MOLECULAR AND CELLULAR BIOLOGY, July 1996, p. 3866–3877 Vol. 16, No. 7
0270-7306/96/$04.00+0

Analysis of Functional Domain Organization in DNA Topoisomerase II from Humans and *Saccharomyces cerevisiae*

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Received 16 February 1996/Returned for modification 8 April 1996/Accepted 22 April 1996

The functional domain structure of human DNA topoisomerase IIα and *Saccharomyces cerevisiae* DNA topoisomerase II was studied by investigating the abilities of insertion and deletion mutant enzymes to support mitotic growth and catalyze transitions in DNA topology in vitro. Alignment of the human topoisomerase IIα and *S. cerevisiae* topoisomerase II sequences defined 13 conserved regions separated by less conserved or differently spaced sequences. The spatial tolerance of the spacer regions was addressed by insertion of linkers. The importance of the conserved regions was assessed through deletion of individual domains. We found that the exact spacing between most of the conserved domains is noncritical, as insertions in the spacer regions were tolerated with no influence on complementation ability. All conserved domains, however, are essential for sustained mitotic growth of *S. cerevisiae* and for enzymatic activity in vitro. A series of topoisomerase II carboxy-terminal truncations were investigated with respect to the ability to support viability, cellular localization, and enzymatic properties. The analysis showed that the divergent carboxy-terminal region of human topoisomerase IIα is dispensable for catalytic activity but contains elements that specifically locate the protein to the nucleus.

Eukaryotic DNA topoisomerase II is an abundant nuclear enzyme involved in regulating the conversion of DNA between different topological isoforms (42, 60). It fulfills essential functions in DNA replication (41, 53) and chromosome segregation during both mitosis (26, 27) and meiosis (46), and it is thought to play a key role in certain types of DNA recombination events (8, 10, 22, 30, 48). The enzyme has furthermore been suggested to constitute a component of nuclear scaffold structures (3, 23), where it may be involved in chromosome condensation (4, 58) and decondensation (45).

Topoisomerase II binds to DNA as a dimer and cleaves its DNA substrate with a 4-bp stagger (40, 60) at sites with loosely defined sequences (54). Upon DNA cleavage, each subunit of topoisomerase II becomes transiently ligated to the 5′ ends of the respective DNA strands through an O^4-phosphotyrosine bond involving a tyrosine residue in the active site of the protein (7, 36). Catalytic activity is ATP dependent, and it is performed by passing one intact DNA helix through the enzyme-mediated double-stranded DNA break, followed by rejoining of the DNA strands (42).

Although the mechanics of topoisomerase II function have been characterized in great detail, little information has emerged about the functional organization of the enzyme. Proteolysis studies on purified topoisomerase II from *Saccharomyces cerevisiae* (35), *Schizosaccharomyces pombe* (50), *Drosophila melanogaster* (34), and humans (9) suggest that the enzyme is composed of three major structural domains, of which two are constituted by the conserved N-terminal and central parts of the protein and a third is constituted by the highly divergent C-terminal region.

The fragment originating from the N-terminal third of topoisomerase II has been predicted to contain the ATPase activity on the basis of its colinear homology to ATP-binding subunit B of the bacterial equivalent of topoisomerase II, DNA gyrase (44). The central part of the eukaryotic enzyme shows homology to subunit A of DNA gyrase, which functions as the catalytic subunit in the bacterial enzyme (44). Therefore, this highly conserved core of topoisomerase II is believed to constitute a catalytic subdomain capable of binding to and cleaving-religating DNA. In accordance with this, a crystal structure of a 92-kDa fragment of *S. cerevisiae* topoisomerase II has recently identified a specific DNA binding motif in this central core region (11), and biochemical experiments have previously identified Tyr-783 as the active-site tyrosine involved in DNA cleavage and religation (67). The hydrophilic C-terminal part of the protein is highly divergent with respect to both sequence and extent and has no equivalent in bacterial gyrase (50, 69). Analyses of mutant topoisomerase II from *S. pombe* (50), *S. cerevisiae* (17), and *D. melanogaster* (20) have shown that a large proportion of the C-terminal region is dispensable for cell viability and has no detectable effect on enzymatic activity in vitro.

Here we report a comparative analysis of the functional importance of the conserved and unconserved regions in human topoisomerase IIα and *S. cerevisiae* topoisomerase II. A yeast plasmid-shuffling system was established to investigate the ability of mutant topoisomerase II proteins to complement yeast *top2* deletion strains and to enable purification of mutant

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proteins for in vitro analysis. By sequence alignments, 13 highly conserved domains separated by unconserved or differently spaced sequences were defined in the topoisomerase II proteins. The importance of spacing between the conserved domains was assessed by insertion of linkers of various lengths into the spacer sequences, and the importance of the conserved domains was tested by individual deletion of these. The specific functions of the C-terminal end of human topoisomerase IIα and *S. cerevisiae* topoisomerase II were studied by C-terminal truncations. The mutant proteins were characterized with respect to complementation ability, cellular localization, and in vitro catalytic activity.

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used in this report: DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; 5-FOA, 5′-fluoroorotic acid; kDNA, kinetoplast DNA; NLS, nuclear localization sequence; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TPI, triose phosphate isomerase.

Yeast strains, media, and plasmids. Yeast strains Y700 (*MAT*α *ura3 leu2 his3 trp1 ade2*) and BJ5462 (*MAT*α *ura3 leu2 pep4*::*HIS3 prb1 trp1 can1*) were used to generate *top2* deletion strains BJ200 and BJ201, respectively. The endogenous *TOP2* gene was disrupted by integration of yeast selection marker *TRP1* into an internal deletion of the *S. cerevisiae TOP2* gene. Yeast cells were transformed by using a modification of the lithium acetate method of Ito et al. (28). The media and other yeast procedures used were essentially those described by Sherman (49). Plasmid pBY105 contains the yeast TPI promoter inserted into the polylinker region of *LEU2*/*ARS-CEN* plasmid pRS315 (52). This low-copy plasmid was used as the plasmid backbone for introduction of all mutant *top2* cDNAs into *S. cerevisiae*. Plasmid pYTO300 contains the wild-type *S. cerevisiae TOP2* cDNA under control of the TPI promoter. The plasmid was constructed by inserting a *Bgl*II-*Not*I topoisomerase II fragment from YEpTOP2-PGAL1 (kindly provided by J. C. Wang) into pBY105 digested with *Bam*HI and *Not*I. Plasmid pYYP7 is a modified version of pYTO300 in which PCR was used to insert a tag encoding the c-Myc epitope, a hexahistidine tail, a stop codon, and a *Not*I restriction site just upstream of the endogenous stop codon. Plasmid pHT300 is identical to pYTO300 but carries the wild-type human α *TOP2* cDNA instead of the yeast *TOP2* cDNA. The human α *TOP2* cDNA was cloned in two steps from pBS-hTOP2 (kindly provided by J. C. Wang) into a modified version of pBSII (KS−). From this plasmid, the *Nhe*I-*Not*I fragment, containing the human α *TOP2* cDNA, was cloned into pBY105, which had been cut with *Spe*I and *Not*I. Plasmid pHT212 is the human counterpart of pYYP7, containing the c-Myc–hexahistidine tag inserted at the C-terminal end of pHT300. Plasmid pSp100 contains the wild-type *S. pombe TOP2* gene under the control of its own promoter. It was constructed by inserting the *Hin*dIII fragment encoding the *S. pombe TOP2* gene (kindly provided by M. Yanagida) into *URA3*/*ARS-CEN* plasmid pRS316 (52).

