Incomplete reversion of double stranded DNA cleavage mediated by *Drosophila* topoisomerase II: formation of single stranded DNA cleavage complex in the presence of an anti-tumor drug VM26

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ABSTRACT

Anti-tumor drug VM26 greatly stimulates topoisomerase II mediated DNA cleavage by stabilizing the cleavable complex. Addition of a strong detergent such as SDS to the cleavable complex induces the double stranded DNA cleavage. We demonstrate here that heat treatment can reverse the double stranded DNA cleavage; however, topoisomerase II remains bound to DNA even in the presence of SDS. This reversed complex has been shown to contain single strand DNA breaks with topoisomerase II covalently linked to the nicked DNA. Chelation of Mg^{++} by EDTA and the addition of salt to a high concentration also reverse the double strand DNA cleavage, and like heat reversion, topoisomerase II remains bound to DNA through single strand DNA break. The reversion complex can be analyzed and isolated by CsCl density gradient centrifugation. We have detected multiple discrete bands from such a gradient, corresponding to protein/DNA complexes with 1, 2, 3, topoisomerase II molecules bound per DNA molecule. Analysis of topoisomerase II/DNA complexes isolated from the CsCl gradient indicates that there are single stranded DNA breaks associated with the CsCl stable complexes. Therefore, topoisomerase II/DNA complex formed in the presence of VM26 cannot be completely reversed to yield free DNA and enzyme. We discuss the possible significance of this finding to the mechanism of action of VM26 in the topoisomerase II reactions.

INTRODUCTION

DNA topoisomerases play an important role in modulating the structure of DNA in cells. Type II topoisomerases interconvert DNA topoisomers by breaking and rejoining both strands of duplex DNA in a more or less concerted manner, and passing a segment of DNA through the transient breaks (1–3). Several reactions are common to the eukaryotic topoisomerase II, such as relaxation of DNA supercoils, catenation/decatenation, and knotting/un knotting of circular DNA molecules. Knowledge of

the biological roles of eukaryotic topoisomerase II comes primarily from genetic studies in yeasts. Topoisomerase II functions in segregating the intertwined daughter chromosomes at the end of DNA replication (4–6), and it may play a role in the condensation of replicated chromosomes at the beginning of mitosis (7). Either topoisomerase I or topoisomerase II is required to relieve the superhelical tension generated during transcription or replication (4, 8–10). In addition, topoisomerase II is implicated as a major component in the nuclear matrix or scaffold structure (11–13).

The suggested mechanism of DNA topoisomerase II, passage of DNA through a reversible double strand break, is strongly supported by the topoisomerase-mediated DNA cleavage reaction. The addition of a potent protein denaturant like sodium dodecyl sulfate (SDS) or strong alkali to a reaction mixture containing topoisomerase II and DNA results in double strand DNA breaks. The DNA cleavage is generated from a putative topoisomerase II/DNA complex termed 'cleavable complex' which may be an intermediate in the strand passage reaction pathway. Detailed structural analysis of the cleavage product revealed that the breaks on the complementary DNA strands are staggered by 4 nucleotides and each subunit of topoisomerase II is covalently joined to the protruding 5' phosphoryl ends at the break (14–16). The double stranded DNA cleavage can be reversed by salt or EDTA before the addition of protein denaturants (15, 16). The salt reversibility of the cleavage reaction suggests that the cleavable complex is in equilibrium with other topoisomerase II/DNA complexes, and topoisomerase II in the cleavable complex can dissociate from DNA after the reversion treatment. The reversibility of the topoisomerase II mediated DNA cleavage is one of the hallmarks of topoisomerase II reactions, which distinguish topoisomerase II mediated DNA cleavage from those catalyzed by nucleases.

