Cellular distribution of mammalian DNA topoisomerase II is determined by its catalytically dispensable C-terminal domain

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ABSTRACT

Mammalian cells express two genetically distinct isoforms of DNA topoisomerase II, designated topoisomerase IIα and topoisomerase IIβ. We have recently shown that mouse topoisomerase IIα can substitute for the yeast topoisomerase II enzyme and complement yeast *top2* mutations. This functional complementation allowed functional analysis of the C-terminal domain (CTD) of mammalian topoisomerase II, where the amino acid sequences are divergent and species-specific, in contrast to the highly conserved N-terminal and central domains. Several C-terminal deletion mutants of mouse topoisomerase IIα were constructed and expressed in yeast *top2* cells. We found that the CTD of topoisomerase IIα is dispensable for enzymatic activity *in vitro* but is required for nuclear localization *in vivo*. Interestingly, the CTD of topoisomerase IIβ was also able to function as a signal for nuclear targeting. We therefore examined whether the CTD alone is sufficient for nuclear localization *in vivo*. The C-terminal region was fused to GFP (green fluorescent protein) and expressed under the *GAL1* promoter in yeast cells. As expected, GFP signal was exclusively detected in the nucleus, irrespective of the CTD derived from either topoisomerase IIα or IIβ. Surprisingly, when the upstream sequence of each CTD was added nuclear localization of the GFP signal was found to be cell cycle dependent: topoisomerase IIα–GFP was seen in the mitotic nucleus but was absent from the interphase nucleus, while topoisomerase IIβ–GFP was detected predominantly in the interphase nucleus and less in the mitotic nucleus. Our results suggest that the catalytically dispensable CTD of topoisomerase II is sufficient as a signal for nuclear localization and that yeast cells can distinguish between the two isoforms of mammalian topoisomerase II, localizing each protein properly.

INTRODUCTION

Eukaryotic DNA topoisomerase II is a ubiquitous nuclear enzyme that can alter the topological structure of DNA and chromosomes, by transiently breaking both strands of the DNA double helix, passing a segment of DNA duplex through the break and religating the two strands (1). The enzyme can relax both positively and negatively supercoiled DNA and can catalyze knotting/ unknotting and catenation/decatenation of closed DNA circles (1). Thus the enzyme is of importance throughout cell proliferation, providing the molecular tool for handling constrained or entangled DNA which will arise during cellular processes such as replication, transcription and recombination (2–5).

In yeasts, in addition to biochemical studies, genetic approaches have been quite successful in elucidating the biological roles of topoisomerase II. *TOP2*, the structural gene for topoisomerase II, is essential for cell viability (6,7). In *top2* mutant cells nuclear division is blocked in mitosis: chromosomes are pulled by the spindle but fail to separate (8–11). Thus topoisomerase II activity is required at the time of cell division, most likely for chromosome segregation. In fission yeast it has also been shown to be required for chromosome condensation (11).

In higher eukaryotes studies using mitotic cell extracts have shown a requirement for topoisomerase II for *in vitro* chromosome condensation (12,13). Also, topoisomerase II has been shown to be essential for replication of SV40 virus chromosomes *in vitro* (14). In addition to these catalytic functions, topoisomerase II has been implicated as a major structural component of the nuclear matrix and/or the chromosome scaffold (15–17). However, in mammalian cells the biological function of topoisomerase II remains unclear owing to a lack of topoisomerase II mutants. Moreover, mammalian cells express two closely related but genetically distinct isoforms of topoisomerase II, designated topoisomerase IIα and topoisomerase IIβ (18–22). Little is known about differences between the respective roles in cellular events that require topoisomerase II activity. However, recent analyses have shown that expression and localization of each isoform are distinct and stage specific during the cell cycle (23–27; Kikuchi *et al.*, in preparation): topoisomerase IIα is expressed

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transiently during mitosis and is a component of the metaphase chromosome scaffold, whereas topoisomerase IIβ is constant in amount and is present in the nuclear matrix as well as in the nucleolus of the interphase nucleus. These observations raise the possibility that topoisomerase IIα is required for chromosome condensation and segregation, while topoisomerase IIβ manages topological constraints caused by replication and/or transcription.

Eukaryotic topoisomerase II protein is composed of three domains: the conserved N-terminal and central domains and the non-conserved C-terminal domain (CTD). Because the N-terminal and central domains are homologous to the B and A subunits of bacterial DNA gyrase respectively, these domains are likely to have critical roles, as do the ATPase and breaking/rejoining domains, essential for enzymatic activity of topoisomerase II (28–30). In contrast, the CTD is divergent and species specific, sharing no homology with the bacterial enzymes, and therefore the biological role of this domain remains unclear.

In this paper we have undertaken a functional dissection of the non-conserved CTD of topoisomerase II using a yeast expression system, in which mammalian topoisomerase II cDNA has been expressed successfully (30–34). We show that the CTD of mammalian topoisomerase II is responsible for nuclear localization, although it is not required for enzymatic activity *per se*. In addition, by using a partial topoisomerase II fragment fused to GFP (green fluorescent protein), we show that the catalytically dispensable CTD of topoisomerase II is sufficient as a signal for nuclear localization. Furthermore, we present data suggesting that yeast cells, although having only one kind of topoisomerase II, can distinguish and localize the two isoforms of mammalian topoisomerase II properly.