Construction of mutant topoisomerase II proteins. Single restriction site insertion mutant forms of the human α and *S. cerevisiae TOP2* cDNAs were constructed by site-directed mutagenesis as described by Kunkel (32). The oligonucleotides used for mutagenesis contain the recognition sequence of restriction enzyme *Sal*I or *Xho*I, thereby resulting in mutant topoisomerase II proteins having two additional amino acids, V and D or L and E, respectively, inserted. For insertion positions, see Fig. 2 and 3. Constructs containing restriction site insertions at two positions in the *TOP2* cDNA were made by site-directed mutagenesis with a single-stranded template from single restriction site insertion mutants. All double restriction site insertion mutants were designed with one *Xho*I site and one *Sal*I site flanking the domain of interest. Deletion mutants were subsequently created by digesting the double restriction site insertion mutants with *Sal*I and *Xho*I, followed by ligation of the compatible ends. Twelve-amino-acid insertion mutants of human topoisomerase IIα were generated from the single restriction site insertion mutants by insertion of the 30-bp linker 5′-TCGACCTGCCTAGGCTCGAGGGGCCCAGAC-3′, which is flanked by *Sal*I and *Xho*I sites and contains a central *Xho*I site. PCR was used to verify the orientation of the inserted linkers. Mutants containing 7-amino-acid insertions were generated from the 12-amino-acid insertion mutants by *Xho*I or *Xho*I-*Sal*I digestion. A panel of carboxy-terminal truncations were generated by introducing a linker encoding the human c-Myc epitope followed by six histidine residues into the *Sal*I or *Xho*I site of the single restriction site insertion mutants. The sequences of the c-Myc–His linker oligonucleotides are 5′-TCGAGGCACAT ATGCCTAGGGAACAAAAGTTGATCTCTGAAGAAGACTTGAACGTG CACCATCACCACCATCATGTGCACTAGC′-3 (top strand), and 5′-GGC CGCTAGTGCACATGATGGTGGTGATGGTGCACGTTCAAGTCTTCTT CAGAGATCAACTTTTGTTCCCTAGGCATATGTGCC′-3 (bottom strand). Truncation mutants hΔC1233, hΔC1263, hΔC1296, and hΔC1352 were generated from pHT300 by introducing an *Xho*I restriction site and a termination codon followed by a *Not*I site at the indicated amino acid positions by PCR. Pfu polymerase from Stratagene was used for the PCR. The *Xba*I-*Not*I fragment from each reaction was used to replace the corresponding *Xba*I-*Not*I fragment of pHT300, within which the 3′ end of the human α *TOP2* cDNA lies. The c-Myc–His linker was subsequently inserted into the *Xho*I site. Plasmid pYYP8 contains the *S. cerevisiae TOP2* cDNA, in which the active-site tyrosine Tyr-783 has been mutated to a serine residue by site-directed mutagenesis with pYYP7 as the template DNA. In plasmid pHT213, the tyrosine at position 804 has been changed to a serine by site-directed mutagenesis with pHT212 as the template DNA. Linker insertion mutants, deletion mutants, and C-terminal truncation mutants of yeast topoisomerase II and human topoisomerase IIα were tested for expression in yeast strains Y700 and BJ201. Expression of linker insertion mutants and yeast deletion mutants was examined in crude yeast extract. Following extraction of cells derived from 50-ml cultures, the protein content was measured with the Bradford assay (12), and equal amounts of protein were loaded onto SDS-protein gels. The C-terminal truncation mutants and human deletion mutants were affinity purified, and eluted fractions were corrected for protein concentration by the Bradford assay prior to gel electrophoresis. SDS-PAGE was performed as described by Laemmli (33). Topoisomerase II mutants containing the c-Myc epitope were visualized by immunostaining with mouse monoclonal antibody MYC1-9E10.2. Yeast topoisomerase II mutants without the c-Myc epitope were detected by immunostaining with an anti-topoisomerase II antibody raised against *Drosophila* topoisomerase II. Human topoisomerase II mutants without the c-Myc epitope were visualized by the commercially available Cambridge anti-topoisomerase II antibody. As indicated by immunostaining, different topoisomerase II mutant proteins are expressed to similar levels within the range of a factor of 2. Mutants unable to complement a *top2* null mutation were further tested by nucleotide sequencing. In cases in which the mutants contained a hexahistidine tail, the enzymes were purified and their ability to decatenate kDNA was tested in vitro.

Oligonucleotides. All synthetic oligonucleotides were synthesized on a 381A DNA synthesizer from Applied Biosystems. The oligonucleotides used for mutagenesis were all 34- to 48-mers (data not shown).

Gene disruption. Haploid yeast strains BJ200 and BJ201, carrying a *top2* disruption, were constructed from Y700 and BJ5462, respectively, by the one-step gene disruption method (47). A linear DNA fragment containing the *TRP1* gene flanked by the N-terminal 163 amino acids and the 391 C-terminal amino acids of *S. cerevisiae* topoisomerase II was used for transformation of Y700 and BJ5462. The cells were cotransformed with plasmid pSp100 carrying the *S. pombe TOP2* gene, which acts as a substitute for topoisomerase II activity. Genomic DNA was isolated from stable *LEU2* transformants and examined by Southern blotting to verify integration of *TRP1* into the endogenous *TOP2* gene.

Complementation assay. Mutant *top2* constructs were transformed into haploid *top2* null strain BJ200 or BJ201, and colonies from selection plates were streaked onto media containing 5-FOA (1 mg/ml) to counterselect against the *URA3* plasmid carrying the *S. pombe TOP2* gene. Colonies were submitted to PCR screening with primers specific to the *S. pombe TOP2* gene to ensure that the *URA3* plasmid was effectively lost from the cells. Generation times of transformed yeast cells were measured on cultures grown to late log phase overnight and subsequently diluted to an optical density at 600 nm of 0.1 in selective medium (without Leu) to allow continued growth. Aliquots were removed at various times; and the optical density at 600 nm was measured.

Purification of topoisomerase II mutants. Human topoisomerase IIα and *S. cerevisiae* topoisomerase II constructs containing the hexahistidine sequence were transformed into protease-deficient strain BJ201. Cells were grown overnight in 50 ml of selective medium (without Leu) containing 2% glucose, transferred to 500 ml of selective medium for further growth, and harvested at an optical density at 600 nm of 0.8 by centrifugation. Crude yeast extract was prepared with 2 volumes of extraction buffer (50 mM Tris [pH 7.8], 1 M NaCl, 1 mM phenylmethylsulfonyl fluoride) and 1 volume of acid-washed glass beads (425 to 600 μm; Sigma) by vortexing at 4°C for 30 min. Glass beads and cell debris were removed by centrifugation at 4,000 × *g* for 10 min, and the supernatant was further centrifuged for 20 min at 15,000 × *g*. After filtration (0.65-μm-pore-size filters), the crude extract was loaded onto an Ni^{2+}-nitrilotriacetic acid-agarose column (matrix purchased from Qiagen). The column was washed with 5 volumes of buffer A (15 mM imidazole [pH 8], 1 M NaCl, 10 mM KPO_4 [pH 8], 10% glycerol) before protein was eluted with buffer B (500 mM imidazole, 1 M NaCl, 10 mM KPO_4 [pH 8], 10% glycerol) in a gradient of imidazole ranging from 15 to 500 mM. Fractions were collected, and the samples were submitted to SDS-PAGE on 8.5% polyacrylamide gels.

In vitro DNA decatenation. Activity of purified mutant topoisomerase II proteins was determined in a DNA decatenation assay with kDNA. Purified topoisomerase II enzyme was incubated with 300 ng of kDNA in 1 mM ATP–5 mM $MgCl_2$–1 mM dithiothreitol–0.5 mM EDTA–50 mM bis-Tris-propane (pH 8.0)–0.12 M potassium glutamate in a total volume of 20 μl. The samples were incubated for 30 min at 37°C and subsequently submitted to electrophoresis on a 0.7% agarose gel.

Indirect immunofluorescence. Wild-type and mutant c-Myc–His-tagged topoisomerase II proteins were expressed in BJ201 and prepared for immunostaining essentially as previously described (6). Mouse monoclonal antibody MYC1-9E10.2 was used to detect the expressed proteins carrying the c-Myc–His tag. The secondary antibody was fluorescein-conjugated $F(ab')_2$ fragments of swine anti-mouse immunoglobulin G (DAKO A/S). Yeast nuclei were counterstained with

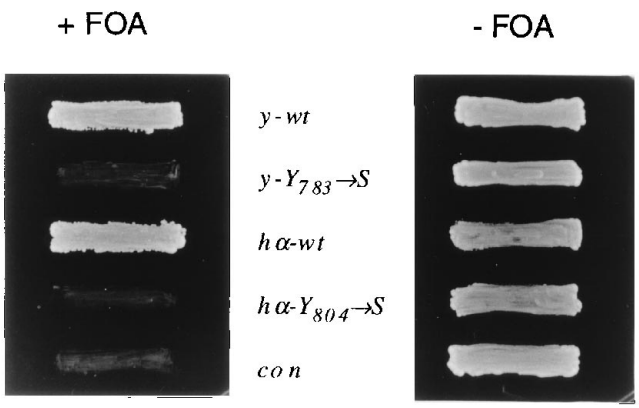

FIG. 1. Human topoisomerase IIα complements functions essential for mitotic growth in *S. cerevisiae* deletion strains. Deletion strain BJ200 was transformed with *LEU2* plasmids carrying TPI-expressed topoisomerase II cDNAs, and cells were grown on selective plates (without Leu) containing 1 mg of 5-FOA per ml (+FOA) or no 5-FOA (−FOA). The expressed topoisomerase II proteins are designated as follows: *y-wt*, wild-type *S. cerevisiae* topoisomerase II; *y-*Y_{783}→*S*, *S. cerevisiae* topoisomerase II with a tyrosine-to-serine mutation at position 783; *h* α-*wt*, wild-type human topoisomerase IIα; *h* α-Y_{804}→*S*, human topoisomerase IIα with a tyrosine-to-serine mutation at position 804; *con*, control in which BJ200 was transformed with pBY105 containing the TPI promoter only. Similar results were obtained with complementation in strain BJ201.