Topoisomerase II has been shown to be the primary target of some anti-tumor drugs (3, 17, 18). The cytotoxicity of these drugs is likely due to the chromosomal DNA breakage. Many *in vivo* and *in vitro* studies have demonstrated that these anti-tumor drugs act by stimulating topoisomerase II mediated DNA cleavage. It has been hypothesized that they stimulate topoisomerase II

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mediated DNA cleavage by stabilizing the cleavable complex (19, 20). The double stranded DNA cleavage mediated by mammalian topoisomerase II in the presence of drugs has been shown to be reversible with respect to salt treatment (20) and heat treatment (21). However, the cytotoxic effects of some of the topoisomerase II-targeting drugs like VM26 (teniposide or 4'-demethylepipodophyllotoxin ethylidene- β -D-glucoside), are noticeably irreversible. For instance, the exposure of human lymphoid cells to VM26 inhibits traversal of the cell cycle and the treated cells that are reincubated in the drug-free medium cannot resume growth (22). During our studies of the interactions between *Drosophila* topoisomerase II and DNA, we investigated if VM26 stimulated double strand DNA cleavage by topoisomerase II could be reversed by salt or heating. We show in this report that the double strand DNA cleavage generated from the cleavable complex can be reversed, but a fraction of the cleavable complexes is converted into another form of cleavable complex in which only one of the topoisomerase subunits can cleave the phosphodiester DNA backbone bond. We discuss the possible significance of this finding to the mechanism of action of VM26 on topoisomerase reaction and its relevance to the cytotoxic action of VM26.

MATERIALS AND METHODS

Enzymes and substrates

The preparation and sources of enzymes and DNA substrates were described previously (23, 24). Radiolabeled DNA fragments were prepared from either pBR322 or 6/122b plasmid DNAs. 6/122b is a pBR322 derivative containing the *Drosophila* heat shock intergenic region. DNA fragments were dephosphorylated with calf intestine alkaline phosphatase, and 5'-ends were labeled with T4 polynucleotide kinase and γ - 32 P-ATP. To prepare labeled negatively supercoiled circular DNA, it was cyclized by the action of T4 DNA ligase in the presence of 6 μ g/ml ethidium bromide. DNAs were separated and isolated from an agarose gel and purified through an NACS-52 column (Gibco-BRL, Gaithersburg, MD) according to the manufacturer's recommended procedures. The calf thymus type II DNA topoisomerase was purified according to the published procedure (25) and was a generous gift from Dr. Leroy Liu.

Double strand DNA cleavage reaction and its reversion

The reaction was performed in 20 μ l of solution containing 10 mM Tris-HCl pH 7.9, 10 mM MgCl₂, 50 mM KCl, 1.25 mM ATP, 50 μ g/ml BSA, 100 μ g/ml VM26, 0.21 nM 1.6 Kb pBR322 *Hinf* I digested linear DNA fragment, and 2.5 nM *Drosophila* topoisomerase II. The reaction mixture was incubated at 30°C for 10 minutes and terminated by adding SDS to 1% and proteinase K to 100 μ g/ml. Incubation was continued at 45°C for 30 minutes. To reverse the double stranded DNA cleavage, the reaction mixture was heated at 65°C for 10 minutes after the addition of 50 mM dithiothreitol, then followed by SDS and proteinase K treatment. The reversion could also be brought about by adding EDTA and NaCl to the reaction mixture to give a final concentration of 12 mM and 0.5 M, respectively and the incubation was continued at 30°C for 10 minutes before the treatment with SDS and proteinase K. For analyzing the topoisomerase II/DNA complexes, samples that were not treated with proteinase were also directly loaded onto an agarose gel containing 0.1% SDS.

CsCl density gradient equilibrium centrifugation

The binding reaction was similar to that used in the cleavage reaction and the reaction volume was scaled up to 1 ml. The molar ratio of enzyme to DNA was increased to 24 (12 nM enzyme/0.5 nM DNA molecules). At the end of the incubation, the binding reaction was terminated by the addition of TE buffer (10 mM Tris-HCl pH 7.9, 0.1 mM EDTA) to a final volume of 1.8 ml. 40 μ l of 0.5 M EDTA, 0.1 ml of 10 mg/ml BSA, and 2.8 ml of a stock solution of saturated CsCl were then added to give a solution with a density of 1.54 g/ml. The CsCl containing solution was spun at 42 k rpm (164,000 g) at 20°C for 44 h. To minimize the adsorption of enzyme/DNA complexes to the centrifuge tubes, it was necessary to coat the centrifuge tube with 0.2 mg/ml BSA for several hours before use. Fractions of about 100 μ l were collected from the bottom of the tube. 10 μ l of each fraction was used to measure the radioactivity by liquid scintillation counting. The compositional densities of the collected fractions were determined by first measuring 10 μ l of sample in a graduated micropipet (Accupette) and then determining its weight with an analytical balance (Mettler AE163) to a precision of 0.01 mg. For the analysis of DNA and complexes isolated from the CsCl gradient, gradient fractions were diluted with 10 volumes of TE buffer before proteinase K digestion or treatment with various protein denaturing conditions.

