human breast cancer cell lines, we measured β_2m levels by Western blotting using ER-negative MDA-MB-231 and ER-positive T-47D cells and their DOX-resistant derivatives, MDA-MB-231A1 and T-47D/Adr-4 (which are 4-fold more resistant to DOX than T-47D cells). No changes in β_2m levels were observed in MDA-MB-231A1 compared to its parental cell line (data not shown). However, β_2m expression was decreased about 55% in T-47D/Adr-4 cells compared to T-47D cells when normalized to their Bax levels (Figure 7A, lanes 2 and 1, respectively).

Interestingly, a similar alteration in ER expression (about 60% decrease) in T-47D/Adr-4 cells was also detected compared to T-47D cells (Figure 7B, lanes 2 and 1, respectively). These results show that the decreased expression of β_2m and ER are positively correlated, and that estrogen or ER may be involved in the regulation of β_2m expression in ER-positive human breast cancer cell lines that are resistant to DOX. To further investigate whether the loss or decreased expression of β_2m is detectable in other nonbreast origin cell lines and their drug resistant sublines, we performed Western blot analysis using the human leukemia cell line HL-60 and its DOX-resistant subline HL-60/Adr (*25*), and the human epidermoid carcinoma cell line KB-3-1 and its sublines resistant to DOX, colchicine, and vinblastine (*26*). No changes in β_2m were detected in any of the KB drug-resistant variants compared to its sensitive parental KB-3-1 cell line as revealed by immunoblotting (data not shown). However, β_2m expression was significantly reduced in HL-60/Adr compared to HL-60 cells (unpublished data). Taken together, these results suggest that (1) the loss or decreased expression of β_2m during the development of drug resistance in ER-positive breast cancer cell lines correlates with the loss of ER and can be initiated by developing drug resistance to drugs including DOX, mAMSA, or AdVp, but not by taxol or cisplatin; and (2) decreased cell surface expression of β_2m may also be detectable in some other drug-resistant cell lines.

Partial Inhibition of β_2m by Antisense RNA in MCF-7 Cells. To determine the significance of altered β_2m expression and its relationship with the drug-resistance phenotype, we partially inhibited β_2m expression using antisense RNA. A 357 bp human β_2m cDNA fragment containing a 5′-end *Bam*HI site and a 3′-end *Hind*III site, generated by RT-PCR, was cloned into the mammalian expression vector pcDNA3 in the antisense orientation as described in Experimental Procedures. The antisense orientation of the cloned β_2m fragment in pcDNA3 was also confirmed by restriction enzyme analysis (data not shown), and the final vector is designated as pcDNA3-αβ_2m. The stable transfectants of MCF-7 cells containing pcDNA3 (control) or pcDNA3-αβ_2m plasmids were selected in 1−2 mg/mL of Geneticin (G418), then β_2m levels were measured by Western blotting. The MCF-7 cells transfected with pcDNA3-αβ_2m, in which decreased β_2m levels were detected compared to controls, were further selected and expanded for cell survival assays. Figure 8A shows a representative Western blot analysis containing MCF-7 cells transfected with pcDNA3-αβ_2m that exhibit about 60% reduction in β_2m levels (lower bands) compared to controls, while their Bax levels (upper bands) are similar (lanes 2 and 1, respectively). Control transfectants (MCF-7/pcDNA3) and the MCF-7 parental cell line demonstrated similar β_2m expression.

Cell Survival Characteristics of MCF-7/pcDNA3 and MCF-7/pcDNA3-αβ_2m. The cell survival assays using MCF-7/pcDNA3 and MCF-7/pcDNA3-αβ_2m with various concentrations of DOX, taxol, and vincristine, as described in Experimental Procedures, revealed that about 60% reduction in β_2m expression by antisense RNA in MCF-7/pcDNA3-αβ_2m resulted in approximately 3-, 2-, and 1.5-fold decreased sensitivity to DOX, VP-16, and mAMSA, respectively, but not to taxol or vincristine (Table 1). MCF-7/Adr cells display high resistance to these drugs compared to MCF-7 cells (Table 1). These results were confirmed in experiments using three different MCF-7/pcDNA3-αβ_2m clones, each of which had about 60% less β_2m, selected from three independent transfections.

