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The Dynamics of DNA Topoisomerase II α in Living Cells

John R. Daum¹, Yin Yuan Mo², and Gary J. Gorbsky^{1,*}

¹Cell Cycle and Cancer Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104

²Department of Medical Microbiology, Immunology and Cell Biology, Southern Illinois University School of Medicine, Springfield, IL 62794

Abstract

Here we describe methods to prepare a mammalian expression plasmid encoding EGFP fused to the amino terminus of human DNA topoisomerase II α (TopoII α). This plasmid is transfected into LLC-Pk cells, a porcine epithelial cell line that remains relatively flat during mitosis. After selection for stable integration, cells are cloned by serial dilution in microwells and used to grow a stable cell line expressing EGFP-TopoII α . This line is termed LPk-GT2. Using photobleaching methods with conventional and patterned photobleaching LPk-GT2 cells are used demonstrate the rapid dynamics of TopoII α exchange in both interphase nuclei and mitotic chromosomes. These rapid dynamics are dependent on enzyme activity since ICRF159 a catalytic inhibitor of TopoII α , slows dynamics significantly.

Keywords

nucleus; chromosome; kinetochore; mitosis; fluorescence; photobleaching; FRAP

1. Introduction

DNA topoisomerase II α (TopoII α) is expressed primarily in actively dividing cells and shows dynamic changes at various stages of the cell cycle. In proliferating cells, both protein and message levels are low during the G1 phase and begin to rise during S phase, with a peak of protein expression during G2 and M phases ^{1–3}. After mitosis TopoII α reconcentrates in the nuclei, and subsequently during G1 message levels decrease and the protein is degraded by the proteasome ^{1–5}. In interphase cells, antibody labeling and expression of tagged forms of the protein show that the enzyme is nuclear with a high concentration in the nucleolus ^{5–7}. As the chromosomes condense during early mitosis, TopoII α concentrates at centromeres and along the axes of the chromosome arms ^{7–10}.

This pattern of association with mitotic chromosomes and the persistence of TopoII α in salt- and DNase-treated chromosome fractions led to the idea that TopoII α might form a stable mitotic chromosome scaffold ¹¹. To test this notion we and others created cell lines that express TopoII α fused to fluorescent proteins that were then used for photobleaching analyses ^{5, 6, 12}. Surprisingly, TopoII α showed rapid turnover in photobleaching experiments in both interphase nuclei and in mitotic chromosomes. When inhibitors of topoisomerase II catalytic activity were applied to cells TopoII α became stably anchored on chromatin in both interphase and mitotic cells ¹⁰. These observations led to the conclusion that TopoII α exhibits rapid

*To whom correspondence should be addressed: Address: Cell Cycle and Cancer Biology Research Program, Oklahoma Medical Research Foundation, MS 48, 825 NE 13th St, Oklahoma City, Ok 73104, Telephone: 1-405-271-8168, Fax: 1-405-271-2037, GJG@omrf.org.

exchange on chromatin that is dependent on enzyme activity. Further studies have implicated TopoII α in a host of important roles for chromatin regulation in interphase and mitosis^{13–17}. The dynamic exchange of TopoII α on chromatin is likely to be a key factor in these functions. Finally several chemotherapeutic agents function through the activity of TopoII α ^{18, 19}.

Overexpression of TopoII α in mammalian cells is toxic and induces apoptosis^{20, 21}. For this reason, establishment of cell lines stably expressing TopoII α tagged with fluorescent proteins is dependent on spontaneous down regulation of the endogenous TopoII α or by purposefully targeting endogenous gene expression^{5, 12}. Keeping in mind this caveat, the techniques described here will facilitate analysis of TopoII α dynamics in living cells under various experimental conditions.

