A Novel Mutant Topoisomerase IIα Present in VP-16-Resistant Human Melanoma Cell Lines Has a Deletion of Alanine 429

Julie A. Campain,[‡,§] Michael M. Gottesman,[∥] and Ira Pastan*[,‡]

Laboratory of Cell Biology, DCBDC, National Cancer Institute, National Institutes of Health, 9000 Rockville Pike, Building 37, Room 1B22, and Laboratory of Molecular Biology, DCBDC, National Cancer Institute, National Institutes of Health, 9000 Rockville Pike, Building 37, Room 4E16, Bethesda, Maryland 20892

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ABSTRACT: The human melanoma cell line FEM-X was selected in multiple steps with VP-16 (etoposide) and an inhibitor of P-glycoprotein (Campain *et al.*, 1993). The resulting clones, FVP1b and FVP3, are highly resistant to the nonintercalative epipodophyllotoxins and exhibit moderate levels of resistance to doxorubicin. The topoisomerase II activity present in crude nuclear extracts from mutant and wild-type cells is similar in amount and equally sensitive to VP-16. However, in live cells, the topoisomerase II from FVP1b and FVP3 is much less susceptible to drug-induced cleavable complex formation than is that from FEM-X. Using reverse transcription followed by the polymerase chain reaction (RT-PCR), we have cloned and sequenced the entire cDNA for topoisomerase IIα from FEM-X and FVP3. The only sequence change unique to the cDNA from drug-resistant cells is a 3 bp deletion of nucleotide 1320–1322, resulting in a deletion of Ala429. Three FEM-X sublines of increasing resistance were tested, and the prevalence of the mutant RNA over wild-type increases in these cells in parallel with their resistance to VP-16. In FVP3, the most highly resistant line, expression of the wild-type allele is barely detectable. Analysis of genomic DNA shows that FEM-X is homozygous for the wild-type topoisomerase IIα sequence and that each of the drug-resistant clones possesses both wild-type and mutant alleles. Although not definitive, these genetic results suggest that the deletion of Ala429 from topoisomerase IIα makes the enzyme less susceptible to drug-induced cleavable complex formation and confers a growth advantage upon cells in the presence of VP-16. Since topoisomerase IIα activity in extracts from these drug-resistant FEM-X lines is normally sensitive to drugs, the deletion of an Ala at residue 429 may alter intracellular localization of the enzyme or change its interaction with other factors, which could then decrease the DNA–topoisomerase IIα complexes trapped in the presence of VP-16. However, proof that the mutant topoisomerase IIα is responsible for drug resistance requires its successful expression in drug-sensitive cells.

Topoisomerase II is a nuclear enzyme that is essential for the regulation of DNA topology [see Wang (1985) for a review]. The protein is a main component of the nuclear matrix and is involved in the segregation of sister chromatids during mitosis and meiosis (DiNardo *et al.*, 1984; Earnshaw & Heck, 1985; Goto & Wang, 1984; Holm *et al.*, 1989; Rose *et al.*, 1990; Uemura *et al.*, 1987). A role for topoisomerase II in regulation of transcription and recombination of DNA has also been suggested [reviewed by Osheroff (1989), Osheroff *et al.* (1991), Wang (1985), and Wang *et al.* (1990)]. There are two genes for human topoisomerase II which code for 170 kDa subunits of two homodimeric, catalytically active protein isoforms (Drake *et al.*, 1987; Miller *et al.*, 1981; Tan *et al.*, 1992; Wang, 1985). The gene for topoisomerase IIα has been localized to chromosome 17 (Tsai-Pflugfelder *et al.*, 1988), and the gene for topoisomerase IIβ is found on chromosome 3 (Tan *et al.*, 1992).

Topoisomerase II is the primary intracellular target of many effective anticancer drugs. Among these drugs are intercalating agents, including the anthracyclines, amsacrine, and mitoxantrone, and the nonintercalative epipodophyllotoxins, VP-16 and VM-26 [see Liu (1989) for a review]. VP-16 and other inhibitory drugs act intracellularly by stabilizing a topoisomerase II–DNA covalent complex. It is the cellular recognition and processing of these complexes, and the ensuing DNA damage, that is thought to be the lethal event in cells treated with inhibitors of topoisomerase II [Chow *et al.*, 1988; Schneider *et al.*, 1989; reviewed by De Isabella *et al.* (1991)]. Quantitative or qualitative alterations in the target enzyme have been strongly implicated in the development of resistance to these classes of drugs [reviewed by Beck (1989), Bugg *et al.* (1991), Chan *et al.* (1993), Cole *et al.* (1991), Hinds *et al.* (1991), Lee *et al.* (1992), and Webb *et al.* (1991)].

