DNA Cleavage and Religation by Human Topoisomerase IIα at High Temperature†

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ABSTRACT: A common DNA religation assay for topoisomerase II takes advantage of the fact that the enzyme can rejoin cleaved nucleic acids but cannot mediate DNA scission at suboptimal temperatures (either high or low). Although temperature-induced DNA religation assays have provided valuable mechanistic information for several type II enzymes, high-temperature shifts have not been examined for human topoisomerase IIα. Therefore, the effects of temperature on the DNA cleavage/religation activity of the enzyme were characterized. Human topoisomerase IIα undergoes two distinct transitions at high temperatures. The first transition occurs between 45 and 55 °C and is accompanied by a 6-fold increase in the level of DNA cleavage at 60 °C. It also leads to a loss of DNA strand passage activity, due primarily to an inability of ATP to convert the enzyme to a protein clamp. The enzyme alterations that accompany the first transition appear to be stable and do not revert at lower temperature. The second transition in human topoisomerase IIα occurs between 65 and 70 °C and correlates with a precipitous drop in the level of DNA scission. At 75 °C, cleavage falls well below amounts seen at 37 °C. This loss of DNA scission appears to result from a decrease in the forward rate of DNA cleavage rather than an increase in the religation rate. Finally, similar high-temperature alterations were observed for yeast topoisomerase II and human topoisomerase II*β*, suggesting that parallel heat-induced transitions may be widespread among type II topoisomerases.

Topoisomerase II is a ubiquitous enzyme that is required for a number of critical DNA processes, including replication, transcription, recombination, and chromosome segregation (*1*−*4*). To carry out its essential cellular functions, topoisomerase II creates a double-stranded break in the DNA backbone, passes an intact double helix through the nucleic acid gate, and religates the break (*3, 5*−*7*).

During its scission event, topoisomerase II forms covalent bonds with the newly cleaved DNA (*8*−*11*). The formation of this covalently joined enzyme-cleaved DNA intermediate (known as the cleavage complex) is critical, as it maintains chromosomal integrity throughout the catalytic cycle of the enzyme. However, the cleavage complex also has the potential to cause a number of deleterious physiological effects. If a DNA tracking system (such as a replication or transcription complex) attempts to traverse a topoisomerase II−DNA bridge, the resulting collision can generate permanent breaks in the genetic material (*12*−*15*). These permanent breaks are recombinagenic and often induce mutagenic events such as chromosomal insertions, deletions, or translocations (*16*−*18*). When their concentration overwhelms the cell, death pathways are triggered (*19*−*21*).

The potentially lethal nature of topoisomerase II has been exploited as a target for several clinically relevant anticancer drugs (*3, 4, 15, 22*−*26*). These agents kill cells by increasing the physiological concentration of enzyme−DNA cleavage complexes (*3, 4, 6, 15, 22*−*26*). While some drugs act by impairing the ability of the enzyme to religate cleaved DNA molecules, others have little effect on the religation reaction and presumably act by enhancing the forward rate of DNA scission (*3, 4, 27*−*32*).

Because of the importance of DNA cleavage and religation to the physiological and pharmacological functions of topoisomerase II, several studies have focused on separating these two interlinked enzyme activities. As a result, three distinct assays have been established that allow religation to be examined independently from cleavage (*33*−*35*).

The first assay that was developed uncouples the DNA cleavage/religation equilibrium of topoisomerase II by substituting Ca^{2+} in lieu of Mg^{2+} (*27, 33*). This system traps the enzyme−DNA cleavage complex in a kinetically competent form, making it possible to monitor a unidirectional religation reaction.

The second assay separates DNA cleavage and religation by positioning a nick or a single-strand/double-strand junction in close proximity to a cleavage site (*34, 36, 37*). Scission of either type of "suicide substrate" by topoisomerase II causes the enzyme to release a segment of the cleaved DNA, allowing ligation of the initial site to a separate acceptor molecule.

The third religation assay takes advantage of the fact that topoisomerase II can religate, but will not cleave DNA at

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suboptimal temperatures (*35*, *38*−*48*). Both high (50−65 °C) and low temperature (<4 °C) ranges have been used for this system.

The temperature-induced religation assay has worked well for a variety of type II topoisomerases, ranging from bacterial topoisomerase IV to mammalian enzymes (*35*, *38*−*48*). However, the shift to high temperature has not been explored for many type II enzymes, including human topoisomerase IIα. Therefore, the effects of high temperature on the DNA cleavage and religation activities of the human α isoform were characterized.

Results indicate that at temperatures commonly employed for religation assays with several type II enzymes (50−65 °C), levels of DNA scission mediated by the human enzyme rise ∼6-fold. This heat-induced transition in the enzyme requires both Mg^{2+} and DNA, is stable for several hours, and leads to a concomitant loss of DNA strand passage activity. Finally, temperature elevation can be used to induce religation with topoisomerase IIα, but in a higher range (70−75 °C) than reported for other species.

EXPERIMENTAL PROCEDURES

Human topoisomerase IIα was expressed in *Saccharomyces cerevisiae* (*10*) and purified by the protocol of Wasserman et al. (*49*) as modified by Kingma et al. (*50*). Negatively supercoiled pBR322 DNA was prepared as described (*51*). [γ-^{32}P]ATP was obtained from Amersham Pharmacia Biotech, etoposide was from Sigma, amsacrine was from Bristol-Myers Squibb, and CP-115,953 was from Pfizer Global Research. Etoposide and amsacrine were prepared as 10 mM stock solutions in 100% DMSO and stored at 4 °C. CP-115,-953 was stored as a 40 mM stock in 0.1 N NaOH at −20 °C and diluted to 8 mM with 10 mM Tris (pH 7.9) immediately prior to use. All other chemicals were analytical reagent grade.

