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Expression of Topoisomerases II α and β in Chinese Hamster Lung Cells Resistant to Topoisomerase II Inhibitors

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SUMMARY

Chinese hamster lung cells resistant to 9-OH-ellipticine, i.e., DC-3F/9-OH-E cells, are several hundredfold resistant to DNA topoisomerase II inhibitors. According to previous studies, this resistance is associated with reduced topoisomerase II activity (about 4-fold) and decreased capacity of the topoisomerase II inhibitors to induce stabilization of the cleavable complex (about 10-fold). In the present work, an antibody was raised against a fragment of human topoisomerase II α. This antibody, which recognizes both isoforms, was used to determine the amounts of topoisomerases II α and β in the sensitive and resistant cells. Northern and immunoblot analyses showed that (i) in the parental DC-3F cells the α enzyme is about 20-fold more abundant than the β enzyme and the enzyme isoforms undergo reciprocal regulation during the cell growth phases, with the expression of the α enzyme dropping at the plateau phase while the expression of the β enzyme increases, and (ii) in the resistant cells the amount of α enzyme is about 4–5-fold smaller than that in the sensitive cells, whereas the β enzyme is almost undetectable. Analysis of DNA restriction sites in several independently selected resistant subclones revealed some rearrangements in the β gene in two clones. However, these gene alterations did not correlate with changes in the resistance level. The relative contribution of these different changes to the resistance phenotype is discussed.

DNA type II topoisomerases are important cellular enzymes that regulate the topological state of DNA by a process in which DNA segments are passed through transient DNA double-strand breaks (for reviews, see Refs. 1 and 2). The physiological role of topoisomerase II, which is a component of the nuclear scaffold (3, 4), includes segregation of chromatids during mitosis (5, 6), DNA replication (7), and introduction or removal of DNA supercoils (8–10). Two forms of functional topoisomerases II, encoded by separate genes, have been identified in murine and human cells (11). They differ in their molecular mass (170 and 180 kDa for topoisomerases II α and β, respectively) and their biochemical and pharmacological properties (12, 13).

The type II topoisomerases are the targets of numerous chemotherapeutic agents, fostering ongoing clinical interest in these enzymes (for reviews, see Refs. 14–16). They are specifically inhibited by several classes of antitumor drugs, including ellipticines, acridines, anthracyclines, and epipodophyllotoxins (for review, see Ref. 17). These inhibitors interfere with the enzyme by trapping a key covalent DNA-topoisomerase intermediate, termed the cleavable complex (17). However, the roles played by topoisomerase isoforms α and β in determining the cellular sensitivity to antitumor agents are not clearly defined. The two enzymes differ by their sensitivity to different inhibitors (12). Reduction in the amount of one isoform may be enough to account for the resistance to drugs such as teniposide (18) or mitoxantrone (19).

We previously described a subline of the Chinese hamster lung cell line DC-3F that was selected for resistance to the DNA-intercalating agent 9-OH-ellipticine (20). The DC-3F/9-OH-E cells display cross-resistance to a variety of antitumor drugs, including topoisomerase II inhibitors and agents sensitive to the multidrug resistance phenotype, and decreased oncogenic potential (20). Analysis of the resistance to 9-OH-ellipticine showed that (i) there is no difference in drug uptake and retention between the sensitive and resistant cells (21), (ii) topoisomerase II activity is about 4-fold lower in the resistant cells, compared with the sensitive cells, as demonstrated by determination of the catalytic activity and by immunoblotting (22), and (iii) DC-3F/9-OH-E nuclear extracts have about a 10-fold reduced capacity to induce the stabilization of the cleavable complex upon stimulation by etoposide and amsacrine (23, 24). However, the precise mechanism of the resistance to topoisomerase II inhibitors in the DC-3F/9-OH-E cells is not presently fully understood.