Moreover, to investigate whether the addition of exogenous purified β_2m protein affects DOX resistance, we incubated MCF-7/pcDNA3 and MCF-7/pcDNA3-αβ_2m cells with or without 5 and 10 μg/mL of β_2m protein in the absence or presence of increasing concentrations of DOX in cell survival assays as described in Experimental Procedures. The IC_{50} concentrations of DOX in these cells with or without exogenous β_2m protein were determined from cell survival plots, and the results are presented in Table 2. As seen in Table 2, the addition of 5 and 10 μg/mL of β_2m protein increased the DOX sensitivity about 2.1- and 3.7-fold in MCF-7/pcDNA, and 1.8- and 2.1-fold in MCF-7/pcDNA3-αβ_2m cells, respectively.

To investigate the mechanisms by which the inhibition of β_2m might exert its effects on drug resistance, we analyzed the growth characteristics of antisense β_2m transfectants as described in Experimental Procedures. As shown in Figure 8B, the growth rate of MCF-7/pcDNA3-αβ_2m was slower than in MCF-7/pcDNA3 cells; however, ER expression was similar in both transfectants (data not shown). In Figure 8B, it is also shown that the growth rate of MCF-7/Adr cells is significantly reduced compared to MCF-7 cells. Since the growth rate of the MCF-7/pcDNA3-αβ_2m transfectants is slower than that of MCF-7/pcDNA3 or MCF-7 cells, questions arise as to whether the resistance levels to DOX, VP-16, and mAMSA in the antisense β_2m transfectants may reflect longer doubling times and possibly lower Topo IIα levels, and/or alterations in the expression of anti-apoptotic proteins such as Bcl-2, and alterations in their cell cycle profile. However, Western blot analysis of Bcl-2 and Topo IIα levels showed that their expression was unchanged in these cells (Figure 8C and D, lanes 1 and 2, respectively). Moreover, analysis of the cell cycle using flow cytometry (Table 3) revealed that the percentage of G_2/M-phase cells in MCF-7/pcDNA3-αβ_2m (95%) was significantly higher than that of MCF-7/pcDNA3 transfectants (4%). As shown in Table 3, the percentages of cells in S-phase and G_0/G_1-phase in MCF-7/pcDNA3-αβ_2m (1.4% and 3%, respectively) were much lower than in MCF-7/pcDNA3 transfectants (42% and 54%, respectively).

DISCUSSION

Molecular Basis of β_2m Loss in MCF-7/Adr Cells. In this report we demonstrate by Western blot analysis that β_2m, a component of the Class I MHC protein, is completely lost in drug-resistant MCF-7/Adr cells but not in sensitive MCF-7 cells. This was confirmed by RT-PCR analysis in which β_2m mRNA was not detectable in MCF-7/Adr cells. Interestingly, in our nuclear run-on transcription assay, human β_2m cDNA was detected by radioactively labeled transcripts of both cell lines. The same results were obtained after high stringency washes following hybridizations, which suggests that the signals are specific to β_2m and that β_2m is transcriptionally active in MCF-7 and MCF-7/Adr cells. In addition, we determined the half-life of β_2m mRNA by act D treatment and found that it is about 10 h in MCF-7 cells. As a control, we measured its half-life in KB-3-1 epidermoid