DAPI (Sigma). The preparations were examined with a Leitz DM epifluorescence microscope coupled to a cooled charge-coupled device camera (PM512; Photometrics) at a magnification of ×1,200. Camera control and image acquisition were done with an Apple Quadra 800 equipped with imaging software from IPLab (IPLabSpectrum). Fluorophores were selectively imaged with filters specially prepared as described by Pinkel et al. (43). Signals from DAPI and FITC were visually distinct and readily identifiable by inspection with appropriate filters.

RESULTS

Human topoisomerase IIα complements mitotic growth in an *S. cerevisiae* topoisomerase II deletion strain. *S. cerevisiae* provides the most efficient eukaryotic system to test large numbers of mutations in an essential gene for effects on functional complementation. We have used a modified version of the plasmid-shuffling technique of Mann et al. (39) to study the ability of wild-type and mutant forms of human topoisomerase IIα and *S. cerevisiae* topoisomerase II to complement a yeast *top2* deletion background. The chromosomal *TOP2* gene was disrupted by insertion of the structural *TRP1* gene (see Materials and Methods), while the essential topoisomerase II activity was provided by the *S. pombe TOP2* gene on a low-copy *URA3*-based plasmid. Wild-type human topoisomerase IIα and *S. cerevisiae* topoisomerase II expressed from *LEU2*-based vectors both complement the genomic disruption following loss of the *S. pombe TOP2* gene by growth on 5-FOA (Fig. 1). The *S. cerevisiae* enzyme allows slightly better growth of the yeast cells than does human topoisomerase IIα (Table 1). The findings on complementation are in agreement with earlier studies, in which wild-type *S. cerevisiae* and a recombinant form of human topoisomerase IIα having the N-terminal part substituted by the equivalent sequence of the yeast enzyme were able to sustain mitotic growth in a *top2* temperature-sensitive strain. Our observation adds to the general conclusion that topoisomerase II enzymes from a wide variety of eukaryotic origins are functionally interchangeable (2, 17, 57, 61, 68).

In the *S. cerevisiae* enzyme, the active-site tyrosine has been mapped to Tyr-783 (67), and in our study, substitution of tyrosine 783 with serine abolished the ability of the yeast cDNA to complement the *TOP2* disruption (Fig. 1). From homology, Tyr-804 has been predicted to be the active-site tyrosine of human topoisomerase IIα. Consistent with this prediction, complementation was abolished when tyrosine 804 was substituted with serine in human topoisomerase IIα, and the resulting enzyme was unable to perform DNA decatenation in vitro (Table 1). This result supports the hypothesis that residue 804 in human topoisomerase IIα is the active-site residue and indicates that the ability to complement is linked to enzymatic activity.

TABLE 1. Characteristics of wild-type and active-site mutant forms of human topoisomerase IIα and *S. cerevisiae* topoisomerase II[a]

Protein	Expression	Decatenation	Generation time (min)
wt-yTOPII	+	+	140
yTOPII-Y_{783}→S	+	ND[b]	
wt-hTOPII	+	+	200
hTOPII-Y_{804}→S	+	−	

[a] Wild-type and active-site mutant forms of human topoisomerase IIα and *S. cerevisiae* topoisomerase II were fused to a bicomposite protein tag encoding the human c-Myc epitope tag (EQKLISEEDLN) recognizable by mouse monoclonal antibody MYC1-9E10.2 and a hexahistidine tail for affinity purification on nickel resin. The purified enzymes were visualized by immunostaining with MYC1-9E10.2. Decatenation with the purified enzyme was performed as described in Materials and Methods. Generation times for yeast cells expressing wild-type human topoisomerase IIα or *S. cerevisiae* topoisomerase II were determined as described in Materials and Methods.

[b] ND, not determined.

Insertion of linkers into unconserved spacer regions of human topoisomerase IIα and *S. cerevisiae* topoisomerase II. Sequence alignments of topoisomerase II enzymes of different eukaryotic origins reveal a complex pattern of conserved regions that cover approximately 70% of the amino acid sequence (57, 69). Conservation is most pronounced in the N-terminal part of topoisomerase II, where highly conserved regions are interspersed with comparatively short "spacer" regions, which vary significantly in sequence between species and, in some cases, by one or a few amino acids in length. Towards the C-terminal end of topoisomerase II, conservation decreases and the most C-terminal 30% of the protein appears to have diverged entirely. Alignment of the amino acid sequences of human topoisomerase IIα (56) and *S. cerevisiae* topoisomerase II (24) has revealed an overall sequence identity of 40%. From alignments of the two sequences with the Pileup program of the Genetics Computer Group package with gap creation and gap extension penalty settings of 3.00 and 0.10, respectively, we have defined 13 conserved regions in topoisomerase II that are separated by unconserved spacer sequences (Fig. 2 and 3). The conserved regions exhibit an average identity of approximately 60%, with the exception of the most C-terminally located of the 13 domains (33% identity), whereas the spacer sequences are defined as regions of low homology, with an average identity of approximately 10%.

As an initial approach to examine the functional organization of topoisomerase II, we probed whether the spacer regions can be assumed to have a more flexible structure of less importance for topoisomerase II function. To investigate this, linkers were introduced into the spacer regions of human topoisomerase IIα and *S. cerevisiae* topoisomerase II and the complementation abilities of the mutant proteins were tested in the yeast deletion strains (Fig. 2 and 3).

In each spacer region of human topoisomerase IIα, a two-amino-acid linker with a DNA sequence corresponding to the recognition sites for restriction endonuclease *Xho*I or *Sal*I was inserted (Fig. 2). In three cases, at amino acid positions 112,