DNA Cleavage. DNA cleavage reactions were carried out as described previously (*52*). Assays contained 220 nM human topoisomerase IIα and 5 nM negatively supercoiled pBR322 DNA in a total of 20 μL of reaction buffer (10 mM Tris-HCl, pH 7.9, 135 mM KCl, 5 mM $MgCl_2$, 0.1 mM NaEDTA, and 2.5% glycerol) in the absence or presence of 1 mM ATP. Reactions were started by the addition of topoisomerase IIα and incubated for 5 min at 37 or 60 °C (unless stated otherwise) to establish DNA cleavage/religation equilibria. Cleavage intermediates were trapped by adding 2 μL of 5% SDS followed by 1 μL of 375 mM NaEDTA, pH 8.0. Proteinase K was added (2 μL of 0.8 mg/mL), and reactions were incubated for 30 min at 45 °C to digest the type II enzyme. Samples were mixed with 2 μL of agarose gel loading buffer (30% sucrose, 0.5% bromophenol blue, 0.5% xylene cyanole FF, and 10 mM Tris-HCl, pH 7.9), heated at 45 °C for 2 min, and subjected to electrophoresis in a 1% agarose gel in TAE buffer (40 mM Tris−acetate, pH 8.3, 2 mM EDTA) containing 0.5 μg/mL ethidium bromide. Cleavage was monitored by the conversion of negatively supercoiled plasmid to linear molecules. DNA bands were visualized by UV light, photographed through Kodak 23A and 12 filters with Polaroid type 665 positive/negative film, and quantitated by scanning photographic negatives with an E-C apparatus model EC910 scanning densitometer in conjunction with Hoefer GS-370 Data System software. Alternatively, DNA bands were quantitated using an Alpha Innotech digital imaging system. In either case, the intensity of the bands was proportional to the amount of DNA present.

In reactions that determined whether DNA cleavage by topoisomerase IIα at high temperature was reversible, 1 M NaCl was added prior to treatment with SDS. To verify that cleaved DNA was protein-linked, proteinase K treatment was omitted. To determine whether the temperature-induced increase in enzyme-mediated DNA scission resulted from global alterations in DNA structure, negatively supercoiled pBR322 DNA was incubated at 60 °C for 5 min prior to the initiation of cleavage.

To examine the stability of human topoisomerase IIα at high temperature, the enzyme was incubated as described above at 60 °C for various times or in the absence of either pBR322 DNA or $MgCl_2$. Following the incubation period, enzyme activity was assessed by DNA cleavage assays (5 min at 60 °C) as described above. For samples that omitted the DNA substrate or divalent cation, assays were initiated by the addition of plasmid (5 nM final concentration) or $MgCl_2$ (5 mM final concentration), respectively.

Site-Specific DNA Cleavage. Topoisomerase II DNA cleavage sites were mapped as described by Burden et al. (*53*). A linear 564 bp *Eag*I/*Bam*HI fragment (residues 375−939) of pBR322 plasmid DNA was prepared and labeled with ^{32}P on the *Eag*I 5′ terminus. Cleavage reactions contained 1.4 nM (25 ng) labeled DNA substrate and 60 nM human topoisomerase IIα in 50 μL of reaction buffer. Assays were carried out in the absence of drugs, or in the presence of 20 μM etoposide, amsacrine, or CP-115,953. Reactions were started by the addition of topoisomerase IIα and incubated for 10 min at 37 or 60 °C. Cleavage intermediates were trapped by adding 5 μL of 10% SDS followed by 5 μL of 250 mM NaEDTA, pH 8.0. Topoisomerase IIα was digested with proteinase K (5 μL of 0.8 mg/mL) for 30 min at 45 °C. DNA products were precipitated twice in ethanol, dried, and resuspended in 40% formamide, 8.4 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanole FF. Samples were subjected to electrophoresis in a denaturing 8% polyacrylamide sequencing gel, which was then fixed in 10% methanol/10% acetic acid for 5 min and dried. DNA cleavage products were analyzed on a Molecular Dynamics PhosphorImager.

DNA Religation. DNA religation by human topoisomerase IIα was monitored by two distinct assays. DNA cleavage/religation equilibria were established at 37 or 60 °C as described above. In the first assay, religation was induced by the addition of 1 M NaCl (final concentration) (*54*). In the second, religation was initiated by shifting samples to 0 °C (*48*). In both cases, reactions were stopped by the addition of 2 μL of 5% SDS followed by 1 μL of 375 mM NaEDTA, pH 8.0. Samples were treated and analyzed as described for topoisomerase II cleavage reactions.

DNA Relaxation. DNA relaxation assays were based on the procedure of Fortune and Osheroff (*52*). Reactions contained 0.5−40 nM human topoisomerase IIα and 5 nM negatively supercoiled pBR322 DNA in a total of 20 μL of reaction buffer. After samples were heated at 37 or 60 °C for 5 min, reactions were started by the addition of ATP (1 mM final concentration). Assays were carried out for 15 min at 37 or 60 °C, respectively, and stopped by the addition of

3 μL of 0.77% SDS, 77 mM NaEDTA, pH 8.0. Samples were mixed with agarose gel loading buffer and subjected to electrophoresis in a 1% agarose gel in TBE buffer (100 mM Tris−borate, pH 8.3, 2 mM EDTA). DNA bands were stained with 1 μg/mL ethidium bromide, and visualized and analyzed as described above.