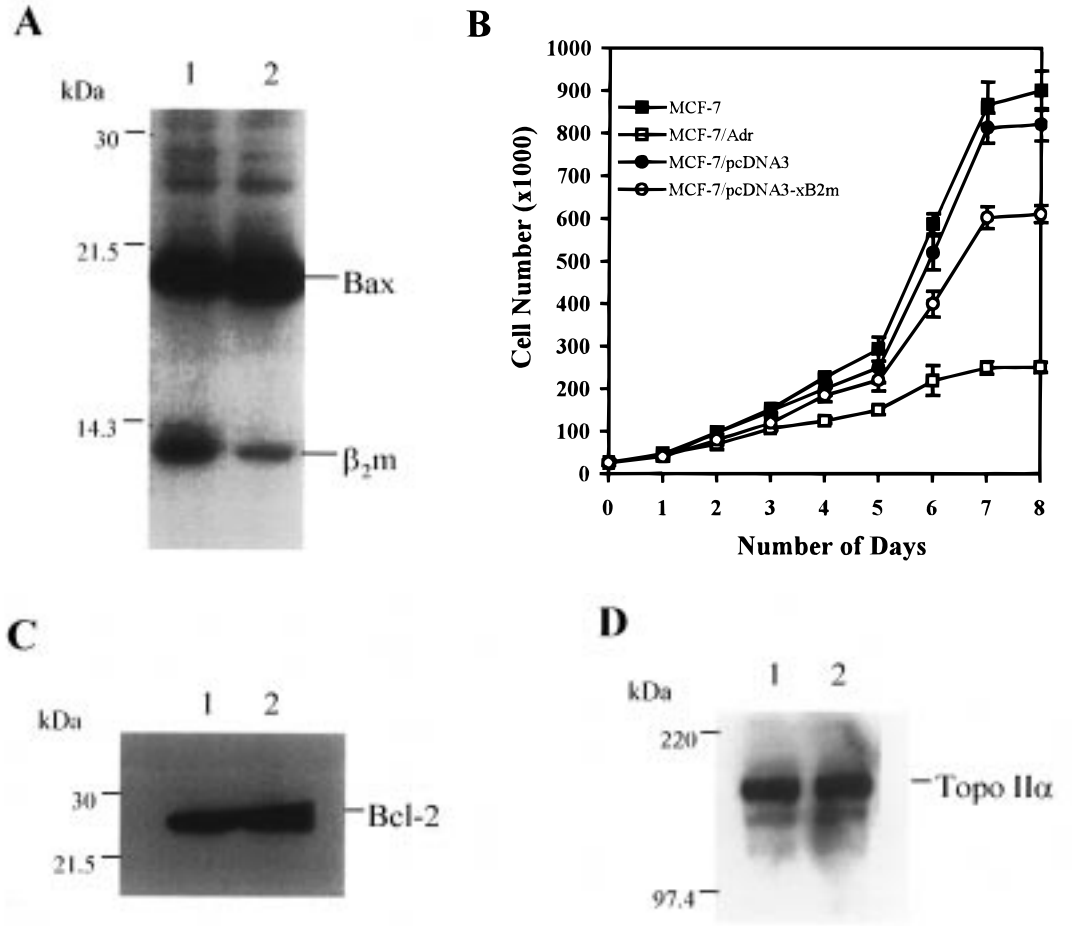

FIGURE 8: Partial inhibition of β_2m expression by antisense RNA and its involvement in the decreased drug sensitivity of MCF-7 cells. (A) β_2m protein expression in MCF-7/pcDNA3 (lane 1) and MCF-7/pcDNA3-αβ_2m (lane 2) were determined by Western blot analysis using rabbit polyclonal anti-β_2m antibody. The protein samples (80 μg/lane) were separated on 12−17% SDS−polyacrylamide gels and transferred to Immobilon membranes prior to Western blotting. Molecular weight markers are indicated on the left. The autoradiogram above is representative of three independent transfections in which approximately a 60% reduction in β_2m levels were observed. Bax protein detected by rabbit polyclonal anti-Bax antibody was used as an internal control (upper band in lanes 1 and 2) to confirm equal loading of the samples. (B) The growth characteristics of MCF-7 (■), MCF-7/Adr (□), MCF-7/pcDNA3 (●), and MCF-7/pcDNA3-αβ_2m (○) were determined as described in Experimental Procedures. The cells were seeded into 24-well plates, and the number of viable cells was determined using a Coulter counter after trypsinization every 24 h for 8 days. Triplicate determinants were used for each point. Error bars represent the standard deviation. The protein levels of Bcl-2 (C) and Topo IIα (D) in MCF-7/pcDNA3 (lane 1) and MCF-7/pcDNA3-αβ_2m (lane 2) were determined by Western blotting as described in Experimental Procedures. Molecular weight markers are indicated on the left. The results shown are representative of at least two independent experiments.

