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# Isolation of genetic suppressor elements, inducing resistance to topoisomerase II-interactive cytotoxic drugs, from human topoisomerase II cDNA

(dominant negative mutants/antisense RNA/retroviral vectors/expression selection)

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ABSTRACT **Many cytotoxic anticancer drugs act at topoisomerase II (topo II) by stabilizing cleavable complexes with DNA formed by this enzyme. Several cell lines, selected for resistance to topo II-interactive drugs, show decreased expression or activity of topo II, suggesting that such a decrease may be responsible for drug resistance. In the present study, etoposide resistance was used as the selection strategy to isolate genetic suppressor elements (GSEs) from a retroviral library expressing random fragments of human topo II (α form) cDNA. Twelve GSEs were isolated, encoding either peptides corresponding to short segments of the topo IIα molecule (2.4–6.5% of the protein) or 163- to 220-bp-long antisense RNA sequences. Expression of a GSE encoding antisense RNA led to decreased cellular expression of the topo IIα protein. Both types of GSE induced resistance to several topo II poisons but not to drugs that do not act at topo II. These results provide direct evidence that inhibition of topo II results in resistance to topo II-interactive drugs, indicate structural domains of topo II capable of independent functional interactions, and demonstrate that expression selection of random fragments constitutes an efficient approach to the generation of GSEs in mammalian cells.**

Selective inhibition of specific genes can be achieved with genetic elements that interfere with the expression or function of the target gene. Genetic suppressor elements (GSEs) may encode inhibitory antisense RNA molecules (1) or mutated or truncated proteins that interfere with the function of the wild-type protein in a dominant fashion (2). A potentially general approach to the isolation of both types of biologically active GSE involves random fragmentation of the target gene or genes, followed by cloning of the resulting fragments in an expression vector and biological selection of clones conferring the phenotype associated with suppression of the target. This strategy was previously tested in bacteria using bacteriophage λ as a model target, resulting in the isolation of many peptide- and antisense RNA-encoding GSEs that blocked phage replication (3).

We have now used a similar approach in mammalian cells, to generate GSEs inhibiting human topoisomerase II (topo II). Topo II, which catalyzes topological isomerization of DNA through breakage–reunion of both DNA strands, is involved in DNA replication (4, 5) and chromosome segregation (6, 7). Human cells contain two isoforms of topo II, topo IIα (170 kDa) and topo IIβ (180 kDa), encoded by different but related genes (8). The better-characterized topo IIα is a 1530-amino acid protein that functions as a homodimer (9, 10).

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Topo II has been identified as a common target for many cytotoxic drugs used in cancer chemotherapy, including epipodophyllotoxins, anthracyclines, and acridines (11). Topo IIα is more sensitive than topo IIβ to at least some of the topo II-interactive drugs (12). Drugs acting as topo II "poisons" stabilize the "cleavable complex," an intermediate product of the topo II-catalyzed reaction, which includes topo II covalently bound to the 5′ termini of broken DNA (10, 11, 13). Accumulation of the cleavable complexes is believed to lead to the eventual cell killing (11). Several mammalian cell lines, isolated by multistep selection for resistance to topo II poisons, show decreased expression or activity of topo II, suggesting that such decrease could cause drug resistance by reducing the number of cleavable complexes formed in the presence of the drug (11, 14, 15). We have hypothesized therefore that GSEs directed against topo II may increase cellular resistance to topo II poisons, thus providing a selective strategy for the isolation of such GSEs. On the other hand, the ability to induce resistance to topo II-interactive drugs by topo II-derived GSEs would constitute a direct proof that a decrease in topo II causes drug resistance in mammalian cells.

In the present study, we have used a library of recombinant retroviruses expressing random fragments of human topo IIα cDNA to isolate clones conferring resistance to etoposide. Twelve GSEs, encoding either peptides corresponding to short segments of the topo II molecule or specific antisense RNA sequences, were isolated by this approach. Both types of GSE induced resistance to several topo II poisons but not to drugs that do not act at topo II. Thus, expression selection of random cDNA fragments constitutes an efficient approach to the generation of GSEs in mammalian cells.