In the present work, we further characterized the topoisom-

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ABBREVIATIONS: SDS, sodium dodecyl sulfate; SSPE, standard saline-phosphate-EDTA; PBS, phosphate-buffered saline.

erase II activities in DC-3F/9-OH-E cells and in the parental cells by Northern and Western blot analysis. The expression of each isoenzyme in the sensitive and resistant cells was thus examined as a function of cellular growth, showing that the α form was about 4-fold decreased and the β form was almost undetectable in the resistant cells. Analysis of the restriction maps revealed structural alterations exclusively in the gene of the β form. However, this rearrangement was not correlated with the resistance level.

Materials and Methods

Cell lines and culture conditions. The Chinese hamster lung cell lines DC-3F and DC-3F/9-OH-E have been described previously (20). Monolayer cultures were maintained in Eagle's minimal medium supplemented with 7% fetal calf serum, 100 IU/ml penicillin, and 50 μg/ml streptomycin. The resistant subline DC-3F/9-OH-E was permanently grown in the presence of 9-OH-ellipticine (0.6 μg/ml). Before each experiment, DC-3F/9-OH-E cells were grown for three passages in the absence of drug.

Growth curves were established as described previously (20). The day before the beginning of the experiment, 140-mm-diameter Petri dishes (Falcon; Becton-Dickinson) were seeded with either 7×10^5 DC-3F cells or 1.8×10^6 DC-3F/9-OH-E cells, in 25 ml of Eagle's minimum medium supplemented with 7% fetal calf serum, and were incubated at 37° in a 5% CO_2 humidified atmosphere for 20, 44, 68, or 96 hr.

Chemicals. All chemicals were of reagent grade and were purchased from commercial sources. [α-^{32}P]dCTP and dUTP were obtained from Amersham. 9-OH-ellipticine was kindly provided by Dr. E. Lescot (Institut Gustave Roussy, Villejuif, France).

cDNA probes. The DNA topoisomerase II partial cDNA clones, obtained from a human Raji-HN2 cDNA library (13), were kindly provided by Dr. K. B. Tan (Smith, Kline, and French Laboratories). The SP1 probe selectively recognizes the 170-kDa α form of the enzyme, whereas SP12 selectively recognizes the 180-kDa β form. A partial cDNA insert, SP1′, was removed from the plasmid pUC13 by digestion with the restriction enzymes *Eco*RI and *Xba*I. A partial cDNA insert, SP12′, was removed from the plasmid pTZ18 by digestion with *Pst*I and *Eco*RI (Fig. 1). For hybridization experiments, these probes were labeled by random priming with [α-^{32}P]dCTP. The 28 S RNA probe (25) was kindly provided by Dr. F. Dautry (Institut Gustave Roussy, Villejuif, France).

DNA extraction and Southern blot analysis. Genomic DNA was extracted from cultured cells as described by Laird *et al.* (26). After digestion with restriction enzymes (Boehringer-Mannheim, Mannheim, Germany), DNA fragments (10 μg/lane) were fractionated by electrophoresis on 0.8% (w/v) agarose gels. After transfer to Hybond-N nylon membranes (no. RPN 3050; Amersham) by vacuum blotting (Vacugene; Pharmacia-LKB), prehybridization was performed for 2 hr at 42° in 40% formamide, 5× standard saline citrate (1× standard saline citrate is 0.15 M NaCl, 15 mM sodium citrate), 50 mM phosphate buffer, pH 6.8, 5× Denhardt's solution, 0.1% SDS. Hybridization was then performed for 20 hr in the same buffer containing the ^{32}P-labeled cDNA probe. The membrane was washed twice at room temperature for 15 min in 0.1% SDS/2× SSPE (SSPE is 10 mM NaH_2PO_4, 0.13 M NaCl, 1 mM EDTA) and twice at 50° for 30 min in 0.1% SDS/1× SSPE. After autoradiography at −70°, the autoradiograms were obtained on Fuji RX film with a DuPont Cronex Lightning-Plus screen.

