Functional compatibility between isoform α and β of type II DNA topoisomerase

Ayako Sakaguchi* and Akihiko Kikuchi‡

Laboratory of Medical Mycology, Research Institute for Disease Mechanism and Control, Nagoya University Graduate School and Faculty of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

*Present address: Department of Biochemistry and Molecular Biology, University of Geneva, Geneva CH-1211, Switzerland

‡Author for correspondence (e-mail: aki@med.nagoya-u.ac.jp)

Accepted 11 November 2003

Journal of Cell Science 117, 1047-1054 Published by The Company of Biologists 2004

doi:10.1242/jcs.00977

Summary

DNA topoisomerase II (topo II) plays a crucial role in controlling the conformation of both DNA and whole chromosomes. This activity is essential for several cellular events such as DNA replication, transcription, chromosome condensation and segregation. In mammals, two genes code for isoforms of topo II, termed α and β. They are similar in primary structure and have almost identical catalytic properties in vitro. We transfected HeLa cells with small interfering RNAs (siRNAs) targeted against either topo IIα or IIβ, and succeeded in knocking down the expression of the corresponding protein. Chromosomes were condensed and aligned at metaphase in topo IIα-knockdown cells. Although some lagging chromosomes were observed, they were still segregated at anaphase despite the absence of topo IIα. When both topo IIα and topo IIβ were removed, the segregation of chromosomes was severely arrested, suggesting that topo IIβ could partially substitute for topo IIα. Double-knockdown experiments also revealed that topo II was required for shortening of the chromosome axis.

Key words: Chromosome condensation, Chromosome segregation, DNA topoisomerase IIα, DNA topoisomerase IIβ, siRNA

Introduction

DNA topoisomerase II (topo II) is a ubiquitous nuclear enzyme that plays a crucial role in controlling the conformation of both DNA and whole chromosomes. This activity is thought to be an essential aspect of several cellular events (Wang, 1996). Mammalian cells are known to possess two isoforms of topo II, α and β; they are similar in primary structure and have almost identical catalytic properties in vitro (Austin and Marsh, 1998; Drake et al., 1987; Jenkins et al., 1992). Several lines of evidence suggest that topo IIα is the main isoform involved in mitotic processes. First, there is a positive correlation between the cellular concentration of topo IIα and the rate of cell proliferation (Drake et al., 1989). Second, the expression of topo IIα mRNA is higher in tissues containing proliferating cells (Capranico et al., 1992). Third, the level of topo IIα protein peaks at G2/M phase during the cell cycle (Woessner et al., 1991) and, finally, topo IIα localizes to the centromeres and axes of metaphase chromosomes (De, 2002). By contrast, the function of topo IIβ at the cellular level remains obscure (Sakaguchi et al., 2001). Topo II inhibitors, such as 2,6-dioxopiperazines (ICRF-159 and ICRF-187) and epipodophyllotoxins (VP-16 and VM-26; Schneider et al., 1990), are commonly used to investigate the roles of topo II (Gorbsky, 1994); however, these drugs inhibit the enzymatic activity of both topo IIα and topo IIβ. To investigate the cellular role of each topo II isoform individually, we transfected HeLa cells with siRNA (a short synthetic duplex of 21 nucleotides with 3′ overhangs of 2 nucleotides) targeted against either topo IIα or topo IIβ. RNAi can be used to suppress selectively the expression of either isoform because siRNAs are ineffective if one or two of the 21 nucleotides are not complementary to their target (Elbashir et al., 2001; Harborth et al., 2001). Using this method, we show that the chromosomes are condensed in the near absence of topo IIα. Surprisingly, although some lagging chromosomes were observed, the cells still managed to segregate them at anaphase. By contrast, topo IIβ was not required for normal mitotic events. Double-knockdown experiments with both topo IIα and topo IIβ siRNAs revealed that topo IIβ was able to substitute partially for topo IIα in chromosome condensation and segregation. In addition, we show that topo II has a crucial role in the shortening of chromosome axes.

Materials and Methods

RNA preparation

21-nucleotide RNAs were purchased from JBioS (Saitama). The siRNAs targeting topo IIα corresponded to the regions 76-96 (α siRNA-1) and 122-142 (α siRNA-2), and the siRNAs targeting topo IIβ corresponded to the regions 73-93 (β siRNA-1) and 86-106 (β siRNA-2) relative to the first nucleotide of the start codon.

Cell culture and transfection

HeLa cells were grown at 37°C in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (FBS), 100 units ml^{-1} penicillin and 100 mg ml^{-1} streptomycin. The day before transfection, cells were trypsinized, diluted with fresh medium without antibiotics and transferred to 35 mm dishes. Transient transfection of siRNAs was carried out using Oligofectamine (Invitrogen). 12 μl OPTIMEM 1 medium (Invitrogen) and 4 μl

Oligofectamine per dish were preincubated for 5 minutes at room temperature. During this incubation, 170 μl OPTIMEM 1 medium was mixed with 10 μl of 20 μM siRNA. The two mixtures were combined and incubated for 20 minutes to allow complex formation. The entire mixture was added to the cells and, 4 hours later, 300 μl FBS was also added.

Immunoblotting and immunofluorescence microscopy

Topo IIα-specific monoclonal antibody (mAb), 8D2, and topo IIβ-specific mAbs, 1A5 and 3B6, were used at 1/20 or 1/50 dilution in hybridoma supernatant for immunofluorescence and 1/50 dilution for immunoblotting (Sakaguchi et al., 2002). The antibodies 6H8 and 7B9, which recognize both topo IIα and β, were used in the same way. Tubulin-specific mAb, YL1/2 (Serotec), and nuclear pore complex protein-specific antibody (BabCO) were used for immunofluorescence at 1/50 and 1/1000 dilutions, respectively. The methods for immunoblotting and immunofluorescence were as described previously (Sakaguchi et al., 2002). Cells were mounted in Vectashield with 4, 6-diamidino-2-phenylindole (DAPI; Vector Labs) and examined with an epifluorescence microscope (Olympus BX-60) using U-MWIG for rhodamine, U-MWIB/GFP for FITC and U-MWU for DAPI filters, respectively. Images were acquired with an ORCA-ER CCD camera (HAMAMATSU, Japan) equipped with IP Lab software (Scananalytics Corp).

