

THE HYDROPHILIC, PROTEASE-SENSITIVE TERMINAL DOMAINS OF EUKARYOTIC DNA TOPOISOMERASES HAVE ESSENTIAL INTRACELLULAR FUNCTIONS

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The amino-terminus of eucaryotic DNA topoisomerase I and the carboxy-terminus of eucaryotic DNA topoisomerase II contain sequences that are enriched in charged amino acid residues, hyper-sensitive to protease digestion, not required for the *in vitro* topoisomerase activities, able to tolerate insertion and deletion mutations, and thus may have a disordered structure. In an interesting contrast to the catalytically essential core domain, the sequences in these terminal hydrophilic domains are not conserved among the topoisomerases from different species. However, many lines of evidence, including those presented here, demonstrate that the topoisomerase tail domains have critical intracellular functions. The biological functions of the amino-terminus of topoisomerase I include the nuclear import and targeting to the transcriptionally active loci. The carboxy-terminus of topoisomerase II also contains the sequences necessary for nuclear localization and possibly sequences necessary for other critical functions.

1. Background and Introduction

DNA structures inside of the cell undergo dynamic changes in order to fulfill its functions in replication, transcription, recombination, and repair. DNA topoisomerases are essential enzymes for facilitating these processes (reviewed in (1)). They work by transiently breaking the DNA backbone bonds utilizing a cycle of transesterification reactions mediated by a specific tyrosine residue in the enzyme. Both the sequence comparison and enzymological considerations have resulted in grouping topoisomerases into two classes: type I enzymes make a reversible single strand break (2) and type II enzymes can make a concerted double strand break and pass another segment of DNA through this transient break (3). The rotation of the DNA strand at the site of break made by type I topoisomerase and the strand passage reaction carried out by the type II enzyme are the key steps in the topological transformation of DNA brought about by these enzymes.

Type I enzymes consist of two distinct families. Type IA are likely the most ubiquitous ones among all topoisomerases and can be found in every organism examined so far. They include for examples, bacterial topoisomerase I (topo I) and topoisomerase III (topo III), archaeobacterial reverse gyrase, and eucaryotic topoisomerase III (topo III). Type IB enzymes are unique in that they are the only topoisomerases which can form the phosphotyrosyl linkage to the 3' end of the break during the transesterification reaction. They consist of all the eucaryotic topoisomerase I (topo I) and possibly, the archaeobacterial topoisomerase V. Members of type II enzymes are the bacterial gyrase and topoisomerase IV, and the

eucaryotic topoisomerase II. While the bacterial and eucaryotic topoisomerase II (topo II) share significant sequence identity (4) and similarity in their three-dimensional structures (5,6), they differ in their number of subunits. Bacterial gyrase and topo IV are heterotypic tetramers, $gyrA_2gyrB_2$ and $parC_2parE_2$, and eucaryotic enzymes are homodimers. In the archaeon *Sulfolobus shibatae* where neither gyrase nor topo II could be found, a new family of type II DNA topoisomerase, topoisomerase VI, was discovered. Topo VI also has a heterotypic tetrameric structure of $top6A_2top6B_2$, but both subunits only show limited identity to the subunits of bacterial and eucaryotic topo II. Instead, the B subunit of topo VI has close homologues from other archaea, *C. elegans*, budding and fission yeasts. In the budding yeast, the homologous protein *spo11* is involved in meiotic recombination. *Spo11* can make a double strand break which is protein-linked, a hallmark reaction for type II enzyme (7). Since this group of proteins may form a distinct family of topoisomerases, it has been proposed that they are named as topo IIB and the previously identified type II enzymes are topo IIA (8).

The reaction of passing double strand DNA through each other suggests that topo II plays a critical role in chromosome condensation and segregation. Such an essential function for topo II is supported by both genetic analysis and cell biological studies (9-12). The biochemical mechanism of type I topoisomerases suggests that they can serve as swivels in relieving supercoiling stress generated during DNA replication and transcription. Inactivation of *E. coli* topoisomerase I, a type IA enzyme, is lethal without a compensatory mutation (13,14). Another type IA enzyme, topo III is not an essential enzyme in *E. coli*, nor in yeast; however, their mutants have a hyper-recombination phenotype (15,16). While *top3* gene is not essential for the viability of yeast cells, the recent mouse knock-out experiment demonstrates the essential role of one of the mammalian topo III genes in early embryogenesis (17). This difference in the essentiality of topoisomerase between a unicellular and multicellular eucaryote is also exemplified in eucaryotic topo I, a type IB enzyme. While yeast topo I has important roles in replication and transcription, its mutation is not lethal, presumably because of the overlapping function between topo I and topo II in yeast (18). However, topo I is essential in both *Drosophila* and in mouse, and one of the critical periods requiring topo I function is early embryogenesis (19,20).