RESULTS

A fraction of the topoisomerase II remains bound to DNA after the reversion of cleavage reaction by either heat or salt treatment

Several clinically important anti-tumor drugs have been shown to target at topoisomerase II (3, 17, 18). These anti-tumor drugs can stimulate topoisomerase II mediated DNA strand cleavage. The drug induced enhancement of DNA cleavage observed *in vitro* has provided a biochemical basis for the mechanism of how the drugs cause cell death and chromosomal rearrangement and fragmentation. DNA cleavage reaction stimulated by the anti-tumor drugs can be reversed by either salt or heat treatment (20, 21). During our studies of topoisomerase II cleavage reaction and its reversion, we made an observation indicating that after the reversion of topoisomerase II cleavage by either salt or heat treatment, a significant amount of DNA is still linked with topoisomerase II. In one of these experiments (shown in Fig. 1), topoisomerase II/DNA complexes were formed in the presence of VM26 at 30°C, followed by the addition of a strong detergent SDS to induce double stranded DNA breaks within the cleavable complex. The DNA products were analyzed by electrophoresis in an agarose gel containing SDS. The DNA cleavable complex was clearly demonstrated in this gel system by the generation of smaller DNA fragments, and the protease treatment produced even smaller DNA fragments spreading toward the high-mobility end of the gel (Fig. 1, lanes 2 and 5). The reversion of the cleavage reaction could be readily monitored by this gel electrophoresis system. Heating the reaction mixture at 65°C for 10 min. prior to the addition of SDS resulted in the disappearance of cleavage complex (Fig. 1, lane 3) and the religation of small DNA fragments into the full-length DNA (Fig. 1, lane 6). In the absence of protease treatment, heating step also converted the heterogeneous labeled DNA fragments of the cleavage complexes into a ladder of discrete bands (Fig. 1, lane 3). Proteolytic digestion converted the ladder of the bands into a

Figure 1. Heat reversion of the topoisomerase II/linear DNA cleavable complex. A 1.6 Kb linear DNA fragment from pBR322 *Hinf*I digestion was 5' end labeled by γ - 32 P-ATP with polynucleotide kinase. A standard 20 μ l binding reaction mixture contained 10 mM Tris-HCl, pH 7.9, 50 mM KCl, 10 mM MgCl₂, 0.1 mM EDTA, 50 μ g/ml BSA, 1.25 mM ATP, 100 μ g/ml VM26, 0.21 nM labeled DNA, and 2.5 nM purified *Drosophila* topoisomerase II homodimer. The reaction mixture was incubated at 30°C for 10 min, after which it was either kept at 30°C (lanes 1, 2, 4, and 5) or heated at 65°C (lanes 3 and 6) for 30 min in the presence of 50 mM dithiothreitol (DTT). The addition of DTT reduces the aggregation of topoisomerase II during the heating step, and it has no effect on the reversion of double strand breaks. 2 μ l of 10% SDS stock solution was then added after the heat treatment. Half of the samples were treated with proteinase K (lanes 4 to 6, marked with +PK). All the samples were analyzed by electrophoresis in 1.5% agarose gel containing 0.1% SDS. The radioautographic intensity in lane 5 is lower because of the spreading of DNA fragments toward the lower end of the gel. Minus enzyme controls are in the lanes 1 and 4. The arrow points to the position of intact substrate DNA.

single band which comigrated with the substrate DNA (Fig. 1, lane 6), suggesting that the radioactive DNA in the ladder, migrating slower than the substrate DNA, were protein linked. While the heat treatment reverses some of the cleavable complexes into free DNA and protein, a significant fraction of the cleavable complex is converted into a protein-linked complex. Protease digestion of the reversion complex releases the full length substrate DNA. Alkaline gel electrophoretic analysis of this DNA indicated that it contained single stranded nicks (data not shown). The reversion complex generated from the cleavable complex therefore contains a nick in the DNA, to which topoisomerase II is covalently linked. Similar results were obtained when the reversion was effected by EDTA and salt treatment (data not shown, also see Fig. 2), suggesting an identical reversion complex can be formed with different reversion procedures.