## MATERIALS AND METHODS

**Random Fragment Retroviral Expression Library.** Human topo IIα cDNA was isolated from a cDNA library of chronic myelogenous leukemia cells in λgt10 (16) (a gift of O. N. Witte) by screening with a partial topo II cDNA clone (a gift of L. F. Liu). Inserts from two phage clones, comprising the entire protein-coding sequence, were fragmented by digestion with DNase I, and the fragment termini were rendered blunt-ended as described (3). The fragments were then ligated with an excess amount of a synthetic adaptor (see Fig. 1*B*). After fractionation by agarose gel electrophoresis, the mixture of 200- to 500-bp DNA fragments was amplified by PCR using the "sense" strand of the adaptor as the PCR primer.

Abbreviations: topo II, topoisomerase II; GSE, genetic suppressor element.

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PCR-amplified DNA was cloned into the *Cla* I site of the pLNCX vector (17) (a gift of A. D. Miller).

The plasmid library (20,000 independent recombinant clones) was transfected into an amphotropic virus-packaging murine cell line, GP+envAM12 (18) (a gift of A. Bank), using the calcium phosphate procedure. Virus-containing tissue culture supernatant was collected 24–72 hr after infection. In another protocol, the plasmid library was transfected into a 1:1 mixture of the amphotropic and ecotropic (GP+E86) (18) packaging cells. Transfected cells ($5 \times 10^5$) were then plated in 100-mm plates in the presence of 350 ng of etoposide per ml. After 3 weeks, cells were placed in drug-free medium, and after 3 more weeks they were used as a source of virus for the infection of HeLa cells.

The representation of topo II cDNA sequences in the retroviral populations was monitored by infecting mouse NIH 3T3 cells with the viral supernatant, followed 24 hr later by rapid DNA extraction and PCR amplification of the proviral inserts. The $^{32}$P-labeled PCR product was then used as a probe in Southern hybridization with *Sau*3a, *Alu* I, and *Dpn* I digests of topo II cDNA. Similar hybridization efficiencies of different restriction fragments indicated relatively even representation of cDNA sequences in a retroviral library.

**Drug Selection and Drug Resistance Assays.** The retroviral libraries were used to infect $2 \times 10^6$ HeLa cells, in the presence of 4 $\mu$g of Polybrene per ml. Two selection strategies were used to isolate etoposide-resistant cells. In the first protocol, HeLa cells were plated at $5 \times 10^5$ per 100-mm plate and, 48 hr after infection, exposed to 0.5 mg of G418 per ml and several different concentrations (200–350 ng/ml) of etoposide (Sigma). In some experiments, etoposide was added 3–5 days after G418. Individual surviving colonies were picked and expanded. In the second protocol, the cells were first selected with G418, and the surviving cell population was allowed to expand for 3–5 weeks. G418-resistant cells were then plated at $10^6$ per 100-mm plate and selected at different concentrations (200–350 ng/ml) of etoposide for 10–15 days. During selection, cells were trypsinized every 3 days and replated in plates of the same size, at the maximum density of $10^6$ cells per plate. The frequent trypsinization did not affect cell growth in drug-free medium, but it allowed for uniform and reproducible etoposide cytotoxicity.

For plating efficiency assays, cells were plated in duplicates on six-well plates, at 500 cells per well, in the presence of increasing concentrations of different drugs. After 10–15 days, colonies were counted. Relative levels of drug resistance were calculated as a ratio of $LD_{50}$ (drug dose resulting in 50% decrease in plating efficiency) of the tested cells relative to that of control HeLa cells carrying the insert-free LNCX provirus.