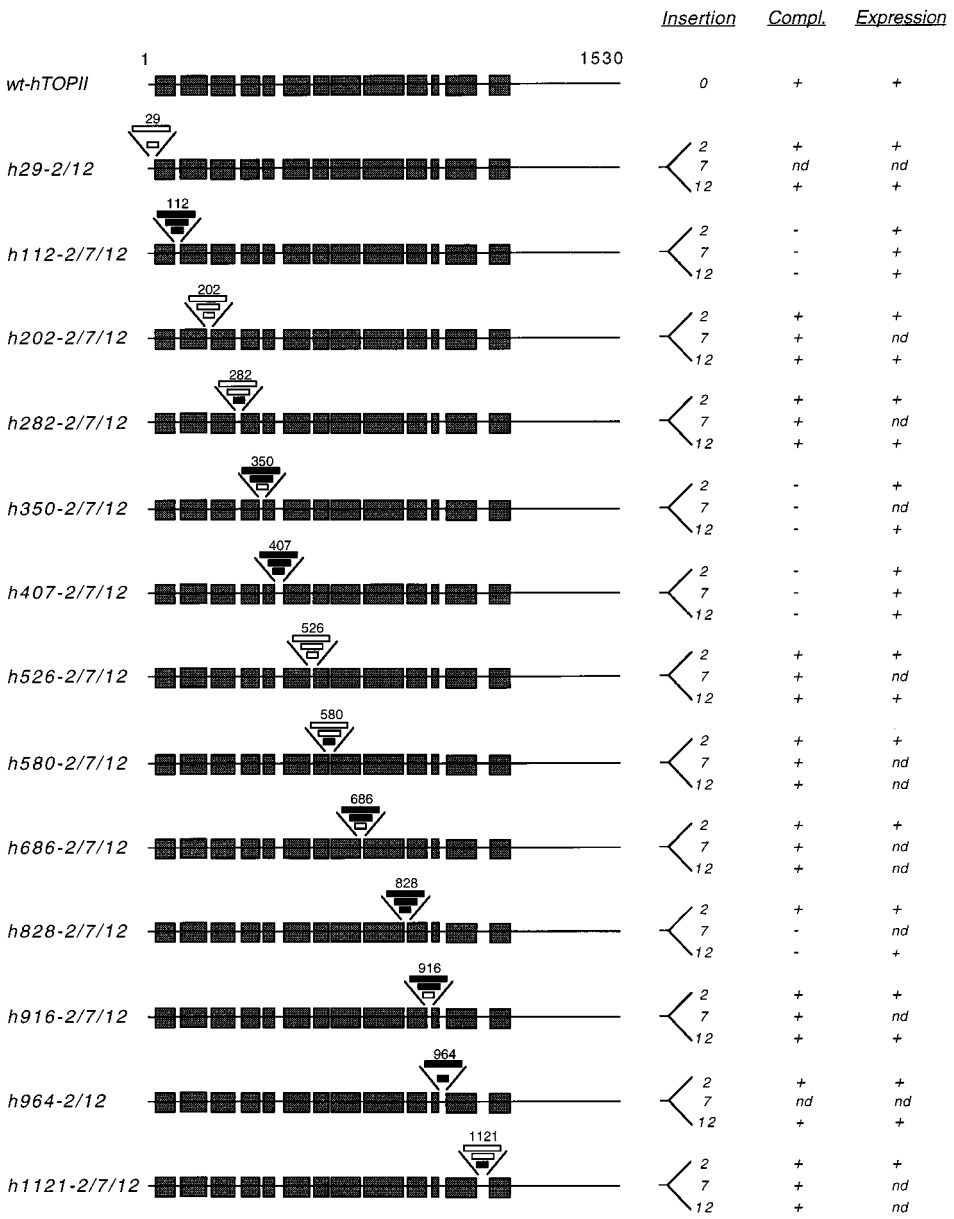

FIG. 2. Linker insertion analysis of regions separating conserved domains in human topoisomerase IIα. Highly conserved regions in topoisomerase II are shown as shaded boxes. They are separated by regions of less homology or different spacing, into which linkers varying in length were inserted at the indicated positions. The two-amino-acid insertions introduced amino acids V and D or L and E, encoded by the recognition sequence of restriction enzyme *Sal*I or *Xho*I, respectively. A short, open bar indicates the insertion of amino acids V and D; a short, solid bar indicates the insertion of amino acids L and E. Medium-size or large bars represent further insertions between the two residues of the respective short linkers as follows: medium-size open bar, ESGPL; medium-size solid bar, DLPRL; large open bar, ESGPLEPRQV; large solid bar, DLPRLEGPRL. The abilities of all constructs to complement (Compl.) for mitotic growth in BJ201 and detection of their expression are shown as plus and minus signs (*nd*, not determined). *wt-hTOPII* illustrates wild-type human topoisomerase IIα; the numbers refer to amino acid residues. Mutant constructs are named with respect to the linker insertion position and the number(s) of amino acids inserted, i.e., 2, 7 or 12, respectively.

350, and 407, insertion causes complete loss of viability for the host strain. The inability to complement is not due to lack of expression or stability of the mutant proteins, since the linker insertion mutants were expressed at a level similar to that obtained with wild-type human topoisomerase IIα (Fig. 2; see Materials and Methods). To rule out the possibility that the minor effect observed after the introduction of two-amino-acid insertions was due to the small size of the linkers, we next investigated the importance of the extent of the inserted linker. Thus, oligonucleotides varying in length were inserted into the already introduced *Xho*I and *Sal*I restriction sites, resulting in total insertions of 7 and 12 amino acids including one and two proline residues, respectively. Insertion of the longer peptides into the spacer regions of human topoisomerase IIα at the positions indicated in Fig. 2 does not change the viability pattern except at one position, residue 828, where both 7- and 12-amino-acid insertions inhibited complementation, in contrast to the 2-amino-acid insertion. This position is in the vicinity of the active-site tyrosine of the enzyme, and a longer insertion here might obstruct the configuration of the active site. The linker insertion analysis thus indicates that most of the domains defined by the sequence alignment can fold as

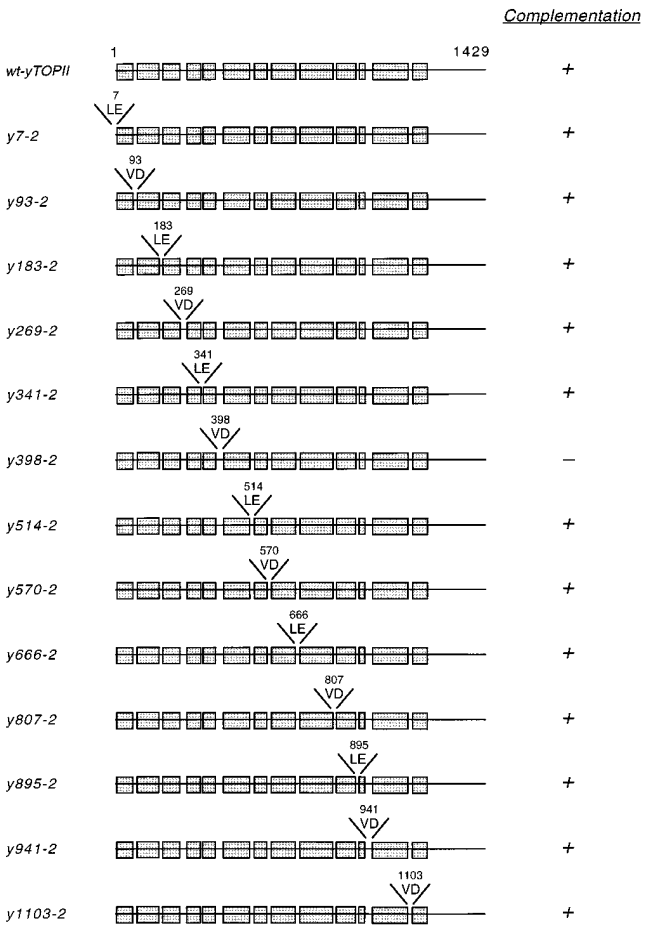

FIG. 3. Linker insertion analysis of regions separating conserved domains in *S. cerevisiae* topoisomerase II. The conserved domains are shown as shaded boxes. They are separated by sequences of less homology or different spacing into which a two-amino-acid linker, VD or LE, was introduced at the indicated positions. The ability of the linker insertion mutant enzymes to complement BJ201 is indicated by plus and minus signs. *wt-yTOPII* illustrates wild-type *S. cerevisiae* topoisomerase II; the numbers refer to amino acid residues. The designation of each mutant construct refers to the insertion position and the extent of the inserted linker. The mutant protein negative for complementation was expressed at a level similar to that of wild-type yeast topoisomerase II, as observed by immunostaining (data not shown), and the construct was sequenced throughout the *TOP2* cDNA to verify its integrity.

individual structural units, since insertion of linkers between them does not destroy folding or function of the entire protein. The notable exceptions to this pattern reside in the N-terminal part of the enzyme, in the spacer between conserved regions 1 and 2 and in the spacers flanking domain 5 (Fig. 2).

On the basis of the very similar patterns obtained with linkers of different lengths in human topoisomerase IIα, only linkers encoding two amino acids were inserted into the spacer regions of *S. cerevisiae* topoisomerase II. As seen in Fig. 3, insertion into the spacers was tolerated in the yeast enzyme, except between domains 5 and 6. Insertion into this spacer is also detrimental in human topoisomerase IIα, and thus, there seems to be a structural dependency between domains 5 and 6 in both enzymes. It is unclear why insertion between domains 1 and 2 does not affect the complementation ability of the yeast enzyme, but the result obtained indicates that the most N-terminal region of human topoisomerase IIα is more restricted than the corresponding region of the yeast enzyme.

Individual deletion of conserved domains. To investigate whether each conserved domain, as defined by the sequence alignment, is necessary for the essential functions of topoisomerase II, the effects of single domain deletions were studied. The 11 most N-terminally located domains in human topoisomerase IIα (Fig. 4A) and *S. cerevisiae* topoisomerase II (Fig. 4B) were deleted individually, whereas the two most C-terminal domains were deleted by C-terminal truncations (see Fig. 5 and 7 for the human and yeast enzymes, respectively). All constructs expressing human topoisomerase IIα (Fig. 4A) or *S. cerevisiae* topoisomerase II (Fig. 4B) with a deletion of one of the conserved domains were unable to complement the yeast deletion strains.

It is noteworthy that none of the human topoisomerase IIα or *S. cerevisiae* topoisomerase II deletion mutants behaved as dominant lethals, since a *TOP2* wild-type strain (BJ5462) expressing the individual deletion mutant enzymes showed no sign of reduced growth (data not shown). This observation indicates that the expressed mutated enzymes do not interfere with the endogenous enzyme to a level detectable in our system. Neither do they interact with DNA in a way that influences the phenotype of the cells.