ATP Hydrolysis. ATP hydrolysis assays were performed as described by Fortune et al. (*55*). Assays contained 50 nM human topoisomerase IIα in 60 μL of reaction buffer and were carried out in the presence or absence of 40 nM negatively supercoiled pBR322 DNA. Reactions were incubated at 37 or 60 °C for 5 min and initiated by the addition of 1 mM [γ-^{32}P]ATP. Samples (2 μL) were removed at time intervals up to 16 min and spotted on poly(ethylenimine)-impregnated thin-layer cellulose chromatography plates (J. T. Baker). Plates were developed by chromatography in freshly made 400 mM NH_4HCO_3. ATP hydrolysis was monitored by quantitating (with a Molecular Dynamics PhosphorImager) radioactive areas corresponding to inorganic monophosphate released by the enzyme.

Topoisomerase IIα Clamp Closing Assay. Clamp closing assays were based on the procedure of Bjergbaek et al. (*56*). Assays contained 5 nM negatively supercoiled DNA and 100 nM human topoisomerase IIα in 20 μL of reaction buffer. Mixtures were incubated for 5 min at 60 °C, APP(NH)P (1 mM final concentration) was added, and reactions were further incubated for 5 min. For some samples, NaCl was added (1 M final concentration), and reactions were incubated for an additional 5 min. Sample volumes were brought to 100 μL with reaction buffer, and 1 volume of phenol (buffered with Tris to pH 7.9) was added. Samples were vortexed and then centrifuged at 14 000 rpm for 5 min. The aqueous phase was removed, precipitated with ethanol, and resuspended in 20 μL of H_2O. The combined phenol and phenol interphase (i.e., organic phase) was washed twice with 500 μL of reaction buffer. The material remaining in the organic phase was precipitated with ethanol and resuspended in 20 μL of H_2O. Resuspended samples were treated with 2 μL of 5% SDS and 2 μL of 0.8 mg/mL proteinase K, and incubated for 30 min at 45 °C to digest the topoisomerase IIα. Final samples were subjected to electrophoresis in a 1% agarose gel in TAE buffer as described above.

RESULTS

Effects of Temperature on DNA Cleavage Mediated by Human Topoisomerase IIα. A commonly used topoisomerase II-mediated DNA religation assay is based on the finding that the enzyme is unable to cut nucleic acids at suboptimal reaction temperatures (either high or low), but is still able to religate enzyme-associated cleaved molecules (*35, 38−48*). Although temperature-induced religation assays have provided valuable mechanistic information for type II enzymes ranging from bacterial topoisomerase IV to mammalian topoisomerase II (*35, 38−48*), high-temperature shifts have not been examined for several species, including human topoisomerase IIα. Therefore, the effects of temperature on the DNA cleavage and religation reactions of the human enzyme were examined.

As seen in Figure 1, topoisomerase IIα goes through two distinct transitions at high temperatures. The first transition, which occurs between 45 and 55 °C, was characterized by a marked increase in DNA scission. During this phase, levels of cleavage (at equilibrium) rose ~6-fold as compared to those observed at 37 °C (optimal temperature for catalytic DNA strand passage). The second transition, which occurs between 65 and 70 °C, was accompanied by a profound decrease in DNA scission. Cleavage dropped dramatically from maximal levels observed at 60 °C and fell well below initial baseline levels seen at 37 °C.

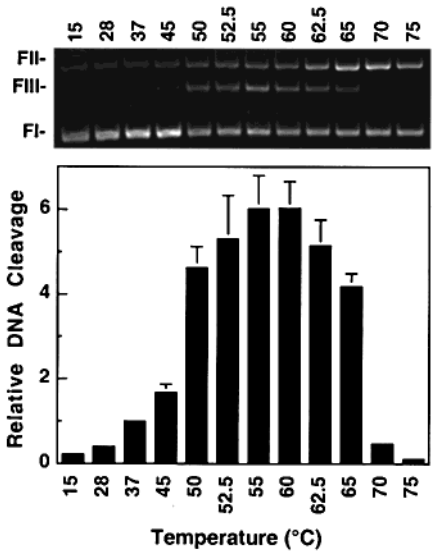

FIGURE 1: Temperature profile of DNA cleavage mediated by human topoisomerase IIα. (Top) An ethidium bromide-stained agarose gel of a typical DNA cleavage assay is shown. Double-stranded DNA cleavage converts negatively supercoiled plasmid (form I, FI) to linear molecules (form III, FIII). The position of nicked circular DNA (form II, FII) is shown for reference. (Bottom) Quantitation of DNA cleavage assays. Levels of DNA scission are relative to the amount of cleavage observed after 5 min at 37 °C (set to 1). Error bars represent standard deviations of three independent experiments.

Since reaction temperatures in the 50−65 °C range have been used to monitor DNA religation by several type II enzymes (*35, 38, 39, 41−43, 45−47*), the temperature profile for human topoisomerase IIα was unexpected. The high levels of DNA scission detected over the midrange (which plateaued at 55−60 °C) have not been reported for a type II topoisomerase. Consequently, the ability of human topoisomerase IIα to cleave DNA at 60 °C was characterized in greater detail.

Type II topoisomerases form covalent attachments with the cleaved DNA molecules that they generate during the scission event (*8−11*). Therefore, to confirm that the DNA scission observed at 60 °C was protein-linked, cleavage complexes trapped by treatment with SDS were not digested with proteinase K (Figure 2). Omission of the protease led to a marked decrease in the electrophoretic mobility of cleavage products, shifting them to the gel origin. This finding confirms that the DNA cleavage observed at 60 °C was mediated by topoisomerase IIα.