**Recovery of Topo II-Derived Inserts from the Infected Cells.** Proviral inserts were isolated from the DNA of individual infected HeLa cell clones by PCR, using primers corresponding to the vector sequences flanking the inserts. The primers (see Fig. 1*A*) were modified to provide unique *Hin*dIII and *Hpa* I restriction sites, which were then used to clone the PCR products into pLNCX in the same position and orientation as the original insert. After cloning, topo II inserts were sequenced in both directions using the dideoxy method and appropriate sequencing primers.

**Topo II Protein Analysis.** Different HeLa cell populations were synchronized by a double thymidine block in late S/$G_2$ phase. Cells were lysed in an equal volume of 2× Laemmli buffer containing 1 mM phenylmethylsulfonyl fluoride, 20 ng of aprotinin per ml, and 1% 2-mercaptoethanol. The lysates were analyzed by PAGE and immunoblotting, using rabbit anti-topo II polyclonal antibody (MAC) (19) or control preimmune serum, as described (20). The single MAC-reactive band in HeLa cells was identified as topo II$\alpha$ by electrophoresis next to a lysate of CEM cells containing $\alpha$ and $\beta$ isoforms of topo II. Topo II$\beta$ was undetectable in HeLa cells under our lysis conditions.

## RESULTS

**Selection of Topo II-Derived GSE.** We have constructed a library of 20,000 recombinant clones containing 200- to 500-bp fragments of topo II$\alpha$ cDNA, generated by random fragmentation with DNase I, in a retroviral vector (LNCX) (17). The vector (Fig. 1*A*) was selected because of high efficiency of retrovirus-mediated gene transfer and because most of the retrovirus-infected clones were expected to contain only one copy of the integrated provirus (21), simplifying subsequent analysis of the inserts. Each fragment was inserted into the *Cla* I site of the vector through an adaptor that supplied it with an ATG translation initiation codon at the 5′ end and with translation termination codons in all possible reading frames at the 3′ end (Fig. 1).

The plasmid library was converted into the form of retroviral particles by transfection into a murine amphotropic virus-packaging cell line. Hybridization analysis showed no major changes in the representation of different topo II sequences in the retroviral population (data not shown). The retroviral library was used to infect human HeLa cells, which were then subjected to selection with G418 and etoposide. In another arm of the experiment, the plasmid library was transfected into a mixture of amphotropic and ecotropic packaging cell lines, so that the virus could spread in this cell population by "ping-pong" infection (22). The transfected packaging cells were selected with etoposide, and the virus produced by the surviving cells was then used to infect HeLa cells. G418-selected HeLa cells carrying insert-free LNCX provirus were used as a control.

Infection of HeLa cells with the retroviral library of topo II$\alpha$ fragments, produced by transient transfection of amphotropic packaging cells, gave rise to ≈10,000 G418-resistant

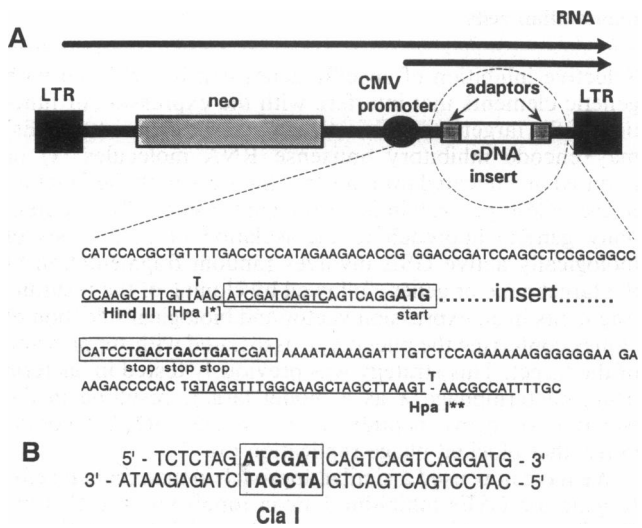


FIG. 1. (*A*) Structure of an integrated provirus containing a fragment of topo II cDNA. The LNCX vector carries the *neo* (G418 resistance) gene transcribed from the long terminal repeat (LTR) promoter, and the cDNA insert is transcribed from the cytomegalovirus (CMV) promoter. Adaptor-derived sequences are bracketed, with translation initiation and termination codons shown in boldface. Sequences used as PCR primers for isolation and recloning of the inserts are underlined; changes in the primer sequences leading to inactivation (*) or creation (**) of the indicated restriction sites are shown above the vector sequence. (*B*) Structure of the synthetic adaptor used for PCR amplification and cloning of DNase I-generated fragments.