To rule out the possibility that the complementation inability of the deletion mutants reflects a lack of protein expression or stability, expression was tested by immunoblotting (Fig. 4 and Materials and Methods). In all of the transformants tested, only a single band of the expected size was observed with the respective antibodies. The intensities of the antibody-specific bands furthermore indicate that the deletion mutants are expressed at similar levels. The human mutants were constructed with a C-terminal bicomposite protein tag consisting of an 11-amino-acid c-Myc epitope and a hexahistidine tail, enabling detection of the proteins by monoclonal antibody MYC1-9E10.2 and purification of mutant proteins by metal-chelating affinity chromatography, respectively. After purification of the individual human topoisomerase IIα enzymes containing a deletion of one of the conserved domains, the enzymes were analyzed for the ability to decatenate kDNA. In accordance with their inability to complement the yeast deletion strains, all of the human deletion mutant enzymes lacked the ability to decatenate kDNA (Fig. 4A). Thus, each conserved domain in topoisomerase II seems either to be essential for folding of a functional protein or to contain sequences that participate in functions essential for enzymatic activity.

C-terminal truncations of human topoisomerase IIα and *S. cerevisiae* topoisomerase II. To assess the importance of the largest unconserved domain in topoisomerase II, the C-terminal region, a series of vectors expressing proteins with C-terminal truncations were constructed. The majority of constructs carried the C-terminal bicomposite protein tag to allow further analysis of the enzymes in vitro.

In human topoisomerase IIα, the variable C-terminal region covers the approximately 300 amino acids extending from amino acid ~1200 (63). When the 31 most C-terminal amino acids were removed by truncation to residue 1499 (hΔC1499), we saw no detectable effect on the ability of the mutant enzyme to complement in *S. cerevisiae*, and truncation to residue 1352 (hΔC1352) only slightly impaired the growth rate of the host strain (Fig. 5). Any further truncations, the least extensive tested being to residue 1296 (hΔC1296), abolished complementation. To rule out the possibility that the observations made on complementation ability were due to low expression or instability of certain truncated proteins, Western blotting (immunoblotting) was performed with monoclonal antibody MYC1-9E10.2 (Fig. 5). From the immunostaining obtained, it appears that the truncated proteins are expressed at similar

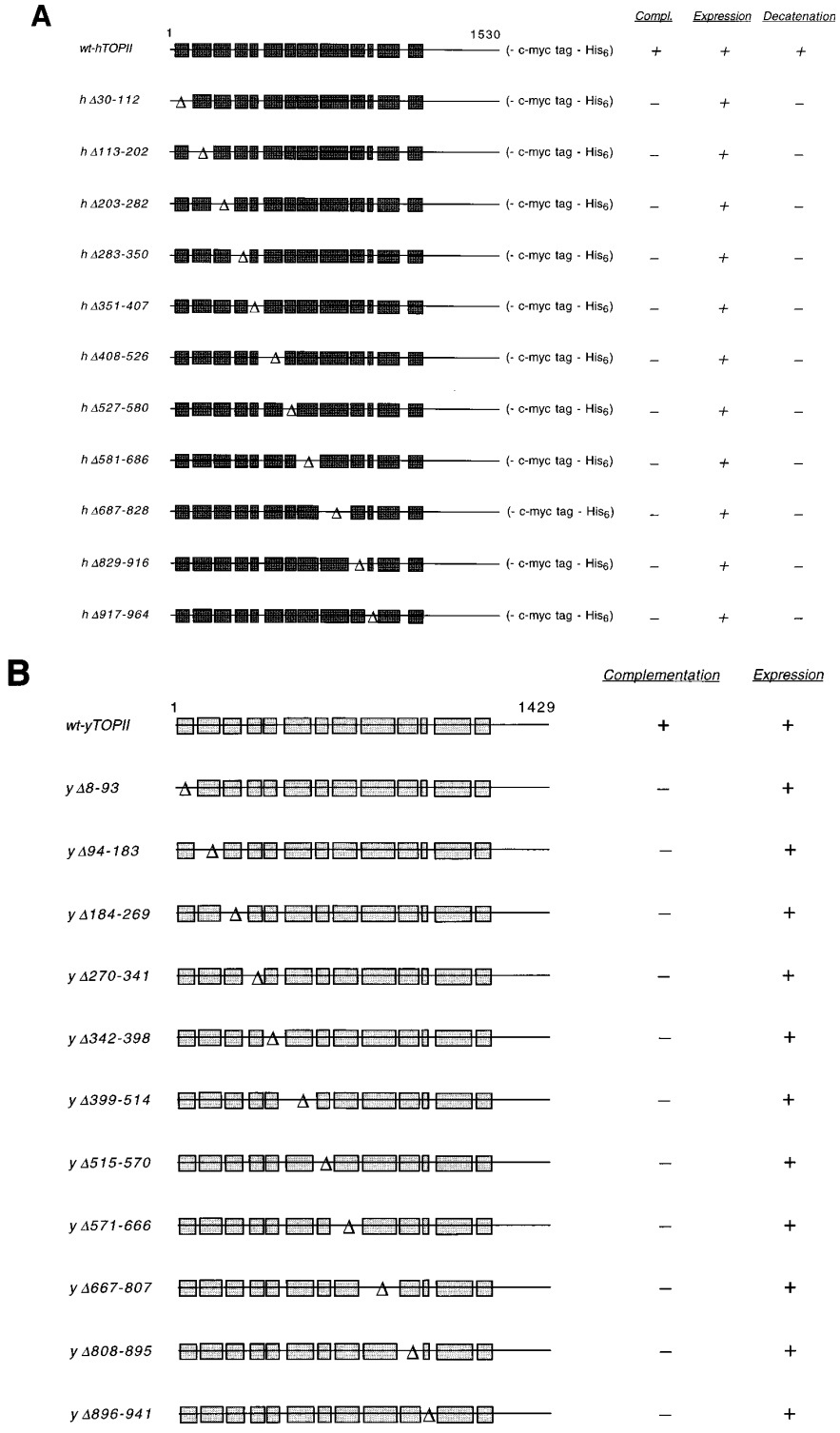

FIG. 4. Deletion analysis of conserved domains in human topoisomerase IIα (A) and *S. cerevisiae* topoisomerase II (B). The ability of deletion mutant enzymes to complement BJ201 was tested as indicated. All human topoisomerase IIα deletion mutant enzymes were constructed with a bicomposite tag (- c-myc tag - His_6) consisting of a c-Myc epitope and a hexahistidine tail as indicated in panel A. Following purification by metal ion affinity chromatography, the mutant proteins were detected by immunostaining with MYC1-9E10.2. The catalytic abilities of the purified enzymes were tested by decatenation of kDNA (see Materials and Methods). The expression and decatenation abilities of the deletion mutant enzymes are indicated by plus and minus signs in panel A. Expression of the yeast deletion mutants was detected in crude extract by immunostaining with a polyclonal antibody raised against *D. melanogaster* topoisomerase II and is indicated in panel B. The extent of the deletion in each topoisomerase II sequence is indicated by the construct name, and it is shown in the schematic presentation as a Δ replacing the conserved domain. Note that deletions also involved approximately half of the less conserved amino acid sequences flanking the particular conserved domain. With each deletion, two new amino acids were introduced as follows: A, LD in deletions starting at positions 113, 283, 408, 581, and 829 and VE in deletions starting at positions 30, 203, 351, 527, 687, and 917; B, LD in deletions starting at positions 8, 184, 342, 515, 667, and 896 and VE in deletions starting at positions 94, 270, 399, 571, and 808. The effects of single deletions of the two most C-terminal conserved regions were tested by C-terminal truncations (see Fig. 5 and 7). Compl., complementation.

levels. Our observations thus show that in the human enzyme only part of the unconserved C-terminal domain is dispensable for complementation in *S. cerevisiae.*

To test whether the ability to complement correlates with enzymatic activity, a number of the truncated proteins were affinity purified on nickel columns. Human topoisomerase IIα remained capable of decatenating kDNA when the truncations only removed unconserved C-terminal sequences (hΔC1352, hΔC1296, hΔC1263, and hΔC1233), whereas enzymatic activity was lost when the most C-terminal of the conserved domains was deleted (hΔC1121) (Fig. 5).

In the proteins expressed from three constructs, hΔC1296, hΔC1263, and hΔC1233, the ability to decatenate kDNA was retained and yet the ability to complement the endogenous topoisomerase II disruption was lost (Fig. 5). The results demonstrate that an enzymatically active truncated human protein, at the expression levels obtained in these experiments, is insufficient to provide the topoisomerase II functions required for mitotic growth.