MATERIALS AND METHODS

Yeast manipulations

Yeast cells were grown in YEPR medium (1% yeast extract, 2% peptone and 2% raffinose) supplemented when appropriate with 2% galactose. For solid medium 2% Bacto-agar was added. 5-FOA medium used to select for Ura⁻ cells was made as described (35). Yeast transformation was performed using standard methods (36).

Construction of topoisomerase IIα deletion mutants

Construction of deletion mutant genes of mouse topoisomerase IIα was performed using the expression plasmid pNA811 (31) containing mouse topoisomerase IIα cDNA under the yeast *GAL1* promoter with the *LEU2* gene as a selectable marker. Briefly, to make the ΔA and ΔB mutants pNA811 was digested with *Aat*II or *Bgl*II respectively followed by blunting and religation. By changing the reading frame, termination codons were introduced just behind the restriction sites. For the ΔE and ΔF mutants PCR was performed on the pNA811 template using oligonucleotides p1 (5′-CGAAGCTTGGATCCATGGAGTTGTC-ACCGCTGCAGCC-3′) and pE (5′-CCCGCATGCTTAGGCT-TTTGCTGCACGACTTCTTTGC-3′) or p1 and pF (5′-CCCG-CATGCTTAATTTTTTGGGGGGGATTTTGGCCTT-3′) respect-ively. The amplified products were digested with *Nde*I and *Sph*I and the resulting *Nde*I–*Sph*I fragments were used to replace the corresponding fragment of topoisomerase IIα cDNA in pNA811.

For construction of the αβ chimeric gene a 1.3 kb *Bgl*II–*Sph*I fragment encoding the extreme C-terminal 444 amino acids of topoisomerase IIβ was used to replace the corresponding fragment of topoisomerase IIα cDNA in pNA811.

Enzymatic assays and indirect immunofluorescent microscopy

Each expression plasmid was transformed into yeast strain RS191 (MAT**a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 top2-1*ᵗˢ). The resulting Leu⁺ cells were isolated and grown in YEPR medium at 28°C. When the culture reached a density of 3×10^7 cells/ml galactose was added to a final concentration of 2%, followed by incubation for a further 4 h. For preparation of crude extracts the cell pellet was suspended in 50 μl lysis buffer (31). A one third volume of glass beads was then added and the cells were lysed by vigorous mixing and brief sonication. After centrifugation at 12 000 r.p.m. for 5 min the supernatant was saved and used for a decatenation assay to measure topoisomerase II activity (31).

To examine the cellular distribution of mutant proteins, cells were fixed with formaldehyde and subjected to indirect immuno-fluorescence microscopy (37). The anti-topoisomerase II antibody 7B9 (Kikuchi *et al.*, unpublished) was used at a 1:10 dilution and FITC-conjugated secondary antibody was used for detection. The nuclear region was counterstained with DAPI, mixed with the mounting medium.

Complementation assays

Each expression plasmid was transformed into yeast strain NAY113 (MAT**a** *lys2-801 ura3-52 trp1 leu2 his3 top2*Δ YCp-ScTOP2 [*TOP2 URA3*]) as described (31). The resulting Leu⁺ transformants were isolated and subsequently plated on galactose-containing 5-FOA medium, in order to examine their ability to give rise to 5-FOA-resistant (Ura⁻) colonies. It should be noted that functional complementation of the yeast *top2* null mutation leads to formation of viable Ura⁻ colonies that have lost the yeast topoisomerase II gene (*TOP2*) on the YCp-ScTOP2 plasmid together with the *URA3* gene (31).

Construction of topoisomerase IIβ–GFP fusion vectors

The mouse topoisomerase IIβ cDNA was cloned from the λgt10 library of fetal mouse brain (kindly provided by Dr Kikuya Kato; 38). Its sequence (EMBL/DDBJ/GenBank accession no. D38046) showed high homology with that of human topoisomerase IIβ and 91.9% of their amino acids were identical (Fig. 1). For expression of mouse topoisomerase IIβ under control of the *GAL1* promoter the entire coding region was placed in the multicloning site of the yeast expression vector pYES2 (Invitrogen). This clone, YES-mTOPO IIβ, was able to complement the yeast *top2* null mutation when expression of mouse topoisomerase IIβ was induced by addition of galactose (unpublished results).

To generate GFP fusion vectors a DNA fragment encoding GFP was cleaved from the pGFP vector (Clontech) and inserted into the *Pac*I site (located at the 3′-end of the topoisomerase IIβ coding region) of the YES-mTOPO IIβ vector. This clone, YES-mTOPO IIβ/GFP, however, gave no GFP signal under the fluorescent microscope and immunoblot analysis using either an anti-topoisomerase IIβ or anti-GFP antibody was unsuccessful (data not shown).