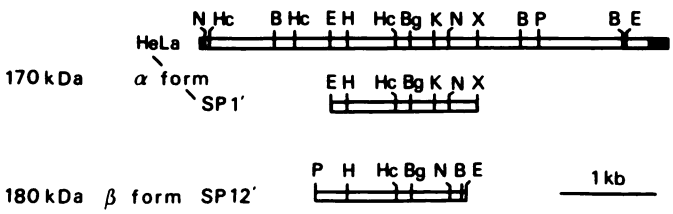

Fig. 1. SP1′ and SP12′ probes. The HeLa topoisomerase II α gene restriction map was generated from the sequence in Ref. 34. SP1′ and SP12′ maps were generated from Ref. 13. Alignment of inserts and restriction sites are indicated. *B*, *Bam*HI; *Bg*, *Bgl*II; *E*, *Eco*RI; *Hc*, *Hin*cII; *H*, *Hin*dIII; *K*, *Kpn*I; *N*, *Nco*I; *P*, *Pst*I; *X*, *Xba*I. *Black regions*, noncoding sequences.

RNA extraction and Northern blot analysis. RNA was extracted by the guanidine thiocyanate technique (27). Polyadenylated RNAs, purified from total RNA using an mRNA purification kit (Pharmacia-LKB Biotechnology), were fractionated by electrophoresis in 1.2% (w/v) agarose gels containing 7% formaldehyde and were transferred to Hybond-N nylon membranes (Amersham) in 150 mM ammonium acetate. Prehybridization, hybridization, and washing were carried out as described above. After autoradiography at −70°, the autoradiograms were analyzed by densitometric scanning using a Joyce-Loebl Chromoscan 3.

Preparation of nuclear extracts. Nuclear extracts from DC-3F and DC-3F/9-OH-E cells were prepared from 3–5 × 10^7 cells harvested at different times during cell growth. Freshly prepared protease inhibitors (phenylmethylsulfonyl fluoride, benzamidine, aprotinin, and soybean trypsin inhibitor) were added to all buffers. The cells were trypsinized, centrifuged, washed with PBS, and pelleted again. The following steps were carried out at 4°. The cell pellets were resuspended in 100 μl of buffer A (10 mM Tris·HCl, pH 7, 1 mM $MgCl_2$) and then lysed with 1 ml of buffer A′ (buffer A containing 1% Nonidet P-40). After gentle stirring for 10 min, the suspension was subjected to Dounce homogenization (20 strokes) and then was layered on a 10-ml cushion of buffer A containing 40% (w/v) sucrose. The nuclei were pelleted by centrifugation at 1900 × *g* for 30 min at 4°. Purified nuclei were gently resuspended in 400 μl of buffer B (10 mM NaCl, 10 mM Tris·HCl, pH 7, 0.6 mM $MgCl_2$). The NaCl concentration was precisely adjusted to 0.35 M with an appropriate volume of buffer C (1.5 M NaCl, 10 mM Tris·HCl, pH 7, 0.6 mM $MgCl_2$). After 30 min, insoluble material was pelleted by centrifugation at 12,000 × *g* for 30 min. Protein concentration in the extract was determined as described previously (28).

Anti-human DNA topoisomerase II antibody. A recombinant fusion protein was generated by expression of an 837-amino acid peptide from human topoisomerase II α (positions 258–1095) fused to β-galactosidase. The topoisomerase II fragment was prepared from the plasmid pBShTOP-2, generously provided by Dr. J. Wang Harvard University, Cambridge, Massachusetts. A 2510-base pair *Bam*HI fragment (positions 775–3285) was subcloned in the pUR291 expression vector (29). A lacZ polypeptide (1022 amino acids) fused at its amino-terminal end with the topoisomerase fragment was produced. Phase and orientation of the inserted fragment were controlled by sequencing the junction region in MP13. Expression of the hybrid protein in *Escherichia coli* TG1 was induced by incubation for 3 hr in the presence of isopropyl β-D-thiogalactoside. The fusion protein was purified by preparative SDS-polyacrylamide gel electrophoresis (5%). After staining with $CuCl_2$ (30), the protein was identified according to the expected molecular mass (210 kDa) and electroeluted from the gel. A polyclonal antibody was then raised by injecting the fusion protein (200 μg) subcutaneously into two rabbits. Injections were repeated twice at 3-week intervals before the first serum collection. The antibody (designated A6) was purified as described by Smith and Fisher (31) and was concentrated about 80-fold at 4° under nitrogen vacuum (Diaflo; Amicon), using an XM50 membrane (Amicon).