Since type II topoisomerases do not release their cleaved DNA products, scission can be reversed if the ionic strength is increased prior to trapping the cleavage complex (*9, 50, 54, 57, 58*). As seen in Figure 2, DNA cleavage products generated at 60 °C were religated following treatment with 1 M NaCl. These data provide additional evidence that DNA scission observed at high temperature is mediated by human

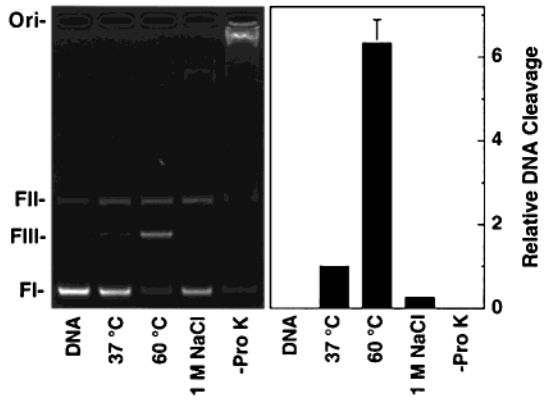

FIGURE 2: DNA scission mediated by human topoisomerase IIα at 60 °C is protein-linked and reversible. (Left) An ethidium bromide-stained agarose gel is shown. A DNA control (DNA) as well as cleavage products generated at 37 and 60 °C are shown. Reversibility of the cleavage reaction was demonstrated by adding salt (1 M NaCl) prior to SDS treatment. To confirm that DNA cleavage at 60 °C was protein-linked, proteinase K treatment was omitted (−Pro K). The gel origin (Ori) and the positions of DNA species are indicated as in Figure 1. (Right) Quantitation of DNA cleavage assays. Levels of DNA scission are relative to the amount of cleavage observed after 5 min at 37 °C (set to 1). The standard deviations of three independent assays are indicated by error bars.

topoisomerase IIα. Moreover, they demonstrate that cleavage is not generated by an abortive scission reaction, since the enzyme still retains religation activity at 60 °C.

Basis for the Increased DNA Cleavage Mediated by Human Topoisomerase IIα at 60 °C. A number of experiments were carried out to determine the underlying basis for the enhanced DNA scission mediated by the human enzyme at 60 °C. First, the increase in cleavage may be due to a temperature-induced alteration in the global structure of the DNA plasmid substrate. This is unlikely, considering that the initial A/T melting transition in pBR322 is not observed until well above 70 °C (*59*). However, to address this issue, a time course for enzyme-mediated DNA cleavage was monitored using plasmid that had or had not been incubated for 5 min at 60 °C prior to the start of assays (Figure 3). If the increase in scission at 60 °C resulted from a transition in the plasmid, it would be expected that cleavage levels would increase more rapidly in reactions that contained preheated DNA. Results indicate that this is not the case. The time course for cleavage induction was unaffected by preheating the plasmid at 60 °C (Figure 3). In either case, DNA cleavage rose rapidly during the first minute after the addition of topoisomerase IIα, and maximal levels were observed by 5 min. Since the increased DNA cleavage is not due to a change in the plasmid, it presumably results from a heat-induced alteration in topoisomerase IIα.

Second, the increase in cleavage at 60 °C may result from a change in the DNA cleavage site-specificity of topoisomerase IIα. To address this possibility, DNA cleavage sites in a fragment of pBR322 were mapped at 37 and 60 °C (Figure 4). The site-specificity of the enzyme was similar at both temperatures. However, levels of cleavage at any given site generally were higher at 60 °C.

In addition, the effects of several anticancer agents on topoisomerase II-mediated DNA scission were determined at the two temperatures (Figure 4). Drugs enhanced DNA scission by the human type II enzyme at 60 °C, albeit to a

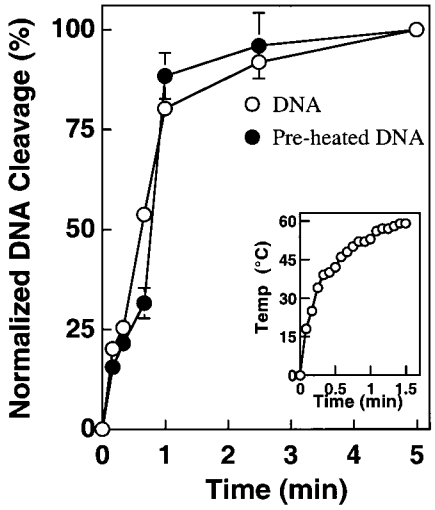

FIGURE 3: Increased DNA cleavage at 60 °C is not due to global alterations in the structure of DNA. A time course for the induction of DNA cleavage by topoisomerase IIα is shown. Cleavage assays employed negatively supercoiled pBR322 DNA that had (●) or had not (○) been heated for 5 min at 60 °C prior to the addition of enzyme. The inset shows the time required for the temperature of reaction mixtures to rise from 0 to 60 °C. Levels of DNA scission observed after 5 min cleavage reactions at 60 °C were normalized to 100%. The standard deviations of three independent assays are indicated by error bars.