Plasmid YES-Δtopo IIβ/GFP, encoding topoisomerase IIβ–GFP devoid of the N-terminal region of topoisomerase IIβ, was

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mtopoIIβ  1172:LWKEDLAAFVEELDKVEAQEREDILAGMSGKAIKGKVGKPKVKKLQLEETMPSPYGRRIVPEIT-A
htopoIIβ  1179:***************S*****V*******************************I****-*
mtopoIIα  1163:*******V*I***EV***K*KQ*EQV*LP***G*A*---G*KAQMCADVL-***R*K*VI*QV*VE
htopoIIα  1166:*******T*+***+A***+*++*++*++*++*G+*+*---+*+T++-+E++-***+*Q*++*RI*I+

mtopoIIβ  1237:MKADASRKLLKKKKGDPDTTVVKVEFDEEFSGTPAEGTGEETLTPSAPVNKGPKPKREKKEPGTRV
htopoIIβ  1244:******K*********L**AA**********A*V**A***A****V*I***************
mtopoIIα  1225:***E*EK*IR**I*SENVE-------------****DGA*PGSLRQRIE------*KQ*****--A
htopoIIα  1227:***+*++*NK**+*N++T+------------*S*Q*++V*LEG+K++L+------*++*R***TKT

mtopoIIβ  1303:RKTPTSTGKTNAKKVKRRNPWSDDESKSESDLEEAEPVVIPRDSLLRRAAAERPKYTFDFSEEEDD
htopoIIβ  1310:******S**PS*****************************T**************************
mtopoIIα  1271:K*QT*LPF*PV-**GRK*****S**DVS*N--*SNVD*P**QK-EQ*SR*AKA*F*V*LDSD**F
htopoIIα  1275:+*++*+A+*+I-**+K*R***P*+**+R*+D--*++F+*+**ET-+P*RA*T+T*+*M*+++*+*+

mtopoIIβ  1369:DA-AAADDSNDLEELKVKASPITNDGEDEFVPSDGLDKDEYAFSSGKSKATPEKSSNDKKSQDFGN
htopoIIβ  1376:**DDDD**N******************************T**P*********LH*********
mtopoIIα  1333:SGLDEK*EDE*------FLPLDATPPKAKIP*KNTKKALKTQG**MSVVDLESDVKDSVPASPGVP
htopoIIα  1337:+DF+++TD++*------+V+S++S+++T+TS*+LSN+E++P+K*VV+DLEAD-+++G+++L+SSP+

mtopoIIβ  1434:PFSFPSYSQKSEDDSAKFDSNEEDTASVFAPSFGLKQTDKLPSKTVAAKKGKPPSDTAPKAKRAPK
htopoIIβ  1442:L*********************************S****S*****************S***V**P******
mtopoIIα  1393:AAD**AETEQSKP-*K--------------------------***GV**TATK*QSSVSTAGTK*
htopoIIα  1396:+TH**D+++ITN+VP+-------------------------*N*T+**+*A**++*T+TT+*A*

mtopoIIβ  1500:QKKIVETINSDSDSEFGIPKKTTTPKGKGRGAKKRKASGSENEGDYNPGRKPSKTASKKPKKTSFD
htopoIIβ  1508:***V**AV***********************************************T***T*********
mtopoIIα  1431:RAAPKGTK-***ALSARVSE*PAPA*A*--NSR***P*S*D-SS*SDFE*AI**G*TS*-*AKGEE
htopoIIα  1435:++PK++++-R*P++NSG++Q*+D++*T*--+R+***+*T*+-D+*+N++KIV**AV++*-*S+++S

mtopoIIβ  1566:QDSDVDIFPSDFTSEPPALPRTGRARKEVKYFAESDEEE-DVDFAMFN
htopoIIβ  1574:************PT***S*************T*****D********
mtopoIIα  1492:**FP**LEDT-----IAPRAKSD****PI**LE***-DDD*L-*----
htopoIIα  1496:D*+HM*FDSA-----V++++++V**K*++**++**--+E+*L-*----
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Figure 1. Comparison of CTDs of mammalian topoisomerase II. The CTD of mouse topoisomerase IIβ (this paper), human topoisomerase IIβ (22), mouse topoisomerase IIα (30,47) and human topoisomerase IIα (20) are aligned arbitrarily. Amino acids identical to mouse topoisomerase IIβ are shown by asterisks (*). In human topoisomerase IIα amino acids identical to mouse topoisomerase IIα are denoted by crosses (+). –, absence of corresponding residues. The typical bipartite nuclear localization signals are shown in the boxes. Possible NLS sequences in mouse topoisomerase IIα are underlined.

constructed from YES-mTOPO IIβ/GFP by removing the upstream region of the *Sca*I site. Similarly, plasmid YES CTD-β/GFP, encoding the topoisomerase IIβ CTD linked to GFP, was made by deletion up to the *Bgl*II site. Both of these clones gave positive GFP signals, as described in the text.

Construction of topoisomerase IIα–GFP fusion vectors

We used a fragment of human topoisomerase IIα cDNA instead of a mouse clone because the amino acid sequence of the topoisomerase IIα CTD is so divergent and species specific that we could not find any antibody capable of recognizing the CTD of mouse topoisomerase IIα, despite our large collection of monoclonal and polyclonal antibodies against human topoisomerase IIα (Kikuchi *et al.*, unpublished results).

To generate GFP fusion vectors the 3.2 kb *Pvu*II fragment of human topoisomerase IIα was inserted in-frame into the YES-GFP vector, in which a *Bsr*BI–*Eag*I fragment containing the GFP coding region had been placed under control of the *GAL1* promoter in the pYES2 vector. The resulting clone, YES-PP158/GFP, lacked 440 amino acids from the N-terminus and 27 from the C-terminus, but contained about two thirds of the topoisomerase IIα coding region. From this clone plasmid YES-CTDα/GFP, encoding the GFP-fused topoisomerase IIα CTD, was constructed by removing one of the *Bam*HI fragments encompassing the upstream region. A plasmid encoding only 61 amino acids of the topoisomerase IIα CTD was also generated by removing all the *Bam*HI fragments.