colonies, some 50 of which were resistant to etoposide. In contrast, none of a similar number of control G418-resistant colonies was able to grow in etoposide. Furthermore, when the mixture of murine ecotropic and amphotropic packaging cells, stably infected with the topo IIα fragment library, was cultured with etoposide, the virus produced by the selected cells induced etoposide resistance in most of the infected HeLa cells. These results have indicated the selection of recombinant viruses conferring etoposide resistance.

The inserts contained in the integrated proviruses were isolated from genomic DNA of etoposide-resistant colonies by PCR amplification using primers corresponding to modified sequences of the LNCX vector (Fig. 1*A*). As shown in Fig. 2, most of the individual infectants contained a single insert, but some of the colonies carried two different inserts. PCR-amplified inserts were recloned into the LNCX vector in the same position and orientation as in the parental construct. Twenty-six plasmid clones carrying putative GSEs were isolated, sequenced, and tested for the ability to induce etoposide resistance in HeLa cells.

The ability of the selected clones to confer etoposide resistance was evaluated by two types of assays. In the first assay, HeLa cells were infected with retroviruses derived from individual plasmid clones and, immediately after infection, scored for colony formation in the medium containing G418 alone or G418 and etoposide. Cells infected with some of the clones showed increased relative plating efficiency in the presence of etoposide. Nevertheless, cells infected with many of the selected clones showed an overall decrease in the plating efficiency and colony size relative to the control, thus complicating the interpretation of the assay. To overcome this problem, we have used a different assay, where infected cells were first selected with G418 and then expanded and tested for the ability to grow in 350 ng of etoposide per ml with repeated trypsinization (Fig. 3*A*). Using the above assays, 12 of the isolated clones were found to confer etoposide resistance, indicating that they contained biologically active GSEs. The orientation and position of the inserts contained in these clones relative to topo IIα cDNA are shown in Table 1 and Fig. 4.

**Sense-Oriented GSE.** Five of seven sense-oriented GSEs were clustered in a 580-bp region of topo II cDNA corresponding to a highly conserved portion of the protein associated with DNA breakage and rejoining (9, 23) (Fig. 4). The sense-oriented GSEs encoded segments of the topo IIα protein, translation of which was initiated either from the internal methionine codons in the topo II sequence or in one case from the ATG codon of the adaptor. The peptides ranged from 37 to 99 amino acids in length, corresponding to 2.4–6.5% of the protein.

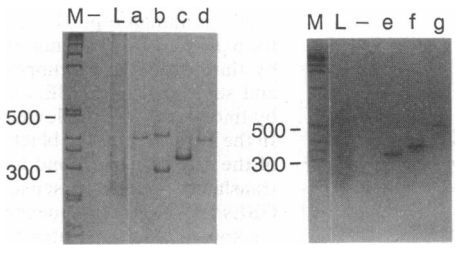


FIG. 2. PCR amplification of proviral inserts from DNA of HeLa cell clones, obtained after infection with the retroviral library and etoposide selection (lanes a–g). Lanes marked L contain PCR products from DNA of the library-infected population of HeLa cells prior to etoposide selection. The "sense" strand of the adaptor shown in Fig. 1*B* was used as the PCR primer. PCR products were separated in polyacrylamide gel and stained with ethidium bromide. Size markers are indicated in base pairs (lanes M).