It is not yet clear how expression of topoisomerase II is regulated in normal cells, and even less is known about the conditions leading to decreased topoisomerase II protein levels in drug-resistant tumor cells. In addition, the structure–function relations of topoisomerase II both in the presence and in the absence of DNA and/or inhibitory drugs and the intracellular factors which may modulate these interactions are still poorly understood. As topoisomerase II inhibitors constitute some of the most effective anticancer agents, there is a critical need for studies in these areas. To this end, we have developed human melanoma cell lines, FVP1b and FVP3, that are resistant to VP-16 (Campain *et al.*, 1993). Several lines of evidence indicated that the topoisomerase IIα in these cell lines was unlike the wild-type enzyme in its interaction with VP-16. The fact that resistance to VP-16 of the FVP1b or FVP3 enzyme could only be demonstrated in live and disrupted cells, and not in crude nuclear extracts, indicated

* Author to whom corresponding should be addressed. Telephone: (301) 496-4797. Fax: (301) 402-1344.

‡ Laboratory of Molecular Biology, NIH.

§ Present address: Department of Medicine, Division of Medical Oncology, University of Colorado, Health Sciences Center, 4200 E. Nineth Ave., Denver, CO 80262.

∥ Laboratory of Cell Biology, NIH.

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that other factors may be involved in the observed resistance, perhaps by modulating the localization of topoisomerase IIα or its interaction with DNA. To elucidate the mechanism which is responsible for VP-16 resistance in the FEM-X cells, we have cloned the complete cDNA for topoisomerase IIα from FEM-X and FVP3, and have identified a 3 bp deletion in FVP3 that appears to be associated with drug resistance in these cells.

MATERIALS AND METHODS

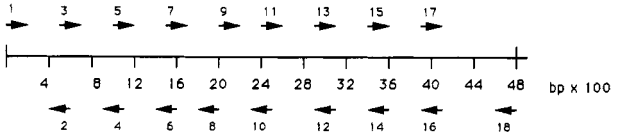
Materials. VP-16 was a gift from Bristol-Myers Laboratories, Syracuse, NY. The tiapamil analog RO-11-2933 was provided by Hoffman LaRoche, Basil, Switzerland.

Cell Culture. FEM-X is a drug-sensitive human melanoma cell line obtained from the Frederick Cancer Research Center, Frederick, MD. All cells were cultured in monolayer in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All drug-resistant clones were maintained in medium containing 1 μg/mL tiapamil analog plus the appropriate concentration of VP-16. These sublines were cultured in drug-free medium for at least 1 week prior to being used for experimentation.

Development of Multidrug-Resistant FEM-X Sublines. The VP-16-resistant sublines were derived from FEM-X as described (Campain *et al.*, 1993). Briefly, cells were mutagenized with ethyl methanesulfonate and then selected in 0.2 μg/mL VP-16. All selections were carried out in the presence of nontoxic levels of the tiapamil analog RO-11-2933, which inhibits the transport of VP-16 by P-glycoprotein (Kessel & Wilberding, 1985; Yin *et al.*, 1989; and personal communication from Dr. J. F. Eliason, Nippon Roche Research Center). Clones were picked from the initial selection and characterized as to their drug-resistance phenotype. A single clone, FVP0.2, was then further selected in 1 μg/mL VP-16. FVP1a was chosen from this second selection based on its drug resistance profile. As this line did not grow well at 1 μg/mL VP-16, further selections were carried out at this drug concentration. After two consecutive selections, a clonal cell line was established which was stably resistant to 1 μg/mL VP-16. This cell line was designated FVP1b. In an earlier description of the selection and characterization of these drug-resistant clones (Campain *et al.*, 1993), FVP1b was designated FVP1; the two are the same cell line. FVP3 was selected directly from FVP1b in 3 μg/mL VP-16.

RNA Isolation. RNA was isolated by the acid–phenol extraction method (Chomczynski & Sacchi, 1987).

DNA Isolation and Southern Analysis. DNA was isolated from all cell types by the salting-out procedure of Miller *et al.* (1988). Purified DNA was digested to completion with *Alu*I, electrophoresed through 2.5% agarose gels, and transferred to nitrocellulose membranes by standard protocols. Southern analysis was carried out by established protocols using either Top50/51A, a fragment of the topoisomerase IIα cDNA spanning nucleotides 1346 through 1528, radiolabeled with ^{32}P by random primer extension or Top52, a 45-mer oligonucleotide (5′GCCAATGATGCAGGGGGCCGAAACTCCACTGAGTGTACGCTTATC3′) corresponding to nucleotides 1366–1410 of the topoisomerase IIα cDNA sequence, radiolabeled with ^{32}P on its 5′-end by T4 polynucleotide kinase. The numbering of oligonucleotides used in this report is modified from that of Tsai-Plugfelder *et al.* (1988). Three bases have been added to the 5′-end of the cDNA as described by Hinds *et al.* (1991) and Lee *et al.* (1992) as for the correct human topoisomerase IIα sequence. For Southerns using the oligonucleotide probe, prehybridization and hybridization were carried out at 42 °C, and the filters were washed in 6× SSC/0.2% SDS twice for 30 min at 45 °C and once for 30 min at 55 °C.