Western blot analysis. Freshly prepared nuclear extracts (10 and 20 μg/lane for DC-3F cells and 40 and 80 μg/lane for DC-3F/9-OH-E cells) were loaded onto a 7.5% SDS-polyacrylamide gel according to the method of Laemmli (32). Protein were transferred (4 mA/cm^2) to nitrocellulose membranes (Gelman Sciences) in 25 mM Tris·HCl, pH 8.3, 92 mM glycine, 20% methanol, for 1 hr at room temperature. The membranes were saturated for 2 hr in PBS with 5% nonfat milk and 0.2% Tween 20 and were then probed at room temperature for 2 hr with the anti-topoisomerase II antibody (1/1000 dilution) in PBS with 2% nonfat dry milk. After rinsing with PBS, the blots were treated for

45 min at room temperature with anti-rabbit immunoglobulin secondary antibody labeled with horseradish peroxidase (1/2000 dilution). The immunoreactive bands were detected by light emission using an enhanced chemiluminescence Western blotting detection system (Amersham).

Results

Immunodetection of DNA topoisomerases II α and β. In a previous work (23), we determined the topoisomerase II catalytic activity in nuclear extracts from sensitive or resistant cells by measuring the initial rate of kDNA decatenation. The enzyme activity was decreased about 4-fold in the resistant cells, compared with the sensitive cells.

To further analyze the topoisomerase II activities in the 9-OH-ellipticine-sensitive and -resistant cells, a polyclonal antibody (A6) was raised against an 837-amino acid peptide fragment from the human topoisomerase II α (positions 258–1095). Immunoblot experiments showed that, in 0.35 M NaCl nuclear extracts from the human lymphoid cell line CEM, A6 recognizes two proteins, with molecular masses of 170 and 180 kDa. In a parallel experiment, the 170-kDa protein was identified as topoisomerase II α using an antibody (Ac22; kindly provided by Dr. F. Drake, Smith, Kline, and French Laboratories) that specifically recognizes this isoform (data not shown). Therefore, this experiment shows that A6 recognizes both topoisomerase II α and β in a 0.35 M NaCl nuclear extract from the human lymphoid cell line CEM. This antibody also recognizes both enzyme forms in the nuclear extracts from DC-3F cells. However, in these cells, the β enzyme appears to be about 20-fold less abundant than the α enzyme, as determined by densitometric scanning of the bands. Finally, Fig. 2 also shows that topoisomerase II α is about 4-fold less abundant in the DC-3F/9-OH-E cells, compared with the parental DC-3F cells. Topoisomerase II β is undetectable in the resistant cells.

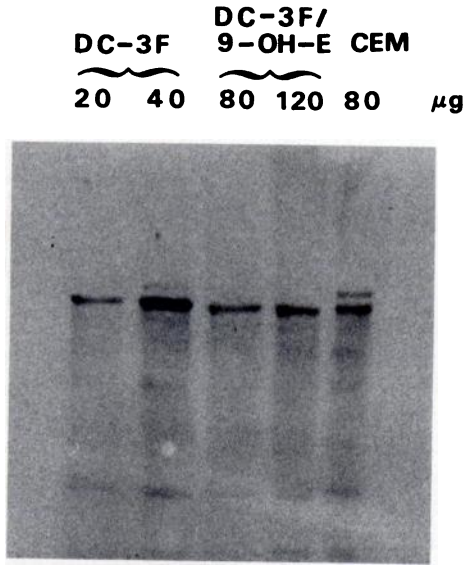

Fig. 2. Immunoblot analysis of the topoisomerase II activities in 0.35 M NaCl nuclear extracts from 9-OH-ellipticine-sensitive and -resistant cells. Nuclear extracts from DC-3F, DC-3F/9-OH-E, and CEM cells were prepared as described in Materials and Methods. The amount of protein loaded in each lane is indicated (in μg). After SDS-polyacrylamide gel electrophoresis and transfer to nylon membranes, topoisomerase II proteins were identified with the A6 antibody.