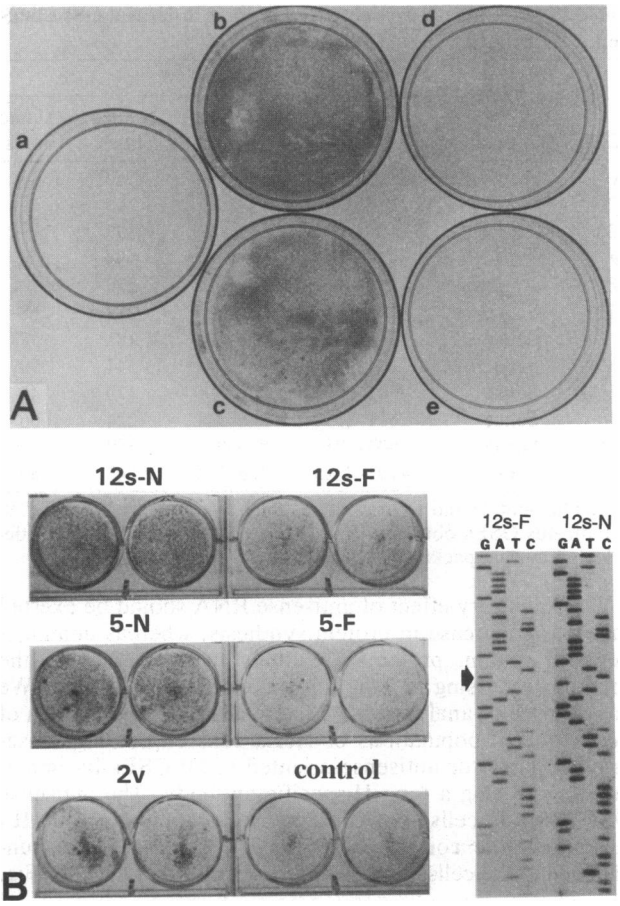


FIG. 3. Induction of etoposide resistance in HeLa cells by topo IIα-derived GSEs. HeLa cells obtained after infection with individual retroviral clones and G418 selection were plated in 250 ng of etoposide per ml (see text). (*A*) Cells infected with a retrovirus produced from LNCX vector without an insert (a), with LNCX carrying a sense-oriented Σ11 (b) or an antisense-oriented Σ20 (c) GSE, or with LNCX with sense- (d) or antisense-oriented (e) topo IIα cDNA fragments without GSE activity (nucleotide positions 1520–1735 and 2799–3009 in topo IIα cDNA, respectively). (*B*) Inactivation of sense-oriented GSEs by frameshift mutations. Cells were infected with GSE clones encoding topo II-derived peptides (5-N or 12s-N) or the corresponding frameshift mutants (5-F or 12s-F). Cells carrying an antisense-oriented GSE 2v or LNCX vector without an insert were used as positive and negative controls, respectively. The frameshift mutation in 12s is indicated with an arrow in the sequencing gel on the right.

To determine whether the biological activity of sense-oriented GSEs was mediated by the corresponding peptides, we have used PCR to generate constructs that expressed topo IIα peptides encoded by two of the GSEs (clones 12s and 5), initiating from the first ATG codon in the transcription unit, as well as frameshift versions of the same GSEs produced by adding a single nucleotide immediately after the initiation codon. The peptide-encoding clones conferred etoposide resistance, but the frameshift mutants no longer possessed this activity (Fig. 3*B*), indicating that sense-oriented GSEs acted through topo IIα-derived peptides.

**Antisense-Oriented GSE.** Five clones with GSE activity expressed topo IIα cDNA fragments in antisense orientation, indicating that they acted through the antisense RNA mechanism (Fig. 4). One of these clones was derived from the 5′ end of topo IIα mRNA including the initiation codon. Four other antisense-oriented GSEs mapped near the 3′ end of the coding region of the cDNA. The length of the antisense-oriented GSEs ranged from 163 to 220 bp.