RT-PCR Primers: Beginning nucleotide:

Top1	5'ATGGAAGTGTCACCATTG3'	37
Top2	5'CTTGTATTCTCTACTGGC3'	580
Top3	5'TGTAACATATTCAGTACC3'	544
Top4	5'GTTGGGTTTTCAATTAAGGC3'	1132
Top5	5'AGAACAAGGGTGGTGTTGCAG3'	1067
Top6	5'GGAACCATCTTGGTCCTG3'	1660
Top7	5'CATTGAAGACGCTTCGTTATG3'	1616
Top8	5' GGTTAACTAATTTCATGG3'	2095
Top9	5'GGTTAACTAATTTCATGG3'	2027
Top10	5'CCCAGTACCGATTCCTTC3'	2596
Top11	5'GCCTGAATGGTACATTCC3'	2547
Top12	5'GAATATCCAACACCGTGTC3'	3073
Top13	5'CACATGCAACTCTATGGTG3'	3021
Top14	5'CCTTGGCTTCAACAGCCTCC3'	3570
Top15	5'GAGTCCATCAGATTTGTG3'	3519
Top16	5'AGCTTCAAGGTCTGACAC3'	4162
Top17	5'ACTTAGTAACAAAGAACTG3'	4124
Top18	5'AAAGCTAAAGAACACTTGGGC3'	4771

FIGURE 1: PCR primers for cloning topoisomerase IIα cDNA's from FEM-X and FVP3. Oligonucleotide primers were designed from the published HeLa toposiomerase IIα cDNA sequence (Tsai-Pflugfelder, 1988) as 18–22-mer's. The nucleotides were numbered starting with the first base of the published cDNA sequence. However, due to the insertion of three bases in the HeLa sequence at positions 363, 374, and 380 [this paper and Hinds *et al.*, (1991) and Lee *et al.* (1992)], three bases have been added to the positions of primers 2–18. Even-numbered primers are antisense, and odd-numbered primers are sense. The primer combinations used were in consecutive order, i.e., Top1 and Top2, Top3 and Top4.

Isolation of Topoisomerase IIα cDNA and Genomic Clones. Reverse transcription was carried out on total cellular RNA using random hexamer primers and MMLV reverse transcriptase for 1 h at 37 °C. The resulting first-strand cDNA was then amplified by 35 cycles of the polymerase chain reaction using primer combinations that spanned the entire topoisomerase IIα cDNA in increments of 500–600 bp (Figure 1). For screening the series of drug-resistant clones for the prevalence of the mutant RNA, primers Top50 (5′GCCATTGGCTGTGGTATTG3′), corresponding to nucleotides 1240–1258, and Top51 (5′GAGAAGCTTCTCGAACATTGAG3′), corresponding to nucleotides 1507–1528, were used. PCR was carried out on 2 μg of DNA from each cell line using Top50 and Top51. The annealing temperature in all cases was 50 °C. The resulting RT-PCR and DNA-PCR products were purified by electrophoresis through low melting point agarose gels and either analyzed by restriction digest with *Alu*I or subcloned into the *Sma*I site of pGEM7Z for subsequent sequencing.

DNA Sequencing. All DNA sequencing was carried out on an Applied Biosystems Model 373A automated DNA sequencer using either T7 or SP6 dye primers or topoisomerase IIα-specific primers and fluorescent terminators as supplied by the manufacturer in prefabricated kits. All sequences were confirmed from both directions.

RESULTS

Drug-Resistant FEM-X Cells Have a Mutant Topoisomerase IIα. Clones derived from the human melanoma cell line, FEM-X, by multistep selection in VP-16 and an inhibitor of P-glycoprotein were described previously (Campain *et al.*, 1993). These clones, FVP1b and FVP3, exhibited resistance to VP-16, as well as to VM-26 and doxorubicin, a

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          1305                                                1350
HeLa      AAACAAGAAGTGTTCAGCTGTAAAACATAATAGAATCAAGGGAATT
FEM-X     AAACAAGAAGTGTTCAGCTGTAAAACATAATAGAATCAAGGGAATT
FVP3      AAACAAGAAGTGTTC --- TGTAAAACATAATAGAATCAAGGGAATT
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FIGURE 2: Comparison of the sequence of topoisomerase IIα cDNA's from FEM-X and FVP3 between nucleotides 1305 and 1350. This region in the topoisomerase IIα cDNA was initially amplified by RT-PCR using primers Top5 and Top6. The deletion of nucleotides 1320–1322 in the topoisomerase IIα cDNA from FVP3 is indicated as compared to cDNA's from both FEM-X and HeLa (Tsai-Pflugfelder, 1988).