2. Materials

2.1. Construction of EGFP-TopoII α plasmid

1. Reagents for Molecular Biology: Restriction enzymes (*Kpn* I, *Bam* H I, *Hind* III and *Sac* II) from New England Biolabs; RNase-free DNase and Triazol reagent from Invitrogen; Gold Taq DNA polymerase from ABI, pCDNA3 plasmid from Invitrogen and pEGFP-C3 plasmid from Clontech.
2. Primers for PCR (Sigma-Genosis)
 - a. N-terminus:
 - i. Ila-B1 (sense) 5'-GGATCCGAAGTGTCACCATTCGAG
 - ii. Ila-3.1 (antisense) 5'-GGTGGATCCAGCAATATCAT
 - b. C-terminus:
 - i. Ila-C1A (sense) 5'-GTGACAGTGAAGAAGACAGCAG
 - ii. Ila-C18.1 (antisense) 5'-GCGGCCGCGGTAAAAACAGATCATCTTCATC
3. Primers to detect the exogenous TopoII α
 - a. T1 (sense), 5'-GGTACCGATACCATGCGGGGTTCT
 - b. T2 (antisense) 5'-TAGCCATTTTCGACCACCTGTCAC

2.2. Transfection of LLC-Pk cells and selection of stable line

1. Culture reagents are obtained from Gibco/Invitrogen. Culture medium for LLC-Pk cells consists of Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% Fetal Bovine serum, 1 $\times$ non-essential amino acids, 20 mM Hepes and antibiotics (penicillin 100 U/L and streptomycin 100 μ g/ml)
2. Transfection medium – Plasmid DNA for transfection is prepared with Lipofectamine plus according the manufacturer's directions in DMEM
3. Selection Medium – Complete culture medium containing 2 mg/ml G148
4. Cloning plates – Corning 96 well tissue culture plates

2.3 Imaging and photomanipulation

1. Medium for cell culture on the microscope: If a stage incubator is used that allows a CO₂ atmosphere on the microscope stage, conventional CO₂-requiring culture medium can be used. In most of our studies this approach has not been available.

Instead we generally use a CO₂-independent medium, L-15 containing 10% FBS and antibiotics.

2. **Microscope logistics and temperature control:** Generally inverted research grade inverted microscopes are suitable for high resolution imaging and photomanipulation of EGFP-TopoII α . For LLC-Pk and other mammalian cells, the temperature of the stage must be maintained at or near 37°C. A number of approaches are possible. One solution is to employ a stage incubator. We have used stage insert heaters obtained from a variety of the microscope manufacturers. In addition we have used dedicated air curtain incubators such as the one provided by Nevtek (Model ASI 400). Surprisingly a simple hair dryer mounted on a ring stand with a variable rheostat to control output works well to maintain stage temperature. An electronic thermometer with a small probe that can be taped to the stage is useful to assess temperature. Finally since high resolution requires oil or water immersion objectives, it is essential to have either a stage incubator that heats the objective or to use a separate objective heater that will maintain temperature of the specimen just over the area being observed. We use one manufactured by Biopetech.
3. **Preparation of glass coverslips for live cell microscopy:** Due to the limited working distance of high resolution, high numerical aperture objectives, it is essential to culture cells on thin glass coverslips for imaging. We prepare 25 mm round number 1.5 glass coverslips (usually one box of approximately 100 coverslips) dropping them one by one by hand into a solution of 95% ethanol in a glass petri dish. Dropping them one by one prevents them from sticking together. The coverslips are rinsed twice with fresh 95% ethanol. The ethanol is removed from the glass petri dish containing the coverslips; the lid is placed on the petri dish, and the dish is placed into a cold drying oven. The heat is then turned on and the ethanol is allowed to evaporate for a few hours to overnight. The oven is then turned off and the petri dish is allowed to cool. The coverslips are now clean and sterile and ready to be used for cell culture.
4. **Culture chambers for live cell microscopy:** A variety of approaches and different types of microscope culture chamber systems have been developed. We use two approaches in our laboratory. We use commercial stainless steel chambers (Molecular Probes/Invitrogen) composed of two halves that screw together clamping a 25 mm coverslip on which cells are plated. The chambers are stored in 95% ethanol to maintain sterility. They are removed with tongs and allowed to dry in a tissue culture hood prior to use. A lower cost solution is to drill holes 18 – 20 mm in diameter in the bottom of a 35 mm plastic petri dish. Using a bead of silicone grease, a 25 mm glass coverslip is applied to the inside of the dish. The dish is sterilized by placing it upside down along with its lid on the surface of a UV light box for 10 minutes. If a high resolution objective with a very shallow shoulder is used that bumps into the cut hole in the dish, the coverslips can be mounted with a grease ring on the outside of the chamber. These chambers should always be placed inside a larger plastic petri dish while in the incubator since they occasionally leak. A final issue is evaporation leading to hypertonicity of the medium in the observation. Our usual solution is to cover the medium with a thin layer of mineral oil that must be spread evenly. An alternative is to apply a large coverslip to the top of the chamber but an air space invites condensation on the top coverslip. With the steel chambers it is possible to fill the medium to the level of a shelf inside the chamber on which a 25 mm coverslip can be rested in contact with the medium surface.
5. **Photobleaching apparatus:** We use two different systems for photobleaching both produced by Photonics Instruments, the Micropoint dye laser system and the Mosaic digital diaphragm system.