The interests in the structural and functional analysis of DNA topoisomerases are heightened by the recent realization that they are the intracellular targets for a number of clinically important antibiotics and anti-cancer drugs (21). Most of these anti-topoisomerase drugs target these enzymes by trapping the intermediate in which the active site tyrosine of the topoisomerase forms a phosphodiester bond with the DNA phosphate at the site of DNA break. However, recent development has also produced novel inhibitors of topoisomerase activities. These new topoisomerase drugs may provide useful reagents for the study of topoisomerase function and mechanism.

2. Eucaryotic topo I and topo II have hydrophilic and protease-sensitive domains at either N- or C- terminus

Topoisomerases are fairly large molecules and can be viewed as biochemical machines with multiple joints. Eucaryotic topo I is a monomeric protein with a size, usually, in the neighborhood of 800 amino acid residues. Some of the notable exceptions include topo I from *Drosophila melanogaster*, *Xenopus* oocytes, and *Arabidopsis thaliana*, all of which have molecular weights greater than 100 kDa (reviewed in (22)). These nuclear topo I have a conserved core domain with a size of about 550 amino acid residues in the central and carboxyl portions of topo I (Fig. 1A).

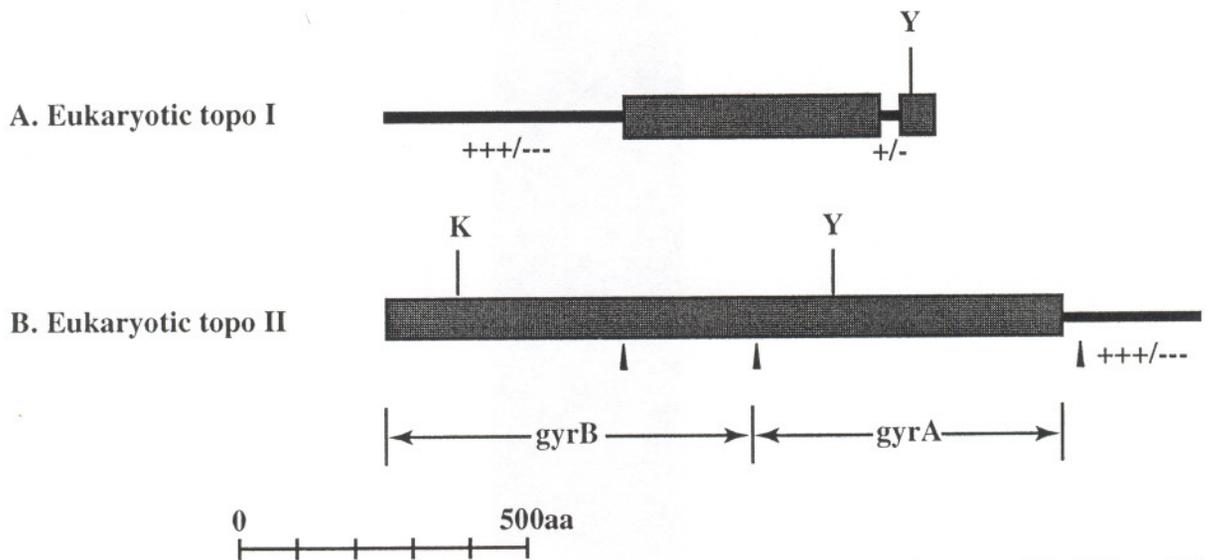


Fig 1. Schematic diagram of eucaryotic topo I (A) and topo II (B). The homologous and the non-conserved regions are represented by *shaded box* and *line*, respectively. The hydrophilic domain is labeled with *+++/--*. The catalytically essential tyrosine residue (close to the C-terminus in topo I and in the *gyrA* domain in topo II) and the lysine residue (in the *gyrB* domain of topo II) are shown as Y and K, respectively. The protease-sensitive sites in topo II are marked with *arrows*.