Detection of GFP signal

GFP fusion vectors were transformed into diploid strain W303 (MAT**a**/α *ade2-1/ade2-1 ura3-1/ura3-1 trp1-1/trp1-1 leu2-3, 112/leu2-3,112 his3-11,15/his3-11,15 can1-100/can1-100*). The cells were cultured in minimal medium (containing 2% galactose and all requirements except uracil) for at least 3 h (usually overnight). This fresh culture was placed under an epifluorescent microscope (Olympus BHS2), followed by detection of GFP signal with either a U or B excitation dichroic mirror.

Staining of DNA with DAPI was performed after fixation of the sample with 3% formaldehyde for 3 min at room temperature. This fixation method causes a several-fold reduction in the intensity of GFP fluorescence. Detection was performed with a U excitation dichroic mirror.

RESULTS

The CTD of topoisomerase IIα is dispensable for enzymatic activity *in vitro* but is required for nuclear localization *in vivo*

We reported previously that mouse topoisomerase IIα could complement the yeast *top2* temperature-sensitive mutation as well as the *top2* null mutation (30,31). This functional complementation enabled us to analyze the functional domains of heterologous topoisomerase II. The mouse protein is efficiently expressed in yeast cells, thus allowing detection of its enzymatic activity (i.e. *in vitro* activity) in crude extracts. In addition, the *in vivo* activity of the mouse enzyme can be determined in terms of functional complementation of yeast *top2* mutations. Therefore, we employed this system to analyze the function of the divergent C-terminal domain (CTD) of mouse topoisomerase IIα.
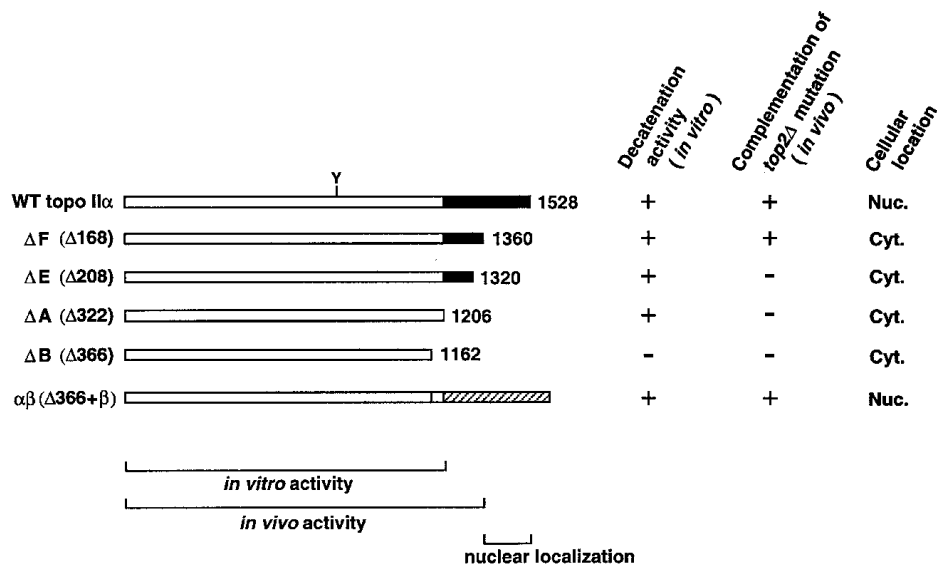
Figure 2. Functional dissection of mouse topoisomerase IIα. The open box shows mouse topoisomerase IIα. The black and hatched boxes represent the poorly conserved CTD of topoisomerase IIα and that of topoisomerase IIβ respectively. Position of the active site tyrosine (Y) is indicated. The wild-type (WT) topoisomerase II protein consists of 1528 amino acids. Each deletion mutant contains the indicated number of amino acids. +, decatenation activity (*in vitro*) and complementation of the yeast *top 2* null mutation (*in vivo*). Nuc., nuclear localiszation; Cyt., cytoplasmic localization. The functional subdomains clarified in this work are shown below.

We constructed several mutant topoisomerase IIα genes, each of which encodes a truncated product owing to a deletion in the CTD (Fig. 2). Each mutant gene, under control of the yeast *GAL1* promoter, was introduced into yeast cells. The cells were grown in galactose-containing medium to allow expression of the mouse protein, followed by preparation of crude extracts that were subsequently used for a decatenation assay. Immunoblot analysis showed that each truncated product was synthesized in a similar amount to the intact protein. Decatenation assays revealed that removal of <322 residues from the C-terminus had no effect on enzymatic activity (ΔA, ΔE and ΔF), whereas removal of 366 residues completely abolished activity (ΔB) (Fig. 2 and data not shown). Thus the C-terminal 322 amino acid region, which encompasses 21% of the whole molecule, was not required for the enzymatic activity of mouse topoisomerase IIα.