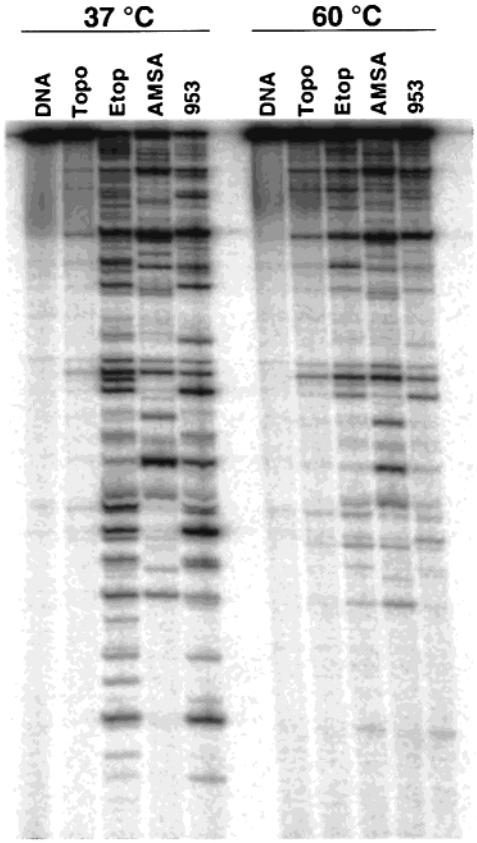

FIGURE 4: Sites of DNA cleavage mediated by human topoisomerase IIα are similar at 37 and 60 °C. Assays containing human enzyme and an end-labeled linear 564 bp substrate were incubated at 37 or 60 °C in the absence (Topo) or presence of 20 μM etoposide (Etop), amsacrine (AMSA), or the quinolone CP-115,953 (953). A DNA standard is shown as a control (DNA).

lesser extent than observed at 37 °C. As above, a similar site-specificity was seen at both temperatures. Taken together,

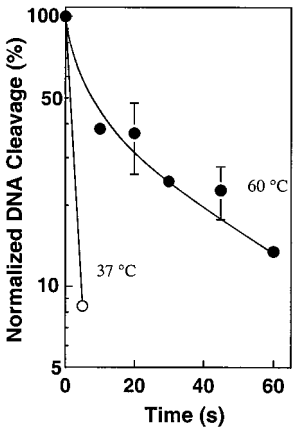

FIGURE 5: DNA religation mediated by human topoisomerase IIα at high temperature. DNA cleavage/religation equilibria were established at 37 (○) or 60 °C (●). Religation was initiated by adding 1 M NaCl (final concentration). The amount of DNA cleavage observed at equilibrium at each temperature was set to 100% at time = 0. Error bars represent standard errors of the mean for two independent assays.

these results indicate that the enhancement of DNA cleavage generated at 60 °C is not due to an alteration in the site-specificity of topoisomerase IIα.

Third, the higher levels of DNA scission observed at 60 °C may reflect a diminished ability to religate cleaved plasmid molecules. Consequently, the ability of human topoisomerase IIα to rejoin DNA at 37 or 60 °C was examined (Figure 5). In these assays, religation of DNA cleavage complexes formed at 37 or 60 °C, respectively, was induced by the addition of 1 M NaCl. The rate of DNA religation at the lower temperature was so rapid that it was difficult to measure an accurate rate. However, >90% of the cleaved plasmids were rejoined by 5 s. In contrast, it took ~1 min to reach a similar level of religation at 60 °C. This finding suggests that the higher levels of cleavage observed at 60 °C result primarily from an impaired ability of the enzyme to religate DNA molecules.

Stability of Human Topoisomerase IIα at 60 °C. To determine the requirements for topoisomerase IIα stability at high temperature, the enzyme was incubated at 60 °C under a variety of conditions prior to monitoring activity by cleavage assays (Figure 6). The human enzyme was stable only when it was incubated in the presence of both plasmid and a divalent cation. Either Mg^{2+} (Figure 6) or Ca^{2+} (data not shown) could be employed for this purpose. When all three components were present, the half-life of topoisomerase IIα at 60 °C was ~44 min. If the DNA or the divalent cation was omitted from incubation mixtures, the half-life of the enzyme dropped to ~0.4 or ~1 min, respectively. Finally, addition of ATP prior to the start of assays, either in the presence or in the absence of DNA, had no effect on the enzyme half-life (data not shown). Thus, the nucleoside triphosphate does not contribute to the stabilization of topoisomerase IIα at high temperature.

Reversion of the First High-Temperature Transition. The alteration in topoisomerase IIα that leads to DNA cleavage enhancement occurs rapidly at 60 °C (see Figure 3). However, it is not known whether this alteration is permanent or reverts upon exposure to temperatures below the first transition point. Two experiments were carried out to distinguish between these possibilities.

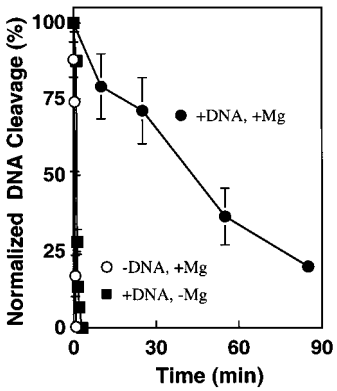

FIGURE 6: Requirements for the stabilization of human topoisomerase IIα at high temperature. Topoisomerase II was incubated at 60 °C in the presence of negatively supercoiled pBR322 DNA and Mg^{2+} (●) or in the absence of either plasmid (○) or divalent cation (■). At the indicated times, DNA or Mg^{2+} was added (as necessary), and a 5 min DNA cleavage assay was carried out at 60 °C. The amount of DNA scission observed without preincubation was set to 100% at time = 0. Error bars represent standard errors of the mean for two independent assays.

