Two COOH-Terminal Truncated Cytoplasmic Forms of Topoisomerase IIα in a VP-16-Selected Lung Cancer Cell Line Result from Partial Gene Deletion and Alternative Splicing†

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ABSTRACT: Topoisomerase IIα is a nuclear enzyme involved in chromosome segregation and other essential cellular processes. It is also the target of several clinically important antineoplastic agents such as the epipodophyllotoxin, VP-16 (etoposide). We have previously described a VP-16-selected lung cancer cell line, H209/V6, that expresses reduced levels of two species of topoisomerase IIα-related mRNAs and a catalytically active, predominantly cytoplasmic topoisomerase IIα-related protein that is 10 kDa smaller than the wild-type protein [Mirski, S. E. L., *et al.* (1993) *Cancer Res. 53,* 4866−4873; Feldhoff, P. W. *et al.* (1994) *Cancer Res. 54,* 756−762]. The smaller H209/V6 4.8 kb mRNA is missing 988 nucleotides of contiguous coding and non-coding sequence at its 3′ end resulting in an mRNA predicted to encode a truncated polypeptide missing three previously unrecognized potential COOH-proximal bipartite nuclear localization signals [Mirski, S. E. L., & Cole, S. P. C. (1995) *Cancer Res. 55,* 2129−2134]. We have now determined the structure of the larger 6.2 kb topoisomerase IIα-related mRNA and show that it is missing 684 nucleotides of contiguous 3′ coding and non-coding sequence between nucleotide positions 4267 and 4951. This sequence is replaced by 847 nucleotides of new sequence, containing an in-frame stop codon after 41 nucleotides. The translation product of the 6.2 kb mRNA is predicted to contain 13 new amino acids replacing the COOH-terminal 109 residues of wild-type topoisomerase IIα, producing a truncated polypeptide of approximately 160 kDa. Immunoblot analyses using antisera against the unique COOH-terminal 13 and 34 amino acids encoded by H209/V6 6.2 kb and 4.8 kb mRNAs, respectively, confirmed that both mRNAs are translated. Restriction enzyme analysis and sequencing of the 3′-proximal region of the *TOP2A* gene in the H209 and H209/V6 DNA revealed that a partial deletion has occurred in H209/V6 and the novel sequence identified in the H209/V6 6.2 kb mRNA is derived from the adjacent 3′ intron as a consequence of read-through at a concensus splice donor site. These observations suggest a mechanism for the generation of the two mutant topoisomerase IIα mRNAs in H209/V6 cells and provide the first reported example of a drug resistant cell line containing two different cytoplasmic forms of topoisomerase IIα.

DNA topo II[1] has been implicated in a variety of cellular functions and is essential for the survival of eukaryotic cells (Adachi et al., 1991; Taagepera et al., 1993). It is believed to play important roles in DNA replication (Clarke et al., 1993; Holm et al., 1989) and recombination (Wang et al., 1990), as well as in chromosome condensation and chromatid segregation at mitosis (Adachi et al., 1991; Holm et al., 1985, 1989). There are two isoforms of mammalian topo II, designated α and *β* (Drake et al., 1987, 1989), which differ from each other with respect to certain biochemical and pharmacological properties. Expression of topo IIα and topo II*β* also appears to be differentially regulated (Austin et al., 1995; Woessner et al., 1991; Drake et al., 1987, 1989). In addition to its essential physiological functions, topo IIα is a target for a variety of antitumor agents including etoposide (VP-16) and doxorubicin, which are two of the most effective drugs used in the treatment of solid tumors. Consequently, it is not surprising that alterations in this nuclear enzyme have been found in cells resistant to these and other drugs which are known to exert their cytotoxicity through interaction with DNA and topo II (Liu, 1989; Sullivan & Ross, 1991; Osheroff et al., 1994; Mirski & Cole, 1996; Cole, 1996; Liu & Wang, 1991; Beck et al., 1994).

We have reported previously the characterization of a VP-16-selected drug resistant small cell lung cancer cell line, H209/V6, that expresses 10-fold lower levels of an apparently normal-sized 6.1 kb topo IIα mRNA (Mirski & Cole, 1995; Mirski et al., 1993). In addition, the H209/V6 cells express low levels of a 4.8 kb topo IIα-related mRNA. Despite the

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[1] Abbreviations: topo II, topoisomerase II; SDS, sodium dodecyl sulfate; Denhardt's solution, 50× = 1% bovine serum albumin, 1% polyvinylpyrrolidone, 1% ficoll; SSC, 1× = 150 mM NaCl, 15 mM sodium citrate, pH 7.0; nt, nucleotide; SSPE, 1× = 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4; PCR, polymerase chain reaction; cDNA, complementary DNA; nt, nucleotide; GST, glutathione *S*-transferase; TBS-T, Tris-buffered saline with Tween (10 mM Tris, 0.15 M NaC1, 0.05% Tween-20, pH 7.5); RT, reverse transcriptase; UTR, untranslated region.

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presence of two H209/V6 topo IIα-related mRNAs, only one immunoreactive topo IIα-related protein is detectable in these cells using an antiserum raised against the 70 kDa recombinant COOH-terminal portion of HeLa cell topo IIα (Sullivan et al., 1989). However, the H209/V6 topo IIα protein is smaller than normal (160 kDa *vs* 170 kDa) and it is present at levels approximately 5-fold lower than the 170 kDa topo IIα in parental H209 cells. Moreover, in contrast to parental cells, the H209/V6 160 kDa protein is primarily localized in the cytoplasm. Nevertheless, the catalytic activities of purified H209 170 kDa topo IIα and H209/V6 160 kDa topo IIα are comparable (Feldhoff et al., 1994).

In a previous study, we determined the structure of the H209/V6 4.8 kb topo IIα mRNA and found that it was missing a contiguous 988 bp sequence corresponding to nt 4267−5255 of the normal topo IIα mRNA (Mirski & Cole, 1995). The absence of this sequence, which is composed of both coding and 3′ untranslated sequences, results in an mRNA predicted to encode a protein lacking the 109 COOH-terminal amino acids of the normal 170 kDa topo IIα enzyme. Within these missing 109 amino acids are several potential bipartite nuclear localization signals (Dingwall & Laskey, 1991; Mirski & Cole, 1995). We therefore concluded that the absence of this sequence from the H209/V6 4.8 kb mRNA might be responsible for the predominantly cytoplasmic localization of the mutant 160 kDa topo IIα (Mirski & Cole, 1995). However, although our analyses showed that the H209/V6 4.8 kb topo IIα-related mRNA could encode the cytoplasmic 160 kDa topo IIα, they did not explain the absence of a normal size 170 kDa protein in H209/V6 cells, which would be expected from the larger size (approximately 6.1 kb) topo IIα mRNA. In the present study we have determined the structure of this larger mRNA and characterized the 3′-proximal region of the H209/V6 *TOP2A* allele which encodes both mutant mRNAs. Our results indicate that a partial gene deletion has occurred in H209/V6 cells and that the mutant *TOP2A* allele gives rise to the two topo IIα mRNA species as a result of alternative splicing. We also show that both H209/V6 topo IIα-related mRNAs are translated into truncated, 160 kDa, cytoplasmic polypeptides which have different COOH-termini and are missing three potential bipartite nuclear localization signals.

EXPERIMENTAL PROCEDURES

Cell Culture. The origin and maintenance of the human small cell lung cancer cell line H209 and its VP-16 selected variant H209/V6 have been described previously (Mirski et al., 1993). Both cell lines were grown in RPMI 1640 medium supplemented with 5% calf serum (Hyclone, Logan, UT) at 37 °C and 5% CO_2. All experiments were performed on day 3 cultures when cells were in exponential growth.