- a. Micropoint dye laser system: This system uses a pulsed UV nitrogen laser to pump a small dye chamber that emits a visible wavelength beam. Different dyes provide different wavelengths that can be adapted for different fluorophores. The primary dye we use is Courmarin 440 which produces light at 440 nm. This wavelength is sufficiently different from the normal excitation wavelength of GFP that a notch filter can be used to pass 440 nm light while still reflecting nearly all the excitation light from the mercury light source used for imaging. Attenuation elements on the Mosaic system are empirically adjusted to allow photobleaching of EGFP in 3 to 10 pulses to be delivered at a repetition rate of 15 – 30 Hz.
 - b. Mosaic digital diaphragm system: This system uses a digital diaphragm to control the spread of light from an argon ion laser that emits at 495 nm. Minimum duration of the photobleaching pulse is empirically determined. For imaging with this system we use a spinning disk confocal microscope coupled with a diode laser having an output of 473 nm. The difference between the two wavelengths allows design of the light path such that filter cubes in the microscope do not move in between photobleaching and observation.
6. Imaging systems: We use ORCA-ER cameras (Hamamatsu). We use two imaging software packages, Metamorph from Molecular devices and Slidebook from Intelligent Imaging Innovations.

3. Methods

3.1 Construction of EGFP-TopoII α plasmid

3.1.1 Construction of His-tagged TopoII α plasmid

1. To tag human TopoII α , we introduced the RGS-6xHis sequence (Qiagen) at *Kpn* I and *Bam*H I sites of pCDNA3 to make an N-terminal tag (pCDNA3-His). A Kozak consensus sequence is also placed in front of the RGS-6xHis tag to improve translation.
2. To facilitate the subcloning of TopoII α cDNA into pCDNA3-His, a *Bam*H I site is introduced at the N-terminus of TopoII α cDNA by PCR amplification, eliminating the first methionine (start codon) of TopoII α . Similarly, the restriction sites *Sac* II and *Not* I are introduced at the C-terminus after the stop codon.
3. Sequences of the PCR products are verified by DNA sequencing. The resultant plasmid is named pT14. It carries full length TopoII α cDNA in pCDNA3-His unidirectionally at *Bam*H I and *Not* I sites without the start TopoII α codon ATG.

3.1.2 Construction of EGFP-tagged TopoII α

1. pT14 is digested to completion with *Sac* II and digested partially with *Hind* III.
2. The full length TopoII α cDNA plus RGS-6xHis region is gel purified and ligated in frame to pEGFP-C3 at the *Hind* III and *Sac* II sites (Fig. 1). Although this construct contains an internal start codon (ATG) from the 6xHis tag, the internal translational product was never detected.

3.2 Transfection of LLC-Pk cells and selection of stable line

1. LLC-Pk cells are grown in 35 mm dishes in DMEM supplemented with 10% FBS and antibiotics until approximately 50% confluent.