Table 2: Cell Survival Analysis Following Doxorubicin Treatments in the Presence or Absence of Exogenous Purified β_2m Protein[a]

	IC_{50} (mM) [relative sensitivity]		
cell line	DOX	DOX + 5 μg/mL β_2m	DOX + 10 μg/mL β_2m
MCF-7/pcDNA3	0.0033 [1.0]	0.0016 [2.1]	0.0009 [3.7]
MCF-7/pcDNA3-αβ_2m	0.0065 [1.0]	0.0035 [1.8]	0.0031 [2.1]

[a] Cells were treated with various concentrations of DOX at 37 °C in 5% CO_2 for 96 h in 24-well plates in the absence or presence of 5 and 10 μg/mL purified β_2m (Sigma). Cell survival after each treatment was determined by MTT cytotoxicity assay as described in Experimental Procedures, and the DOX concentrations that reduced cell survival by 50% (IC_{50}) were calculated from cell survival plots. The average of triplicate determinants were used for the analysis. Standard deviations for IC_{50} values in each treatment in MTT assays were between 0.00005 and 0.00008.

Table 3: Cell Cycle Distribution of MCF-7/pcDNA3 and MCF-7/pcDNA3-αβ_2m Cells[a]

cell line	G_0/G_1-phase	S-phase	G_2/M-phase
MCF-7/pcDNA3	54 (1.6)	42 (2.5)	4 (0.1)
MCF-7/pcDNA3-αβ_2m	3 (0.6)	1.4 (0.5)	95 (3.2)

[a] Cells were stained with propidium iodine and analyzed for cell cycle distribution by flow cytometry as described in Experimental Procedures. Duplicate samples were used in two independent experiments for each cell line. Standard deviations are presented in parentheses.

carcinoma cells with HeLa origin, and the result was comparable to that of MCF-7 (data not shown). These results are very similar to that of HeLa cells in which the half-life of β_2m mRNA is reported as 10.5 h (*27*). However, no β_2m expression was detected in MCF-7/Adr cells following act D treatment. These results and the presence of β_2m transcriptional activity suggest that its mRNA is rapidly degraded after synthesis in MCF-7/Adr cells, indicating that the loss of β_2m expression in MCF-7/Adr cells is controlled by post-transcriptional mechanisms. The regulation of β_2m at the RNA level has been shown to be a major mechanism in adult human organs and during development by Drezen et al. (*1*). Similarly, it has been reported that human Daudi

lymphoid cells produce β_2m mRNA that is inactive in protein synthesis (*28*). In later studies, Rosa et al. (*29*) have shown that the expression of β_2m in human Daudi cells is inhibited by a mutated initiation codon. The mechanism(s) of the rapid degradation of β_2m mRNA after its synthesis in MCF-7/Adr cells are not known. However, it is known that mutations in the polyadenylation signal can cause rapid degradation of mRNAs (*30*, *31*). Whether such mutations in the polyadenylation signal of β_2m in these cells might be involved in deregulating β_2m transcription remains to be found.