To seek an explanation for the role of the sequences in the nonconserved C-terminal region that are required for complementation but not for kDNA decatenation, we next studied the cellular distribution of the truncated human proteins in yeast cells (Fig. 6). Immunofluorescence studies showed that overexpressed full-length human topoisomerase IIα is located mainly in the nucleus (Fig. 6A). Thus, specific nuclear localization of human topoisomerase IIα appears to be functional in yeast cells. Truncation of the human protein to residue 1352 (Fig. 6B) decreased nuclear staining and increased cytoplasmic staining. An even higher level of cytoplasmic staining was obtained with a mutant protein truncated to residue 1296 (data not shown), whereas only cytoplasmic staining was evident with a protein truncated to residue 1233 (Fig. 6C).

In conclusion, it appears that in human topoisomerase IIα, sequences between residues 1352 and 1233 are essential for specific nuclear localization. According to our observations, even partial impairment of the localization mechanism, by C-terminal truncations beyond residues 1352 to 1296, correlates with loss of complementation. Thus, failure to complement may be correlated with the lower content of the truncated human enzyme in the yeast nucleus.

In *S. cerevisiae* topoisomerase II (Fig. 7), truncations until the most C-terminal of the conserved domains (yΔC1236 and yΔC1208) had no detectable effect on the ability to complement, and the purified recombinant enzymes were capable of decatenation in vitro. Further truncations removing one, two, or three of the most C-terminal conserved domains (yΔC1103, yΔC941, and yΔC895) caused complete loss of complementation ability. The enzyme truncated at residue 1103 was affinity purified, but no decatenation activity was detectable. As the truncated proteins are expressed at similar levels, the inability to complement correlates with loss of decatenation activity in the yeast enzyme. It has previously been pointed out that the unconserved C-terminal region of *S. cerevisiae* topoisomerase II contains a sequence motif that may act as a nuclear localization signal in the region between residues 1171 and 1334 (17, 21). In our study, proteins truncated to residue 1236 gave a specific immunofluorescence staining of the nucleus, whereas further deletion of 28 amino acids to residue 1208 abolished specific nuclear staining, and this truncated enzyme was found mainly in the cytoplasm. Thus, a major nuclear localization signal seems to be located between residues 1236 and 1208. Because the construct with a deletion from residue 1208 still complemented (Fig. 7), it appears that with the expression level obtained with the TPI promoter, specific localization of the yeast enzyme to the nucleus is not a prerequisite for performance of the functions essential for mitotic growth.

DISCUSSION

Domain organization in eukaryotic topoisomerase II as deduced by linker insertion mutagenesis. Conservation between human topoisomerase IIα and *S. cerevisiae* topoisomerase II is distributed in 13 different regions as defined in our study, where the first 8 cover the N-terminal 650 amino acids in topoisomerase II, showing colinear homology to bacterial gyrase subunit B (44, 50, 63), and the last 5 domains cover the central part of the eukaryotic enzyme, stretching from approximately amino acid 650 to amino acid 1200. This part shows homology to subunit A of the bacterial enzyme (38) and is flanked in the surrounding spacers by proteolytic cleavage sites (34, 35, 50). In the present study, insertion of a two-amino-acid linker into 10 of the 13 spacer regions in human topoisomerase IIα (Fig. 2) and 12 of the 13 spacer regions in *S. cerevisiae* topoisomerase II (Fig. 3) had no detectable effect on the complementation of a *top2* null strain. Linkers abolishing complementation were all located in the approximately 400 N-terminal amino acid residues. Insertion of more extensive linkers containing proline residues into the human topoisomerase IIα protein produced results similar to those obtained with the two-amino-acid insertions. The only exception was insertion of the linkers at position 828 in the vicinity of the active site, which gave rise to a noncomplementing enzyme. The results obtained thus indicate that the majority of the defined spacer regions are of minor importance for topoisomerase II function and are more likely to represent flexible joints between conserved domains. It is noteworthy that in a recent investigation by Lee and Hsieh (34) in which random linker insertion was used to study the organization of *Drosophila* topoisomerase II, the majority of insertion points corresponding to the conserved domains defined in this study were found to abolish complementation of a *top2* temperature-sensitive yeast strain. In agreement with our observations, this work also indicated that the structure of the N-terminal part of the protein is highly constrained.

The five most N-terminal linker insertion positions in our study are located in a region corresponding to the crystallized 43-kDa N-terminal fragment of gyrase subunit B that forms a dimer in the presence of an ATP analog (66). The crystal structure reveals two internal subdomains separated by a hinge at amino acid position 220. One of our insertion positions (residues 269 in the *S. cerevisiae* enzyme and 282 in the human enzyme) is located in this hinge region, and the complementation ability of mutants containing up to 12 amino acids at this position indicates that a similar structural domain organization exists in eukaryotic topoisomerase II enzymes. Intriguingly, complementation was also achieved with mutant enzymes containing a linker at positions 7 and 29 in the yeast and human enzymes, respectively, corresponding to Tyr-5 in gyrase subunit B, which, from the crystal structure, has been shown to be directly involved in ATP binding and dimerization of the N-terminal claws (66). The complementation abilities of these insertion mutants indicate that this region, no matter its important function, can sustain the perturbations derived from linker insertion. Two human mutant enzymes carrying insertions at positions 112 and 350 were unable to complement. These positions fall into the region constituting the interior walls of the cavity enclosing DNA in gyrase subunit B, and insertions here would therefore be expected to be detrimental for enzyme function. It is, however, puzzling that linker inser-

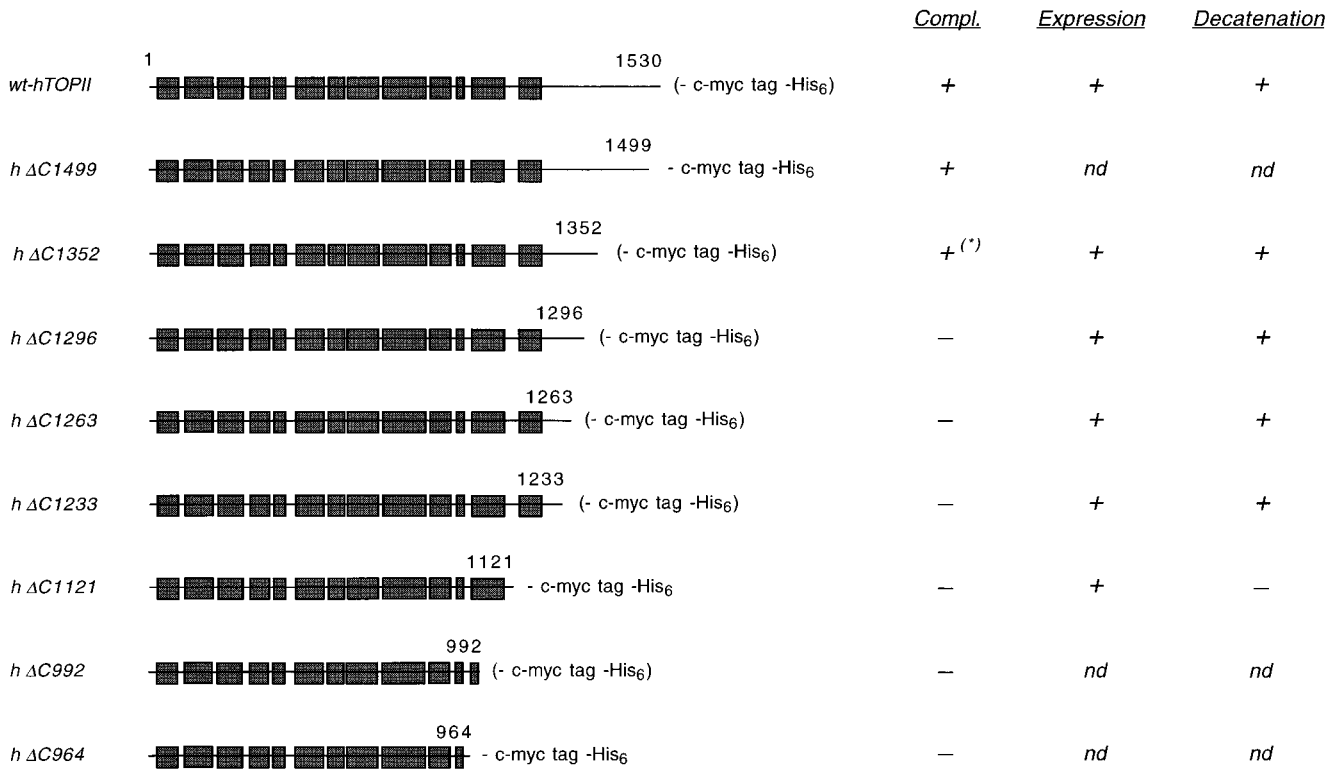

FIG. 5. Effects of C-terminal truncations of human topoisomerase IIα on complementation (Compl.) and enzymatic activity. C-terminally truncated topoisomerase II constructs were fused to a bicomposite tag (- c-myc tag - His_6). The abilities of the fusion constructs to complement were tested in strain BJ201. Brackets indicate that identical complementation results were obtained with constructs both with and without the bicomposite tag. Lack of brackets indicates that only the depicted construct was tested. Expression of C-terminally truncated constructs with the synthetic linkers was tested by immunostaining with MYC1-9E10.2. Affinity-purified proteins were tested for decatenation abilities as indicated. C-terminal endpoints of the topoisomerase II sequences are indicated above each construct. *wt-hTOPII* with and without the tag refer to plasmids pHT212 and pHT300, respectively. *, cells showing a slightly reduced growth rate compared with cells expressing wild-type topoisomerase II (*nd*, not determined).

tion at the equivalent position in yeast topoisomerase II gave a functional enzyme.