2. To produce a stable cell line expressing EGFP-TopoII α , LLC-Pk cells are transfected with pT104 plasmid encoding EGFP-TopoII α at 1 μ g/ml using Lipofectamine Plus according to the manufacturer's instructions. Following 3 hours in transfection medium, complete culture medium is added and the cells are cultured for 24 hours.
3. Cells are examined by brief exposure to fluorescent light to assess the overall transfection efficiency. If efficiency is 25% or greater, selection of stable lines is performed.
4. To select for cell lines expressing stably integrated plasmid, cells are then transferred to selection medium containing 2 mg/ml G418 and, after they have reached 80% confluence passaged once into 100 mm tissue culture dishes and cultured for an additional three days under selection.
5. To isolate colonies, cells are trypsinized and plated at an average density of 1/well in 96 well dishes. A portion of the remaining cells are frozen and the rest are plated to 100 mm dishes as a backup should the cloning be unsuccessful.
6. To identify single cell colonies, 96 well dishes are observed with a tissue culture microscope and wells with single cells marked. These cultures are maintained until individual cells in wells grow to confluence. Cells are transferred to 12 well dishes. Each well contains a 12 mm sterile circular coverslip covering a portion of the well. When cells from individual have populated the wells of the dish, the coverslip is removed and cells are assessed for expression and proper localization of EGFP-TopoII α fluorescence (Fig. 2).
7. Wells containing colonies with proper expression of EGFP-TopoII α in interphase nuclei and mitotic chromosomes are grown to confluence, FACS sorted to confirm and enrich EGFP-TopoII α expressing cells and then transferred to larger dishes for expansion and freezing¹.

3.3 Imaging and photomanipulation

1. Cells are observed on a warm stage on an inverted microscope (e.g. Zeiss Axio Observer). When used with high numerical aperture water or oil immersion objectives, an objective heater is used.
2. Cells are imaged by fluorescence, phase contrast or DIC microscopy for targeting regions for photobleaching. To limit phototoxicity and photobleaching of EGFP, fluorescence exposures are minimized. Thus we generally target with phase contrast microscopy and capture a prebleach fluorescence image immediately before photobleaching.
3. When using the nitrogen laser-pumped dye laser (Micropoint, Photonic Instruments), we generally bleach by delivering 3–8 pulses at a repetition rate of 30 Hz. The laser is attenuated to the appropriate level with neutral density filters. Levels that cause photobleaching without cytotoxicity are determined empirically (Fig. 3).
4. When using pattern photobleaching with the digital light processor-modulated system (Mosaic, Photonic Instruments), we typically collect fluorescent images every 500 milliseconds prior to and after the photobleaching pulse. We create a photobleaching pattern template to target specific regions prior to data collection so that any delay during image acquisition due to the argon laser pulse is minimal. The template is drawn using a phase contrast or fluorescence image of the sample captured immediately before photobleaching. The photobleaching pulse duration of the argon laser is empirically determined, but generally ranges between 200 and 600 milliseconds (Fig. 4).

3.4 Data analysis

1. Metamorph and Slidebook software were used for data collection and analysis. An area that includes but is larger than the target area is used for subtraction of local cytoplasmic background using the procedures described Howell et al.²² according to the equation $F_S - F_{BG} = F_S - (F_L - F_S) \times A_S / (A_L - A_S)$ where F_S is the average fluorescence intensity within the target bleach zone, F_L is the average intensity in the larger background area, A_S is the area of the target bleach zone and A_L is the area of the larger background zone. This generates the final intensity minus the background $F_S - F_{BG}$.
2. Acquisitions are also normalized to adjust for reduction of total cellular fluorescence due to photobleaching and image acquisition according to the procedures of Phair and Misteli²³ using the equation $F_n = F_t \times T_0 / F_0 \times T_t$ where F_n is the normalized fluorescence intensity in the bleach zone, F_t is the average intensity in the bleach zone at time point t , F_0 is the average intensity in the target bleach zone before photobleaching, T_t is the total cellular intensity at time point t and T_0 is the total cellular intensity before photobleaching and long term imaging. Integrated intensities of the target and background regions and their associated area measurements are logged to Excel spreadsheets.
3. Analysis of photobleaching recovery half-times and percent recovery is carried out using the methodology described by Howell et al.²² using normalized relative intensity values. The equation $F_{inf} - F(t) = [F_{inf} - F(0)]e^{-kt}$ describes the perturbation-relaxation for fluorescence recovery after photobleaching where F_{inf} is the normalized intensity of the photobleached area after maximum recovery, $F(0)$ is the normalized intensity at $t = 0$ after photobleaching. Normalized relative fluorescence intensity values are exported to GraphPad Prism software and the rate constant k is determined by nonlinear regression.
4. Half time for recovery is calculated using $t_{1/2} = \ln 2/k$. Percent recovery is determined by the equation $F_{inf} - F(0) / F(p) - F(0)$ where $F(p)$ is the prebleach intensity at the target (Fig. 5).