LSC2 measurements

Transfected cells grown on Akura films (Nisshin EM) were fixed in 100% ethanol for 1 hour at 4°C. After being rinsed well in PBS, they were incubated with RNase (1 mg ml^{-1}; Sigma) for 1 hour at 37°C. The films were dipped in a 50 mg ml^{-1} solution of propidium iodide (PI; Sigma) and mounted in PermaFluor Mounting Medium (Shandon).

DNA content was measured using a laser scanning cytometer (LSC2; Olympus). Three parameters were employed to define nuclear characteristics, namely nuclear area, fluorescence value and fluorescence peak; we show data using two-parameter dots of possible permutations of these three parameters. The fluorescence value corresponds to nuclear DNA content; the fluorescence peak represents the state of chromosome condensation (Kawasaki et al., 1997).

DNA topoisomerase II assay

Extracts for topo II assay were prepared from 4×10^5 cells 3 days after transfection with siRNAs, as described in 'Small Scale Preparation of Topo I and II Extracts from Tissue Culture Cells (Optimized for Hela Cells)' on the TopoGEN website (http://www.topogen.com/html/extracts.html). Topo II activity was measured by a decatenation assay using kinetoplast DNA as a substrate (topoisomerase II assay kit; TopoGEN) according to the manufacturer's instructions. Decatenation products were analyzed by agarose gel electrophoresis using 1.0% agarose in TBE buffer (89 mM Tris borate, pH 8.2, 2 mM EDTA). One unit activity of topo II decatenates 0.2 μg of kinetoplast DNA in 30 minutes.

Chromosome preparation

For metaphase chromosome spreading, cells grown on Akura films in 35 mm dishes were washed once in PBS and soaked in a hypotonic solution of 0.075 M KCl for 5 minutes at room temperature. Metaphase chromosomes were spread by centrifugation for 5 minutes at 2000 rpm. Cells were fixed in freshly prepared 4% paraformaldehyde in PBS for 20 minutes at room temperature before examination using immunofluorescence procedures.

To measure the condensation of each chromosome, cells were incubated in 0.05 μg ml^{-1} nocodazole (Sigma) for 2 hours, then treated with 0.075 M KCl hypotonic solution for 20 minutes at 37°C before fixation with acetic acid:cooled methanol (1:3). An aliquot of the cell suspension (about 0.1 ml) containing approximately 1×10^4 mitotic cells was taken up into a syringe (1 ml) with a 22-gauge needle, and the cells were ruptured by pumping at least 10 times. The single-chromosome suspensions were then spread on Akura films, air-dried and analyzed under the fluorescent microscope.

Results

Knockdown of each topo II isoform

Two RNA duplexes of 21 nucleotides in length for each of topo IIα (α siRNA-1 and α siRNA-2) and topo IIβ (β siRNA-1 and β siRNA-2) cDNAs were prepared. HeLa cells were transfected with each siRNA, or mock transfected with buffer, and cells were assayed 2, 3 and 4 days after transfection by immunoblotting (Fig. 1A,B). Transfection with α siRNA-1 or α siRNA-2 resulted in specific silencing of topo IIα but not of topo IIβ (Fig. 1A). Most endogenous topo IIα disappeared between the second and the fourth days after transfection with α siRNA-1, and optimal silencing of topo IIα was reached on the third day after transfection. To quantify the remaining topo IIα protein, crude extracts from cells on the third day after transfection with α siRNA-1 were assayed by immunoblotting together with 1/5, 1/10, 1/20, 1/40 and 1/80 dilutions of extracts from control cells. The remaining level of topo IIα corresponded to about 4.2±0.3% of the control value. Similarly, most topo IIβ disappeared over the same period after transfection with β siRNA-2 (Fig. 1B), and optimal silencing of topo IIβ was also reached on the third day after transfection. The remaining level of topo IIβ protein corresponded to about 3.8±2.1% of the control value. Gene silencing was also confirmed by immunofluorescence. The topo IIα-specific mAb 8D2 (Sakaguchi et al., 2002) stains both mitotic and interphase nuclei in control cells; in cells transfected with α siRNA-1, most nuclei were not stained and only nonspecific fluorescence was seen throughout the cytoplasm (Fig. 1C, upper panel). On the third day after transfection, 92 of 1254 nuclei were stained by 8D2, indicating that the efficiency of α siRNA-1 transfection was approximately 92.7%. In the same manner, silencing by β siRNA-2 was confirmed using the topo IIβ-specific mAb 1A5 (Sakaguchi et al., 2002); the efficiency of β siRNA-2 transfection was approximately 94.2% (Fig. 1C, lower panel).

Topo IIα-knockdown cells are delayed in early mitotic stages

The DNA content of control and topo II-knockdown cells was measured on the third day after transfection, when the silencing of topo II by either α siRNA-1 or β siRNA-2 was highly efficient. DNA content was measured using a laser scanning cytometer (LSC2; Olympus), which functions as both a fluorescence microscope and a cytometer (Darzynkiewicz et al., 1999; Kamentsky and Kamentsky, 1991). The upper panels of Fig. 2B show the fluorescence values and the peak values of each PI-stained body; the sum of the number of spots of each value range is shown in the histogram of DNA content for each spot (Fig. 2B, lower panels). Using LSC2, cells can be sorted into cell-cycle stages (Kawasaki et al., 1997); cells in the red boxes are in prophase and metaphase, and those in the blue boxes are in anaphase and telophase (Fig. 2A).