The variation in the molecular size is attributed to the variation in size and sequence at the N-terminal domain, and to a lesser extent from the variation in the linker domain that bisects the conserved core domain near the active site tyrosine residue (Fig. 1A). The N-terminal domains of eucaryotic topo I, despite their lack of sequence conservation, are characterized by an abundance of both acidic and basic residues. The *Drosophila* topo I is unique in that it contains not only the longest N-terminal domain (430 residues), but also several clusters of histidines and serines. In general, eucaryotic topo I N-terminal domains have a higher density of prolines and serines. This plus the results that topo I N-terminus is hypersensitive to proteolytic cleavage suggest that the hydrophilic tail lacks any ordered structures. Biophysical measurements from hydrodynamic experiments and circular dichroism using the holoenzyme and the protein with N-terminal truncation indeed indicate that

this domain does not have a high proportion of secondary structures (23). Since this domain is dispensable for the DNA relaxation and cleavage activity of topo I, the essential catalytic activity of topoisomerase must reside entirely in the conserved core domain. Recent high resolution crystal structures of the conserved core domain complexed with DNA duplex have provided important insight into the structure and mechanism of this catalytically essential domain (24). Although the hydrophilic N-terminus apparently lacks any ordered structures, it may assume an ordered structure inside the cell when topo I is bound with chromatin or complexed with other specific proteins. There is also evidence demonstrating the important intracellular functions of the N-terminal domain (see section 3 and reference (25)).

In an interesting parallel, eucaryotic topo II also contains a hydrophilic, protease sensitive tail. Eucaryotic topo II is a homodimer of a subunit with a size of about 1,400 amino acid residues, two thirds of which are highly conserved (26). The amino terminal one third is homologous to the *gyrB* subunit of bacterial DNA gyrase and the *parC* of topo IV, and at least one of its functions is in the ATP binding and hydrolysis which is required for the strand passage activity of topo II (Fig. 1B). The central portion of topo II is homologous to *gyrA* of gyrase and *parE* of topo IV, and contains the catalytically essential tyrosine residue and together with the C-terminal portion of *gyrB* domain, it forms the site for DNA breakage and rejoining (Fig. 1B). The C-terminal domain is not conserved among the topo II. However, they share the following important features: enriched in charged amino acid residues, hypersensitive to protease digestion, and dispensable for all the catalytic activities for topo II (27-29). These properties therefore suggest that the C-terminal domain of topo II lacks any ordered structures. Our linker insertion mutagenesis experiments strongly support this notion (30). We have introduced a short stretch of amino acids into various locations in topo II in order to gain some information regarding the flexibility in the topo II structure. The C-terminal domain of topo II can readily tolerate insertion mutations and neither the *in vitro* catalytic activities nor the *in vivo* functions were affected by such mutations. Both the tolerance to insertional mutations and protease hypersensitivity suggest that eucaryotic topo II contains a flexible carboxyl tail which lacks any ordered structures.

3. Important intracellular functions associated with the hydrophilic N-terminal tail of eucaryotic topo I

Despite that the N-terminal domain of topo I is not required for its *in vitro* activities, like the supercoil relaxation and DNA cleavage, it is possible that this terminal domain has some critical *in vivo* functions. One of the proposed functions is the regulation of topo I activity through the phosphorylation of the N-terminal domain. The hydrophilic domain contains several consensus sequences for phosphorylation by a number of protein kinases. Topo I is shown to be a phosphoprotein in cell lines for examples, Novikoff hepatoma cells (31) and fibroblast cells activated by phorbol ester (32). One of the phosphorylation sites

maps to a serine residue in the N-terminal domain (33). The phosphorylation of topo I by either casein kinase II or protein kinase C can greatly stimulate topo I activity (34,35).

A second function of topo I N-terminal domain is in the nuclear targeting. Using a heterologous expression system, full-length human topo I expressed in yeast cells can be imported into nuclei, whereas removing a 70-residue segment in the N-terminus results in its mislocalization in the cytoplasm (36). We have established transgenic fly strains in which we engineered a transgene capable of driving the expression of a fusion protein with topo I N-terminus linked to a reporter molecule, β -galactosidase (37). While the bacterial β -galactosidase can not be imported into the nuclei, fusion of the N-terminal 315 residues from the *Drosophila* topo I can result in efficient recruitment of the chimeric protein into the nuclei. These data demonstrate a critical function of topo I N-terminal tail in targeting a protein to the nuclear import complex.