Alterations of β_2m Expression in Drug-Resistant MCF-7 Sublines. Our results demonstrate that β_2m is completely abolished in the highly resistant MCF-7 subline MCF-7/Adr. To determine whether β_2m expression is also altered in MCF-7 cells with a low level of resistance, we developed a new subline, MCF-7/Adr-5, which expresses approximately 5-fold resistance to doxorubicin. β_2m expression in these cells was decreased about 68% compared to MCF-7 cells as determined by Western blotting. Analysis of β_2m by RT-PCR in the cells above showed that its mRNA is decreased about 35% compared to MCF-7 cells. The difference between the measured levels of β_2m in these cells by Western blotting (about 68% reduction) and RT-PCR (about 35% reduction) suggests that β_2m expression in this cell line might be regulated both at the RNA and protein levels. Moreover, to investigate the mechanisms of altered β_2m mRNA expression, we inhibited protein synthesis using CHX in MCF-7, MCF-7/Adr, and MCF-7/Adr-5 cells. The β_2m mRNA remained undetectable following CHX treatments in MCF-7/Adr cells. However, the induction of β_2m mRNA levels after 12 h in MCF-7 and 36 h in MCF-7/Adr-5 cells, and its similar half-life in each cell line, suggest that altered levels of a negative regulatory protein might be involved in the decreased transcription of β_2m; however, the identity of this labile protein remains unknown. This was supported by our nuclear run-on transcription analysis, showing that the rate of β_2m transcription was decreased about 30% in MCF-7/Adr-5 compared to MCF-7 cells. Our results, therefore, reveal that a decreased level of β_2m mRNA expression can be initiated in MCF-7 cells expressing a low level of doxorubicin resistance at the transcriptional level. The control of β_2m expression at the protein level in MCF-7/Adr-5 cells, however, remains to be determined. Similar results were obtained when we examined β_2m status in MCF-7 sublines resistant to various drugs by Western blotting. Our results show that the inhibition of β_2m is not restricted to DOX but can also be detected in cells independently selected for resistance to mAMSA and AdVp. Furthermore, RT-PCR analysis demonstrates that the inhibition of β_2m is regulated mainly at the protein level in these cells, since the differences in β_2m mRNA levels compared to controls were not as striking as the differences in β_2m protein levels. Taxol and cisplatin did not affect the β_2m levels in these cells.

The mechanism of deregulation of β_2m expression by DOX is not known. We (*12*) and others (*11*) have shown that MCF-7/Adr cells lose their ER during MDR development. In this report, a decreased level of ER was also detected in MCF-7/Adr-5 cells compared to MCF-7 cells. Similarly, decreased levels of ER in cells resistant to mAMSA and AdVp seem to correlate with their decreased β_2m levels. Analysis of β_2m expression in the estrogen-independent human breast cancer cell line MDA-MB-231 and its DOX-resistant derivative MDA-MB-231A1 showed no changes in its protein levels, while the coordinated inhibition of β_2m and ER expression was detected in DOX-resistant T-47D/Adr-4 cells compared to sensitive T-47D cells. Our results suggest that ER and/or estrogen may control β_2m expression in MCF-7 and its DOX-, mAMSA-, and AdVp-resistant sublines, and this control is not limited to MCF-7 cells but can be detected in other ER-positive human breast cancer cells that express resistance to DOX. Moreover, the absence of the reported estrogen consensus element 5′-GGTCANNNTGACC-3′ (*32*) in the entire β_2m genomic DNA sequence (*33*), in both translated and untranslated regions, indicates that estrogen might be involved indirectly in regulating β_2m expression. Alternatively, the deregulation of both ER and β_2m expression in DOX-resistant cells may be controlled by the same factor(s) activated by drug treatment. In addition, we could not detect any P-gp expression in MCF-7/Adr-5 cells, suggesting that P-gp expression might be regulated by mechanisms which are related to a higher degree of DOX resistance, unlike decreases in β_2m, Topo IIα, and ER expression which seem to be controlled in the early stages of DOX resistance in MCF-7 cells. Undetectable P-gp levels in MCF-7/Adr-5 cells might also suggest that the low degree of drug resistance in these cells can be induced by non-P-gp-mediated resistance mechanisms such as decreased levels of Topo IIα and/or β_2m. In addition, it is not known whether the reduction of β_2m expression to undetectable levels in highly resistant MCF-7/Adr cells by post-transcriptional mechanisms is somewhat associated with P-gp expression.

Our analysis of β_2m levels in KB-3-1 cells and their drug-resistant sublines showed no detectable changes in total cellular β_2m expression, suggesting that β_2m is controlled differently in these cells than in ER-positive breast cancer cell lines. This is somewhat similar to clinical studies which reported the importance of β_2m levels in human breast tumors and melanomas (*8*, *9*), but not in renal carcinomas (*34*).