Northern Blot Analyses. Poly(A)$^+$ RNA was prepared using a Micro-FastTrack mRNA isolation kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. RNA was separated on 1% agarose gels containing 0.66 M formaldehyde and blotted onto ZetaProbe membrane (Bio-Rad, Mississauga, Ontario, Canada) by pressure transfer with 10× SSC/10× SSPE as described previously (Mirski & Cole, 1995). The DNA probes used for these studies included the 3.1 kb *Eco*RI/*Sca*I SP-1 cDNA specific for topo IIα (provided by Dr. K. B. Tan) (Chung et al., 1989) and probes corresponding to selected segments of coding and 3′ non-coding sequence of normal topo IIα mRNA. These latter probes [designated probe I (nt 4328−4645), probe II (nt 4665−5203), and probe III (nt 5188−5589), respectively] were generated by PCR using the plasmid *hTOP2* (American Type Culture Collection #59748, Rockville, MD) as template and *Pfu* DNA polymerase (Stratagene). Probe IV was generated by PCR using the H209/V6 2.2 kb reverse transcriptase (RT)-PCR product as template (see below) and sense primer 5′-CTTTGAGATGGGTTAATGTT-3′ and antisense primer 5′-AAAGATCGAAAAGAAATTGC-3′. The PCR products were gel purified using GeneClean (Bio 101, La Jolla, CA). All DNA probes were labeled with [α-^{32}P]dCTP (NEN-Dupont) to a specific activity of approximately 1×10^8 cpm/μg using BRL Random primers DNA labeling system (GIBCO-BRL). Blots were hybridized with probes and then exposed to Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) at −70 °C with enhancing screens for 1−10 days as required. In some experiments, membranes were stripped in a 0.5% SDS/0.1× SSC solution at 95 °C for 20 min and then subjected to hybridization with a second probe.

Reverse Transcription and PCR Amplification of the H209/V6 6.1 kb Topo IIα mRNA. Single stranded cDNA was synthesized by reverse transcription of poly(A)$^+$ RNA from H209 and H209/V6 cells using avian myeloblastosis virus reverse transcriptase (Life Science Inc., St. Petersburg, FL) and random hexamers (Amersham, Oakville, Ontario, Canada). The cDNAs were subjected to PCR using *Taq* DNA polymerase and *Taq* extender (Promega, Madison, WI) according to the manufacturer's instructions. In these reactions, the sense primer was 5′-CCACCTTCAACTATCTTCT-3′ (nt 3367−3387) and the antisense primer was 5′-GCTGAGCATGGTTATCAA-3′ (nt 5381−5399). These primers were selected because they lie 5′ and 3′, respectively, of the missing sequence in the H209/V6 4.8 kb mRNA (Mirski & Cole, 1995). Confirmation of the identity of the PCR products was determined by southern blot analyses using appropriate oligonucleotide probes labeled with [γ-^{32}P]dATP (4500 Ci/mmol; ICN Biomedical, Cleveland, OH) and T4 kinase (GIBCO-BRL).

Subcloning and Sequencing of the H209/V6 2.2 kb RT-PCR Product. The H209/V6 2.2 kb product obtained from the RT-PCR experiments described above was ligated into the pBluescript SK$^-$ plasmid (Stratagene) by blunt-ended cloning (Hitti & Bertino, 1994). Recombinant plasmids were transformed into competent DH5αF′ *Escherichia coli* cells (GIBCO-BRL) by electroporation. The individual bacterial colonies containing plasmid with insert DNA were identified by hybridization with ^{32}P-labeled oligonucleotide probe nt 5188−5208 as well as by restriction enzyme analysis. Plasmid DNAs from 10 positive colonies were purified, pooled, and sequenced by the dideoxy method using [^{35}S]dATP (Amersham) and Sequenase version 2.0 DNA polymerase (United States Biochemical, Cleveland, OH). Sequence data was compiled and analyzed using the Eyeball Sequence Editor software (Eric Cabot).

Immunoblot Analyses. Preparation of H209 and H209/V6 total cell lysates and subsequent electrophoretic separation and transfer of these proteins to nylon membranes were performed as described previously (Mirski & Cole, 1995; Liu & Wang, 1991). For detection of topo IIα, blots were incubated with rabbit antiserum raised against the recombinant 70 kDa COOH-terminus of HeLa topo IIα (kindly

provided by Drs. W. Ross and D. Sullivan) (Sullivan et al., 1989), or with rabbit antiserum A160 raised against the synthetic peptide [Cys]-Lys-Pro-Lys-Ser-Leu-Arg-Trp-Val-Asn-Val-Ala-Ile-Thr (kindly provided by Dr. W. G. Harker) (Harker et al., 1995b), or with rabbit antiserum QU10 raised against a glutathione *S*-transferase (GST) fusion protein containing the COOH-terminal 34 amino acids of the putative polypeptide encoded by the H209/V6 4.8 kb topo IIα-related mRNA. To generate this fusion protein, a PCR product corresponding to the sequence between nt 5256 and 5356 in the 3′-UTR of topo IIα cDNA was synthesized using the plasmid *hTOP2* as template and sense primer 5′-GCG-GATCCACTCTTGACCTGTCCCCTCTG-3′ and antisense primer 5′-CGGAATTCAAATGTTGTCCCCGAGTCTT-3′. The PCR product was digested with *Bam*HI and *Eco*RI and subcloned in-frame into the *Bam*HI/*Eco*RI site of a pGEX2T expression vector (Pharmacia Biotech, Uppsala, Sweden) as described previously (Hipfner et al., 1996). The fidelity of the construct was confirmed by DNA sequence analysis. Expression of the GST-fusion protein was induced in DH5α F′ bacteria by adding isopropyl thio-β-galactoside (2 mM) for 5 h. After cell lysis by sonication, the expressed GST-fusion protein was affinity purified by using glutathione cross-linked agarose (Sigma) as described (Smith, 1993). Rabbit antiserum was raised against the purified fusion protein by Cocalico Biologicals (Reamstown, PA). Antisera were diluted appropriately in blocking solution [4% non-fat Carnation milk in TBS-T (10 mM Tris, 0.15 M NaC1, 0.05% Tween 20, pH 7.5)] and then incubated with the blots for 1−2 h. After washing 3 × 5 min in TBS-T, horseradish peroxidase-conjugated $F(ab')_2$ fragments of goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch, West Grove, PA) (diluted in blocking buffer) were added and blots were incubated for 1 h. After washing 5 × 5 min in TBS-T, antibody binding was determined by enhanced chemiluminescent detection (Dupont NEN, Boston, MA) and exposure on Kodak X-Omat AR film.

Southern Blot Analyses and Amplification of Genomic DNA. High molecular weight genomic DNA was isolated from exponentially growing H209 and H209/V6 cells using a Cell Culture DNA kit (Qiagen, Chatsworth, CA) following the manufacturer's instructions. DNA (10 μg) was digested with selected restriction enzymes, size fractionated on a 0.7% agarose gel and transferred to Zeta Probe nylon membrane. The membranes were prehybridized at 42 °C for 4 h in a solution containing 50% formamide, 5× SSPE, 5× Denhardt's, and 1% SDS and hybridized with ^{32}P-labeled topo IIα-specific SP-1 cDNA in the same solution at 42 °C for 12−18 h. After hybridization, the membranes were washed once in 1× SSC/0.1% SDS at room temperature for 15 min and twice at 42 °C in 0.1× SSC/0.1% SDS for 15 min. The membranes were exposed to film at −70 °C with enhancing screens for 7−10 days.