Table 1: Frequency of Mutant Topoisomerase IIα RNA As Detected in cDNAs from FEM-X and VP-16-Resistant Clones

cell line	x-fold VP-16 resistance	no. of clones sequenced	wild-type	del(1320–1322)	% mutant
FEM-X	1	12	12	0	0
FVP1b	28	11	4	7	64
FVP3	56	11	0	11	100[a]

[a] Upon Southern analysis of RT-PCR products from the three cell lines cut with *Alu*I, a very low level of the wild-type RNA could be detected in FVP3.

pattern of drug resistance that suggested involvement of the target enzyme for these drugs, topoisomerase II. Reverse transcription of RNA followed by the polymerase chain reaction (RT-PCR) was used to clone the full-length cDNA for topoisomerase IIα from both FEM-X and FVP3. Sequencing of cDNA's from FEM-X revealed several differences from the published sequence for human topoisomerase IIα derived from HeLa cells (Tsai-Pflugfelder *et al.*, 1988). However, the sequence of the FEM-X clone within these regions was identical to that reported for topoisomerase IIα from HL-60 cells (Hinds *et al.*, 1991; Lee *et al.*, 1992). Three base additions were present in the topoisomerase IIα cDNA from FEM-X as compared to that from HeLa: a T at position 363, an A at 374, and a T at 380. These changes alter the amino acid sequence in this region from Met-Ile-Arg-Lys-Gln to Ile-Asp-Pro-Glu-Asn-Asn. In addition, nucleotides 3100 and 3919 were T's instead of C's. These base changes alter amino acid 1021 in topoisomerase IIα from Leu to Phe and residue 1294 from Pro to Ser, and have also previously been described (Hinds *et al.*, 1991; Lee *et al.*, 1992).

The cDNA isolated from the highly drug-resistant cell line FVP3 had the same changes as that from FEM-X plus a unique 3 bp deletion (Figure 2). In the cDNA from FVP3, nucleotides 1320–1322 had been deleted, thus deleting Ala429. To confirm the presence of this mutation in the VP-16-resistant cell lines, new RNA was made from FVP1b (moderately drug resistant) and FVP3 (highly drug resistant) cells, and the RT-PCR for this region was repeated. After subcloning the PCR products, 10–12 clones were picked from each cell type and their sequences compared to 12 clones derived from the parental FEM-X. The results of this analysis and the corresponding levels of VP-16 resistance exhibited by the three cell lines are shown in Table 1. All of the clones derived from FEM-X were wild-type and did not have the 1320–1322 deletion. In contrast, 64% (7/11) of the clones from FVP1b were mutant, and 36% were wild-type. Of the 11 clones sequenced from FVP3, all were mutant; we did not detect any wild-type sequences in this cell line.

To identify where in the multistep selection the deletion of nucleotides 1320–1322 first appeared, RNA from the earliest clones, FVP0.2 and FVP1a, in addition to RNA from FEM-X, FVP1b, and FVP3, was used for RT-PCR with primers Top50 and Top51 (see Materials and Methods for a description of these primers). Amplification of this region in the topoisomerase IIα cDNA gave a 289 bp fragment (Figure 3a,b). The deletion of nucleotides 1320–1322 destroys an internal *Alu*I site within this fragment. The products of the RT-PCR were then digested extensively with *Alu*I and analyzed by electrophoresis through 2.5% agarose gels. Wild-type cDNA's with an intact *Alu*I site at 1320 are cleaved by the enzyme into two fragments of 81 and 201 bp. Mutant cDNA's lack the internal *Alu*I site, and, therefore, the resulting fragment is 282 bp.