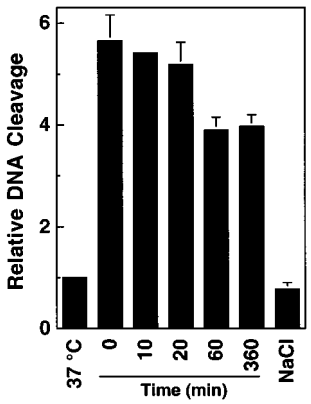

FIGURE 7: Reversion of the first high-temperature transition. After establishing a DNA cleavage/religation equilibrium at 60 °C, samples were shifted to 37 °C for the times shown. To confirm that topoisomerase II was still active after the 360 min shift to 37 °C, 1 M NaCl (NaCl) was added. A DNA cleavage assay carried out at 37 °C is shown as a control. Levels of DNA cleavage are relative to the amount of cleavage observed after 5 min at 37 °C (set to 1). The standard deviations of three independent assays are indicated by error bars.

First, following a 5 min incubation at 60 °C, assay mixtures were shifted to 37 °C (Figure 7). Even after 360 min at the lower temperature, cleavage remained high and did not revert to initial baseline levels. To ensure that topoisomerase IIα was active after the long incubation period, 1 M NaCl was added to samples after the 360 min shift to 37 °C. The majority of cleaved plasmids were religated by this process, indicating that the enzyme retained activity over the course of the experiment.

Second, all type II topoisomerases examined to date will religate, but will not cleave DNA at 0 °C (*32*, *35*, *40*, *44*, *48*). Thus, shifting preexisting cleavage complexes to 0 °C results in a unidirectional religation of cleaved DNA molecules. This religation assay was used to further probe the stability of the high-temperature alteration in human topoisomerase IIα. The rationale underlying the experiment is as follows: the rate of salt-induced religation at 60 °C was considerably slower than that observed at 37 °C (see Figure 5). Therefore, if the high-temperature alteration in

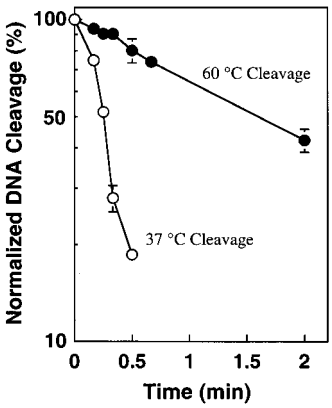

FIGURE 8: Cold-induced DNA religation mediated by human topoisomerase IIα. DNA cleavage/religation equilibria were established at 37 (○) and 60 °C (●), and samples were shifted to 0 °C to initiate religation. The amount of DNA cleavage observed at equilibrium at each temperature was set to 100% at time = 0. Error bars represent standard errors of the mean for two independent assays.

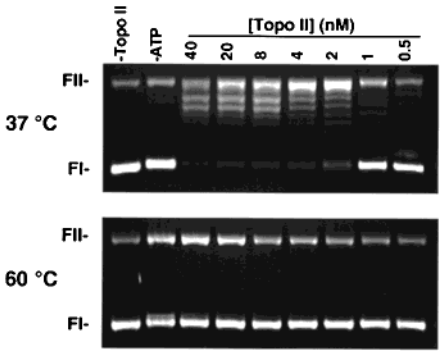

FIGURE 9: Effects of temperature on DNA relaxation catalyzed by human topoisomerase IIα. Ethidium bromide-stained agarose gels are shown. DNA relaxation was examined at 37 (top) or 60 °C (bottom). Control reactions were carried out in the absence of enzyme (−Topo II) or ATP (−ATP). The relative mobilities of negatively supercoiled plasmid DNA (form I, FI) and nicked circular plasmid (form II, FII) are shown. Relaxed topoisomers migrate with a mobility intermediate to that of the FI and FII DNA species.

the enzyme is stable, rates of religation at 0 °C for DNA cleavage complexes formed at 60 °C should remain slower than for those formed at 37 °C. As seen in Figure 8, the rate of DNA religation (at 0 °C) that followed cleavage at 60 °C was ~6-fold slower than that observed for plasmid cleaved at 37 °C.

Taken together, these results indicate that the alteration in topoisomerase IIα induced at 60 °C is long-lived and does not readily revert at lower temperatures.

Catalytic Activity of Human Topoisomerase IIα at 60 °C. To further characterize the effects of high temperature on human topoisomerase IIα, the ability of the enzyme to carry out its overall catalytic reaction at 60 °C was assessed. In these experiments, topoisomerase IIα was incubated at 37 or 60 °C for 5 min with all reaction components except for ATP prior to the start of assays.

In the initial experiment, the ability of the enzyme to relax negatively supercoiled plasmid DNA at 60 °C was determined. In contrast to reactions carried out at 37 °C (the optimal temperature for overall catalytic activity), no relaxation was observed at 60 °C (Figure 9). Even at enzyme concentrations that were 40-fold higher than that of the

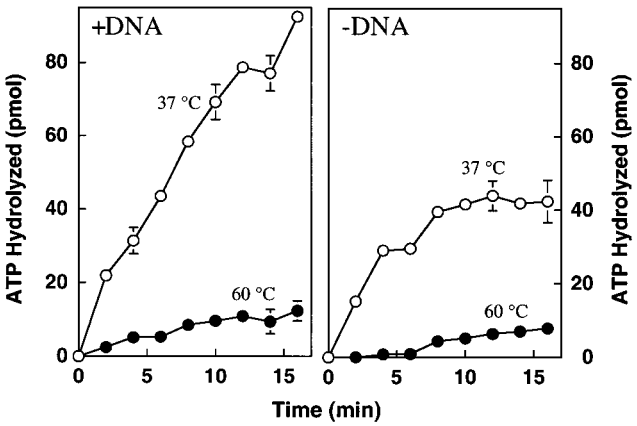

FIGURE 10: Effects of temperature on ATP hydrolysis catalyzed by human topoisomerase IIα. ATP hydrolysis was monitored by the release of free phosphate from [γ-^{32}P]ATP. Reactions were carried out at 37 (○) or 60 °C (●) in the presence (left) or absence (right) of DNA. Error bars represent standard errors of the mean for two independent assays.

plasmid, no relaxed DNA topoisomers were observed (data not shown). Since the latter conditions do not require the enzyme to recycle after a round of strand passage, these results indicate that topoisomerase IIα loses its ability to catalyze the double-stranded DNA passage event at high temperature.