The third function is related to the possible protein/protein interactions between topo I N-terminus and other chromosomal proteins. Since this domain is flexible and extended, it may be proficient in providing an interacting surface to allow for binding with other proteins. Recent biochemical experiments have shown that a 44-residue segment in the human topo I N-terminus can bind to an abundant nucleolar protein nucleolin (38). The function of nucleolin is not entirely clear, but it may play important roles in the rRNA transcription, processing and ribosome assembly. Since topo I is known to be enriched in nucleoli and associated with rDNA, it is possible that nucleolar import and targeting are mediated through a specific interaction between nucleolin and the topo I N-terminal tail.

Eucaryotic topo I may interact with other nuclear proteins as well. For example, human topo I can bind to the SV40 large T antigen, a virus-specific replicative helicase, and one of T antigen binding sites is in the N-terminal domain of topo I (39). However, no information is available with respect to the topo I interaction with any cellular helicase or other proteins involved in DNA replication. Topo I is also involved in the transcriptional process; topo I can interact with TFIID and function both as a coactivator for specific gene expression and as a repressor for the basal transcription in the *in vitro* transcription system (40,41). The coactivation function of topo I is mediated through the formation of an active TFIID and TFIIA initiation complex (42). A catalytically inactive mutant topo I with the active site tyrosine replaced by phenylalanine can still function in the transcriptional coactivation reaction (42). This result suggests that the function of topo I in transcriptional initiation may not require its catalytic activity. However, during the transcription elongation step, topo I activity is involved in relieving the torsional stress generated by the movement of transcriptional fork. Many lines of evidence including the immuno-localization experiments and the use of camptothecin to trap and map topo I cleavages, have demonstrated the specific association of topo I at the transcriptionally active loci (1). However, the mechanism by which this specific

localization occurs remains to be determined. Neither is it known whether topo I N-terminus plays any role in this process.

We have investigated the possible function of topo I N-terminus in the targeting to the transcriptionally active loci on chromatin. We took advantage of the *Drosophila* transgenic lines containing the chimeric gene of topo I N-terminal domain linked to a reporter molecule, bacterial β -galactosidase. In these transgenic flies, a novel fusion molecule of topo I and β -galactosidase was synthesized and its intracellular distribution was readily monitored with immunochemical methods using an antibody specific for the β -galactosidase. Because of the wealth of information in both genetics and cytology regarding the polytene chromosome in salivary gland, we examined the localization of the fusion protein in the salivary gland tissue. Not only were these fusion proteins efficiently imported into the nuclei, but they were targeted to the transcriptionally active loci known as the developmental puffs. A detailed experimental protocol and discussion of the results were presented in a separate publication (37). But a typical result from double localization experiment using immunofluorescence is shown here (Fig. 2).

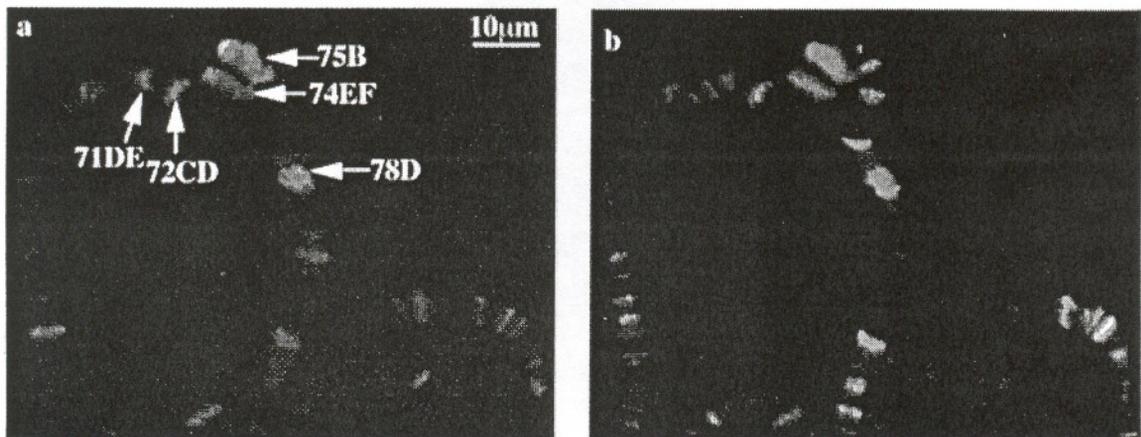


Fig. 2. Colocalization of topo I N-terminal fusion protein and RNA pol II on developmental puffs. Polytene chromosome squashes were prepared from the salivary gland of the third-instar larva and reacted with goat antibody against RNA pol II and rabbit antibody against β -galactosidase. Species-specific secondary antibody with different fluorescent conjugate was used to allow the visualization of RNA pol II (a) and topo I N-terminal fusion protein (b) by the fluorescence microscopy. The major developmental puffs to which RNA pol II and top1/lacZ fusion protein are colocalized are marked by arrows in (a).