Expression of the topoisomerase genes during the cell proliferation phases. Topoisomerase II expression and activity are known to be cell proliferation dependent (18, 33, 34). We then examined the expression of both topoisomerase II α and β genes at different growth stages of the 9-OH-E-sensitive and -resistant cells. Fig. 3A shows the growth curves for the DC-3F and DC-3F/9-OH-E cells. Polyadenylated RNAs and nuclear extracts were prepared from samples taken at the indicated times.

After fractionation by agarose gel electrophoresis, the RNAs were successively hybridized with the SP1′ and SP12′ probes, which specifically recognize the α and β transcripts, respectively (13). Fig. 3B shows that the amount of α transcripts in DC-3F cells was increased during the exponential phase of growth and was down-regulated when the cells reached the plateau phase. In the resistant cells, α transcripts remained almost constant throughout the different growth phases and at the end of the exponential phase were about 5-fold less abundant than in the sensitive cells. Expression of the topoisomerase β gene in DC-3F cells continued to increase even when the cells had reached the plateau phase of growth. In the DC-3F/9-OH-E cells, expression of the β gene was barely detectable throughout all growth phases.

The amounts of each enzyme present in the nuclear extracts from the sensitive or resistant cells were analyzed by immunoblot using the antibody described above (Fig. 4A). The peak area for each band was determined by scanning, and the peak area per microgram of protein was plotted in the curves shown in Fig. 4B. In the sensitive cells, the amount of enzyme was relatively constant during the exponential phase of growth and then decreased at the plateau phase, as expected from the

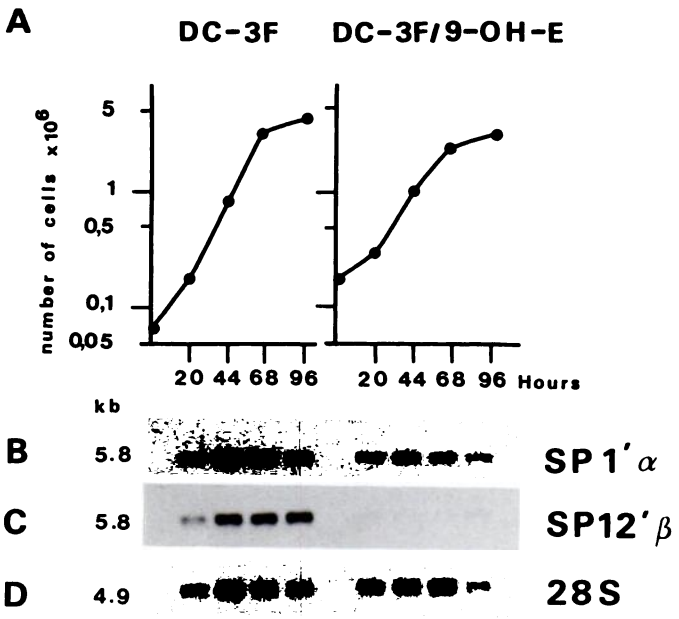

Fig. 3. Proliferation dependence of the expression of the topoisomerase II genes. A, Growth curves for DC-3F and DC-3F/9-OH-E cells. Total RNAs and nuclear extracts were prepared from culture aliquots collected at the indicated times. B-D, Northern blot analysis of the topoisomerase II transcripts at different growth stages. After fractionation by agarose gel electrophoresis (1.2%) and transfer to nylon membranes, the RNAs were sequentially hybridized with the SP1′ (B), SP12′ (C), and 28 S RNA (D) probes. The 28 S RNA probe was used as an internal control for the quantity of RNA loaded in each lane.