In parallel, each mutant gene was introduced into yeast *top2* mutant cells to examine whether or not it could rescue the lethality caused by disruption of the yeast topoisomerase II gene (Fig. 2). The ΔF mutant protein, which had lost the C-terminal 168 residues, retained complementation activity, indicating that this region is not required for *in vivo* activity of topoisomerase IIα. However, the other mutant proteins lacking >208 residues completely lost *in vivo* activity. Thus, surprisingly, the ΔA and ΔE proteins failed to complement the yeast *top2* mutation despite retaining enzymatic activity. This suggests that the extreme 208 amino acid region has some essential cellular function(s) other than enzymatic activity.

We presumed that the CTD might be involved in interaction with chromatin, nuclear structures or other cellular protein(s) and that the mutant proteins might show a distinct localization compared with wild-type enzyme. We therefore examined the distribution of these mutant proteins in yeast cells by indirect immunofluorescent microscopy using antibodies against mouse topoisomerase IIα. In cells expressing the full-length protein

(WT) the antibody strongly stained the nuclear region, which was identified by staining with DAPI (Fig. 4). In contrast, in cells expressing proteins devoid of the CTD (ΔA, ΔE and ΔF) only the cytoplasm was intensively stained and there was no detectable signal in the nucleus (Fig. 3). From these results it was suggested that the enzymatically active ΔA and ΔE mutant proteins failed to enter the nucleus, resulting in a loss of complementation activity. Furthermore, the results indicate that the extreme 168 amino acid region of the CTD was indispensable for nuclear targeting of mouse topoisomerase IIα.

The CTD of topoisomerase IIβ functionally substitutes for that of topoisomerase IIα

It was of interest to examine whether or not the CTD of mouse topoisomerase IIα might be substituted by the corresponding topoisomerase IIβ sequence, because the CTDs of both isoforms have a similar hydrophilic nature, despite lacking significant homology. For this purpose we made a chimeric mutant, designated αβ, by adding the topoisomerase IIβ CTD to the ΔB mutant protein of topoisomerase IIα (Fig. 2). This mutant was then introduced into yeast cells in order to examine whether or not its product was active.

In contrast to the ΔB protein, which had no enzymatic activity and failed to complement the yeast *top2* mutation, the αβ mutant protein displayed enzymatic activity as well as complementation activity (Fig. 2 and data not shown). Furthermore, indirect immunofluorescence revealed that cells expressing the αβ protein gave a strong signal in the nuclear region, similar to that found with wild-type topoisomerase IIα protein (Fig. 4). From these results it was suggested that, at least in yeast cells, the divergent CTDs of the two isoforms have a similar enzymatic function and a signal for nuclear localization.
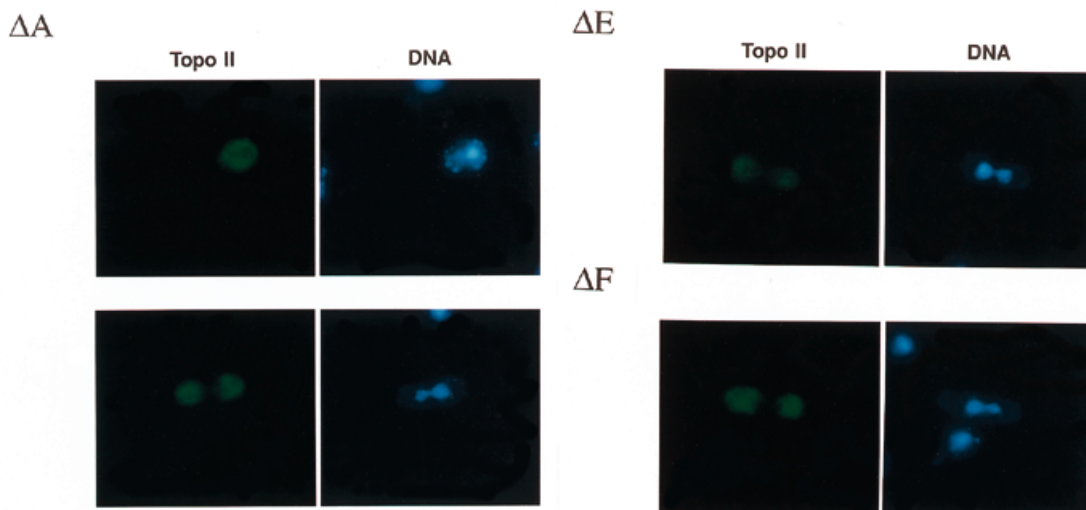
Figure 3. Cytoplasmic localization of mutant proteins devoid of the CTD. Yeast cells expressing the mutant protein (ΔA, ΔE or ΔF) were fixed with formaldehyde and subjected to indirect immunofluorescent microscopy using the anti-topoisomerase IIα antibody, followed by detection with FITC-conjugated secondary antibody (left). The nuclear region was identified by staining with DAPI (right). For the ΔA protein both G_1 (upper) and M phase (lower) cells are shown.