From Figure 3c,d, it is apparent that in FEM-X the vast majority of the parental fragments are cleaved by *Alu*I. Despite repeated digestion with *Alu*I, there was consistently a slight amount of uncut 282 bp fragment. From analysis of genomic DNA (described below), it appears that FEM-X does not possess a mutant topoisomerase IIα allele, and we were unable to detect mutant cDNA's during sequencing of multiple clones, so it is unclear as to what is preventing complete digestion of this residual parental fragment. FVP0.2 appeared similar to FEM-X in its prevalence of wild-type RNAs. The first clone in which a distinct prevalence of mutant topoisomerase IIα RNA (cDNA's uncut by *Alu*I) appeared was FVP1a, the cell line isolated at the second step of the selection. The percentage of mutant RNA's as compared to wild-type increased in the subsequent clones FVP1b and FVP3 in parallel to the increase in drug resistance in these cells. In FVP3, the expression level of the wild-type RNA was so low that it could only be consistently visualized by hybridization with labeled DNA probes covering this region (Figure 3d and data not shown). To confirm that the DNA fragments were indeed topoisomerase IIα-specific, the gel was transferred to a nitrocellulose membrane and probed separately both with the 45-mer Top52 (Figure 3d) and with the fragment Top50/51A as described under Materials and Methods (data not shown). The results using both probes were indistinguishable. When the same RT-PCR products from each cell type were cut with *Hinf*I, which should not be affected by the deletion at 1320–1322, the resulting restriction fragments were identical in all of the samples (data not shown).

The Mutation in Topoisomerase IIα Can Be Detected in Genomic DNA from Resistant Cells Only. To determine if the 1320–1322 deletion was present in the topoisomerase IIα gene in the VP-16-resistant clones, a portion of the topoisomerase IIα locus in genomic DNA was amplified by PCR using primers Top50 and Top 51. This amplification gave rise to a 651 bp fragment which was subsequently purified from low melting point agarose, digested to completion with *Alu*I, and analyzed by electrophoresis using 2.5% agarose gels. Figure 4a shows the Southern analysis of a representative gel where the hybridization probe used was ^{32}P-labeled Top52. Digestion of the genomic fragment from FEM-X gave two bands of 570 and 81 bp (Figure 4a,b). The 81 bp fragment was not detected as the oligonucleotide probe used for hybridization did not encompass the region 5′ of nucleotide 1366. Sequencing of multiple clones from this region confirmed the presence of an intact *Alu*I site at nucleotide 1320 and showed that one intron of 368 bp was present in the genomic clone between nucleotides 1378 and 1379 of the cDNA sequence.

The genomic fragments isolated from the drug-resistant clones gave three fragments upon *Alu*I digestion. The first two fragments were the 570 and 81 bp fragments seen in FEM-X and represent the wild-type allele. The third fragment is 651 bp and represents the mutant allele with the deletion which destroys the internal *Alu*I site. The presence of both the wild-type allele and the mutant allele with the 1320–1322 deletion was confirmed by sequencing two and five genomic clones from FVP1b and FVP3, respectively.