In both human topoisomerase IIα and *S. cerevisiae* topoisomerase II, linker insertion between conserved domains 5 and 6 at positions 407 and 398, respectively, yielded enzymes unable to sustain mitotic growth. This spacer sequence is located in a region between the ATP binding domain and the remaining part of the gyrase subunit B-homologous region in eukaryotic topoisomerase II (the B′ fragment [11]). It is supposed to be involved in the conformational changes that take place during ATP binding because it is sensitive to proteolytic degradation only in the absence of ATP. Although the spacer is protease sensitive, our observation indicates that positioning of these respective domains is highly important for ATP-derived transitions in the enzyme.

The seven most C-terminally located linker insertion positions fall into the region from residues 400 to 1200 in *S. cerevisiae* topoisomerase II, and recently the crystal structure of this 92-kDa fragment has been revealed (11). None of the linkers are inserted in secondary structural entities such as α helices and β pleated sheets but rather lie in relative hydrophilic segments separating such structures.

The crystal structure presented by Berger et al. (11) reveals two dimerization regions, where the primary dimer interface is present in the C-terminal part of the fragment involving amino acids 1031 to 1046 and 1114 to 1130 and the other involves residues in the B′ region stretching from amino acids 420 to 680. One of our linker insertion sites maps to position 1103 in *S. cerevisiae* topoisomerase II, between the two identified C-terminal dimerization regions. Since this region tolerates linker insertion in both yeast topoisomerase II and human topoisomerase IIα, our results indicate that exact spacing is not a prerequisite for functional dimerization, and this area might constitute a more flexible structure. The fact that a fragment spanning residues 1109 to 1163 in *S. cerevisiae* topoisomerase II recently has been found to interact specifically with Sgs1, a eukaryotic homolog of the bacterial helicase RecQ (64), suggests that this C-terminal region plays a major role in protein-protein interactions, further supporting a more accessible and flexible structure of this region. To this end, it is likely that the lack of catalytic activity of C-terminal truncation mutants yCΔ1103 and hCΔ1121 in our study is due to absence of subunit dimerization.

On the basis of the crystal structure, it has been suggested that ATP-dependent dimerization of the B′ fragments involves residues Ile-540, Leu-551, and Leu-557 on one subunit and Tyr-602 on the other (11). In this respect, it is interesting that two of our linker insertions, at positions 514 and 570 in *S. cerevisiae*, located in the same structural domains as the interacting residues, were tolerated without functional consequences. Our observation implies either that this region is not sensitive to perturbations or that the interaction seen in the crystal structure is merely a result of crystal packing and is absent in the intact enzyme. The latter explanation is supported by a previously reported linker insertion mutant of *Drosophila* topoisomerase II that retained its catalytic ability

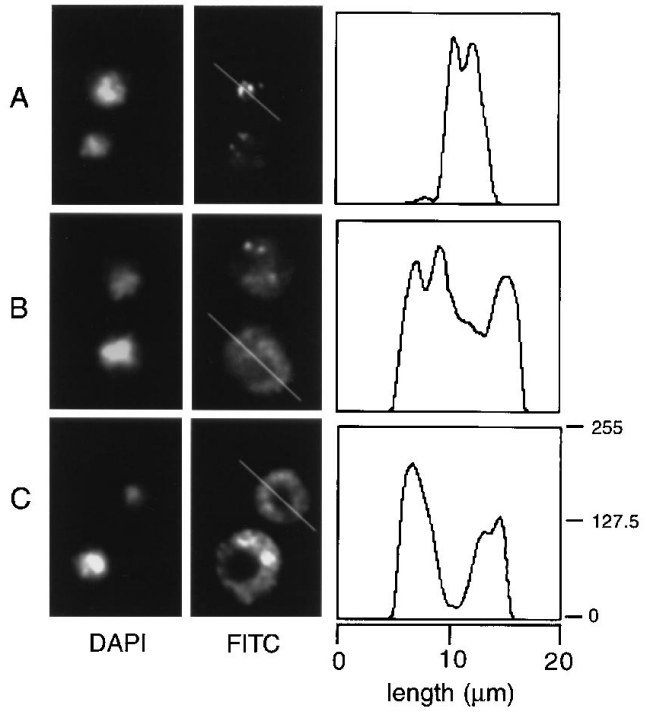

FIG. 6. Immunofluorescent localization of c-Myc–His-tagged human topoisomerase IIα and C-terminally truncated mutant enzymes expressed in BJ201. Yeast cells transformed with human c-Myc–His-tagged wild-type or mutant constructs were prepared as spheroblasts, fixed, and probed with antibody MYC1-9E10.2. Visualization of the bound primary antibody was done with FITC-conjugated swine anti-mouse $F(ab')_2$ fragments, and DAPI staining was subsequently performed to highlight nuclei. Details of constructs are shown in Fig. 5. Images were taken at an original magnification of ×1,200. The pictures generated by software depict the distribution of light intensity. Images of cells expressing full-length human topoisomerase IIα, wt-hTOPII (A), hΔC1352 (B), and hΔC1233 (C) were captured, and the fluorescence signal was quantified along the line in the FITC images. Values on the *x* axis show cell length, and FITC signal strength is indicated on the *y* axis.

following insertion of a hydrophilic linker at a position equivalent to amino acid 597 of *S. cerevisiae* topoisomerase II (34).

The only spacer in the region from amino acids 400 to 1200, where a structural dependency seems to exist between the flanking conserved domains, is the spacer between domains 9 and 10. Insertion of more extensive linkers at position 828 in human topoisomerase IIα was detrimental to enzyme function. Since this position is in the vicinity of a general DNA binding motif detected from the crystal structure, it is plausible that insertion of 7 or 12 residues at this point might influence the stability of the enzyme-DNA complex by interfering with this motif.

The two most C-terminally located insertion positions are present in a region of the enzyme which, deduced from the crystal structure, forms the upper part of the DNA binding cavity and might be involved in further stabilization of the protein-DNA complex. These sites tolerate perturbations derived from insertions of up to 12 amino acids, suggesting that this region folds into a more flexible structure. On the basis of the architecture of the DNA binding cavity and the fact that all contacts between DNA and the enzyme occur on one side of the DNA helix (5), it seems reasonable to assume that these insertion positions are located outside the actual enzyme-DNA contact region and thus are of minor importance for enzyme-DNA interactions.

Functional domains in eukaryotic topoisomerase II. It is not clear to what extent it will be possible to ascribe different functions of topoisomerase II to particular domains of the primary sequence. Deletion of individual conserved domains in human topoisomerase IIα and *S. cerevisiae* topoisomerase II in all cases led to inactive, noncomplementing proteins, indicating that the conserved domains are critical for topoisomerase II function. However, the possibility that deletion of conserved domains, in some cases, may have obstructed correct folding of a functional enzyme cannot be ruled out.