Partial Inhibition of β_2m Results in Decreased Sensitivity of MCF-7 Cells to Doxorubicin. The multidrug resistance phenotype in MCF-7/Adr cells is linked to several mechanisms, including overexpression of P-gp, glutathione S-transferase (GSPi), glutathione peroxidase (GSPx) (*35*), and mutated p53 (*16*). To show specifically whether the inhibition of β_2m contributes to drug resistance, we transfected MCF-7 cells with the antisense β_2m expression vector, rather than transfecting MCF-7/Adr cells with an expression vector containing a full-length β_2m cDNA. After transfecting MCF-7 cells with the β_2m antisense expression vector, only partial inhibition of β_2m expression (maximum inhibition of β_2m was about 60% compared to controls) was obtained in three independent transfections. Usually, inhibition of protein expression resulting from the use of an antisense expression vector is about 50−70% (*36*, *37*). Our cytotoxicity assays, following transfections with the β_2m antisense RNA expression vector, demonstrate that a 60% decrease in β_2m results in about 2−3-fold decreased sensitivity to DOX, VP-16, and mAMSA, but not to taxol or vincristine, in MCF-7 cells. This low degree of drug resistance as a result of decreased β_2m is comparable to the degree of resistance usually observed in clinical tumor samples (*38*).

In addition, we report here for the first time that adding exogenous β_2m protein to the growth media at 5 and 10 μg/mL, near its physiological serum levels in humans (*7*), results in 2−3-fold increased DOX sensitivity in MCF-7 antisense β_2m transfectants and controls. Therefore, these results support the idea that the altered β_2m expression is involved in the drug-resistant phenotype in these cells. Our results are in agreement with clinical studies in which β_2m expression levels are positively correlated with response and survival during chemotherapy in patients with breast cancer (*39*). Reduced β_2m expression has been observed in many solid tumors including kidney, germ cell testicular, prostate, gastric, and colon cancer, and it is associated with poor survival in bladder cancer (*40*). In addition, cell surface expression levels of β_2m are suggested as a significant prognostic indicator in human lymphoma (*41*). The mechanism(s) by which the inhibition of β_2m decreases the sensitivity of MCF-7 cells selectively to DOX, VP-16, and mAMSA, drugs known to act on S-phase cells (*42*), need to be determined. It is possible that the growth inhibition of cells by decreased levels of β_2m, as shown in Figure 6C, might alter the cellular functions of these drugs and result in decreased cellular sensitivity to these DNA interacting agents. This is similar to a recent report (*43*) that Bcl-2 contributes to cell survival by diminishing the rate of cell proliferation. In fact, Wosikowski et al. (*13*) reported that a decreased growth rate is frequently observed in drug-resistant human breast cancer sublines. Cell cycle arrest, usually from delays in the G_1−S and G_2−M transitions of the cell cycle, is reportedly necessary to repair DNA damage (*13*), and cells with unrepaired DNA are directed to apoptosis. Therefore, inhibition of growth might decrease cell sensitivity to the DNA damaging function of the drugs. Interestingly, analysis of the cell cycle profiles of MCF-7 antisense β_2m transfectants by flow cytometry revealed that the partial inhibition of its expression results in a significant increase in G_2/M and decrease in S-phase cell populations compared to controls. These results suggest that β_2m inhibition might exert its effects on the development of drug resistance by arresting cells in G_2/M of the cell cycle. This is somewhat similar to overproduction of retinoblastoma (Rb) protein after the G_1/S boundary causing G_2 arrest (*44*), and human immunodeficiency virus type 1 viral protein (Vpr) which arrests cells in G_2 and delays apoptosis of infected cells to produce more virus (*45*). The role of β_2m in the cell cycle, however, needs further in-depth investigation.