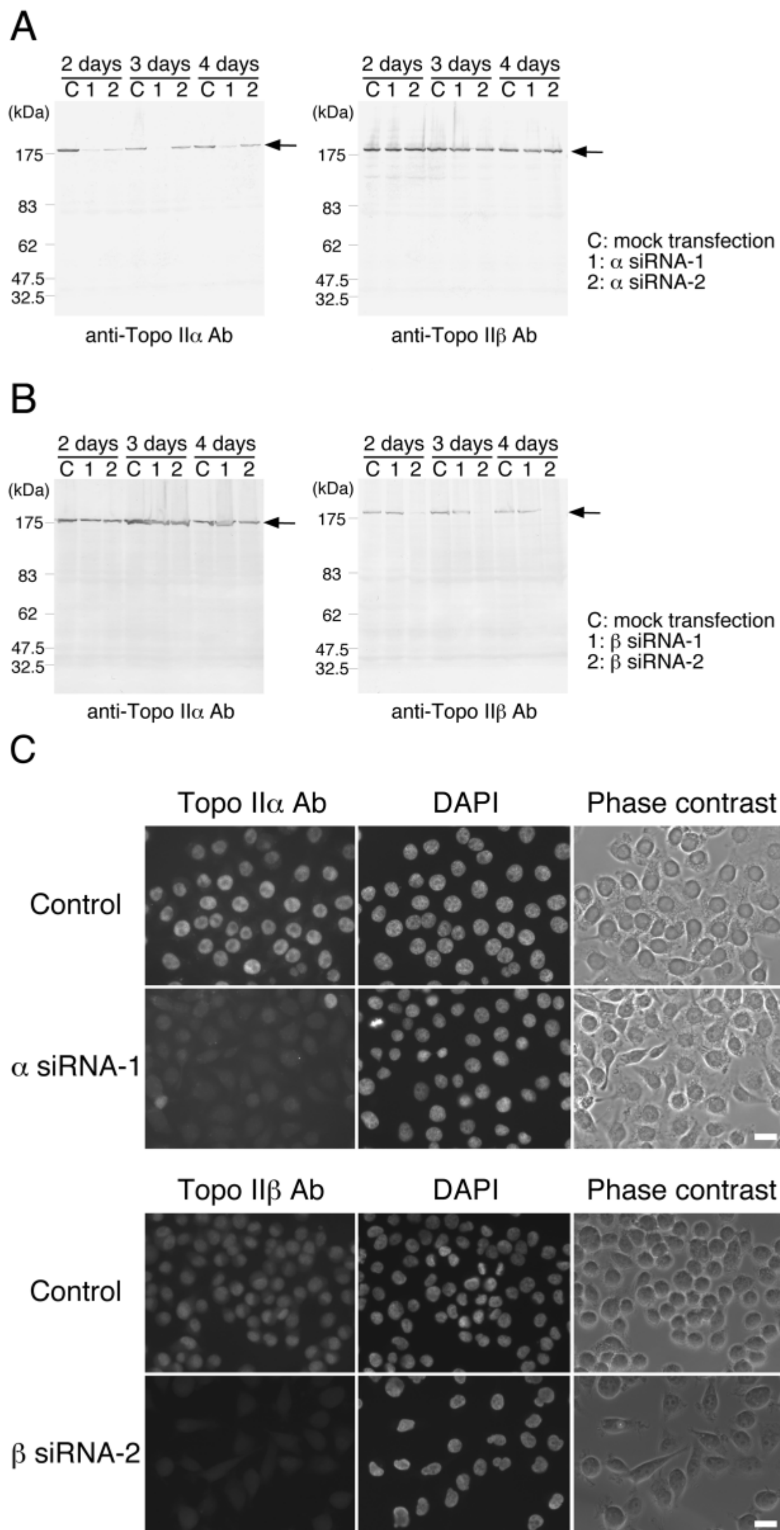


Fig. 1. Knockdown of each topo II isoform by siRNA. (A,B) HeLa cells were transfected with α siRNA-1 (A1), α siRNA-2 (A2), β siRNA-1 (B1) and β siRNA-2 (B2) or buffer as a control (A and B; C). Cells were assayed on the second, third and fourth days after transfection by immunoblotting using the anti-topo IIα antibody 8D2 (left panels) and the anti-topo IIβ antibody 3B6 (right panels). (C) Gene silencing was also confirmed by immunofluorescence using the anti-topo IIα antibody 8D2 (for α siRNA-1-knockdown cells, upper panels) or the anti-topo IIβ antibody 1A5 (for β siRNA-2-knockdown cells, lower panels); cells were also stained with DAPI. Ab: antibody. Bar, 20 μm.

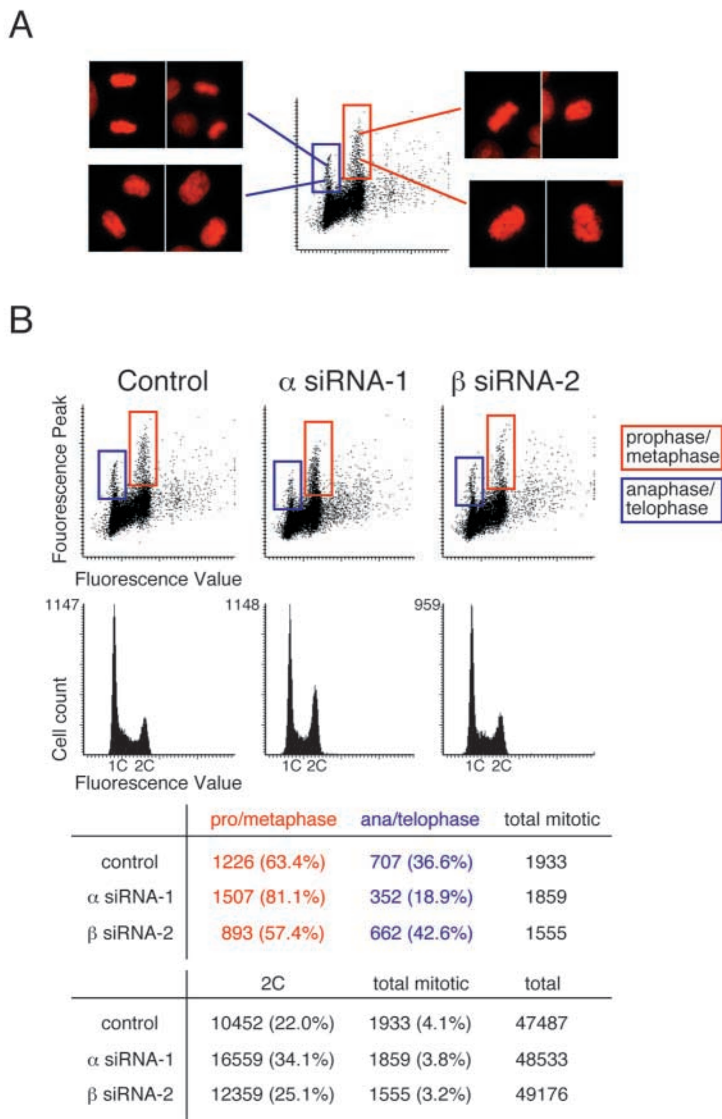
In topo IIα-knockdown cells, the population of cells in prophase or metaphase was greater than that of control cells (Fig. 2B, red boxes). Whereas the proportion of mitotic cells was almost identical in the control and topo IIα-knockdown populations, the proportions of cells in prophase/metaphase and anaphase/telophase were different (Fig. 2B, table). In topo IIα-knockdown cells, the population of cells in prophase/metaphase was about 80% of total mitotic cells; by contrast, in control cells, it was about 60%. In addition, the 2C peak position of topo IIα-knockdown cells was higher than that of control cells. In control and topo IIα-knockdown cells, the 2C peak comprised 22.0 and 34.1% of total cells, respectively (Fig. 2B, lower table), indicating that there is also an increase in the number of cells in G2 phase in topo IIα-knockdown cells. These results suggest that topo IIα-knockdown cells are delayed in the early mitotic stages relative to normal topo IIα-expressing cells. By contrast, we found that the knockdown of topo IIβ did not appear to have any effect on the cell cycle.