In such an experiment, the transcriptional loci were revealed by the fluorescence from the conjugated secondary antibody against the goat antibody specific for RNA pol II (Fig. 2a). A rabbit antibody raised against the bacterial β -galactosidase was used to react with the fusion protein on the polytene chromosome, and its location was shown by the fluorescence from the anti-rabbit secondary antibody conjugated with a fluorophore emitting light with a different color (Fig. 2b). Both the fusion

protein and RNA pol II are co-localized in the major developmental puff sites and most of the interbands along the polytene chromosome. Some of the major, well characterized puffs like 71DE, 72CD, 74EF, 75B, and 78D are marked here (Fig. 2a). Upon heat shock treatment, the regular transcription is halted and a specific set of genes known as heat shock genes are turned on. In the heat treated samples, these developmental puffs regressed and the heat shock puffs appeared. The distribution of the fusion protein followed closely with that of RNA pol II in the heat shock chromosome. When the heat treatment was stopped, the heat shock puffs regressed and the normal developmental puffs resumed. Again in these samples, the fusion protein is localized in a manner like the RNA pol II. Therefore, these experiments demonstrate that not only can the hydrophilic N-terminal domain direct a protein through a nuclear import machinery and to the transcriptionally active loci, but it also allows for an appropriate response to cellular process during the re-programming of gene expressions. While the biochemical mechanism underlying this process is unknown, it is likely that the flexible and charged tail may interact with various target proteins and direct topo I to the chromatin region where its function is critically needed.

4. The hydrophilic carboxyl terminus of eucaryotic topo II is essential for its in vivo functions

Previous experiments have shown that the non-conserved sequence at the C-terminal domain of topo II can tolerate insertional mutagenesis and is exquisitely sensitive to protease digestion (30). It is therefore likely that this domain lacks any ordered structures. To ascertain if this carboxyl tail is essential for the catalytic functions of topo II, serial truncations at the topo II terminus have been generated and tested for their effects on the DNA strand passage activity. Interestingly, removal of most or all of the hydrophilic tail does not affect the in vitro topoisomerase activity (27-29). However, the DNA/protein interactions may be attenuated as the mode of topoisomer distribution during the supercoil relaxation is shifted from being processive to distributive.

Since topo II is an essential enzyme in yeast, it is relatively straightforward to test the in vivo function of the truncated yeast topo II by a simple viability assay. A heterologous expression system has also been established to test for the in vivo functions of the truncated topo II from *Drosophila* and mouse (43,44). This is based on the following two observations: one is that the metazoan topo II can be functionally expressed in yeast cells, and the heterologous topo II can complement the lethality in the yeast *top2* mutation. Two main conclusions have emerged from these experiments. The first is that the C-terminus harbors one or several nuclear localization signal sequences. The removal of the carboxyl tail results in the poor import of the truncated proteins in the nuclei and thus interferes with the biological functions of topo II. Secondly, it is also clear that there are important in vivo functions associated with the terminal domain other than nuclear localization. This was demonstrated in the budding yeast using either a truncated *Drosophila* topo II or

yeast topo II (27,44). While these engineered topo II proteins are active in the in vitro and in vivo strand passage assays, they are not fully functional in complementing the yeast *top2* mutations, suggesting an impaired intracellular function for the truncated proteins. Since we could detect supercoil relaxation in the yeast nuclei, at least some of the truncated topo II can be imported into the nuclei. These results suggest that an impaired nuclear localization function cannot account for the loss of biological functions of the truncated molecules, and other possible essential functions such as protein/protein interaction may reside in the C-terminus of topo II. However, one cannot rule out the possibility that the chromosome segregation function requires a higher intranuclear concentration of topo II and thus a reduced nuclear import of the truncated topo II is responsible for the loss of in vivo function.