Genomic DNA was amplified using the Expand Long Template PCR System (Boehringer Mannheim, Germany) for 35 cycles. For these reactions the sense primer 5′-CCATCAGATGCTAGTCCACCTAAGACC-3′ (nt 4048−4074) derived from the topo IIα coding sequence and the antisense primer 5′-CCAGAGGGGACAGGTCAAGAG-3′ (nt 5256−5277) from the topo IIα 3′-untranslated sequence were used. PCR conditions were as follows: for the first cycle, denaturation at 94 °C for 5 min; annealing at 54.1 °C for 5 min; and elongation at 72 °C for 40 min. For subsequent cycles, denaturation was at 94 °C for 1 min; annealing at 54.1 °C for 1 min; and elongation at 72 °C for 10 min. Final extension was at 72 °C for 15 min. The genomic PCR products were gel purified using Geneclean and then sequenced with the 373A DNA Sequencing System using DyeDeoxy Terminators (Applied Biosystems, Mississauga, Ontario, Canada). Sequence data were compiled and analyzed as before.

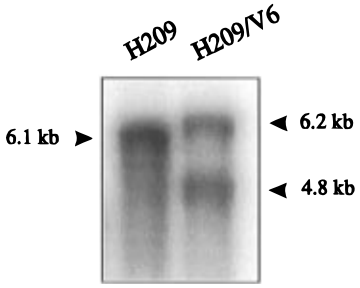

FIGURE 1: Northern blot analysis of topo IIα mRNA expression in H209 and H209/V6 cells. Poly(A)$^+$ RNA was prepared from H209 and H209/V6 cells, electrophoretically separated on formaldehyde-agarose gels, and blotted onto nylon membranes. Hybridizations were performed using the random primed ^{32}P-labeled topo IIα-specific SP-1 cDNA probe. Approximately 1 μg of H209 poly(A)$^+$ RNA and 3 μg of H209/V6 poly(A)$^+$ RNA were loaded on the gel.

RESULTS

Northern Blot Analyses of Topo IIα mRNAs in H209/V6 Cells. The first objective of this study was to characterize the previously described apparently normal sized 6.1 kb topo IIα mRNA in H209/V6 cells. In some RNA blots it was noted that this mRNA might not be exactly the same size as its counterpart in parental H209 cells. By increasing the amount of H209/V6 mRNA loaded on the gel, it was possible to obtain a topo IIα mRNA signal of similar intensity from the two cell lines. These experiments clearly demonstrated that the topo IIα mRNA in H209/V6 was approximately 6.2 kb and larger than the normal 6.1 kb mRNA in H209 (Figure 1).

Northern blots of H209 and H209/V6 mRNAs were hybridized with PCR-generated probes derived from the 3′-proximal region of the normal topo IIα mRNA (Figure 2, probe III) or from within the region we previously determined to be absent from the H209/V6 4.8 kb topo IIα-related mRNA (Figure 2, probes I and II) (Mirski & Cole, 1995). Neither probe I nor probe II hybridized with the 4.8 kb mRNA in H209/V6, as expected (Figure 2). Probe I also failed to detect the 6.2 kb mRNA in H209/V6, whereas probes II and III both hybridized with this mRNA. It was concluded that a 5′ portion of the region previously found to be missing from the topo IIα-related 4.8 kb mRNA was also absent from the 6.2 kb mRNA in H209/V6 cells.

Reverse Transcriptase-PCR, Cloning, and Sequencing of the 3′-Proximal Region of the H209/V6 6.2 kb Topo IIα mRNA. To compare the 3′-proximal region of the H209/V6 topo IIα 6.2 kb mRNA with the corresponding region of the H209 topo IIα 6.1 kb mRNA, PCR was performed on cDNA that had been reversed transcribed from H209 and H209/V6 mRNAs. For these reactions, primers were selected such that the region spanning nt 3367−5396 would be amplified. A 2 kb PCR product was obtained from H209 cDNA as expected (Figure 3). However, PCR of H209/V6

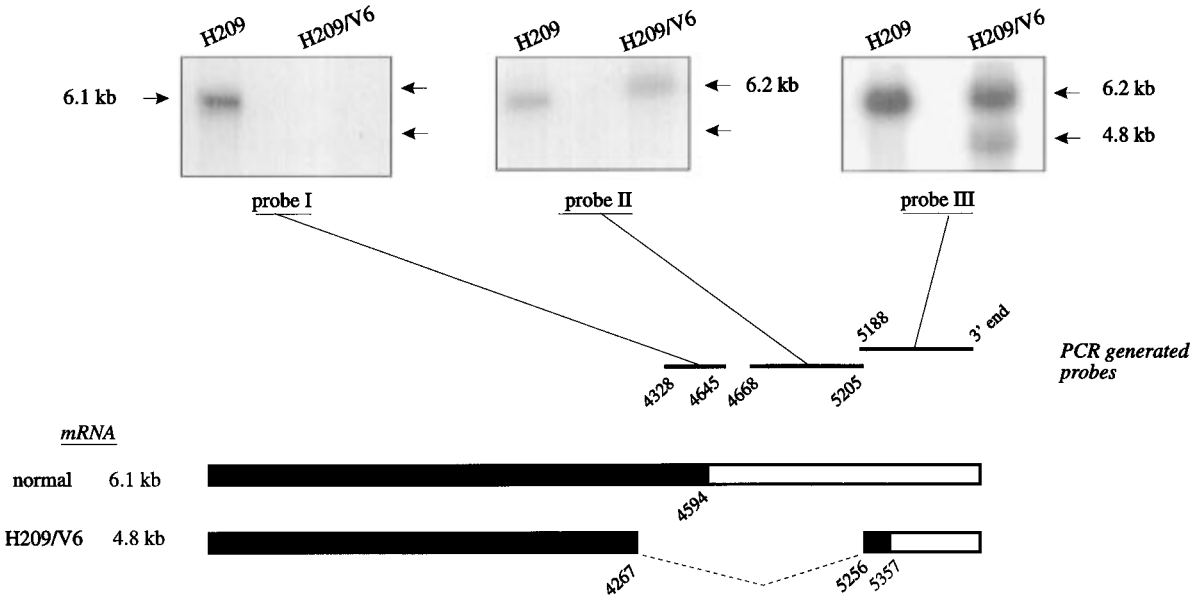

FIGURE 2: Northern blot analyses of topo IIα mRNA in H209 and H209/V6 cells with selected DNA probes. Poly(A)$^+$ RNA was isolated and blots prepared as described in Figure 1 and Experimental Procedures. Probes I, II, and III were generated by PCR using the human topo IIα h*TOP2* cDNA as template and correspond to the sequences indicated on the figure. Shown below the blots is a schematic diagram of the 3′-proximal region of the normal 6.1 kb topo IIα mRNA aligned with the corresponding region in the H209/V6 4.8 kb topo IIα-related mRNA (Mirski & Cole, 1995). The solid portion represents coding sequence while the open portion represents non-coding sequence. The broken line in the H209/V6 4.8 kb mRNA indicates the normal topo IIα sequence missing from this mRNA.