Chromosome segregation in topo IIα-knockdown cells

Control (Fig. 3A) and topo IIα-knockdown cells (Fig. 3B) were stained with DAPI, topo IIα-specific mAb (8D2) and tubulin-specific mAb (YL1/2) on the third day after transfection. The chromosomes were condensed and the mitotic spindles were assembled in topo IIα-knockdown cells (Fig. 3Bb). However, in some topo IIα-knockdown cells, the alignment of metaphase chromosomes was distorted and their segregation towards daughter cells was affected, which may be indicative of difficulties in chromosome segregation (Fig. 3Bc). In addition, whereas compact chromosomes were maintained until telophase in control cells (Fig. 3Ac), the chromosomes of topo IIα-knockdown cells became slightly thicker and less compact in the meta-anaphase (Fig. 3Bd,e). Although some lagging chromosomes were observed, they still managed to separate (Fig. 3Bf). In some topo IIα-knockdown cells, the two daughter nuclei were connected by a thread of DNA (Fig. 3C, arrowheads); this connection remained until the G1 phase, when the nuclear lamina reformed (Fig. 3D). In control cells, we seldom saw such threads of DNA. Among all topo IIα-knockdown cells, 21.4% were connected by DNA (Fig. 3E). The DNA content of connected nuclei was measured using the LSC2: we found that DNA was evenly partitioned in the 75.2% of all connected nuclei. Therefore, even in the near absence of topo IIα, most chromosomes can segregate evenly; however, the catenanes of intertwined DNAs cannot be removed completely at the onset of G1 phase.

Topo IIβ partially substitutes for topo IIα in chromosome segregation

To investigate whether topo IIβ could substitute for



	pro/metaphase	ana/telophase	total mitotic
control	1226 (63.4%)	707 (36.6%)	1933
α siRNA-1	1507 (81.1%)	352 (18.9%)	1859
β siRNA-2	893 (57.4%)	662 (42.6%)	1555

	2C	total mitotic	total
control	10452 (22.0%)	1933 (4.1%)	47487
α siRNA-1	16559 (34.1%)	1859 (3.8%)	48533
β siRNA-2	12359 (25.1%)	1555 (3.2%)	49176

Fig. 2. Cell-cycle analysis using the LSC2. (A) Using LSC2, cells can be sorted into cell-cycle stages (Kawasaki et al., 1997). Cells in red boxes are at prophase and metaphase, and those in blue boxes are at anaphase and telophase. (B) Topo IIα-knockdown, topo IIβ-knockdown and mock-transfected cells were sorted using the LSC2 3 days after transfection (plots). The tables show the number of cells in each category; red boxes correspond to prophase/metaphase cells, blue boxes to anaphase/telophase cells, 2C to G2 phase cells and 'total mitotic' is the sum of prophase/metaphase and anaphase/telophase cells. Each transfection was repeated independently at least three times and samples were prepared in four separate Akura films for every experiment.

topo IIα in the topo IIα-deficient cells, the expression of both topo IIα and topo IIβ was knocked down simultaneously. HeLa cells were transfected with both α siRNA-1 and β siRNA-2, resulting in the silencing of both topo IIα and topo IIβ (Fig. 4A). The levels of topo IIα and topo IIβ remaining on the third day after transfection corresponded to approximately 4.3±0.7% and 3.6±0.2% of the control values, respectively.

By immunofluorescent staining of topo IIαβ-knockdown cells, the number of topo IIα-positive cells as seen in the upper right corner in Fig. 4C seems to account for the larger proportion (285/1185) than that in the single topo IIα-knockdown case (92/1254; Fig. 1C). Immunostaining with isoform-specific antibodies was also carried out; 19.1% (233/1218) were topo IIα-positive cells, whereas 18.5% (180/971) were topo IIβ-positive cells. This large difference between single- and double-knockdown experiments is because, in the first case, the cells continue to divide, whereas most of the topo IIαβ-knockdown cells could not divide any further and the number of the cells did not increase. In topo IIαβ-knockdown cells, the 2C population increased dramatically and a large 4C peak appeared (Fig. 4B). As shown by DAPI staining (Fig. 4C, upper panel), many large and multi-lobed nuclei were observed and it was confirmed by LSC2 measurements that most of these were 4C, indicating that these cells were a result of the failure of chromosome segregation. Some cells were observed in which nearly all of the chromosomes were segregated into one of the two daughter cells (Fig. 4C, lower panel). We therefore concluded that double-knockdown cells were unable to segregate chromosomes to daughter cells. Because they managed to separate in topo IIα-knockdown cells, this result suggests that, in topo IIα-knockdown cells, topo IIβ can assume the essential catalytic roles required for chromosome segregation.