To address this issue and probe the biological function of the hydrophilic carboxyl tail of topo II, we have applied a functional test for the expression of either homologous or heterologous topo II in the yeast system. To increase the intranuclear concentration of the truncated topo II, we introduced a short nuclear localization signal sequence, a basic heptapeptide sequence originally identified in the SV40 T-antigen, into the truncated topo II molecules. These engineered topo II proteins were then tested for their ability to complement the yeast *top2* mutations. In the first series of experiments, we have tested the truncated *Drosophila* topo II with the SV40 nuclear localization sequence for its in vivo functions in yeast. Since the topo II gene is controlled by the GAL1 promoter, the expression of the recombinant topo II can be turned on by including 2% galactose in the growth media or can be shut off with 2% glucose. In the presence of glucose to repress any expression of recombinant topo II, no growth could be observed under restrictive temperature when the temperature-sensitive yeast *top2* was inactivated (Fig. 3B). Under the permissive temperature when the chromosomal *top2* remained active, all the strains tested here showed identical growth with respect to each other (Fig. 3, A, C, and E). The complementation of the recombinant topo II was assayed by monitoring the yeast growth under restrictive temperature in the presence of galactose (Fig. 3, D and F). While the C-terminal truncation mutant cannot complement the yeast *top2* mutation under the restrictive temperature (Fig. 3D, $\Delta 240$), the inclusion of a nuclear import signal sequence allows the chimeric topo II to rescue the temperature-sensitive *top2* mutation (Fig. 3D, $\Delta 240$ -NLS). However, the ability of the truncated protein with nuclear import sequence in complementing *top2* mutation is still not at the same level as the full length topo II protein (Fig. 3D, Dm topo II). This difference in the complementation efficiency was enhanced when the expression of recombinant topo II was partially turned off by including glucose in the galactose medium (Fig. 3F). These results therefore suggest that whereas the hydrophilic C-terminus of topo II has a critical function in the nuclear localization, it also harbors other functions which have an important part in the physiological role of topo II.