4. Notes

1. Because overexpression of TopoII α is toxic, selection for EGFP-TopoII α expression also selects for down regulation of endogenous TopoII α expression.
2. A supplementary means for selection for lines expressing appropriate levels of EGFP-TopoII α is fluorescence-activated cell sorting (FACS). FACS is also useful if cells are cultured for many passages which sometimes leads to the development of non-expressing cells that retain G418-resistance.

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Fig. 1.

Production strategy used to create pT104 plasmid containing EGFP-TopoIIα for transfection to produce GT2-LPk cells. Primers used for amplifying the N- or C-terminal regions of TopoIIα by RT-PCR are shown as arrows. Restriction sites (*Eco* 0109I and *Eco*R I) are used for convenient cloning of PCR products. As indicated, both constructs use the same CMV promoter (Pcmv), but pT104 carries EGFP fused to TopoIIα. (Adapted from 24)

Fig. 2. EGFP-TopoII α localization in a panel of living GT2-LPK cells at various stages of mitosis. EGFP-TopoII α concentrates in the nucleus and particularly in the nucleoli of cells in interphase. It associates with chromosomes in prophase, becoming concentrated at kinetochores (closed arrows) and along chromosome arms in prometaphase and metaphase. During anaphase the kinetochore concentration decreases but EGFP-TopoII α remains and is associated with the chromosome arms. In late anaphase and telophase, EGFP-TopoII α remains associated with the decondensing chromosomes. Some TopoII α appears in the cytoplasm with a portion becoming concentrated in cytoplasmic foci (open arrow). Bars = 10 μ m. (Adapted from 5)

Fig. 3.

Recovery of EGFP–TopoII α fluorescence on kinetochores and chromosome arms after photobleaching occurs rapidly and is slowed by catalytic inhibitors of TopoII α enzyme activity. (A) Targeting of kinetochores (arrows) and chromosome arms at prophase using point photobleaching with the Micropoint dye laser. (B) The topoisomerase II inhibitor ICRF187 shows a general reduction in the extent of chromosome condensation in prophase nuclei. ICRF187 treatment greatly slows recovery after photobleaching. Images include a prebleach image, one taken just after photobleaching (0 time) and images taken during recovery. Time is depicted as minutes:seconds after photobleaching. Bar = 10 μ m. (Adapted from 5)

Fig. 4.

Recovery of EGFP-TopoII α fluorescence in interphase nuclei using pattern photobleaching with the Mosaic argon laser system. (A) Control cell nucleus shows rapid recovery of fluorescence after photobleaching (B) The topoisomerase II inhibitor ICRF159 causes a change in TopoII α distribution in interphase nuclei resulting in a more granular appearance and a relative decrease in concentration of TopoII α in the nucleolus. ICRF159 greatly slows recovery of photobleaching in the nucleus although recovery is somewhat more rapid within the nucleolus even in the presence of the drug. Images include a prebleach image, one taken just after photobleaching (0 time) and images taken during recovery. Time is depicted as minutes:seconds after photobleaching. Bar = 10 μ m.

Fig. 5. Data analysis of photobleaching and recovery of EGFP-TopoII α in metaphase chromosome arm. Images show a prebleach image and images taken at photobleaching and after for an isolated chromosome arm extending from the metaphase arrayed chromosomes. The graph shows the fitting of corrected intensity measurements in the photobleached region to determine recovery half times ($t_{1/2}$) and total degree of recovery (recovery). Bar = 10 μ m. (Adapted from 5)