The enzymatic activity of topo II in knockdown cells

The use of siRNAs enabled us to eliminate the activities of topo IIα or topo IIβ individually. The absence of topo II in knockdown cells was shown by both immunoblotting and immunostaining. To confirm the absence of topo II, its enzymatic activity in knockdown cells was analyzed by an ATP-dependent decatenation assay using kinetoplast DNA as a substrate (Fig. 5). We inoculated 4×10^5 cells and prepared extracts for the topo II assay from cells 3 days after transfection. In the presence of ATP, the products of topo II decatenation activity appear as two bands on agarose gels. The topo II activity in crude extract from control cells was found to be 1.38×10^4 units mg^{-1} protein (1 unit of topo II activity can decatenate 0.2 µg of kinetoplast DNA in 30 minutes.) (Fig. 5A). The topo II activities in topo IIα- and topo IIαβ-knockdown cell extracts were 1.72×10^3 and 0.86-1.29×10^3 units mg^{-1}, respectively, which correspond to 12.5% and 6.23-9.35% of the activity of the control cells (Fig. 5B,C). Compared with the difference in the activity of control and topo IIα-knockdown cells, only a small difference was detected between topo IIα- and topo IIαβ-knockdown cells. Additionally, immunoblotting showed that the relative protein level of topo IIβ was about 13.0% of topo IIα (Fig. 5D), indicating that most of the residual decatenating activity in topo IIαβ-knockdown cells must be owing to the activity of topo IIα. The residual topo II decatenation activity in topo IIαβ-knockdown cells was approximately 6.23-9.35% of the topo II activity in mock-transfected cells, which corresponds to the proportion of cells stained by anti-topo IIα antibody in Fig. 1C (Note that in the cell mass used in the topo II assay, about 7% of cells were topo IIα-positive.) The remaining activities of topo II in topo IIα- and topo IIαβ-knockdown cells were at a similar level. Nevertheless, the phenotype of topo IIαβ-knockdown cells was quite different

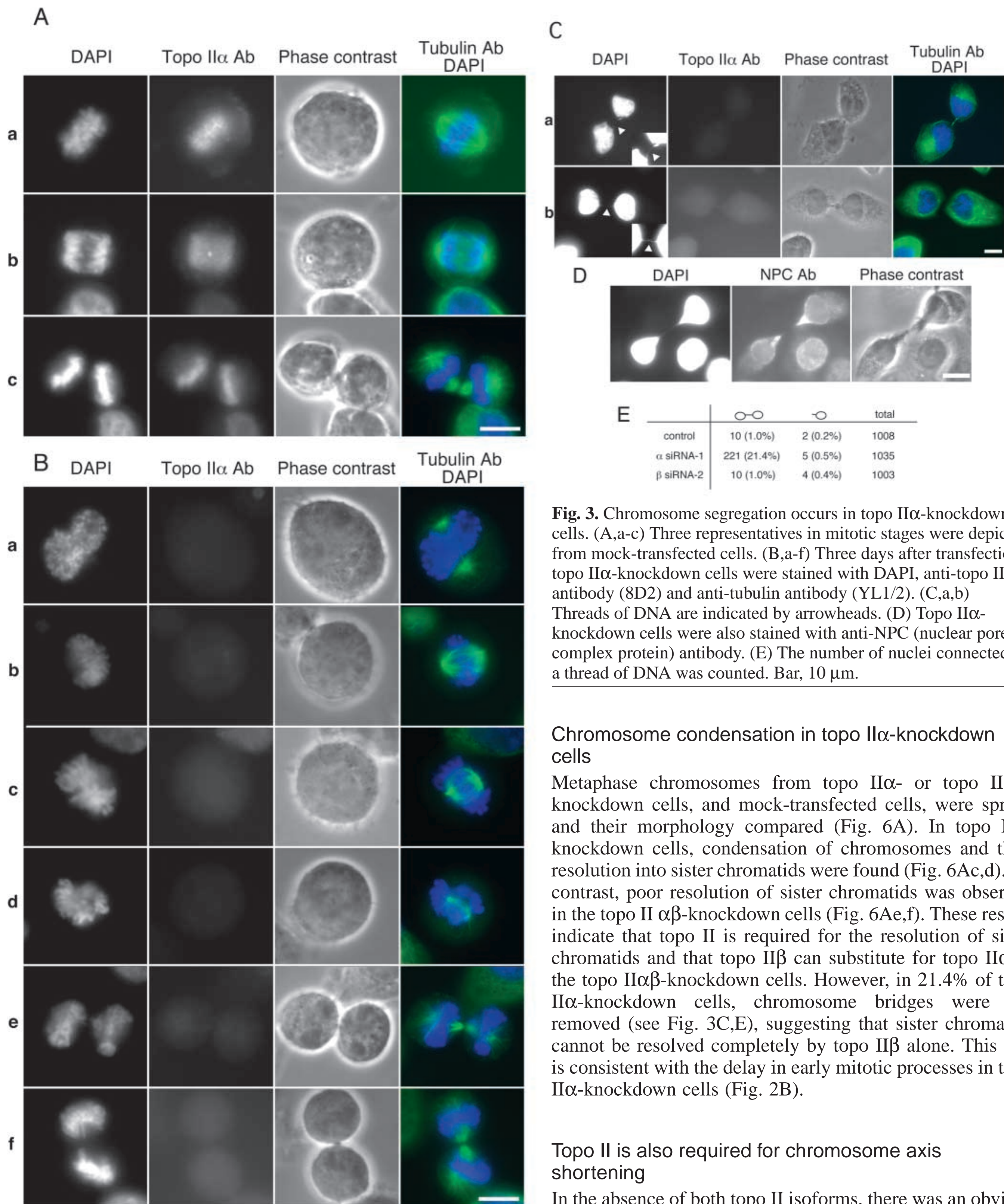


Fig. 3. Chromosome segregation occurs in topo IIα-knockdown cells. (A,a-c) Three representatives in mitotic stages were depicted from mock-transfected cells. (B,a-f) Three days after transfection, topo IIα-knockdown cells were stained with DAPI, anti-topo IIα antibody (8D2) and anti-tubulin antibody (YL1/2). (C,a,b) Threads of DNA are indicated by arrowheads. (D) Topo IIα-knockdown cells were also stained with anti-NPC (nuclear pore complex protein) antibody. (E) The number of nuclei connected by a thread of DNA was counted. Bar, 10 μm.

from that of single topo IIα-knockdown cells. Therefore, it is reasonable to suppose that the different phenotype (i.e. whether chromosomes could separate to daughter cells or not) must be the direct consequence of the presence of topo IIβ activity, rather than the residual amount of topo IIα.