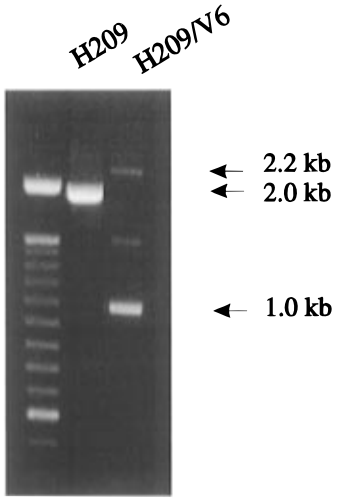

FIGURE 3: Gel electrophoresis analysis of RT-PCR products generated from the 3′ proximal region of topo IIα-related mRNAs in H209 and H209/V6 cells. cDNA was synthesized from poly(A)$^+$ RNA from both H209 and H209/V6 cells and subjected to PCR. To amplify the region which spans the sequence known to be missing from the H209/V6 topo IIα 4.8 kb mRNA, primers that lie on either side of the missing sequence were used. The PCR products obtained were resolved by agarose gel electrophoresis and stained with ethidium bromide. The 2 kb PCR product from H209 cDNA and the 2.2 kb and 1 kb PCR products from H209/V6 cDNA are indicated. A minor product of 1.5 kb was also observed. The 100 bp DNA ladder is shown on the left.

cDNA generated two major products of approximately 2.2 kb and 1 kb. It seemed probable that the 2.2 kb PCR product was derived from the H209/V6 topo IIα-related 6.2 kb mRNA because it was somewhat larger than the 2 kb product obtained with H209 cDNA. Evidence that this was the case was obtained from southern analyses of the RT-PCR products. As shown in Figure 4, probe II hybridized with the 2.2 kb RT-PCR product, whereas probe I did not. This hybridization pattern is consistent with the Northern blot analyses of H209/V6 6.2 kb mRNA using the same probes (Figure 2). Southern analyses of the H209/V6 2.2 kb RT-PCR product with six different oligonucleotides (corresponding to sequences between nt 3367 and 5381) gave positive signals with oligonucleotide probes 3367, 3560, 5188, and 5256 but not with oligonucleotide probes 4272 and 4897 (Figure 4). These results indicated that the H209/V6 2.2 kb RT-PCR product was missing at least 0.6 kb of topo IIα sequence located somewhere between nt 4264 and 4914. Subcloning and sequencing of the 2.2 kb RT-PCR product revealed that it contained the normal topo IIα sequence from nt 3367 to 4267 and from nt 4951 to 5401 but 684 bp of coding and non-coding topo IIα sequence between nt 4267 and 4950 were replaced by 847 nt of non-topo IIα cDNA sequence (Figure 5). To confirm that the novel sequence identified in the 2.2 kb product was unique to the H209/V6 6.2 kb topo IIα-related mRNA, northern analysis was performed using a PCR probe encompassing 146 nt of the 5′-proximal region of the novel sequence following nt 4264 (probe IV). As expected, this probe hybridized with the H209/V6 6.2 kb mRNA but not with either the H209/V6 4.8 kb mRNA or the H209 6.1 kb mRNA (not shown).

Predicted Structure of the H209/V6 Mutant Topo IIα 6.2 kb mRNA and Encoded Protein. The structure of the H209/V6 6.2 kb topo IIα-related mRNA and its encoded protein are depicted in Figure 6, aligned with the structure of the 4.8 kb mRNA we described previously (Mirski & Cole, 1995). The region of the normal topo IIα sequence missing from the H209/V6 6.2 kb mRNA contains the normal stop codon at nt 4594 and encodes the 109 COOH-terminal amino acids of the normal topo IIα enzyme which are consequently predicted to be absent from the protein encoded by this mRNA (Figure 6A). In addition, the 847 nt of new sequence in the H209/V6 6.2 kb mRNA contain an in-frame stop codon after 41 nt and thus are predicted to add 14 new amino acids in place of the 109 terminal residues of normal topo IIα (Figure 6B). The smaller topo IIα-related protein

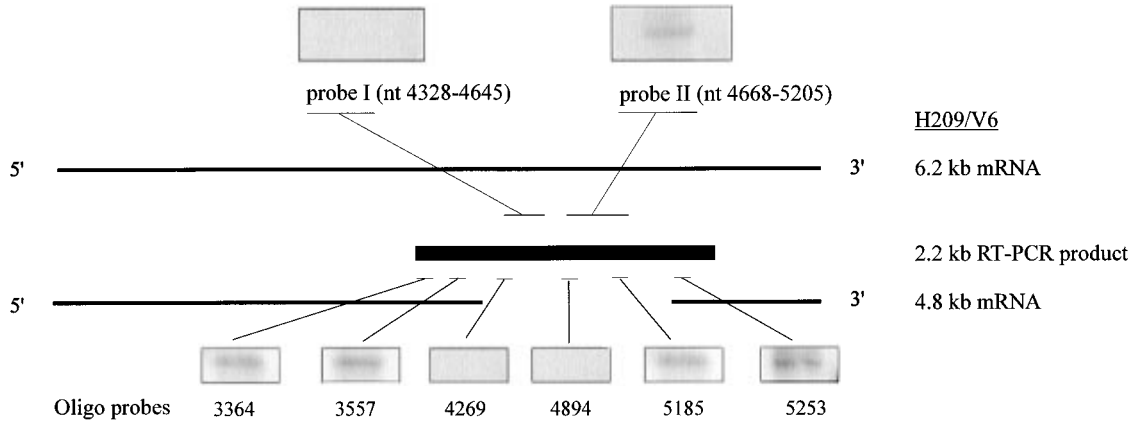

FIGURE 4: Southern analyses of the H209/V6 2.2 kb RT-PCR product. Blots of the 2.2 kb RT-PCR product derived from the H209/V6 6.2 kb mRNA were hybridized with ^{32}P-labeled PCR probes I (nt 4328−4645) and II (nt 4668−5205) generated using the wild-type *hTOP2* cDNA as template and with six wild-type topo IIα-specific ^{32}P-labeled synthetic 19−21 mer oligonucleotide probes. The approximate positions of these probes in the normal topo IIα mRNA sequence are indicated on the diagram. With respect to the oligonucleotide probes, the numbers under the blots indicate the nucleotide position in normal topo IIα mRNA of the first nucleotide of the probe. The 3′-proximal regions of the H209/V6 2.2 kb and 4.8 kb topo IIα-related mRNAs (narrow lines) are indicated above and below, respectively, the H209/V6 2.2 kb RT-PCR product (wide line). Conditions for radiolabeling of DNA probes and hybridization are described in Experimental Procedures.