In addition, heat-treated topoisomerase IIα did not regain its DNA relaxation activity after a further 6 h incubation at 37 °C (data not shown). This result provides further evidence that the temperature-induced transition in the enzyme is stable.

The DNA strand passage activity of type II topoisomerases requires the two catalytic centers of the enzyme to work in concert (*3*, *6*, *7*, *56*, *60*, *61*). While one center cleaves the DNA, the second interacts with ATP. The interaction with ATP triggers a conformational change in topoisomerase II that opens the DNA gate, converts the enzyme into a protein clamp (which becomes topologically linked to its nucleic acid substrate), and induces the strand passage event (*3*, *6*, *62*−*68*). Since levels of DNA cleavage by human topoisomerase IIα are remarkably high at 60 °C, the lack of strand passage may result from a deficiency either in the ATP catalytic center or in the ability of the two centers to coordinate their actions.

As a first step toward understanding the lack of strand passage activity at high temperature, the ability of topoisomerase IIα to hydrolyze ATP at 37 and 60 °C was compared (Figure 10). Samples were incubated at the appropriate temperature for 5 min prior to starting the hydrolysis reaction by the addition of the nucleoside triphosphate. Over the course of assays, no hydrolysis was observed in the absence of enzyme. Topoisomerase IIα hydrolyzed ATP at the high temperature, and the reaction was stimulated by the presence of DNA. However, rates of hydrolysis were significantly (~8−10-fold) slower at 60 °C than at 37 °C.

While these findings demonstrate that high temperature impairs the ATPase activity of the human enzyme, it is not clear that the slow rates of hydrolysis can explain the total absence of strand passage at 60 °C. Therefore, the ability of APP(NH)P (a nonhydrolyzable ATP analogue) to induce protein clamp closing at high temperature was determined.

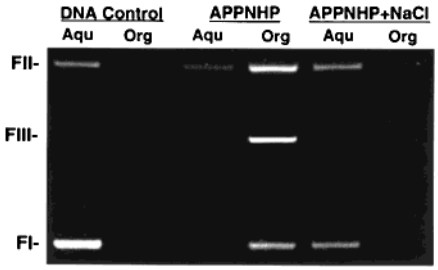

FIGURE 11: The protein clamp of human topoisomerase IIα cannot close at 60 °C. An ethidium bromide-stained agarose gel is shown. Reactions containing the enzyme and negatively supercoiled pBR322 DNA were incubated for 5 min at 60 °C. The nonhydrolyzable ATP analogue APP(NH)P was then added, and mixtures were incubated further for 5 min. In some samples, 1 M NaCl was added following the ATP analogue (APPNHP+NaCl). Mixtures were extracted in buffered phenol, and both the aqueous (Aqu) and organic (Org) phases were precipitated in ethanol and subjected to electrophoresis. A DNA control is shown for reference. The positions of DNA species are as in Figure 1.

The clamp closing assay is based on the following: upon treatment of topoisomerase II−DNA mixtures with buffered phenol, free DNA segregates into the aqueous phase, while protein-bound plasmids are extracted into the organic phase (*56*). Plasmid molecules that are peripherally bound to topoisomerase II can be distinguished from those that are topologically linked to the enzyme (as a result of clamp closure) by the inclusion of 1 M NaCl prior to extraction (*63*). While peripherally bound DNA is released by the enzyme and segregates into the aqueous phase, topologically linked molecules remain complexed with topoisomerase II and are extracted with the enzyme into the organic phase.

Results of a 60 °C clamp closing assay for human topoisomerase IIα are shown in Figure 11. In the presence of APP(NH)P, the majority of the plasmid substrate (both cleaved and noncleaved molecules) was extracted into the organic phase. However, when high salt was added prior to phenol extraction, virtually all of the plasmid segregated into the aqueous phase. These findings demonstrate that topoisomerase IIα binds DNA at high temperature, but is incapable of ATP-induced clamp closing. Thus, the DNA cleavage and ATP catalytic centers do not act in concert at 60 °C. This lack of coordination appears to be the mechanistic basis for the lack of DNA strand passage at high temperature.

DNA Religation at 75 °C. Many type II topoisomerases (ranging from bacterial to mammalian) can be induced to religate DNA when reaction conditions are shifted from optimal catalytic temperatures (25−37 °C) to high temperatures (50−65 °C) (*35*, *38*, *39*, *41*−*43*, *45*−*47*). Although human topoisomerase IIα displays a large increase in DNA scission (compared to 37 °C) in this elevated temperature range, cleavage drops precipitously at 75 °C (see Figure 1). Therefore, the human enzyme may undergo this same induction of religation, only at a higher temperature than reported for other species.