Nuclear localization of GFP fused to the CTD of topoisomerase II

Next we examined whether the CTD alone is sufficient for nuclear localization *in vivo*. To accomplish this we put each CTD fragment under control of the *GAL1* promoter, to which a GFP gene was fused in-frame at the C-terminus. Each construct was introduced into yeast cells and the GFP signal analyzed under the microscope. As shown in Figure 5A, in cells expressing the GFP-fused topoisomerase IIα CTD the signal was completely localized to the nucleus, coincident with DAPI staining. Very similar results were obtained when the topoisomerase IIβ CTD was fused to GFP (Fig. 5B). Thus, no significant difference was observed between CTD fragments of topoisomerase IIα and IIβ. In contrast, GFP with no topoisomerase II fragment or GFP with a subfragment of the extreme C-terminus (61 residues) gave a uniform green fluorescent signal in the cytoplasm with some occasional nuclear staining (Fig. 5C; data not shown). From these results we conclude that the CTD is necessary and sufficient for nuclear localization.

Cell cycle-dependent localization of topoisomerase IIα–GFP and topoisomerase IIβ–GFP

Although we have shown that the CTD of both topoisomerase II isoforms is responsible for nuclear targeting *in vivo*, it remained unclear whether these proteins are localized in the nucleus irrespective of cell cycle progression or whether they show distinct localization even in the nucleus, as has been observed in mammalian cells. To investigate this we attempted to express a full-length topoisomerase II fused to GFP. However, this was unsuccessful: no GFP signal under the microscope nor protein product by immunoblotting were detected (data not shown). Therefore, we removed an N-terminal region from the construct. Deletion of this region resulted in stable products that allowed us to detect the GFP signal in yeast cells (see below). Presumably the N-terminal region has some sequences which affect stable maintenance of the product *in vivo*.

As shown in Figure 6A, in cells expressing GFP fused to N-terminally truncated topoisomerase IIα (topoisomerase IIα–GFP) the signal was detected in the mitotic, but not in the interphase nucleus. On the other hand, topoisomerase IIβ–GFP was localized predominantly in the interphase nucleus and less in the mitotic nucleus (Fig. 6B). This localization pattern is reminiscent of the cell cycle-dependent localization of topoisomerase IIα and IIβ in higher eukaryotic cells, namely topoisomerase IIα is localized in mitotic chromosomes, while topoisomerase IIβ is found in interphase nuclei. Thus it was suggested that the catalytically dispensable CTD of topoisomerase II is important not only for nuclear targeting but also, in concert with the central domain containing the active site, for cell cycle-dependent and isoform-specific localization of this protein. The results also suggest that yeast cells can distinguish and localize the two isoforms of mammalian topoisomerase II properly. We note that overexpression of topoisomerase IIα–GFP in the nucleus seems to be harmless, since the various mitotic stages were observed and cell cycle progression was not significantly blocked (Fig. 6C).

DISCUSSION

Function of the CTD of topoisomerase II

Using a yeast expression system we have analyzed the functions of the CTD of mouse topoisomerase IIα. We constructed several mutants devoid of the CTD and examined their enzymatic activity *in vitro* as well as their biological activity *in vivo*. We showed that truncation of <322 residues from the C-terminus had no effect on enzymatic activity. The results are in good agreement with the observation that the C-terminal 322 amino acid region has no homology with bacterial type II DNA topoisomerases (30). As observed in the ΔA and ΔE mutant proteins, enzymatically active protein failed to complement the yeast *top2* null mutation. This conflicting result is probably due to a lack of topoisomerase II activity in the nucleus: indirect immunofluorescence revealed that none of the truncated mutant proteins were transported to the nucleus. Thus our results suggest that a potent nuclear localization
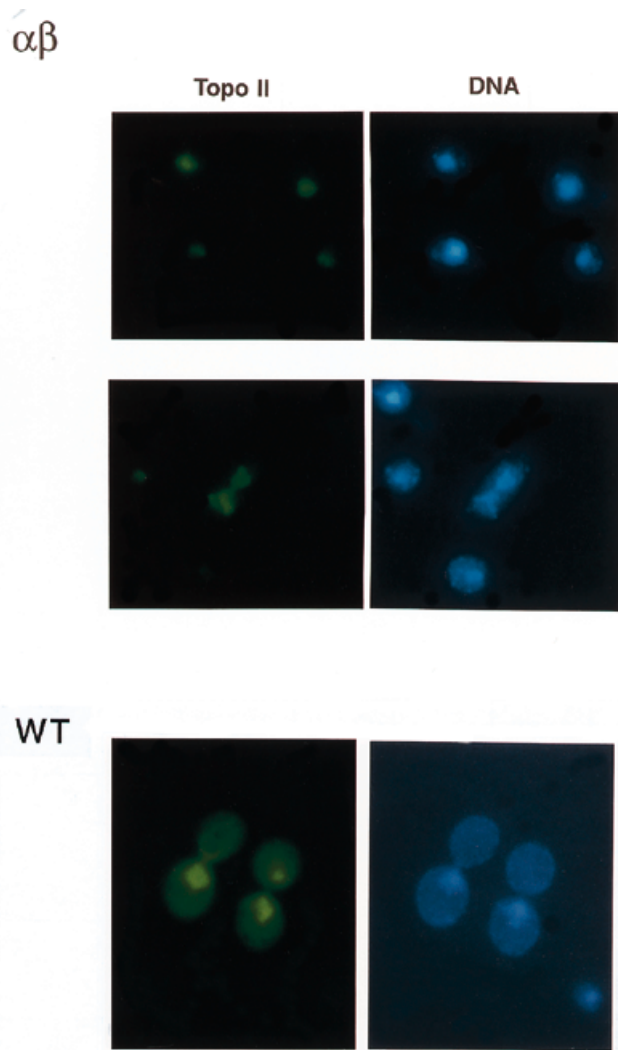
Figure 4. Nuclear localization of the αβ chimeric protein and wild-type topoisomerase IIα protein. Yeast cells were stained as in Figure 3. For the αβ protein both G_1 (upper) and M phase (lower) cells are shown.