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CAGCAAAAA*GTAAGCCTAAATCTTTGAGATGGGTTAATGTTGAATTACCTAACTGGTTTC*

CACGTGTCTATTTCAATTTTTTTATTGCCAAAACTTACTATTGATATTACAGATTAAATATTTT

CAATTGGAAGCAATTTCTTTTCGATCTTTATAATCAAAATTAGTAGTCAAGGCTGTTCCAAAA

CAGTAAGTTATCTCTATTGATTGTTCAGTTACAGATCAACTCCTTGTTCTACTCTTTTCCCTC

CTTCTCACTACTGCACTTGACTAGTCAAAAAACAAAACAAAAGCCAGGCAGAGTGGCTCAT

GCCTGTAATCCCAGCACTTTGGGAGGCTAAAACAAGAGGATTGCTTGAGGCCAGGAGTTC

AAGGCTGCAGTGAGCTATATGATCAAGCCACTGCACTCAGCCTGAGTGAGAGAGCAAGAC

CCTGTCTCCAAAAATAAGGTAGTCCAAATGTTTAAAATCAGTGAGTTTCTCTCAGTACCAGG

CTTCATCTAGTTCATTTCTGTGACAAGGATAGGATTTATAAGCAATTGCAATGTTTAACGTA

AAACGTATTCTTGAAATTGAATTAAGTTTAAGGCTGGGTGCAGTGGCTCATGCTGTAATCC

CAGCACTTTGGGAGGCCAAGGTGGGTGGATCACCTGAGGTCAGGAGTTCGAGACTAGCC

TGGCCAACATAGTGAACCCTCATCTCTACTAAAAACACAAAATTAGCCAGGTGTGGTGGCA

CATGCCTGTAGTCAGCTACTCGGGAGGCTGAAACAGGAGAATCACTTGAACCCAGGAGGC

*AGAGGCTACAGTGAGCCGAGATCGTGCCACTGCACTCCAGCCTGGGGGCGAGATAGAGA***T**

▲

GATTTAAAAGTGTCACTCTTCCTCCTTTTCTACTTTCAGTAGATATGAGATAGAG

CAT

5000

FIGURE 5: Nucleotide sequence of the divergent region of the H209/V6 2.2 kb RT-PCR product. The region between the two triangles (italics type) represents the non-topo IIα mRNA sequence. The regions 5′ and 3′ of the triangles (normal and bold type) correspond to wild-type topo IIα mRNA sequence. The location of the new predicted stop codon, TAA, in the H209/V6 6.2 kb topo IIα-related mRNA is indicated by underlining.

encoded by the 6.2 kb mRNA is thus expected to have a molecular weight of approximately 160 kDa.

Relationship between the H209/V6 4.8 kb and 6.2 kb mRNAs and the 160 kDa Topo IIα. Comparison of the COOH-terminal deduced amino acid sequences of the H209/V6 6.2 kb and 4.8 kb topo IIα-related mRNAs shows that they could encode polypeptides that differ in size by only 21 amino acids. Thus if both mRNAs are translated, they would not be distinguishable on an immunoblot with the antiserum against the recombinant COOH-terminal 70 kDa topo IIα polypeptide (Sullivan et al., 1989). The single immunoreactive band observed previously on topo IIα immunoblots of H209/V6 cells using this antiserum could therefore correspond to two co-migrating proteins rather than just one (Mirski et al., 1993). To determine if the 160 kDa topo IIα in H209/V6 cells was encoded by the 6.2 kb mRNA or the 4.8 kb mRNA or both, immunoblotting experiments were performed. Proteins from whole cell lysates of H209 and H209/V6 cells were resolved by electrophoresis, blotted and probed with an antiserum (A160) (Harker et al., 1995b)

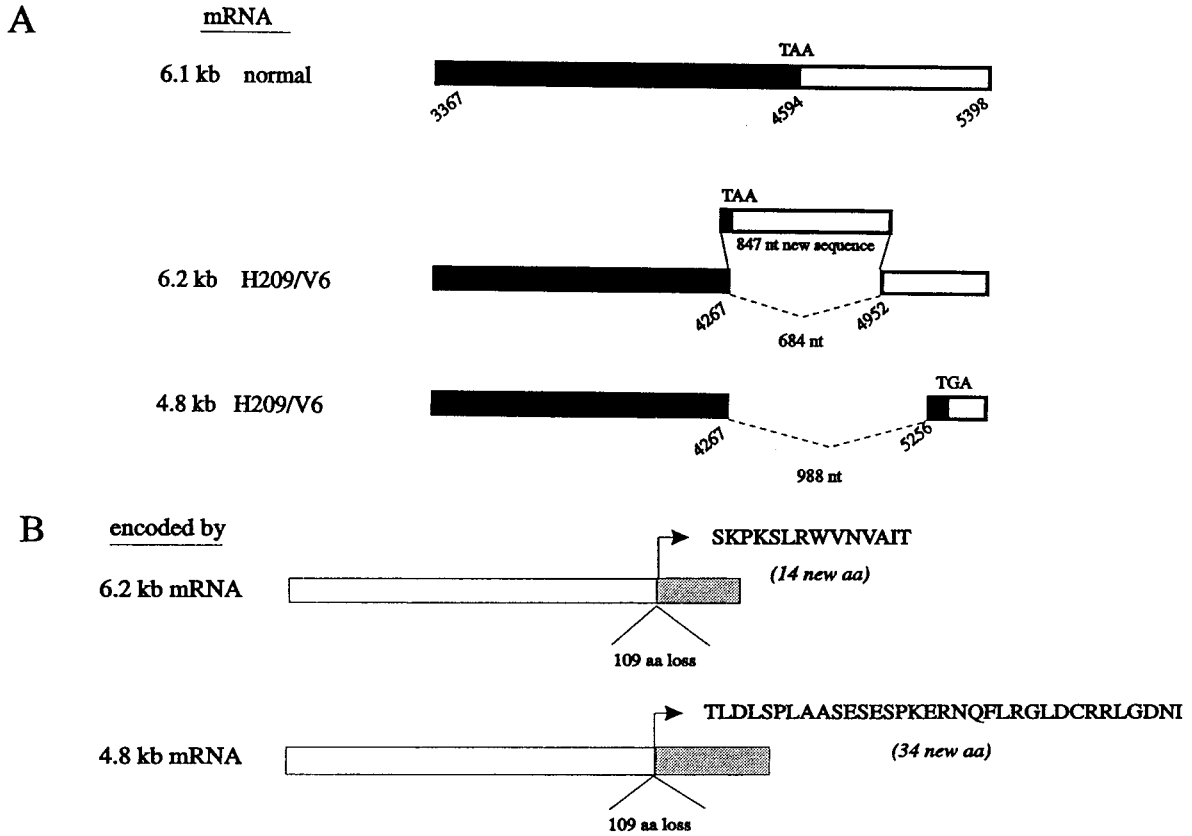

FIGURE 6: Predicted structures of the 3′-proximal region of the H209/V6 topo IIα 6.2 kb and 4.8 kb mRNAs and encoded proteins. (A) Predicted structure of the 3′-proximal region of the H209/V6 6.2 kb mRNA is aligned with the H209/V6 4.8 kb mRNA and H209 normal 6.1 kb topo IIα mRNA. The solid portion represents coding sequence while the open portion represents non-coding sequence. The regions connected by broken lines represent the missing normal topo IIα sequence in the H209/V6 4.8 kb and 6.2 kb mRNAs. Predicted stop codons in these two mRNAs are shown as TAA and TGA, respectively. (B) Schematic diagram of proteins predicted to be encoded by the H209/V6 6.2 kb and 4.8 kb mRNAs. The open portions represent normal topo IIα amino acid sequence while the shaded portions represent the new COOH-terminal peptides in the two proteins. In the case of the H209/V6 6.2 kb mRNA, 14 new amino acids are predicted while for the 4.8 kb mRNA 34 new amino acids are predicted. The sequences of the new COOH-terminal peptides are indicated by the single-letter amino acid code; aa, amino acids.