To address this point, DNA cleavage complexes formed between human topoisomerase IIα and pBR322 at 37 °C were shifted to 75 °C (Figure 12). As the temperature in reaction mixtures rose to 60 °C (which took ∼0.5 min), cleavage levels rose markedly. This cleavage enhancement corresponds to the first heat-induced transition in the enzyme (see Figure 1). However, as the temperature surpassed the

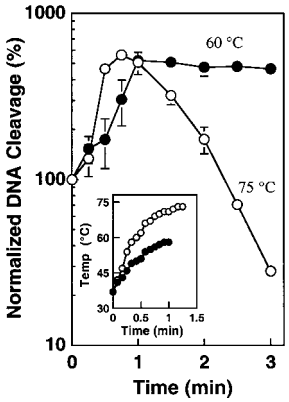

FIGURE 12: DNA religation mediated by human topoisomerase IIα at 75 °C. A DNA cleavage/religation equilibrium was established at 37 °C, and DNA religation was initiated by shifting reaction mixtures to 75 °C (○). As a negative control for religation, mixtures were shifted from 37 to 60 °C (●). The inset shows the time required for the temperature of reaction mixtures to rise from 37 to 60 (●) or 75 °C (○). The amount of DNA cleavage observed at equilibrium at 37 °C was set to 100% at time = 0. Error bars represent standard errors of the mean for two independent assays.

70 °C threshold (at ∼1 min), significant DNA religation was observed. Over the following 2 min, ∼95% of the cleaved DNA molecules that were generated following the first transition were religated.

As a control, the experiment was repeated with a shift from 37 to 60 °C. Once again, DNA scission rose dramatically as temperatures approached 60 °C (which took ∼1 min). Maximal cleavage levels were similar to those observed during the 75 °C shift, but no religation was observed over the time course of the assay. These data support the conclusion that human topoisomerase IIα undergoes two distinct transitions at high temperatures. Furthermore, they suggest that the human enzyme can be induced to religate cleaved DNA molecules at elevated temperatures, but at a higher range than reported for other type II topoisomerases.

Effects of Temperature on DNA Cleavage Mediated by Yeast Topoisomerase II and Human Topoisomerase IIβ. The high-temperature transitions described for human topoisomerase IIα have not been reported for other type II enzymes. Therefore, the effects of temperature on the DNA cleavage activities of yeast topoisomerase II and human topoisomerase IIβ were analyzed.

As seen in Figure 13, the yeast enzyme and the human β isoform displayed temperatures profile that paralleled that of human topoisomerase IIα (see Figure 1). Both showed a significant rise (∼5-fold) in DNA scission that peaked in the 50−60 °C range. This increase was followed by a dramatic drop in cleavage at 65−70 °C. These results demonstrate that other type II topoisomerases display the high-temperature transitions observed for human topoisomerase IIα, and suggest that they may be common among type II enzymes.

DISCUSSION

Previous studies have utilized temperature shifts to characterize the DNA cleavage and religation reactions of several type II topoisomerases (*35*, *38*−*48*). However, the effects of high temperature on human topoisomerase IIα have not been explored. Therefore, the present study examined DNA

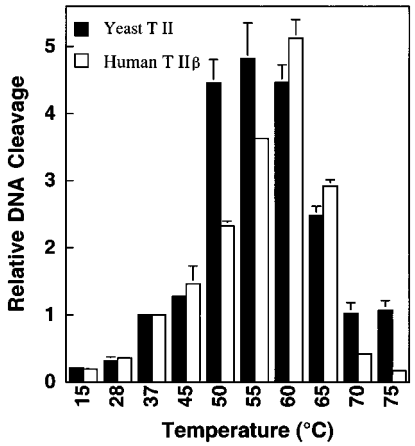

FIGURE 13: Effects of temperature on DNA cleavage mediated by yeast topoisomerase II (solid bars) and human topoisomerase IIβ (open bars). Levels of DNA scission are relative to the amount of cleavage seen after 5 min at 37 °C (set to 1). Error bars represent standard errors of the mean for two independent assays.

cleavage and religation mediated by the human enzyme over the temperature range of 37−75 °C.

Results indicate that topoisomerase IIα undergoes two distinct transitions at high temperatures. The first transition occurs between 45 and 55 °C and correlates with a large increase (~6-fold) in the level of enzyme-mediated DNA cleavage. It also leads to a loss of DNA strand passage activity, due at least in part to the inability of ATP to convert the enzyme into a protein clamp. The alterations in the enzyme that accompany this first transition appear to be stable and do not revert even after several hours at lower temperature.

The high levels of DNA scission that accompany the first heat-induced transition correlate with a decreased ability of topoisomerase IIα to religate cleaved DNA. The specific spatial alterations in the enzyme that impair DNA religation are not known. Decreased religation may reflect heat-induced conformational changes in the vicinity of the cleavage/religation catalytic center. Alternatively, it may result from changes in the ATPase domain of the enzyme. This latter possibility is supported by two findings. First, ATP hydrolysis by human topoisomerase IIα is impaired at 60 °C. Second, specific mutations in the ATPase domain of human topoisomerase IIα (*69*) and bacterial topoisomerase IV (*70*) that perturb ATP interactions lead to higher levels of DNA cleavage and decreased rates of religation.

The second heat-induced transition in human topoisomerase IIα occurs between 65 and 70 °C and correlates with a precipitous drop in levels of DNA scission. At 75 °C, cleavage is well below baseline amounts seen at 37 °C. We propose that the decrease in DNA scission is caused by a drop in the forward rate of DNA cleavage rather than a reinitiation of high rates of religation. This conclusion is based on the fact that the rate of temperature-induced DNA religation at 75 °C is comparable to that of salt-induced religation at 60 °C. While it is not known whether religation rates measured by these different systems are completely equivalent, clearly the results of the two assays do not differ dramatically.

The high-temperature alterations in the activity of human topoisomerase IIα are not limited to this species. They also are observed for yeast topoisomerase II and human topoisomerase IIβ. This finding indicates that similar temperature-induced transitions may be widespread among type II topoisomerases and suggests that they may be useful tools for dissecting enzyme function.

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