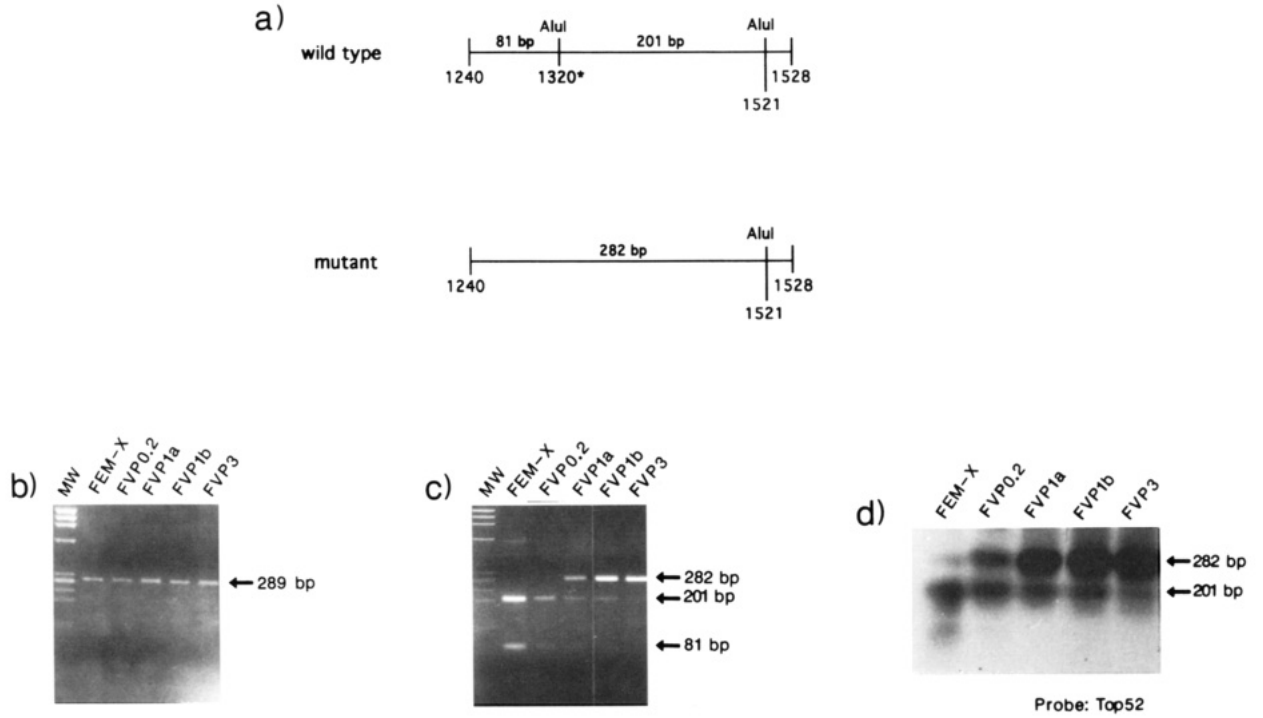

FIGURE 3: *Alu*I restriction analysis of topoisomerase IIα cDNA's from FEM-X- and VP-16-resistant clones. Reverse transcription of RNA from FEM-X and the four drug-resistant sublines was followed by the polymerase chain reaction using primers Top50 and Top51 as described under Materials and Methods. (a) Schematic diagram of the topoisomerase IIα cDNA between nucleotides 1240 and 1528. The *Alu*I sites within this region are shown, as are the expected restriction fragment sizes if the *Alu*I site at 1320 is intact (wild type) or deletd (mutant). The products of the RT-PCR were electrophoresed through 2.5% agarose gels either before (b) or after (c) *Alu*I digestion. The sizes of the resulting fragments are indicated. The molecular weight markers shown (MW) are phage λ DNA digested with *Hin*dIII mixed with φX174 DNA digested with *Hae*III. (d) Southern analysis was carried out on the gel from (c) using the oligonucleotide Top52 as a probe as described under Materials and Methods. The 81 bp fragment is not detected by Southern analysis since the hybridization probe does not encompass the region between 1240 and 1320.

DISCUSSION

We have used reverse transcription followed by the polymerase chain reaction to clone the entire topoisomerase IIα cDNA from both FEM-X and its drug-resistant counterpart, FVP3. Sequence analysis of these clones and comparison with the sequence for human topoisomerase IIα cloned from HeLa (Tsai-Pflugfelder *et al.*, 1988) revealed a deletion of nucleotides 1320–1322 which was unique to the cDNA from FVP3. The result of this sequence change was deletion of Ala429 from the topoisomerase IIα protein. This deletion appears to be present in the majority of the topoisomerase IIα mRNA molecules from FVP3, since we did not detect the wild-type sequence in 11 independent cDNA clones from this region. The fact that FEM-X does not have this mutant allele, and that FVP1b, with its intermediate drug resistance, expresses both wild-type and mutant mRNA's, strongly supports a role for this sequence change in the VP-16 resistance observed in the selected cell lines.

To understand the selection process that led to the development of the highly resistant clone, FVP3, it was imperative that we determine at what step the mutation in the topoisomerase IIα gene had occurred and whether this single change could manifest itself as numerous different levels of drug resistance. We took advantage of the fact that the deletion at 1320–1322 destroyed an *Alu*I site to go back to the earliest clones in the multistep selection and analyze them for the presence of the mutant RNA. The first clone in which the mutant mRNA was expressed preferentially was FVP1a, which is approximately 7-fold resistant to VP-16. The proportion of the total topoisomerase IIα RNA represented by the mutant species continued to increase in the more highly resistant FVP1b and FVP3. These results support the idea of an initial mutation in topoisomerase IIα that renders the enzyme less susceptible to the effects of VP-16. Since expression of the wild-type enzyme is deleterious in the presence of drug, isolation of clones resistant to higher and higher concentrations of VP-16 selected for cells in which the level of wild-type RNA was decreased and expression of the mutant RNA increased. The net effect was to maintain a constant level of topoisomerase IIα mRNA and protein (Campain *et al.*, 1993). We have shown by sequencing and restriction analysis that the drug-resistant clones have both wild-type and mutant topoisomerase IIα alleles.