Since the immunological function of β_2m is well-known in the recognition of MHC class I antigens by cytotoxic T cells, altered expression of β_2m may cause deficiencies in immune surveillance (*46*). In fact, Kaklamanis et al. (*8*) observed a higher incidence of HLA class I loss with complete loss of β_2m and TAP-1 expression in lymph node metastases, suggesting that HLA class I antigens could have an important role in the metastatic evolution of breast cancer. The MHC class I heavy chain and *TAP*-1 genes are located on a different chromosome than β_2m, suggesting that their expression might be regulated by a common factor (*8*). Similar results were also reported by Restifo et al. (*9*), in which the loss of β_2m in human metastatic melanomas after immunotherapy was correlated with the tumor aggressiveness and suggested as a possible mechanism of immunoresistance. Therefore, our results support the idea that the prognostic significance of β_2m as an indicator of chemoresistance in some types of tumor cells that can escape immunosurveillance should be further investigated.

ACKNOWLEDGMENT

We thank Drs. Susan E. Bates, Melvin S. Center, Kenneth H. Cowan, Michael W. DeGregorio, and Igor B. Roninson for providing us with the various different human cancer cell lines and their drug-resistant sublines. The direct sequencing of β_2m cDNA generated by RT-PCR was performed by the DNA Sequencing Facility of Medical University of South Carolina, Charleston, SC.

REFERENCES

1. Drezen, J. M., Babinet, C., and Morello, D. (1993) *J. Immunol. 150*, 2805−2813.
2. Dixon, B., Stet, R. J. M., van Erp, S. H. M., and Pohajdak, B. (1993) *Immunogenetics 38*, 27−34.
3. D'Urso, C. M., Wang, Z., Cao, Y., Tatake, R., Zeff, R. A., and Ferrone, S. (1991) *J. Clin. Invest. 87*, 284−292.
4. York, A. I., and Rock, K. L. (1996) *Annu. Rev. Immunol. 14*, 369−396.
5. Kristoffersen, E. K., and Matre, R. (1996) *Eur. J. Immunol. 26*, 1668−1671.
6. Santos, M., Schilham, M. W., Rademakers, L. H., Marx, J. J., de Sousa, M., and Clevers, H. (1996) *J. Exp. Med. 184*, 1975−1985.
7. Rowley, D. R., Dang, T. D., McBride, L., Gerdes, M. J., Lu, B., and Larsen, M. (1995) *Cancer Res. 55*, 781−786.
8. Kaklamanis, L., Leek, R., Koukourakis, M., Gatter, K. C., and Harris, A. L. (1995) *Cancer Res. 55*, 5191−5194.
9. Restifo, N. P., Marincola, F. M., Kawakami, Y., Taubenberger, J., Yannelli, J. R., and Rosenberg, S. A. (1996) *J. Natl. Cancer Inst. 88*, 100−108.
10. Scaddan, P. B., and Dufresne, M. J. (1993) *Invasion Metastasis 13*, 301−313.
11. Vickers, P. J., Dufresne, M. J., and Cowan, K. H. (1989) *Mol. Endocrinol. 3*, 157−164.
12. Ogretmen, B., and Safa, A. R. (1996) *Int. J. Cancer 67*, 608−614.
13. Wosikowski, K., Regis, J. T., Robey, R. W., Alvarez, M., Buters, J. T. M., Gudas, J. M., and Bates, S. E. (1995) *Cell Growth Differ. 6*, 1395−1403.
14. Fleming, G. F., Amato, G. M., Agresti, M., and Safa, A. R. (1992) *Cancer Chemother. Pharmacol. 29*, 445−449.
15. Catapano, C. V., Carbone, G. M. R., Pisani, F., Qiu, J., and Fernandes, D. J. (1997) *Biochemistry 36*, 5739−5748.
16. Ogretmen, B., and Safa, A. R. (1997) *Oncogene 14*, 499−506.
17. Qiu, J., Catapano, C. V., and Fernandes, D. J. (1996) *Biochemistry 35*, 16354−16360.
18. Ogretmen, B., and Safa, A. R. (1995) *BioTechniques 19*, 374−376.
19. Ogretmen, B., Ratacjzak, H., Kats, A., Stark, B. C., and Gendel, S. M. (1993) *BioTechniques 14*, 932−935.
20. Noonan, K. E., Beck, C., Holzmayer, T. A., Chin, J. E., Wunder, J. S., Andrulis, I. L., Gazdar, A. F., Willmann, C. L., Griffith, B., Von Hoff, D. D., and Roninson, I. B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7160−7164.
21. Groudine, M., Peretz, M., and Weintraub, H. (1981) *Mol. Cell. Biol. 1*, 281−288.
22. Greenberg, M. E., and Ziff, E. B. (1984) *Nature 311*, 433−437.
23. Gant, T. W., Silverman, J. A., and Thorgeirsson, S. S. (1992) *Nucleic Acids Res. 20*, 2841−2846.
24. Muller, C., Goubin, F., Ferrandis, E., Cornil-Schwartz, I., Bailly, J. D., Bordier, C., Benard, J., Sikic, B. I., and Laurent, G. (1995) *Mol. Pharmacol. 47*, 51−56.
25. Marquardt, D., McCrone, S., and Center, M. S. (1990) *Cancer Res. 50*, 1426−1430.