It is interesting that domain 9 is consistent with a structural domain observed from the crystal structure to contain the active-site tyrosine in a CAP-like DNA binding region stretching from positions 702 to 790 in *S. cerevisiae* topoisomerase II. It has previously been suggested that Y-804, located in the equivalent domain of the human enzyme, constitutes the ac-

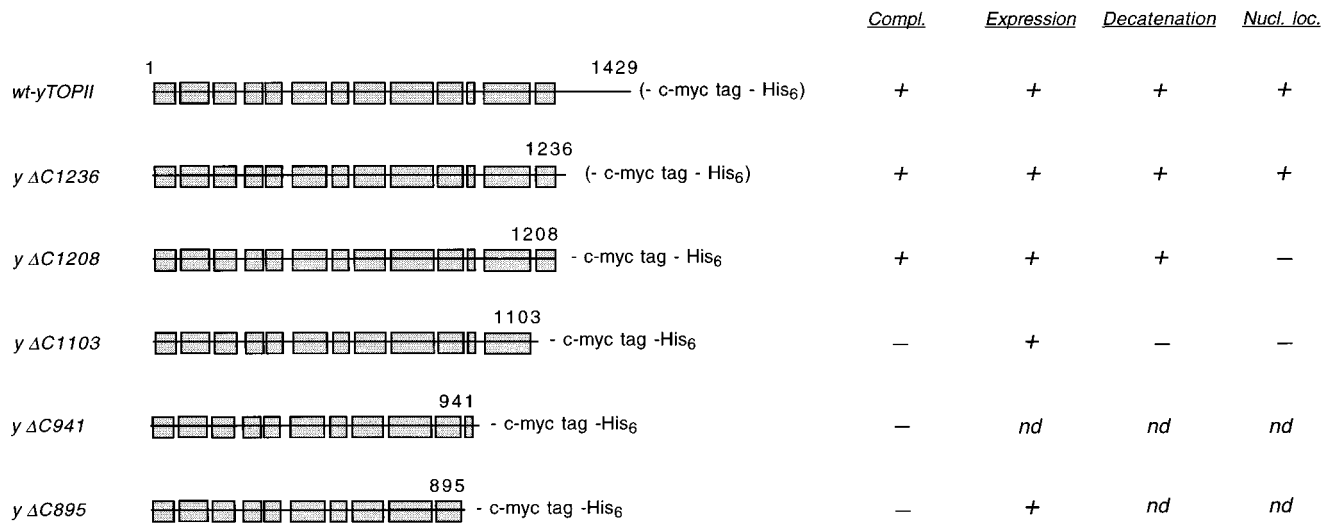

FIG. 7. Effects of C-terminal truncations of *S. cerevisiae* topoisomerase II on complementation (Compl.) and enzymatic activity. Experiments and conventions are described in the legend to Fig. 5. The C-terminal truncation mutant proteins were examined for the ability to localize to the nucleus by indirect immunofluorescence (see Materials and Methods). *wt-yTOPII* with and without the tag refer to pYYP7 and pYTO300, respectively. Nucl. loc., nuclear localization. *nd*, not determined.

tive-site tyrosine (67). Here we have demonstrated that a point mutation changing the tyrosine at position 804 to a serine residue abolishes complementation of the *top2* null mutants and yields an inactive enzyme in vitro, indicating that Y-804 is the active-site tyrosine in human topoisomerase IIα.

Domain 10, stretching from residues 808 to 895 in yeast topoisomerase II, covers a region in which a multitude of mutations giving rise to a temperature-sensitive or cold-sensitive phenotype have been mapped (29, 34, 55). The fact that mutations scattered in this region exhibit the same phenotype suggests that the region executes a common function. Alignment of all eukaryotic topoisomerase II sequences has furthermore shown that the length of this particular region is highly conserved (29). From the crystal structure, domain 10 constitutes a connecting region important for linking two major structural domains in the related gyrase subunit A fragment (11). It is thus tempting to speculate that the sensitivity of domain 10 is due to involvement in the stabilization of DNA binding.

A number of studies have focused on identifying domains in topoisomerase II interacting specifically with anti-topoisomerase II agents (59). This has led to the characterization of a multitude of mutations conferring resistance towards drugs such as etoposide and amsacrine. It is striking that the majority of these mutations are clustered in specific regions of topoisomerase II corresponding to domain 6 (13, 18, 62) in the N-terminal ATPase region and domains 9 (37) and 10 (29, 55) in the central region of the enzyme flanking the active site. That these specific domains interact with the topoisomerase II agents has been further supported by a genetic suppressor study in which peptides located in domains 9 and 10 represented the majority of genetic suppressor elements conferring drug resistance (25).

Although some of the 13 domains defined in this study seem to constitute functional domains, the present analysis does not demonstrate whether any of these domains contain autonomous subfunctions or whether these depend on the presence of a multitude of domains and their accurate folding into larger structural entities. Further biochemical studies of our linker and deletion mutant proteins are necessary to elucidate the actual correspondence between enzymatic functions and structural domains in eukaryotic topoisomerase II.

Functions of the unconserved C-terminal domain in human topoisomerase IIα and *S. cerevisiae* topoisomerase II. Only part of the divergent C-terminal region of human topoisomerase IIα is dispensable for complementation in *S. cerevisiae*, as a truncation mutant deleted to position 1352 supported viability whereas this was lost with further truncation to position 1296. Loss of complementation does not correlate with loss of catalytic activity, as three constructs truncated to residues 1296, 1263, and 1233 exhibited decatenation activity yet failed to support viability. An explanation for this may be found in the altered localization pattern of these truncated proteins, as shown by immunostaining experiments with yeast cells. The enzyme truncated at residue 1352 was partially impaired with respect to nuclear localization, whereas the noncomplementing enzyme truncated at residue 1233 appeared only in the cytoplasm (Fig. 6). This observation is in correlation with the existence of a suggested NLS (KKQTTLAFKPIKKGKKR) between residues 1274 and 1290 (21). Two additional mutants flanking this sequence, hCΔ1296 and hCΔ1263, were tested for cellular localization. Mutant hCΔ1296 was less abundant in the nucleus than was hCΔ1352, whereas hCΔ1263 gave a staining pattern similar to that obtained with hCΔ1233 (data not shown), in support of an NLS at the indicated position. Although the mutant truncated to residue 1296 was still detectable in the nucleus, it is likely that the inability of this enzyme to support viability reflects the slightly lower concentration of this enzyme than of hCΔ1352 in the nucleus. Identification of a region responsible for nuclear localization of human topoisomerase IIα in the highly variable C-terminal part correlates with previous studies of topoisomerase II from *S. pombe* (50, 51), *D. melanogaster* (19, 20), and *S. cerevisiae* (17).

In topoisomerase II from *S. cerevisiae*, the region between amino acids 1171 and 1334 has been shown to be important for nuclear transport of the enzyme (17). In our study, we found that a mutant truncated to residue 1208 was still catalytically active although specific nuclear localization was lost in this mutant. A mutant truncated only to residue 1236 still located to the nucleus, indicating the importance of the residues between positions 1208 and 1236 for nuclear localization. The sequence between residues 1195 and 1211 in yeast topoisomerase II (RKLVDDEDYDPSKKNKK) has previously been identified as a potential NLS (21). The observation that the construct ending at residue 1208, from which three residues of the proposed NLS have been removed, is located in the cytoplasm supports the suggested role of this sequence. It is possible, however, that since the bipartite nature of the NLS is not entirely destroyed, some specific localization to the nucleus still takes place. Our data are in agreement with earlier results obtained by Caron et al. with a mutant enzyme truncated to position 1208. In that case, the mutant enzyme was found to be marginally functional in vivo (17).

Besides nuclear localization signals, the C-terminal region of eukaryotic topoisomerase II contains several potential phosphorylation sites (15, 65), and this region has been assigned a function in the regulation of enzymatic activity via differential phosphorylation throughout the cell cycle (1, 14, 15, 31). However, as the present study demonstrates, this type of regulation cannot be essential for the complementation ability of human topoisomerase IIα and *S. cerevisiae* topoisomerase II during mitotic growth, since the phosphorylation sites (15, 65) can be deleted with no significant effect. Phosphorylation may, instead, fine-tune activity or relieve negative regulation during particular stages in the cell cycle, as suggested by Cardenas and Gasser (16).

In summary, we have established a catalog of human topoisomerase IIα and *S. cerevisiae* topoisomerase II linker insertion mutants in which the presence of pairs of unique compatible restriction sites conveniently enables the isolation, expression, and characterization of single conserved domains or arrays of conserved domains. The system can furthermore be applied for construction and analysis of chimeric proteins in which one or more domains have been exchanged between the yeast and human enzymes. Such studies will be valuable for further examination of the functional organization of eukaryotic topoisomerase II.

ACKNOWLEDGMENTS

We thank K. Andersen and B. Dahl for technical assistance and M. Etzerodt for valuable discussions. We are grateful to G. Evan for the kind gift of anti-c-Myc antibody MYC1-9E10.2 and to S. Rasmussen for kindly providing the anti-*Drosophila* topoisomerase II antibody. We also thank J. C. Wang for kindly supplying the *S. cerevisiae* and human *TOP2* cDNAs and M. Yanagida for kindly providing the *S. pombe TOP2* gene.

This work was supported by Danish Cancer Society grants 93-004 and 78-5000, the Human Genome Research Programme, the Danish Center for Molecular Gerontology, the Danish Center for Respiratory Physiological Adaptation, the Danish Research Academy, the Aarhus University Research Foundation, the Thaysen Foundation, and Deutsche Forschungsgemeinschaft grant Bi 541/1-1.

The first two authors contributed equally to this work.

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