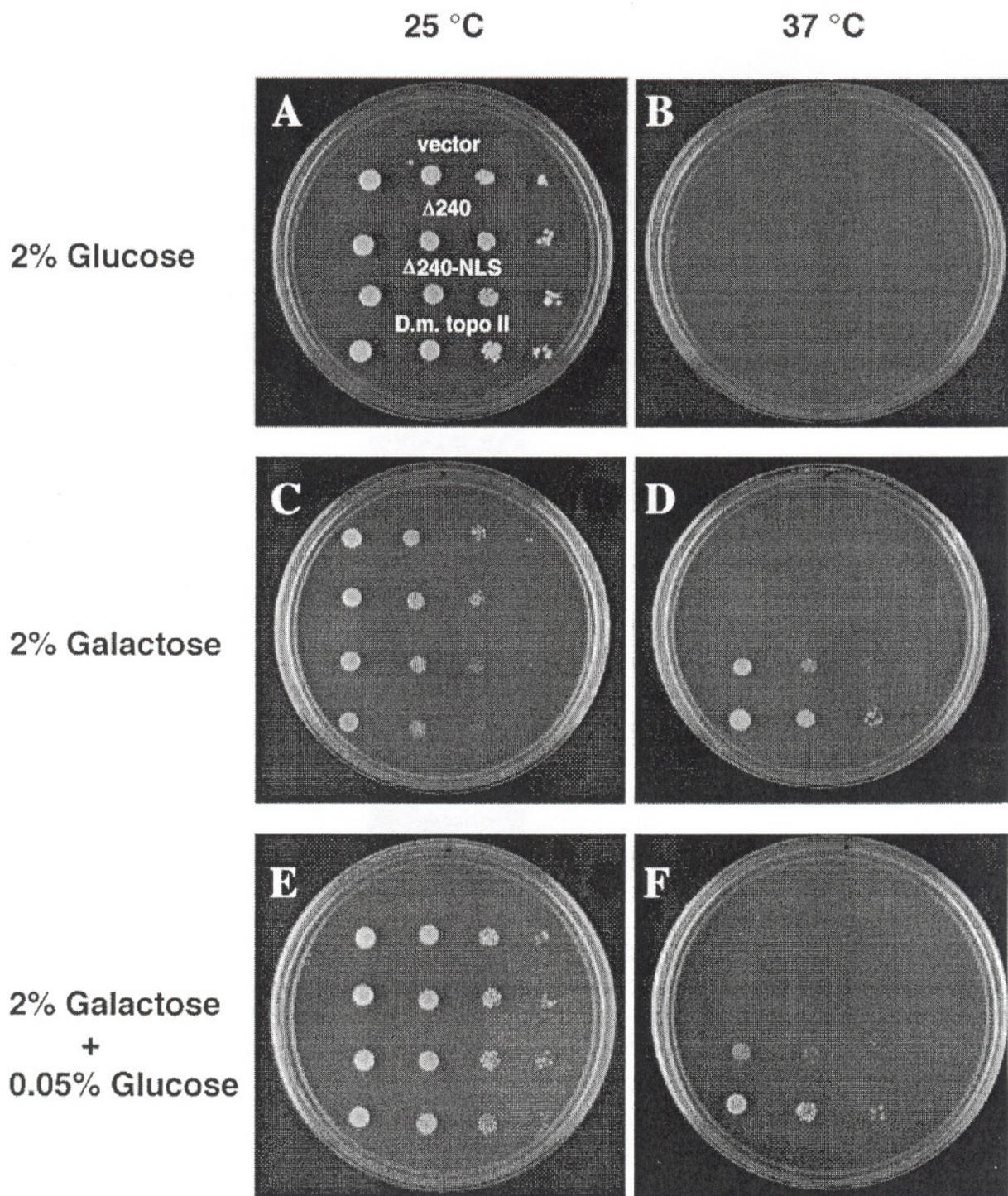


Fig 3. Complementation of yeast *top2* temperature-sensitive mutations with *Drosophila* topo II with C-terminal truncations. Yeast expression vector YEPG and the expression constructs containing $\Delta 240$, $\Delta 240$ -NLS, and wildtype *D.m.* topo II (from the top row to the bottom on each plate) were respectively transformed into a temperature-sensitive yeast strain DY-1 (*MATa*, *his4*⁻⁵³⁹, *lys2*⁻⁸⁰¹, *ura3*⁻⁵², *top2*^{-4^{ts}}). The transformed cells were plated on agar support with a selective media containing 2% glucose (A and B), 2% galactose (C and D), or 2% galactose and 0.05 % glucose (E and F). For each construct, 4 serial dilutions were applied on the plate

such that each spot contained 3×10^4 , 3×10^3 , 3×10^2 , and 3×10^1 cells (from left to right). Duplicate plates were incubated either in 25°C (A, C, and E), or 37°C (B, D, and F).

We have applied the similar analysis with the yeast topo II truncation construct which Caron et al have shown earlier to be catalytically active but to have a compromised *in vivo* activity (27). A similar conclusion was arrived at using the truncated yeast topo II with or without nuclear localization signal sequence. Taken together, both the homologous and heterologous topo II expression systems yield concordant results and suggest that the C-terminus of topo II contains critical biological functions including, but not limited to nuclear localization.

5. Summary and Prospectives

Eucaryotic topo I and topo II contain a hydrophilic terminal sequence in addition to the catalytically essential core domain. Interestingly, these termini share many common features: they are not conserved in sequence but are enriched in charged amino acid residues; they are extremely sensitive to protease digestion; they are totally dispensable for the catalytic activities; and they can tolerate insertional and deletional mutations and possibly lack any ordered structures. However, many lines of evidence demonstrate that they have critical intracellular functions. For topo I, it is clear that the hydrophilic N-terminus contains the signal sequence for nuclear import and for targeting to the transcriptionally active loci. It is possible the topo I N-terminal tail may contain the targeting function to other chromatin loci that need the swivel function of topo I, for example, DNA replication complex. The non-conserved C-terminus of topo II apparently contains the nuclear localization signal sequence and possibly other important intranuclear function. These extended and flexible sequences located at the termini of eucaryotic DNA topoisomerases may serve as an interface for efficient protein/protein interactions which can play a regulatory role for topoisomerase activity and/or can target the enzyme to the region of chromatin where topoisomerase functions are needed. For the protein targeting function, the extended terminal sequence may have a kinetic role in its ability to quickly locate a proper partner protein. It may also have a thermodynamic role in that it may adjust to the template surface and form a unique structure upon binding with the target protein, thus enhancing the stability of the protein complex. With the advent of many biochemical and genetic techniques to search for and identify those nuclear proteins which can interact with topoisomerases, we should be able to further characterize these protein complexes involving topoisomerases and determine the specific structures for the terminal domains which would otherwise remain disordered in the free state.

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