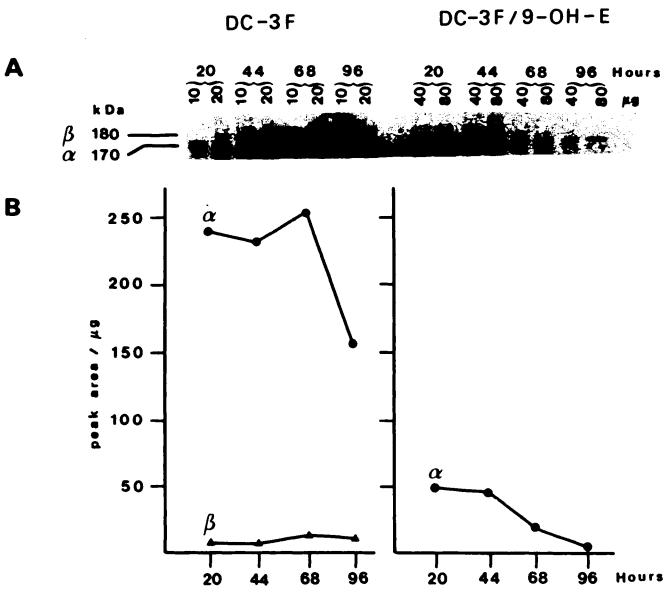

Fig. 4. Proliferation dependence of the amounts of topoisomerase II α and β. At different times of cell growth (see Fig. 3A), nuclear extracts were prepared and topoisomerase II proteins were immunoblotted with the A6 antibody. For each time, two amounts of proteins were loaded. The molecular mass of the immunoreactive proteins is indicated (A). Band intensities on autoradiograms were determined by densitometer scanning and the peak area/μg of protein was plotted versus time (B). ●, α enzyme; ▲, β enzyme.

Northern blot experiments. In these cells, the amount of the β form was about 20-fold smaller than that of the α form but was not down-regulated at the plateau phase. These data are consistent with previous reports demonstrating differential cellular regulation of the two enzymes (12). In the resistant cells, the amount of topoisomerase II α was about 5-fold smaller than in the sensitive cells and progressively decreased with time. Topoisomerase II β was undetectable, which is consistent with the very low level of transcription observed in the Northern blot experiments.

Anatomy of the topoisomerases II genes. As an approach to the identification of genetic alterations possibly associated with the resistance phenotype, we compared the restriction patterns of the α and β genes in the sensitive and resistant cells. Fig. 5 shows a Southern blot experiment in which genomic DNAs from the parental DC-3F cells and from two independent resistant subclones were digested with the restriction enzymes *Eco*RI, *Bam*HI, and *Hind*III and then hybridized sequentially with the SP1′ and SP12′ probes. Fig. 5A shows that the α-specific probe recognizes the same fragments in the three cell lines. In contrast, Fig. 5B shows that the restriction patterns of the resistant subclones with the three enzymes were different from those observed with the parental cells. The restriction fragments identified in each resistant clone were different both qualitatively and quantitatively. However, the resistance to topoisomerase II inhibitors, such as 9-OH-ellipticine, amsacrine, and etoposide, was identical in both sublines. Clone C was used in all of the experiments described in this work.

Discussion

A variety of cell lines resistant to DNA topoisomerase II inhibitors have been isolated and characterized. In some of them, the resistance phenotype was associated with either normal (35) or altered catalytic activity (23, 36), as well as with normal or low levels of the protein (36, 23). Using an antibody raised against the mouse α enzyme, the amount of topoisomerase II protein in the DC-3F/9-OH-E cells was found by immunoblot analysis to be about 2-fold smaller than that in the parental DC-3F cells (37). Determination of the initial rate of kDNA decatenation revealed a 4–5-fold decreased catalytic activity (23). However, none of these techniques discriminated between the α and β isoforms of the enzyme. α and β topoisomerases II, identified by Drake *et al.* (11), were shown to be the products of separate genes (13) that are differentially regulated in cells (11, 12). Further studies on the mechanism of resistance to topoisomerase II inhibitors in DC-3F/9-OH-E cells then required a detailed examination of the expression of both topoisomerase II isoforms in these cells.

Transcription of the α and β genes was analyzed using two specific probes, SP1′ and SP12′, derived from the SP1 and SP12 probes previously described by Chung *et al.* (13). In Southern blot experiments, the DNA fragments recognized in the Chinese hamster genome by the SP1′ and SP12′ probes were identical to those initially recognized by the SP1 and SP12 probes (13), thus demonstrating that these probes have retained the capacity to specifically recognize the α and β characteristic sequences, respectively. The amounts of topoisomerase II proteins present in the sensitive and resistant cells were determined using an antibody raised against an 837-amino acid fragment corresponding to a *Bam*HI fragment (positions 775–3285) in the human α cDNA. In human cells, this antibody clearly recognized two protein bands, with molecular masses of 170 kDa and 180 kDa. The 170-kDa band was identified as the α enzyme using another antibody that was previously shown to specifically recognize this isoform (12). The band at 180 kDa is then very likely to represent the β enzyme.