Chromosome condensation in topo IIα-knockdown cells

Metaphase chromosomes from topo IIα- or topo IIαβ-knockdown cells, and mock-transfected cells, were spread and their morphology compared (Fig. 6A). In topo IIα-knockdown cells, condensation of chromosomes and their resolution into sister chromatids were found (Fig. 6Ac,d). By contrast, poor resolution of sister chromatids was observed in the topo II αβ-knockdown cells (Fig. 6Ae,f). These results indicate that topo II is required for the resolution of sister chromatids and that topo IIβ can substitute for topo IIα in the topo IIαβ-knockdown cells. However, in 21.4% of topo IIα-knockdown cells, chromosome bridges were not removed (see Fig. 3C,E), suggesting that sister chromatids cannot be resolved completely by topo IIβ alone. This fact is consistent with the delay in early mitotic processes in topo IIα-knockdown cells (Fig. 2B).

Topo II is also required for chromosome axis shortening

In the absence of both topo II isoforms, there was an obvious defect in the resolution of chromosomes (Fig. 6Ae). Chromosome condensation can be divided into two components, namely the resolution of sister chromatids and the shortening of the longitudinal axis of the chromosome (Steffensen et al., 2001). To investigate whether double-knockdown cells also have defects in chromosome axis

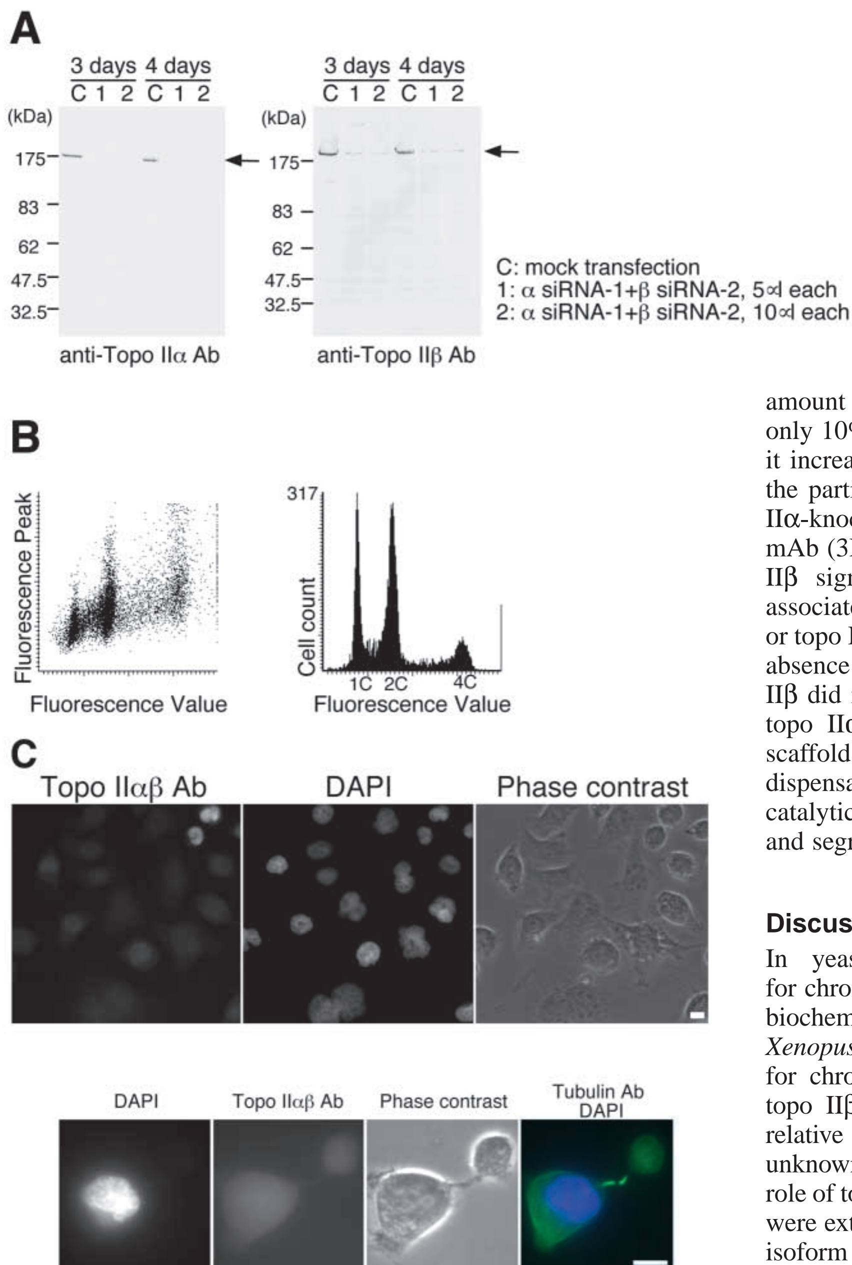


Fig. 4. Knockdown of both topo II isoforms by siRNAs. (A) HeLa cells were transfected with both α siRNA-1 and β siRNA-2 (1; 5 µl each siRNA was added per 35 mm dish; 2; 10 µl each) or buffer as a control. Cells were assayed on the third or fourth day after transfection by immunoblotting using the anti-topo IIα antibody 8D2 (left) and the anti-topo IIβ antibody 3B6 (right). (B) The topo IIαβ-knockdown cells were analyzed using the LSC2 3 days after transfection. (C) Topo IIαβ-knockdown cells (3 days after transfection) were stained with DAPI, anti-topo IIαβ antibody (6H8) and anti-tubulin antibody (YL1/2). Bar in C, 20 µm (upper panels) and 10 µm (lower panels).

shortening, we measured the length of arms of metaphase chromosomes in control cells and that in topo IIαβ-knockdown cells (Fig. 6B). The average length of each axis of metaphase chromosomes in control cells was 6.05±1.47 µm. By contrast, its axis length in topo IIαβ-knockdown cells was 7.51±1.60 µm. Thus, the extent of axial compaction of metaphase chromosomes in topo IIαβ-knockdown cells was clearly reduced, indicating that topo II is also required for the chromosome axis shortening as well as for the resolution of chromosomes.