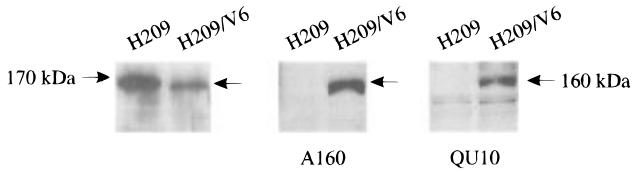

FIGURE 7: Immunoblot analyses of H209 and H209/V6 whole cell lysates. Proteins from H209 and H209/V6 whole cell lysates were resolved by SDS−PAGE and blotted onto nylon membrane as described in Experimental Procedures. Protein equivalent to 1×10^6 cells was loaded per lane. The blots were incubated with the following: (left panel) rabbit antiserum raised against the recombinant 70 kDa COOH-terminal portion of HeLa topo IIα (Sullivan et al. 1989); (centre panel) rabbit antiserum A160 raised against a synthetic peptide corresponding to the COOH-terminal 13 amino acids predicted to be encoded by the H209/V6 6.2 kb topo IIα-related mRNA (Harker et al. 1995b); and (right panel), rabbit antiserum QU10 raised against a GST-fusion protein containing the COOH-terminal 34 amino acids predicted to be encoded by the H209/V6 4.8 kb topo IIα-related mRNA. The blots were processed as described in Experimental Procedures.

raised against a synthetic peptide corresponding to the COOH-terminal 13 amino acids predicted from the H209/V6 6.2 kb topo IIα mRNA sequence, or with an antiserum (QU10) raised against a GST fusion protein containing the COOH-terminal 34 amino acids predicted from the H209/V6 4.8 kb topo IIα mRNA sequence. As shown in Figure 7, both antisera react with a 160 kDa topo IIα in the H209/V6 whole cell lysate but not with the 170 kDa topo IIα in the H209 whole cell lysate. These results indicate that both the H209/V6 mutant 6.2 kb and 4.8 kb mRNAs are translated. The resultant mutant 160 kDa topo IIα proteins may localize differently from each other because they differ at their COOH termini. However, immunoblots of crude nuclear and cytoplasmic fractions of H209/V6 cells prepared (Feldhoff et al., 1994) and probed with each of the three different topo IIα antisera, show that both 160 kDa topo IIα proteins are similar in their inefficient localization to the nucleus (not shown).

Restriction Enzyme Analysis of the 3′-Proximal TOP2A Gene in H209 and H209/V6 Cells. To begin analysis of the genetic basis for the altered topo IIα expression in H209/V6 cells, genomic DNAs from H209 and H209/V6 cells were digested with a series of restriction endonucleases and analyzed by southern blotting with the topo IIα-specific SP-1 cDNA probe. The enzymes used included *Bam*HI, *Eco*RI, *Hin*dIII, *Hpa*II, *Sst*I, *Ava*I, *Bgl*I, *Dra*I, *Mse*I, *Nde*I, *Pvu*II, *Sma*I, *Stu*I, and *Bgl*II. Different restriction patterns were observed for the H209 and H209/V6 genomic DNAs after digestion with seven of these enzymes, *viz.*, *Bam*HI, *Hpa*II, *Sst*I, *Nde*I, *Pvu*II, *Stu*I, and *Bgl*II, and four of these are shown in Figure 8. Extra bands were observed with H209/V6 DNA digests in addition to the fragments seen with H209 genomic DNA digests (Figure 8). These results suggested the presence of at least two alleles which differ at the 3′ end, one of which has sustained a rearrangement or deletion.

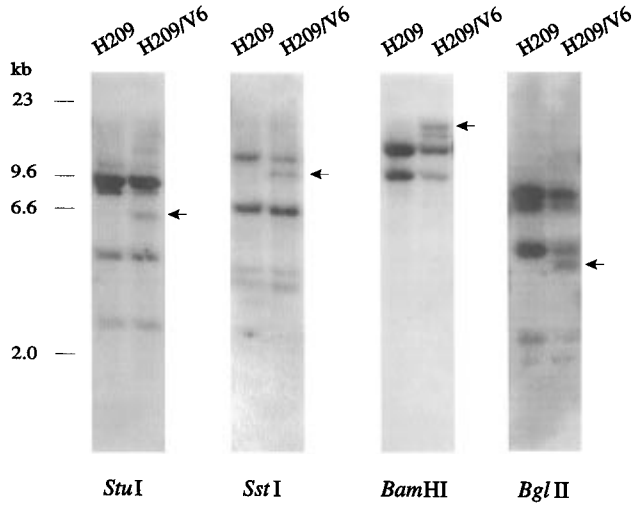

FIGURE 8: Topo IIα restriction enzyme analysis of genomic DNA from H209 and H209/V6 cells. Genomic DNA was prepared from H209 and H209/V6 cells and 10 μg digested with the indicated restriction enzymes. After electrophoresis in 0.7% agarose, the DNA was blotted onto a nylon membrane and hybridized with ^{32}P-labeled SP-1 topo IIα cDNA. In addition to the fragments detected in the H209 DNA, H209/V6 DNA showed extra fragments, which are indicated by the arrows. The autoradiographs shown are 5-day exposures. Positions and sizes of *Hin*dIII-digested λ DNA fragments are shown on the left.

Sequence and Structure Analyses of the 3′ Region of the TOP2A Gene in H209 and H209/V6 Cells. A portion of the 3′-proximal region of the *TOP2A* gene in H209 and H209/V6 cells was amplified from genomic DNA by PCR using a pair of primers which lie 5′ and 3′ of the region containing the mutations in the H209/V6 topo IIα-related 4.8 kb and 6.2 kb mRNAs. Amplification of H209 genomic DNA generated a 4 kb product whereas amplification of H209/V6 genomic DNA generated both a 4 kb product and a 1.8 kb product (not shown). This observation suggested that a deletion of approximately 2.2 kb had occurred in one allele of the *TOP2A* gene in H209/V6 cells. Sequence analyses of these genomic PCR products revealed that this deletion consisted of an entire exon and intron and portions of a second exon and intron as illustrated in Figure 9A. Comparison of the genomic sequence with the sequence of the H209/V6 6.2 kb topo IIα-related mRNA revealed that the 847 nt of new sequence in the mRNA were derived from the intron immediately 3′ to the end of the exon where the mutation begins.

DISCUSSION

Reduced expression of topo IIα or mutations in conserved regions of this enzyme have been associated with drug resistance in numerous resistant tumor cell lines (Liu, 1989; Mirski & Cole, 1996; Sullivan & Ross, 1991; Osheroff et al., 1994; Cole, 1996). More recently, cytoplasmic localization of topo IIα has also been suggested as a mechanism leading to drug resistance (Cole, 1996; Feldhoff et al., 1994; Harker et al., 1995; Mirski & Cole, 1995; Harker et al., 1991). Previous studies from this laboratory identified quantitative and qualitative alterations of topo IIα in the VP-16-selected H209/V6 small cell lung cancer cell line (Mirski et al., 1993). In addition to the presence of a 160 kDa topo IIα-related protein in H209/V6 cells was the appearance of a new species of topo IIα-related mRNA of approximately 4.8 kb. We found that 988 nucleotides of 3′-proximal normal topo IIα sequence were absent from this mutant mRNA and that the missing sequence consisted of both coding and 3′ untranslated sequence. Analysis of the missing sequence indicated that it contained three potential bipartite nuclear localization signals (Mirski & Cole, 1995). We therefore suggested that the H209/V6 4.8 kb mRNA encoded the H209/V6 160 kDa cytoplasmic immunoreactive topo IIα protein (Mirski & Cole, 1995). In agreement with this conclusion was the inability of an antiserum against the terminal 17 amino acids of normal topo IIα to detect the H209/V6 160 kDa protein. However, the fact remained that H209/V6 cells contained an apparently normal sized 6.1 kb topo IIα mRNA but no detectable normal size 170 kDa protein. In the present study, we have determined that this larger H209/V6 topo IIα-related mRNA is slightly bigger than its counterpart in H209 cells (Figure 1). This observation provided the first indication that the H209/V6 6.2 kb topo IIα-related mRNA might be different from the H209 6.1 kb topo IIα mRNA. Further evidence that this was the case was obtained from Northern analyses with probes corresponding to 5′ and 3′ portions of the region previously determined to be missing from the H209/V6 4.8 kb mRNA (Mirski & Cole, 1995). While the 3′ proximal probes hybridized to the H209/V6 6.2 kb topo IIα-related mRNA, the 5′ proximal probe did not (Figure 2), indicating that the H209/V6 6.2 kb mRNA is also missing a portion of the region that we previously showed was absent from the H209/V6 4.8 kb mRNA (Mirski & Cole, 1995).