Both Northern and immunoblot analysis demonstrated that (i) in the parental DC-3F cells the α enzyme is about 20-fold more abundant than the β enzyme and thus essentially accounts for the overall topoisomerase II activity in these cells, (ii) in the resistant cells the amount of topoisomerase II α is about 4–5-fold smaller than in the sensitive cells, which is consistent with the decrease of the catalytic activity (23), and (iii) the β enzyme is undetectable in the resistant cells. We also observed that the amount of α enzyme dropped as the cells reached the plateau phase, whereas the amount of β enzyme increased at the plateau. A similar reciprocal regulation has been previously observed in human U937 cells (12).

The relative contribution of these topoisomerase II α and β alterations to the resistance phenotype remains difficult to ascertain. Recently, Gudkov *et al.* (38), using genetic suppressor elements, induced in human cells a decrease of the topoisomerase II α activity comparable to that observed in the DC-3F/9-OH-E cells and demonstrated that it resulted in resistance to topoisomerase II-interactive drugs. However, in that system the resistance to the tested inhibitors was only about 3–5-fold, compared with several hundredfold in the DC-3F/9-OH-E cells. Topoisomerase II α is a very abundant cellular protein. For example, in different cell types, including mouse L1210 cells and several human colon adenocarcinoma lines, levels of topoisomerase II ranging from about 0.7 to 7×10^6 copies/cell have been reported (39). Therefore, the residual amount of topoisomerase II α in the DC-3F/9-OH-E cells still represents a large number of enzyme copies, which allows nearly normal cell