Figure 5. Nuclear localization of GFP fused to the topoisomerase IIα CTD (**A**) and fused to the topoisomerase IIβ CTD (**B**). Each product was expressed in yeast cells and the GFP signal was detected with a microscope (upper). The position of the nucleus was visualized by simultaneous staining with DAPI (lower). The bar indicates 5 μm. (**C**) Cytoplasmic localization of GFP fused to the extreme C-terminal 61 amino acids of topoisomerase IIα. GFP signal (left), DAPI staining (right) and double exposures (center) are shown.

signal (NLS) resides in the extreme 168 amino acid residues of mouse topoisomerase IIα. Indeed, some NLS-like sequences are found in this region (see Fig. 1). Importantly, mouse topoisomerase IIα has two bipartite NLS sequences, one of which is located in the CTD (30,39). It should be noted, however, that these NLS sequences are unlikely to be fully functional, since the ΔE and ΔF proteins retained these sequences but failed to localize to the nucleus. Nevertheless, the ΔF protein was able to complement the yeast *top2* mutation, while the ΔE protein was not. It is possible that a small amount of ΔF, but not ΔE, protein entered the nucleus, although they were not detectable. Indeed, Jensen *et al.* have recently observed similar results with truncated human topoisomerase IIα proteins (33). However, other explanations are also possible. For example, the catalytically dispensable 40 amino acid region between the ends of the ΔE and ΔF proteins may play a crucial role *in vivo* and therefore the ΔE (and ΔA) protein would be intrinsically inert *in vivo*, even in the nucleus. This idea is supported by the fact that the region contains several putative phosphorylation sites which have been implicated in the regulation of topoisomerase II activity (30). Thus it would be interesting to
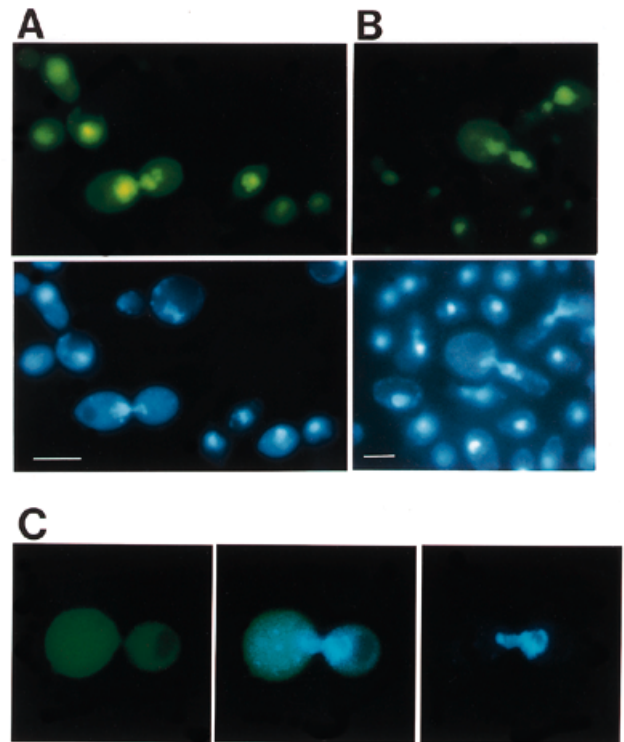
examine whether or not the ΔE protein would become active *in vivo* when fused to a functional NLS such as that of SV40 T antigen.

We constructed a chimeric mutant, designated αβ, containing the CTD of topoisomerase IIβ instead of that derived from topoisomerase IIα and found that enzymatic activity as well as nuclear localization of this chimeric protein were comparable with those of the wild-type topoisomerase IIα protein. These results indicate that the CTD of topoisomerase IIβ can substitute for the corresponding domain of topoisomerase IIα, despite little sequence homology between them. Thus the CTDs of topoisomerase IIα and IIβ have a common ability, at least in yeast cells, to act as NLS sequences. This idea is further supported by our experiments in which we have demonstrated that, using GFP as a tag, CTD alone is sufficient for nuclear localization of topoisomerase IIα and topoisomerase IIβ. GFP fused to the CTD of topoisomerase IIα was completely localized to the nucleus. This was also the case with the CTD of topoisomerase IIβ, suggesting no significant difference, with respect to NLS function, between the two CTDs.