When cDNA corresponding to the 3′-proximal region of H209/V6 6.2 kb mRNA was amplified, two major RT-PCR products were obtained (Figure 3). These products (2.2 and 1 kb) corresponded in size to those expected from cDNAs derived from the H209/V6 6.2 kb and 4.8 kb mRNAs, respectively (Figure 3). The 2.2 kb RT-PCR product from H209/V6 cDNA is approximately 0.2 kb longer than the corresponding product from H209 cDNA, consistent with our observation that the H209/V6 6.2 kb topo IIα-related mRNA is a little larger than its counterpart in H209. Furthermore, Southern blot analyses of the H209/V6 2.2 kb RT-PCR product with several probes showed similar hybridization patterns as the Northern blot analyses with the same probes (Figure 4). Taken together, these data provided convincing evidence that the 2.2 kb RT-PCR product was indeed derived from the H209/V6 6.2 kb topo IIα mRNA. The subsequent finding that this H209/V6 RT-PCR product was missing at least 0.6 kb of normal topo IIα sequence seemed inconsistent with the observation that it was approximately 0.2 kb longer than the corresponding RT-PCR product from H209 cDNA. A possible explanation for this finding was that the larger H209/V6 RT-PCR product contained non-topo IIα sequence. This was confirmed by cloning and sequencing which revealed that 684 nt from the 3′-coding and non-coding region of the normal topo IIα mRNA sequence were no longer present in the H209/V6 6.2 kb mRNA but were instead replaced by 847 nt of unknown sequence (Figure 5). This alteration results in a net gain of 163 nucleotides which is consistent with the decreased electrophoretic mobility of H209/V6 6.2 kb mRNA compared to the H209 6.1 kb mRNA, and with the larger size (2.2 kb) of the H209/V6 RT-PCR product compared to the H209 RT-PCR product (2 kb). Northern analysis with a PCR probe corresponding to a 143 nt sequence within the 5′-proximal region of the novel sequence of the H209/V6 2.2 kb RT-

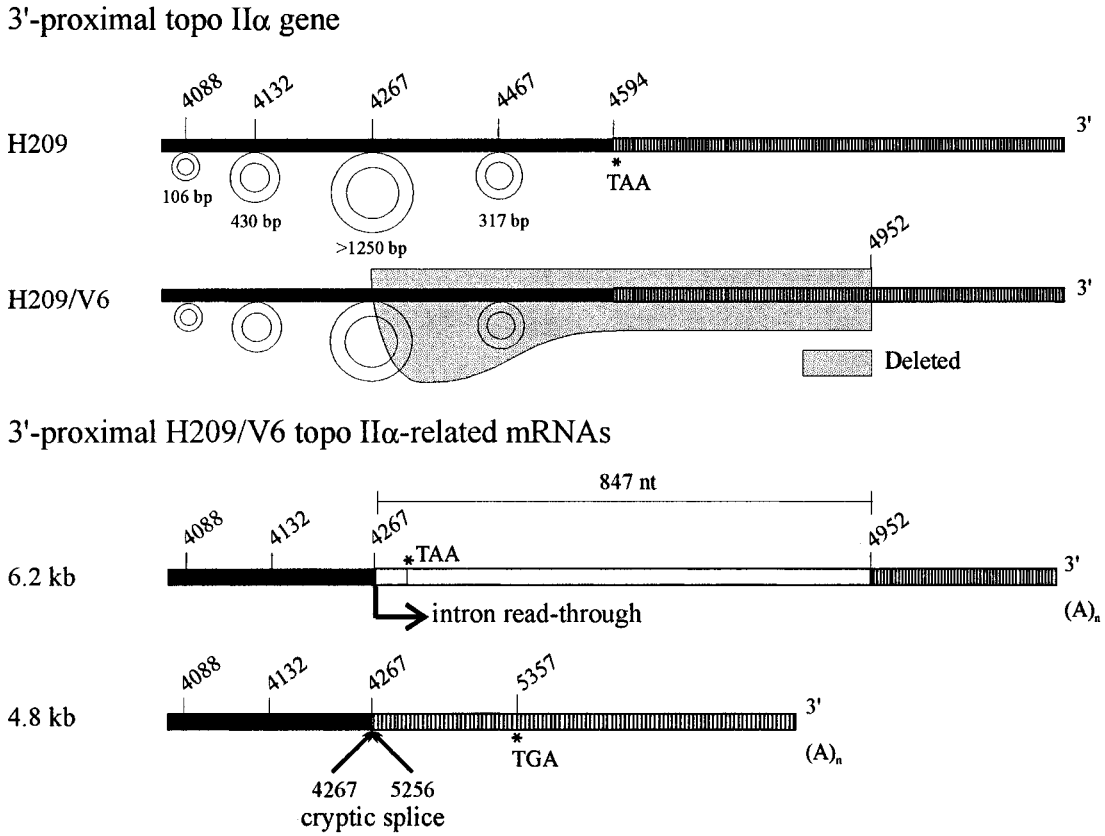

FIGURE 9: Schematic relationship of the 3′-proximal regions of the H209/V6 6.2 kb and 4.8 kb topo IIα mRNAs to the 3′-proximal region of the topo IIα gene in H209 and H209/V6 cells. (A) Exon/intron organization of the 3′-proximal region of the topo IIα gene in H209 and H209/V6 as determined by the sequencing of genomic PCR products is shown. The solid portions represent the exons (coding sequence), the open circles below represent the introns, and the hatched portions represent 3′-untranslated sequence. The numbers at the top show the nt position of the intron/exon boundaries within the topo IIα mRNA sequence. The normal H209 stop codon TAA at nt 4495 is indicated by an asterisk. The portion of the gene deleted in H209/V6 is indicated by shading. (B) Structures of the 3′-proximal H209/V6 topo IIα 6.2 kb and 4.8 kb mRNAs as predicted from the partial gene deletion shown in A. The solid portions represent coding sequence with the position of the exon/intron boundaries indicated by the numbers above. For the 6.2 kb mRNA (top), the open portion indicates intron sequence which is transcribed; the first stop codon TAA encountered 41 nt into the 847 bp intron sequence is noted by an asterisk. The hatched region beginning at nt 4952 indicates the position at the end of the deletion where normal 3′-UTR sequence resumes. For the 4.8 kb mRNA (bottom), the solid portion represents normal 3′-proximal topo IIα coding sequence with intron/exon boundary positions indicated. The hatched portion represents what was formerly normal 3′-UTR and the sequence between nt 4267 and 5256 has been spliced out by utilization of a cryptic 3′ acceptor splice site located at nt 5256. The first stop codon after the nt 5256 acceptor splice site, TGA, is located at nt 5357 in the former 3′-UTR and is indicated by an asterisk.

PCR product directly confirmed that the H209/V6 6.2 kb mRNA contains the novel sequence (not shown).

In the RT-PCR analysis of H209/V6 topo IIα mRNA, a low abundance product of approximately 1.5 kb was observed in addition to the expected 2.2 and 1 kb products which correspond to the 6.2 and 4.8 kb topo IIα-related mRNAs, respectively (Figure 3). Southern analysis of this product demonstrated a hybridization pattern similar to that of the 2.2 kb RT-PCR product (data not shown). One possible explanation for this finding is that the 1.5 kb product represents another species of topo IIα-related mRNA. This seems unlikely since it was not detected in Northern analyses. A second and more probable explanation is that the 1.5 kb PCR product is an artifact resulting from heteroduplex formation of single strands from the 1 and 2.2 kb RT-PCR products (Zacharias et al., 1994). The occurrence of such heteroduplexes would not be surprising given the partial complementarity of the two amplified DNA sequences.