26. Safa, A. R., Agresti, M., Bryk, D., and Tamai, I. (1994) *Biochemistry 33*, 256−265.
27. Cosgrove, D. E., and Cox, G. S. (1990) *Arch. Biochem. Biophys. 280*, 95−102.
28. Preval, C., and Mach, B. (1983) *Immunogenetics 17*, 133−140.
29. Rosa, F., Berissi, H., Weissenbach, J., Maroteaux, L., Fellous, M., and Revel, M. (1983) *EMBO J. 2*, 239−243.
30. Bernstein, P., and Ross, J. (1989) *Mol. Cell. Biol. 9*, 659−670.
31. Jackson, R. J., and Standard, N. (1990) *Cell 62*, 15−24.
32. Teh, M., and Hui, K. M. (1989) *J. Immunogenet. 16*, 397−405.
33. Gussow, D., Rein, R., Ginjaar, R., Hochstenbach, F., Seemann, G., Kottman, A., and Ploegh, H. L. (1987) *J. Immunol. 139*, 3132−3138.
34. Buszello, H., and Ackermann, R. (1992) *Eur. Urol. 21*, 70−74.
35. Fairchild, C. R., Moscow, J. A., O'Brien, E. E., and Cowan, K. H. (1990) *Mol. Pharmacol. 37*, 801−809.
36. Ohga, T., Koike, K., Ono, M., Makino, Y., Itagaki, Y., Tanimoto, M., Kuwlanesano, M., and Kohno, K. (1996) *Cancer Res. 56*, 4224−4228.
37. van der Krol, A. R., Mol, J. N. M., and Stuitje, A. R. (1988) *BioTechniques 6*, 958−976.
38. Giaccone, G., and Pinedo, H. M. (1996) In *Multidrug Resistance in Cancer Cells* (Gupta, S., and Tsuruo, T., Eds.) pp 473−492, John Wiley and Sons Ltd., New York.
39. Klein, T., Levin, I., Niska, A., Koren, R., Gal, R., Schacter, J., Kfir, B., Narinski, R., Warchaizer, S., and Klein, B. (1996) *Eur. J. Immunogenet. 23*, 417−423.
40. Klein, B., Levin, I., and Klein, T. (1996) *Isr. J. Med. Sci. 32*, 1238−1243.
41. Swan, F., Ordonez, N., Manning, J., Rodriguez, M. A., Redman, J., Velasquez, W., Hagemeister, F., McLaughlin, P., and Cabanillas, F. (1988) *Blood 72*, 258a.
42. Gorczyca, W., Gong, J., Ardelt, B., Traganos, F., & Darzynkiewicz, Z. (1993) *Cancer Res. 53*, 3186−3192.
43. Borner, C. (1996) *J. Biol. Chem. 271*, 12695−12698.
44. Karantza, V., Maroo, A., Fay, D., and Sedivy, J. M. (1993) *Mol. Cell. Biol. 13*, 6640−6652.
45. He, J., Choe, S., Walker, R., Di Marzio, P., Morgan, D. O., and Landau, N. R. (1995) *J. Virol. 69*, 6705−6711.
46. Sunday, M. E., Isselbacher, K. J., Gattoni-Celli, S., and Willett, C. G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4700−4704.

BI980573C