The localization of topo IIβ is mostly unchanged in topo IIα-knockdown cells

To investigate how topo IIβ substitutes the roles of topo IIα in its absence, we examined the quantity and localization of topo IIβ. It is noticed from the right panel of Fig. 1A, the amount of topo IIβ protein, which usually accounts for only 10% of total topo II protein, did not change, nor did it increase after the knockdown of topo IIα. To examine the particular localization change, both control and topo IIα-knockdown cells were stained with topo IIβ-specific mAb (3B6) (Fig. 7). In mitotic cells, the majority of topo IIβ signal diffused into the cytoplasm and was not associated with metaphase chromosomes in either control or topo IIα-knockdown cells. This result shows that, in the absence of topo IIα, the localization of the bulk of topo IIβ did not change during the cell cycle to substitute for topo IIα as the major constituent of the chromosome scaffold. Therefore, we suggest that topo IIα might be dispensable in the chromosome scaffold but that its catalytic activity is essential for chromosome condensation and segregation.

Discussion

In yeast, a single copy of topo II is essential for chromosome segregation (Uemura et al., 1987). In the biochemical and immunological depletion study of *Xenopus* egg extracts, topo IIα was shown to be required for chromosome condensation, whereas the function of topo IIβ remained obscure because its localization and relative amount to topo IIα in the egg extracts were unknown (Hirano and Mitchison, 1993). In mammals, the role of topo IIα and topo IIβ, and their cellular localization, were extensively studied using specific mAbs against each isoform (Cobb et al., 1999; Tsutsui et al., 2001; Turley et al., 1997; Yabuki et al., 1996). This idea was challenged by the recent observation that there might exist residual but sizable amounts of heterodimers of topo IIα/topo IIβ in the cultured cell extract (Christensen et al., 2002).

The knocking-out of topo IIβ does not affect embryonic development because topo IIα can substitute for it until the birth of the fetus (Yang et al., 2000), indicating that topo IIβ might be dispensable in cell proliferation. The knocking-out of topo IIα causes early embryonic death (Akimitsu et al., 2003) and the activity of topo IIβ, if there is any, cannot sufficiently compensate for the absence of topo IIα. In cultured HeLa cells, this may not be the case.

Although 90% of topo IIα was removed by specific siRNA, cells continued to divide. In these cells, we could not detect any signal of topo IIα in the centromeric region and axis of each metaphase chromosome (Fig. 6Ad), nor could we detect any sign of dramatic relocation of topo IIβ by immunofluorescent staining. In fact, topo IIβ was dispersed into the cytoplasm after

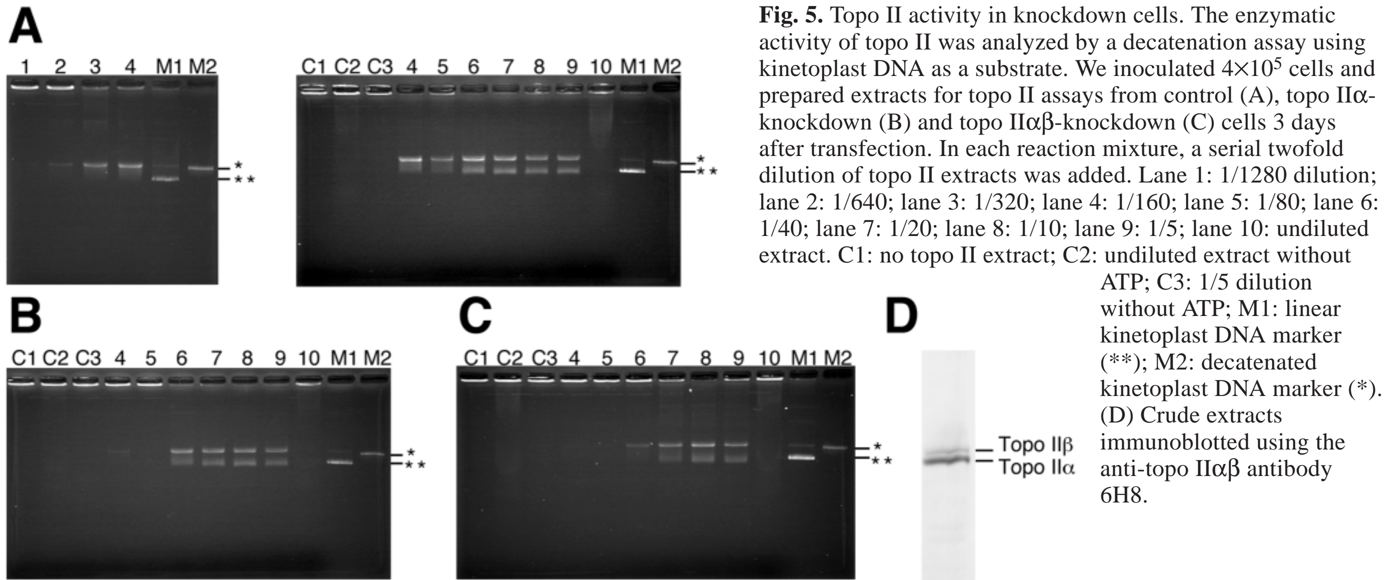


Fig. 5. Topo II activity in knockdown cells. The enzymatic activity of topo II was analyzed by a decatenation assay using kinetoplast DNA as a substrate. We inoculated 4×10^5 cells and prepared extracts for topo II assays from control (A), topo IIα-knockdown (B) and topo IIαβ-knockdown (C) cells 3 days after transfection. In each reaction mixture, a serial twofold dilution of topo II extracts was added. Lane 1: 1/1280 dilution; lane 2: 1/640; lane 3: 1/320; lane 4: 1/160; lane 5: 1/80; lane 6: 1/40; lane 7: 1/20; lane 8: 1/10; lane 9: 1/5; lane 10: undiluted extract. C1: no topo II extract; C2: undiluted extract without ATP; C3: 1/5 dilution without ATP; M1: linear kinetoplast DNA marker (**); M2: decatenated kinetoplast DNA marker (*). (D) Crude extracts immunoblotted using the anti-topo IIαβ antibody 6H8.