Several mutant forms of topoisomerase IIα from drug-resistant cell lines have been identified in other laboratories (Bugg *et al.*, 1991; Chan *et al.*, 1993; Hinds *et al.*, 1991; Lee *et al.*, 1992). These mutations all lie within the highly conserved region between amino acids 450 and 500 of the topoisomerase IIα sequence which contains several motifs important in nucleotide binding. The amino one-third of the topoisomerase IIα protein is homologous to the B-subunit of the bacterial DNA gyrase and is thought to function as an ATPase (Wyckoff *et al.*, 1989). Bugg *et al.* (1991) showed that the topoisomerase IIα from CCRF-CEM cells resistant to teniposide is altered in its ATP-binding capacity. This biochemically distinct enzyme differs by a single amino acid from the wild-type; Arg450 has been changed to Gln. An *m*-AMSA-resistant form of topoisomerase IIα isolated from HL-60/AMSA cells has been found to contain an Arg to Lys mutation at residue 487, a site previously implicated in the sensitivity of the enzyme to various inhibitory drugs (Hinds *et al.*, 1991; Lee *et al.*, 1992). Chan *et al.* (1993) have also identified a change at amino acid 493 in the Chinese hamster ovary (CHO) topoisomerase IIα from Arg to Gln which is associated with resistance of the enzyme to the epipodophyllotoxins. Mutations within this region have also been

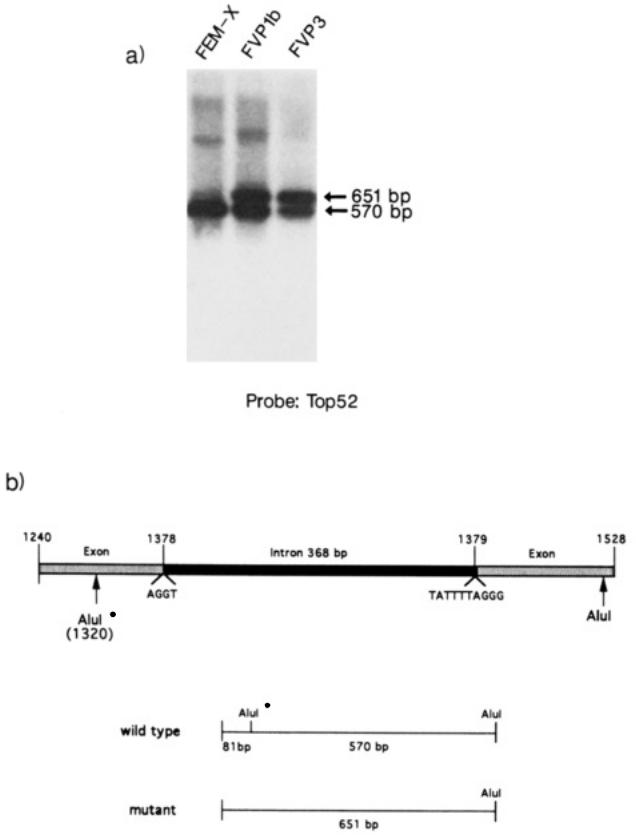

FIGURE 4: Screening of the topoisomerase IIα locus in FEM-X and the VP-16-resistant subclones for the presence of wild-type and mutant alleles. (a) The region between nucleotides 1239 and 1528 of the topoisomerase IIα cDNA was amplified from genomic DNA from FEM-X, FVP1b, and FVP3 using primers Top50 and Top51. The primary product of this amplification was purified from a low melting point agarose gel and digested to completion with *Alu*I. The resulting restriction fragments were then analyzed by electrophoresis through a 2.5% agarose gel, transferred to a nitrocellulose membrane, and probed for topoisomerase IIα-specific sequences with the ^{32}P-labeled Top52 oligonucleotide. (b) Schematic view of this region within the topoisomerase IIα locus as determined by both restriction and sequence analysis of multiple independent clones from FEM-X, FVP1b, and FVP3. Although *Alu*I digestion of the wild-type parental fragment should yield 570 and 81 bp fragments, the latter was not detected due to the fact that the hybridization probe does not encompass the region between 1239 and 1320. The position and size of the single intron within this domain are shown as are the consensus donor and acceptor splice sites (Mount, 1982).

linked to nalidixic acid resistance in the *E. coli gyr*B gene product (Yamagishi *et al.*, 1986).

Although the deletion identified in the VP-16-resistant clones is fairly close to these other identified mutations, it does not lie within this highly conserved domain, and does not correspond to other sites known to be important for topoisomerase IIα function. Ala429 is not conserved among a wide group of topoisomerase II enzymes including those from *Drosophila* and yeast and the bacterial gyrases (Wyckoff *et al.*, 1989). This residue is the same in the human and CHO enzymes (Chan *et al.*, 1993), but is not conserved between human topoisomerases IIα and β (Chung *et al.*, 1989).