It has recently been reported that the ASFV (African swine fever virus) genome has a putative gene for topoisomerase II (40). Interestingly, the predicted protein consists of only 1191 amino acids, being equivalent to the ΔA mutant protein of mouse topoisomerase IIα in that it lacks a region corresponding to the CTD of eukaryotic topoisomerase II. Provided that this putative topoisomerase II is involved in ASFV genome replication, which
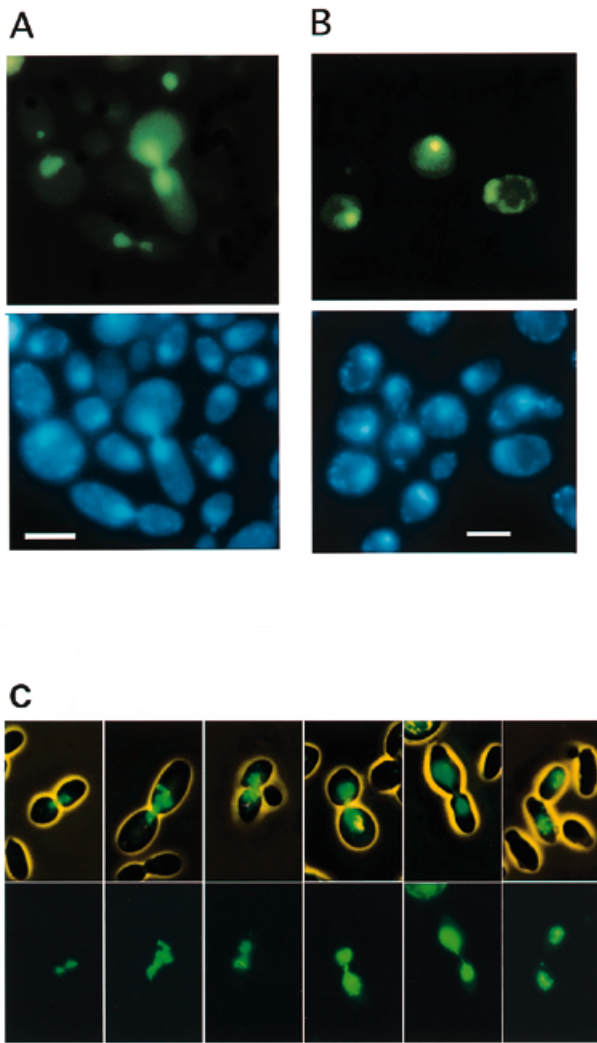
Figure 6. Cell cycle stage-specific nuclear localization of topoisomerase IIα–GFP (**A**) and topoisomerase IIβ–GFP (**B**). Each topoisomerase II cDNA devoid of an N-terminal region was fused to GFP and expressed in yeast cells, followed by detection of GFP (upper) and DAPI (lower) fluorescence. The bar indicates 5 µm. (**C**) Representative images of topoisomerase IIα–GFP in mitotic cells. The pictures were taken using a phase contrast microscope with epifluorescent attachment. In the lower panels GFP signal alone is shown.

occurs in the cytoplasm, this may support the notion that the primary function of the long CTD of eukaryotic topoisomerase II is to act as an NLS. Consistent with this, it has been reported that the CTD of topoisomerase II from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Drosophila* and human is responsible for nuclear localization *in vivo* (33,41–43). Taken together, the divergent CTDs of eukaryotic topoisomerase II are most likely to have a common function in transporting the enzyme into the nucleus.

Cell cycle-dependent localization of mammalian topoisomerase II in yeast

In higher eukaryotes, in addition to its catalytic function, topoisomerase II has been implicated as a structural component of the chromosome scaffold and the nuclear matrix (15–17).

Moreover, recent studies have revealed that expression and localization of each isoform are distinct and cell cycle stage specific (23–27; Kikuchi *et al.*, in preparation): topoisomerase IIα is expressed transiently during mitosis and is a component of the metaphase chromosome scaffold, whereas topoisomerase IIβ is constant in amount and is present in the nuclear matrix as well as in the nucleolus of the interphase nucleus. Hence, it was of great interest to examine whether these proteins are continuously localized in the nucleus irrespective of the cell cycle or whether they exhibit distinct localization in the nucleus. To accomplish this we employed GFP, which has been utilized successfully in yeast cells as a tag to follow protein localization (see for example 44–46). We showed that in contrast to GFP fused to the CTD alone, which showed continuous localization in the nucleus, topoisomerase IIα–GFP was localized mostly in the mitotic nucleus and absent from the interphase nucleus. Conversely, topoisomerase IIβ–GFP was localized predominantly in the interphase nucleus and less in the mitotic nucleus. Thus addition of a further upstream region of topoisomerase II resulted in nuclear localization that is cell cycle dependent. Interestingly, this pattern is reminiscent of that in higher eukaryotic cells.

We cannot rule out the possibility that our results might be caused by an artificial condition: for example, because an N-terminal region was removed from the topoisomerase II moiety, the product, otherwise properly degraded, accumulated in the nucleoplasm. Nevertheless, we prefer the interpretation that the central domain of topoisomerase II has some additional role in distinguishing mitotic from interphase nuclei. Given that this is the case, yeast cells are capable of distinguishing between the two mammalian isoforms and thereby localizing them properly, so that each isoform should be fully functional. Considering the fact that yeast cells possess only one form of topoisomerase II, it is also possible that, even in a heterologous organism, mammalian topoisomerase II by itself can be properly localized in the nucleus, presumably through a direct interaction with DNA and/or chromosomes.

We failed to detect signals of GFP fused to a full-length topoisomerase II, despite extraordinary stability of intact GFP. This is most likely due to the existence of the N-terminal region of topoisomerase II, since in our experiments deletion of this region resulted in stable GFP-fused products. Irrespective of any additional role of the N-terminus, the data presented here suggest that the catalytically dispensable CTD of topoisomerase II is important not only for nuclear localization, but also, in concert with the central domain, for cell cycle-dependent and isoform-specific localization of this protein.

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