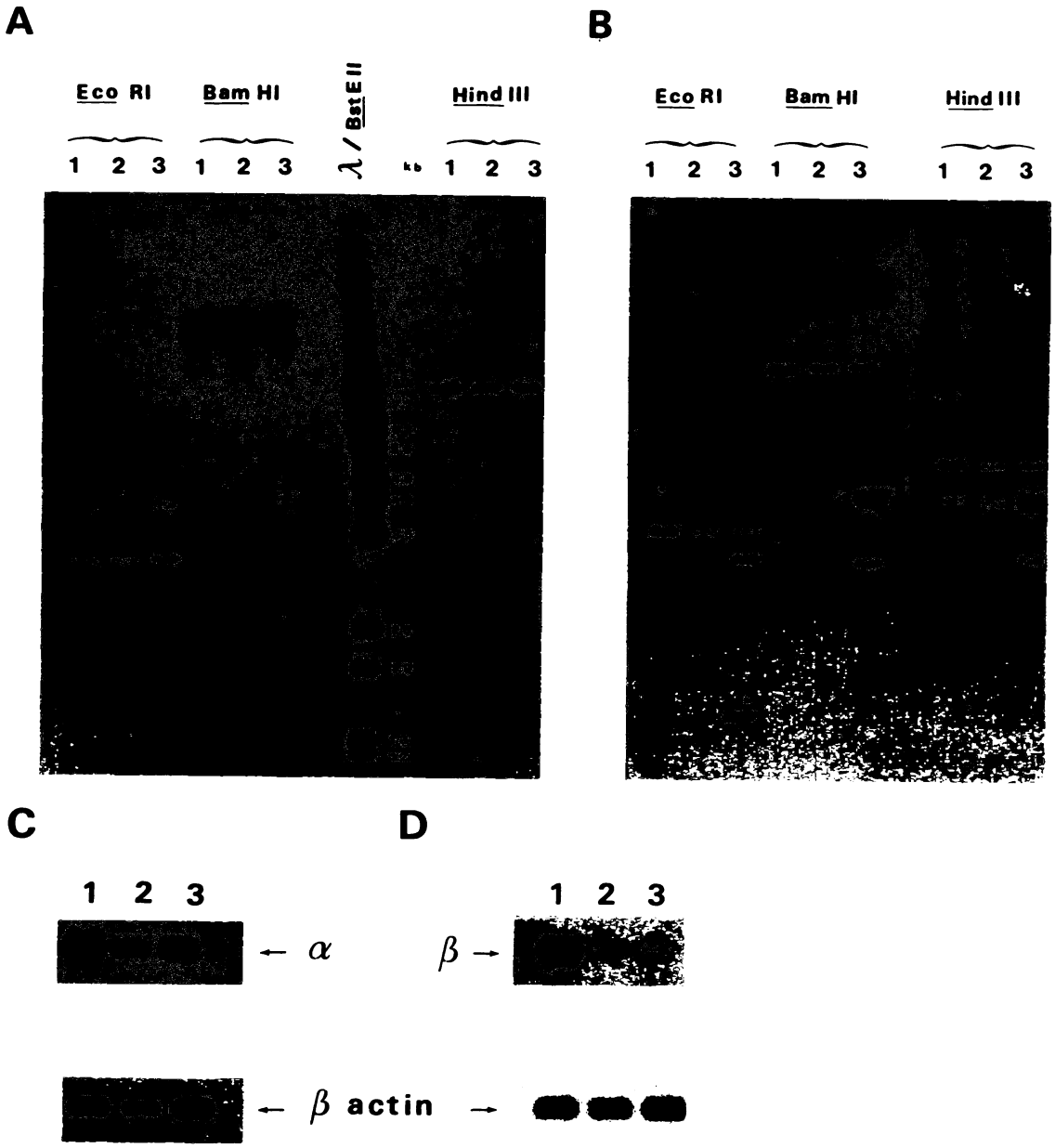

Fig. 5. Southern blot analysis of genomic DNA from DC-3F and DC-3F/9-OH-E cells. A and B, After digestion with the indicated restriction enzymes, genomic DNAs from DC-3F and DC-3F/9-OH-E cells were fractionated by agarose gel elctrophoresis and transferred to a nylon membrane. The membrane was then sequentially hybridized with the SP1′ (A) and SP12′ (B) probes. C and D, Northern blot analysis shows that the α (C) and β (D) enzymes are expressed at the same level in both resistant clones. *Lane 1*, DC-3F; *lane 2*, clone A; *lane 3*, clone C.

growth and also is likely to mediate nearly normal toxicity of topoisomerase II inhibitors. This suggests that high resistance levels, such as that in the DC-3F/9-OH-E cells, would require additional genetic alteration(s). If one assumes that the β enzyme is mainly responsible for the toxicity of these compounds, then the apparent loss of this isoform in the resistant cells might account for the resistance phenotype. For example, 35-fold resistance to mitoxantrone has been found to be associated with the loss of β enzyme activity (40). However, one would expect the resistance phenotype to be completely recessive in hybrids between the sensitive and resistant cells, because the sensitive parent carries normal levels of both α and β enzymes. In 13 clones of such hybrids, we previously observed that the resistance to 9-OH-ellipticine was actually partially dominant (41). Other possibilities should then be considered; the stabilization of the cleavable complex triggers a lethal pathway in the cell that is not presently understood, and obviously alterations in one or several steps of this pathway might also be involved in high levels of resistance to topoisomerase II inhibitors.

Finally, restriction sites in the α and the β genes were analyzed after digestion of the genomic DNA from sensitive and resistant cells with the restriction enzymes *Eco*RI, *Bam*HI, and *Hind*III. The sizes of the fragments generated in the α gene were identical in all of the tested clones. In contrast, in two independently selected DC-3F/9-OH-E subclones this study revealed several changes in the β gene that are different for each clone. These changes did not correlate with any mod-

ification of the resistance level. Therefore, they are probably not associated with functional alterations of the β gene. Further characterization of genetic alterations in the topoisomerase II genes of DC-3F/9-OH-E cells must await the cloning and sequencing of the corresponding cDNAs presently in progress in our laboratory.

Acknowledgments

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