According to its deduced amino acid sequence, the H209/V6 6.2 kb topo IIα-related mRNA is predicted to encode a protein that no longer contains the 109 COOH-terminal amino acids of the normal enzyme but instead contains 14 new amino acids encoded by the new sequence (Figure 6). In our earlier study, we determined that the protein encoded by the H209/V6 4.8 kb mRNA is missing the same 109 amino acids but is predicted to contain 34 different amino acids encoded by sequence that was previously in the 3′-untranslated region (UTR) of the mRNA (Mirski & Cole, 1995). Thus, both the 4.8 kb and 6.2 kb mRNAs could encode proteins of approximately 160 kDa which differ in length by only 21 amino acids or in molecular mass, by 2 kDa. In both cases, the encoded polypeptides have lost three putative bipartite nuclear localization motifs that begin at amino acid positions 1416, 1441, and 1448. Consequently, both proteins might be expected to localize predominantly in the cytoplasm. We previously observed only one topo IIα immunoreactive protein in H209/V6 cells despite the presence of two mRNAs and suggested that only one of the mRNAs was translated (Mirski & Cole, 1995). However, in view of the 3′-proximal sequences of the H209/V6 4.8 kb and 6.2 kb mRNAs, it became clear that the 160 kDa cytoplasmic protein could be the translation product of either or both of these mRNAs. Immunoblot analyses using an antiserum generated against a 13 amino acid COOH-terminal peptide predicted to be encoded by the H209/V6 6.2 kb mRNA confirms that this mRNA is indeed translated into a

160 kDa polypeptide (Figure 7). Antiserum against the GST-fusion protein containing the 34 COOH-terminal amino acids predicted to be encoded by the 4.8 kb mRNA also reacts with a 160 kDa protein in H209/V6 cells (Figure 7). These observations indicate that both H209/V6 topo IIα-related mRNAs are translated and provide the first reported example of a drug resistant cell line containing two different cytoplasmic forms of topo IIα.

Southern analyses of genomic DNA from H209 and H209/V6 cells digested with several different restriction enzymes and probed with a 3′-proximal topo IIα-specific cDNA probe showed different hybridization patterns (Figure 8), suggesting that at least one of the *TOP2A* alleles in H209/V6 cells had sustained a deletion. Sequence analysis of the corresponding 3′-proximal region of the *TOP2A* gene in H209 and H209/V6 DNA confirmed that a deletion consisting of both intron and exon sequences had occurred (Figure 9A). As a consequence of this deletion, the 5′ donor splice site at the exon/intron boundary located at nt 4267 (AA/GT) remains intact but the normal 3′ acceptor splice site is disrupted, allowing intron read-through and the inclusion of 847 nt of intron sequence in the H209/V6 6.2 kb mRNA (Figure 9B). The H209/V6 4.8 kb and 6.2 kb topo IIα mRNAs could be encoded by the same or different alleles; however, given that the mutations in the two mRNAs begin at exactly the same position, the most likely explanation is that they arise from differential splicing of a primary transcript from the single mutant *TOP2A* allele described here. Consistent with this interpretation is the observation that the H209/V6 4.8 kb mRNA sequence resumes at a potential cryptic 3′ acceptor splice site (TTTCAG/CTC) located at nt 5256 in the 3′-UTR of the normal topo IIα mRNA sequence (Figure 9B). Thus, the H209/V6 6.2 kb mRNA is produced as a consequence of intron read-through while the H209/V6 4.8 kb mRNA is produced from the 6.2 kb mRNA by utilization of a cryptic 3′ acceptor splice site. Finally, although the limited southern analysis data presented here does not indicate abnormalities in the second *TOP2A* allele of H209/V6 cells, this allele is clearly not normal since no normal topo IIα 170 kDa protein or its cognate 6.1 kb mRNA are detectable. Further investigation of the 5′ end of the *TOP2A* gene is ongoing to understand why the normal topo IIα mRNA is not expressed in H209/V6 cells.

Another mutant topo IIα lacking a normal COOH-terminus has been reported in the mitoxantrone-selected human leukemia cell line, HL-60/MX2 (Harker et al., 1995b). However, in contrast to H209/V6 cells, HL60/MX2 cells contain a normal nuclear 170 kDa protein in addition to a mutant cytoplasmic 160 kDa protein (Harker et al., 1995a). Similiar to H209/V6, HL60/MX2 cells express a 4.8 kb mRNA but they also express the normal 6.1 kb topo IIα mRNA. It was determined that the HL60/MX2 topo IIα-related 4.8 kb mRNA encodes a protein identical to that encoded by the H209/V6 6.2 kb mRNA (Harker et al., 1995b). The HL60/MX2 4.8 kb mRNA contains 725 fewer bp of topo IIα intron sequence in its 3′-UTR than the H209/V6 6.2 kb mRNA and uses an alternate polyadenylation signal within its 122 bp intron sequence, thus accounting for the difference in size of the mRNAs from the two cell types. Intron sequence was included in both the H209/V6 6.2 kb and HL60/MX2 4.8 kb topo IIα-related mRNAs because of a failure to splice correctly at the exon/intron junction site (AA/GT) at nt 4267 of the normal topo IIα mRNA. In H209/V6 cells we have shown that this occurs because the portion of the *TOP2A* gene deleted in these cells includes the normal 3′ acceptor splice site. Whether or not the HL60/MX2 cells contain the same deletion in one of its *TOP2A* alleles is unknown although comparison of southern analyses of HL60/MX2 and H209/V6 genomic DNAs suggest that the genetic alterations in the two cell lines differ (Harker et al., 1995a). Nevertheless, the fact that both the mitoxantrone-selected HL60/MX2 cells and the VP-16-selected H209/V6 cells have acquired resistance by producing aberrantly localized topo IIα suggests that resistance via this mechanism occurs as a result of exposure to several drugs which target this enzyme.

The observation that the H209/V6 mutant 160 kDa topo IIα proteins display similar catalytic activity to the wild-type topo IIα protein indicates that the COOH-terminal region does not play a critical role in human topo IIα catalytic function (Feldhoff et al., 1994). This finding is consistent with other reports where it has been demonstrated that the COOH-proximal domain of topo II in several simpler eukaryotic species is not required for activity *in vivo* (Crenshaw & Hsieh, 1993; Caron et al., 1994; Shiozaki & Yanagida, 1991). Instead, it has been suggested that this region acts as a target for post-translational modification (Wells et al., 1994; Cardenas et al., 1992). One study has shown that the major phosphorylation site in human topo IIα is serine 1524 located in the extreme COOH-terminus (Wells et al., 1994). Since this site is no longer present in either of the H209/V6 mutant 160 kDa proteins, and it has been shown that nuclear targeting of proteins can be modulated by phosphorylation, it raises the possibility that reduced phosphorylation may also contribute to their inefficient nuclear localization. Site-directed mutagenesis studies are in progress to determine which of the three bipartite nuclear localization signals in the COOH-terminal 109 amino acids of topo IIα are functional and whether they are interdependent. We have also identified at least six potential nuclear localization signals in the NH_2-proximal portion of topo IIα and their functionality is under investigation since mutations in these motifs might also result in resistance by reducing nuclear localization of this important drug target.

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