Table 1. Positions and orientations of topo II-derived GSEs and peptides encoded by sense-oriented GSEs

| GSE | Orientation | Position | | Size | |
|---|---|---|---|---|---|
| | | Nucleotides | Amino acids | Nucleotides | Amino acids |
| 2v | Antisense | −18–145 | | 163 | |
| Σ2 | Antisense | 3150–3343 | | 194 | |
| Σ20 | Antisense | 3486–3692 | | 207 | |
| 39 | Antisense | 3934–4127 | | 194 | |
| Σ8 | Antisense | 4123–4342 | | 220 | |
| Σ11 | Sense | 393–605 | 134–201 | 213 | 68 |
| 6 | Sense | 2352–2532 | 808–844 | 181 | 37 |
| ΣVPs2 | Sense | 2494–2834 | 846–944 | 341 | 99 |
| ΣVM | Sense | 2501–2670 | 846–890 | 170 | 45 |
| 5 | Sense | 2511–2734 | 846–911 | 224 | 66 |
| Σ28 | Sense | 2603–2931 | 879–977 | 329 | 99 |
| 12s | Sense | 4102–4343 | 1368–1447 | 242 | 80 |

The nucleotide and amino acid residues are numbered as in ref. 9. Σ indicates GSEs obtained from the virus produced by etoposide-selected murine packaging cells.

The inhibitory effect of antisense RNA should be exerted through a decrease in protein synthesis, whereas dominant negative mutant proteins are likely to interfere with the protein processing or function rather than expression. We have therefore analyzed the content of topo IIα in lysates of synchronized populations of HeLa cells expressing sense-oriented (Σ11) or antisense-oriented (Σ20) GSEs by immunoblotting using a topo II-specific antibody. The antisense GSE-carrying cells showed decreased amounts of topo IIα relative to the control, but topo IIα expression was unchanged in the cells expressing the sense-oriented GSE (Fig. 5).

**Drug Resistance of GSE-Expressing Cells.** To determine the pattern of drug resistance induced by topo IIα-derived GSEs, we initially assayed populations of HeLa cells, obtained after infection with individual GSEs and G418 selection, for plating efficiency in increasing concentrations of etoposide. Analysis of the drug response curves indicated, however, that most of these populations were heterogeneous, with the majority of the cells remaining as sensitive to etoposide as the control population, but a minority (5–10%) showing apparently undiminished plating efficiency at concentrations 2- to 3-fold higher than $LD_{10}$ of the control cells (data not shown). For further analysis, the resistant subpopulations were isolated by a single step of selection in 300 ng of etoposide per ml. These populations were then tested for resistance to cytotoxic drugs with different mechanisms of action. Table 2 shows the relative levels of resistance for two cell populations, one carrying a sense- and the other an antisense-oriented GSE. Both populations were cross-resistant to the drugs known to act as topo II poisons, including etoposide, teniposide, amsacrine, and doxorubicin, but not to Actinomycin D, which according to some reports (24) may also interact with topo II. GSE-carrying cells remained sensitive to all of the tested drugs that do not act at topo II, including colchicine, methotrexate, and camptothecin (a topoisomerase I poison). Essentially the same patterns of cross-resistance were observed in cell populations carrying several other topo II-derived GSEs (data not shown).

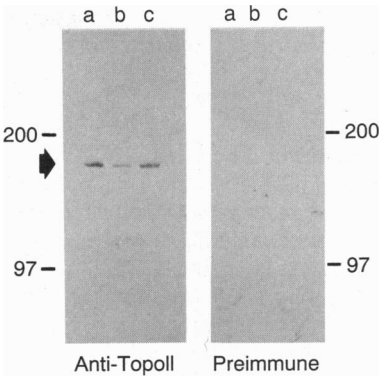


FIG. 5. Immunoblot analysis of topo IIα protein in synchronized HeLa cell populations carrying sense-oriented Σ11 (lanes a) or antisense-oriented Σ20 (lanes b) GSEs or control uninfected HeLa cells (lanes c). Each lane contains a lysate from $5 \times 10^5$ cells. Immunoblotting was carried out with an anti-topo II antibody, MAC (19) (*Left*) or control preimmune rabbit serum (*Right*). The arrow indicates topo IIα (see text). Size markers are indicated in kDa.