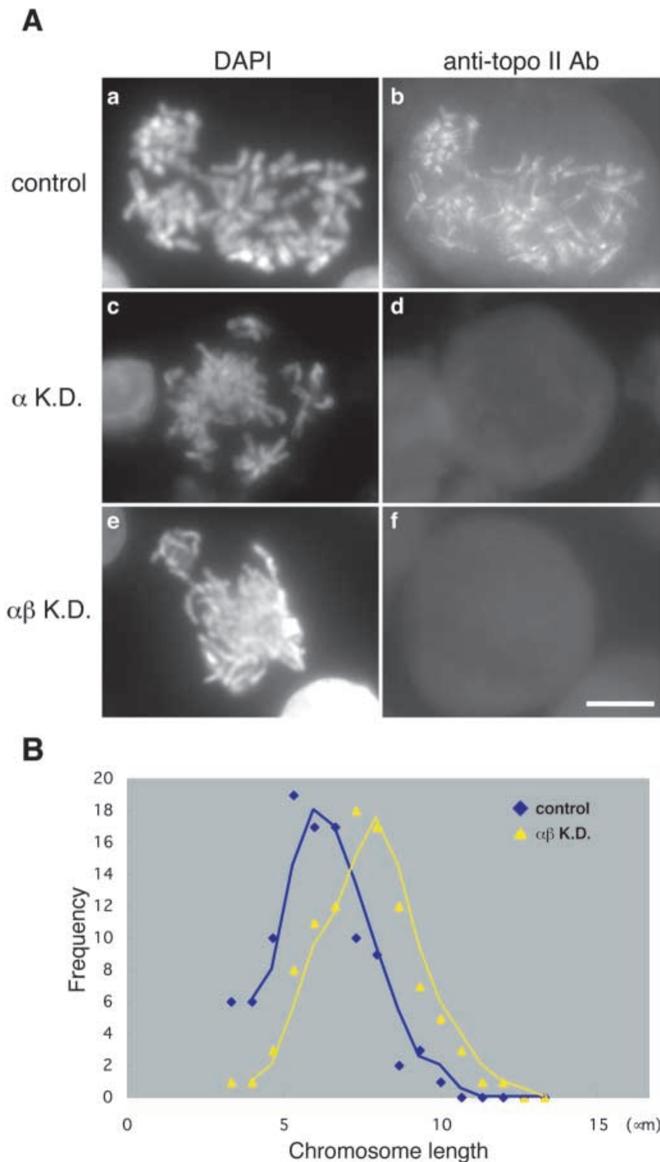
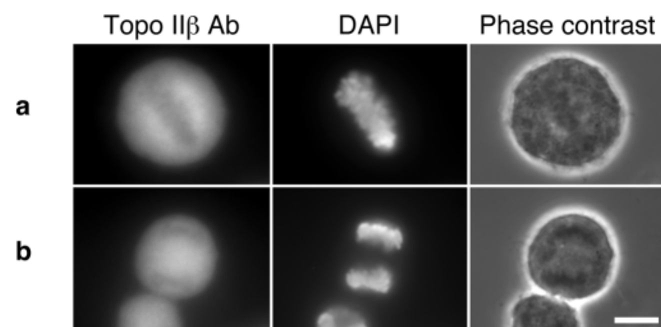


Fig. 6. Chromosome condensation and chromosome axis shortening. (A) Metaphase chromosomes of control (a,b), topo IIα-knockdown (c,d) and topo IIαβ-knockdown (e,f) cells were spread and stained with DAPI (a,c,e) and the anti-topo IIαβ antibody 7B9 (b,f) or the anti-topo IIα antibody 8D2 (d). In control cells, chromosome axes and centromeres were stained with anti-topo IIαβ antibody. Bar, 10 μm. (B) Frequency distribution of the length of metaphase chromosomes in control cells and topo IIαβ-knockdown cells.

nuclear membrane breakdown in topo IIα-knockdown cells (Fig. 7c,d), as in mock-transfected cells. The total amount of topo IIβ did not change in the topo IIα-knockdown cells (Fig. 1A). Thus, we might conclude that the majority of topo IIα at the metaphase chromosomes could be dispensable for chromosome condensation and segregation. When topo IIβ was removed from the topo IIα-knockdown cells, we could detect several phenotypic changes, such as the appearance of 4C cells and anuclear cells, indicating that chromosome segregation was severely affected. Also, the metaphase chromosomes formed in these cells were morphologically altered. They were more slender and slightly longer by 10%. Thus, we could conclude that topo IIβ partially substituted for topo IIα deficiency in cell division, namely during chromosome condensation and segregation. It is noticed here that we could still see the morphologically recognizable chromosomes in the double-knockdown cells (Fig. 6Ae), even if they were very sick, indicating that their formation does not require normal protein levels of topo IIα or β.

In conclusion, we have demonstrated for the first time in cultured human cells the selective suppression of the expression of topo IIα, topo IIβ or topo IIα/topo IIβ using siRNAs, and that topo IIα can be largely removed and yet chromosomes look normal and segregate reasonably well. Furthermore, the double-knockdown studies show that mitotic chromatin condensation and formation of morphologically recognizable mitotic chromosomes certainly does not require normal levels of topo IIα or β. It seems to be true that, although the loss of topo II causes minor problems for mitotic chromosome structure, these chromosomes look remarkably normal compared with the attempts at chromosome



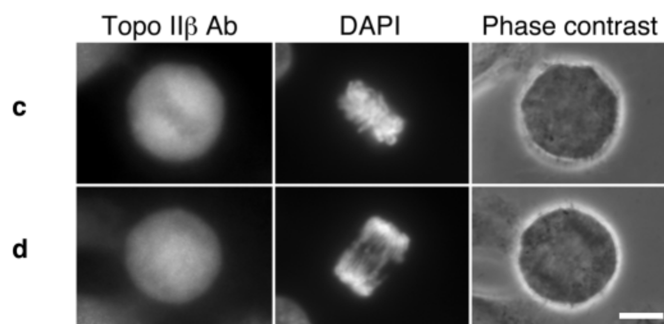


Fig. 7. Localization of topo IIβ in mitotic chromosomes of topo IIα-knockdown cells. Mitotic chromosomes of both control (a,b) and topo IIα-knockdown (c,d) cells on the third day after transfection were stained with anti-topo IIβ antibody (3B6) and DAPI. Bar, 10 μm.

condensation in the absence of topo II obtained by Adachi and Laemmli (Adachi et al., 1991).

We thank K. Sekimizu and N. Akimitsu for discussions. We are particularly grateful to T. Akashi and W. Earnshaw for advice and discussions, and to K. Okada for his help in reproducing the knockdown experiment several times by himself. The topo II assay kit was a gift from M. Suzuki and N. Suda. We also thank Y. Kiyomatsu for technical advice on the LSC2. Part of this work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology, Japan. A.S. thanks the Ito Scholarship Foundation for their support.

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