Studies of topoisomerase II from both *Drosophila* and yeast have shown that the region between approximately amino acids 400 and 450 is particularly sensitive to protease digestion when the protein is folded in its active form (Lindsley & Wang, 1991; Shiozaki & Yanagida, 1991; Lee & Hsieh, 1994). This region of eukaryotic topoisomerase II is within the N-terminal portion of the protein which is homologous to the B-subunit of bacterial DNA gyrase. Partial proteolysis of gyrase B gives rise to two peptides: the N-terminal half possesses ATPase activity, and the C-terminal half interacts with the gyrase A subunit (Brown *et al.*, 1979; Gellert *et al.*, 1979; Adachi *et al.*, 1987). It has been suggested that these two defined domains within gyrase B are separated by a more flexible region that is exposed to the outside and readily accessible. It is likely that the protease-sensitive domain within the gyrase B homology region of the eukaryotic enzyme is also exposed and, thus, may be readily available to interact with other proteins in the nucleus (see below). Deletion of Ala429 from the human enzyme may directly or indirectly affect the structure and/or accessibility of this domain.

The exact role played by the Ala429 deletion in the drug resistance of the FEM-X clones is uncertain at this time. However, the genetics of the system are very clear, and show a progressive increase in the expression of the mutant topoisomerase IIα allele with increasing drug resistance. It is likely that the sequence change in this area of the protein prevents it from being trapped in a drug-induced covalent complex with DNA. Topoisomerase IIα from FVP3 is highly resistant to the effects of VP-16 when assayed in live cells. However, the enzyme present in nuclear extracts from these cells is similar to the wild-type enzyme in its sensitivity to drug-stimulated cleavable complex formation. One possibility is that the deletion of Ala429 may not directly affect the interaction of the drug and topoisomerase IIα. Rather, the mutation may alter localization of the enzyme or change its interaction with DNA and/or other factors, any of which could decrease indirectly the DNA–topoisomerase IIα complexes trapped in the presence of VP-16. Structural changes that allow topoisomerase II to localize differently within the nucleus or alter its capacity for binding DNA may modulate the sensitivity of the enzyme to inhibitory drugs (Boege *et al.*, 1992; Fernandes *et al.*, 1990). Quantification of matrix-associated topoisomerase IIα in FEM-X and FVP3 has shown similar levels of the mutant and wild-type enzyme within the insoluble fraction (data not shown). However, this finding does not preclude the possibility that the mutant enzyme interacts differently with either the matrix itself or the DNA substrate. Also, topoisomerase II could associate with other factors in a manner which modulates its activity, drug resistance, or both (Darkin & Ralph, 1989; Darkin-Rattray & Ralph, 1991; Rowe *et al.*, 1986). Topoisomerase II has been shown to interact with proteins such as CREB which contain a leucine zipper for protein dimerization (Kroll *et al.*, 1993). These interactions modulate both the drug-induced DNA cleavage activity and the strand passing activity of the topoisomerase II.

Several lines of evidence have indicated that the susceptibility of topoisomerase II to inhibitory drugs and cleavable complex formation depends on numerous external factors. Among these, posttranslational modification of the enzyme [by phosphorylation or poly(ADP)ribosylation] is known to alter its catalytic activity (Ackerman *et al.*, 1985; Darby *et al.*, 1985; Rottman *et al.*, 1987; Sahyoun *et al.*, 1986). As the toxicity of anti-topoisomerase II poisons depends on the activity of the enzyme, these types of alterations could reasonably be assumed to play a role in drug resistance. In support of this idea, DeVore *et al.* (1992) have shown that phosphorylation of topoisomerase II by either casein kinase II or protein kinase C decreases the levels of cleavable complexes stimulated by VP-16 or amsacrine. Indications that this may also be the case *in vivo* come from the finding that VP-16-resistant tumor cells lines show enhanced levels of topoisomerase II phosphorylation (Takano *et al.*, 1991). To address this possibility for our VP-16-resistant melanoma cell lines, we have looked at the steady-state levels of

topoisomerase IIα phosphorylation in both FEM-X and FVP3. Under these conditions, we were unable to demonstrate consistent differences in phosphorylation between the enzymes in the two cell lines (data not shown).

Whatever the mechanism of action of the Ala429 deletion mutant, the genetic evidence presented here from five clonal cell lines shows a clear association of the expression of mutant topoisomerase IIα with resistance to VP-16 and other inhibitory drugs. As is true, however, with all putative drug resistance genes, the definitive experiment in this case is to express the mutant topoisomerase IIα in drug-sensitive cells and have it confer resistance to VP-16. We have attempted to carry out these experiments but were unable to get the mutant cDNA expressed at significant levels (greater than approximately 1% of the endogenous level of topoisomerase IIα) in FEM-X and saw no change in the drug resistance phenotype. The genetic means by which the topoisomerase IIα mutant mRNA becomes predominant in these drug-resistant melanoma cells, the prevalence of this mutation in clinical cancer isolates, and further exploration of its mechanisms of action promise to be fruitful areas for further study.

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