## DISCUSSION

It was previously hypothesized that decreased levels of topo II can induce resistance to topo II-interactive drugs in mammalian cells (10, 15), but this suggestion could not be directly tested in the absence of experimental systems for manipulating the intracellular levels of topo II. We have now tested this hypothesis by using the strategy for selecting random cDNA fragments that act as GSEs selectively interfering with the function of the target gene (3). Starting from topo IIα cDNA, we isolated a set of GSEs, encoding specific antisense RNA or peptide sequences, that induced resistance to topo II-interactive drugs. In the case of antisense RNA-encoding GSE, drug resistance was accompanied by a decrease in the topo IIα protein expression. These results provide direct evidence that the inhibition of topo II causes resistance to drugs that poison this enzyme.

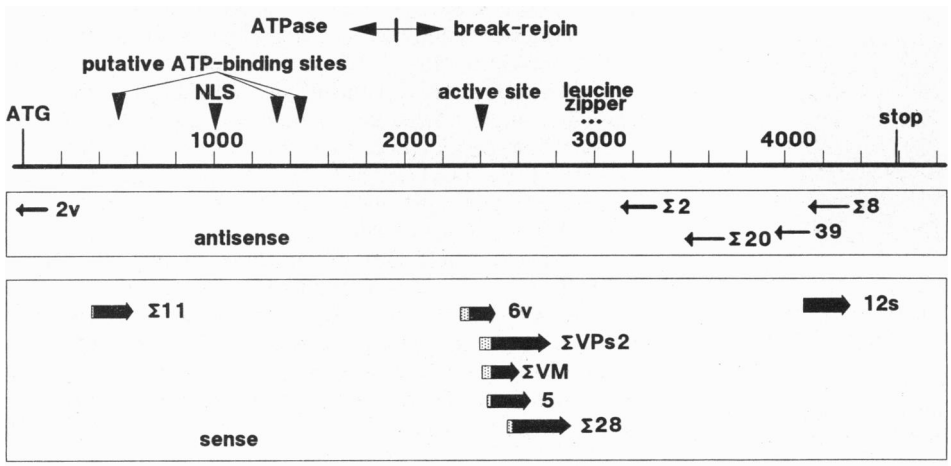


FIG. 4. Position of the isolated GSEs in human topo IIα cDNA. Antisense-oriented elements are shown by thin arrows in the upper bracket and sense-oriented GSEs are shown by thick arrows in the lower bracket. In the latter group, the black portions of the arrows correspond to putative translated regions in sense-oriented GSEs. Positions of sequences encoding known functional sites of topo IIα are indicated, including translation initiation (ATG) and termination (stop) codons, the ATPase domain including putative ATP-binding sites and nuclear-localization signal (NLS), the breakage-rejoining domain including the "active site" tyrosine residue and the "leucine zipper" region (see refs. 9 and 23).

Table 2. Relative resistance to different drugs in HeLa cells carrying antisense-oriented (Σ20) or sense-oriented (Σ11) GSE

| Compound | Relative resistance | |
|---|---|---|
| | Σ20 | Σ11 |
| Etoposide | 4.70 ± 0.23 | 5.51 ± 0.33 |
| Teniposide | 3.40 ± 0.22 | 4.33 ± 0.24 |
| Amsacrine | 2.38 ± 0.18 | 3.15 ± 0.16 |
| Doxorubicin | 2.49 ± 0.29 | 2.92 ± 0.19 |
| Actinomycin D | 0.58 ± 0.29 | 1.02 ± 0.11 |
| Colchicine | 0.66 ± 0.17 | 1.10 ± 0.11 |
| Methotrexate | 0.88 ± 0.25 | 0.91 ± 0.21 |
| Camtothecin | 1.12 ± 0.18 | 0.95 ± 0.20 |
| *o*-AMSA | 1.08 ± 0.23 | 1.12 ± 0.18 |

The resistance is calculated as the ratio between $LD_{50}$ of GSE-carrying cells and control G418-selected cells carrying insert-free LNCX provirus. *o*-AMSA, 4′-(9-acridinyl)aminomethanesulfon-*o*-anisidide. Data are presented as mean ± SEM.

Our results also provide some further information on the mechanisms of toxicity of individual drugs. Thus, it has been suggested that effects other than topo II poisoning may be responsible for the cytotoxicity of doxorubicin (25). Nevertheless, topo II-GSE expressing cells in our study showed similar resistance to doxorubicin as to the undisputed topo II poisons. On the other hand, though it was suggested by some biochemical studies that Actinomycin D may also act at topo II (24), GSE-expressing cells were not resistant to this drug. GSE-expressing cells showed no cross-resistance to the drugs that do not interact with topo II, indicating that such cells can be used as a "clean" tissue culture model for the analysis of topo II-mediated drug resistance. This model should be useful for the identification of new topo II-interactive drugs and agents that would overcome topo II-mediated drug resistance.

The selection and characterization of topo II-derived GSEs were complicated by the reduced plating efficiency and growth rate of GSE-expressing cells, suggesting that GSE-mediated inhibition of topo IIα could be detrimental to the cell growth. A selective disadvantage for cells expressing topo II-derived GSEs may explain at least in part why only a minority of GSE-transfected cells displayed the GSE-associated phenotype in the absence of etoposide selection. Thus, it is possible that the most efficient GSEs for topo II were missed by our selection procedure because of their growth-inhibitory effect. The use of inducible promoters for GSE selection may help to overcome this type of problem in future studies.

The GSEs isolated from topo IIα cDNA included sense- and antisense-oriented elements. Some GSEs from both classes were initially selected in virus-packaging cell lines of murine origin, indicating that they were efficient against human and mouse topo II. Most of the GSEs encoding antisense RNA (163–220 bp long) were derived from the 3′ end of the cDNA, although one of the elements was complementary to the 5′ end of mRNA, the region most commonly utilized by antisense RNA investigators. The apparently nonrandom distribution of biologically active antisense RNA sequences is in agreement with previous findings (3). As expected, expression of an antisense-oriented GSE led to a decrease in the intracellular level of topo IIα protein.

Most of the topo II-derived GSEs encoded peptides rather than antisense RNA. The peptide-encoding GSEs are of special interest as indicators of protein domains that are capable of independent functional interactions. The sense-oriented GSEs of topo II encoded peptides from 37 to 99 amino acids in length, corresponding to just 2.4–6.5% of the protein molecule. As shown in Fig. 4, one of these peptides (Σ11) corresponds to the region encoding the most N-terminal putative ATP-binding site (26). Five GSEs are clustered in the area between the "active site" (a sequence that includes the tyrosine residue involved in covalent interaction with DNA) and the "leucine zipper" region, which may be involved in protein dimerization (9). One GSE peptide (12s) is located near the C terminus, in a region not associated with any known functions. These locations suggest the potential steps in protein processing or function with which the corresponding GSEs may interfere.

Our results show that the random fragment selection strategy, previously tested in bacteria (3), can be efficiently utilized in mammalian cells to isolate biologically active GSEs. The high efficiency of retroviral gene transfer makes it possible to apply this strategy to targets that are much more complex than a single cloned cDNA, used in the present study. Specifically, random fragment libraries derived from total cellular cDNA can be used to isolate GSEs originating from previously unknown genes and conferring different selectable phenotypes (A.V.G., A.R.K., R.T., and I.B.R., unpublished data). Thus, the random fragment strategy for GSE selection provides a general approach to the identification and analysis of recessive genes associated with important biological phenomena.

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