To further support the idea that the reversion product of the cleavable complex is a protein/DNA complex containing single stranded DNA breaks with a covalently linked topoisomerase subunit rather than free, intact DNA, we carried out similar reactions with radioactively labeled circular DNA substrates which should allow a more sensitive detection of the nicked DNA product in the reversion reaction. Topoisomerase II/DNA cleavage complexes were induced by adding SDS to a reaction mixture containing VM26, and the reaction products were treated either with or without proteinase K. The protease treated samples were analyzed by agarose gel electrophoresis in the presence of ethidium bromide to distinguish between nicked and covalently closed circles (Fig. 2B), while the samples without protease treatment were analyzed by SDS agarose gel electrophoresis to reveal tightly linked protein/DNA complexes (Fig. 2A). Formation of cleavable complexes at multiple sites generated DNA fragments of various sizes, appearing as smears in the gel electrophoresis (Fig. 2A&2B, lanes 1 and 3). With an increasing

Figure 2. EDTA/salt reversion of the topoisomerase II/circular DNA cleavable complex. A 1.6 Kb DNA fragment from the heat shock intergenic region (24) was 5' end labeled and circularized in the presence of ethidium bromide by T4 ligase to generate negatively supercoiled DNA. Labeled circular DNA was isolated from the agarose gel and purified through a NACS-52 column. The cleavage condition was the same as described in Fig. 1 except that it contained 0.046 nM labeled DNA and 0.63 nM topoisomerase II (lanes 1 and 2), or 1.26 nM topoisomerase II (lanes 3 and 4). Reversion was effected by the addition of EDTA to 12 mM and NaCl to 0.5 M and the incubation continued for 10 minutes (lanes 2 and 4). Marker lanes indicate the positions of NC (nicked circle), SC (supercoiled circle), RC (relaxed circle), KNC (knotted, nicked circle), and L (linear DNA). A: Samples were analyzed by electrophoresis in a 1.5% agarose gel containing 0.1% SDS. B: Samples were treated with proteinase K and then analyzed on a 3% agarose gel containing 0.5 μ g/ml ethidium.

topoisomerase II/DNA ratio, the sizes of the cleavage products decreased due to the multiple cleavage events per DNA molecule (compare lanes 1 and 3 of Fig. 2A&2B). Another aliquot of the same reaction mixture was treated with EDTA and salt to reverse the DNA cleavage reaction (Fig. 2A&2B, lanes 2 and 4). Similar to the results from the reversion products of linear DNA substrate (see Fig. 1), the reversion complex from circular DNA also showed multiple bands migrating slower than the nicked circles in the SDS/agarose gel (Fig. 2A, lanes 2 and 4). These multiple bands likely correspond to the circular DNA molecules with different amounts of covalently linked topoisomerase moiety. After the treatment of the reaction mixtures containing the reversion complexes with the proteinase K, we detected the formation of various DNA species including linear, nicked circular, and covalently closed circular molecules in the agarose gel containing ethidium bromide (Fig. 2B, lanes 2 and 4). The nicked circles include both unknotted and knotted DNA molecules. The detection of nicked, knotted DNA using this type of gel electrophoresis system was described earlier (26). All of the nicked DNA species account for more than 50% of the reversion products based on the densitometric analysis of the autoradiographs similar to the one shown in Fig. 2B. These results therefore suggest that most of the topoisomerase II/DNA complexes reversed from the cleavable complex contain DNA nicks with covalently attached topoisomerase II. We also detected some linear DNA after proteinase digestion (Fig. 2B, lanes 2 and 4). This is apparently due to some of the residual cleavable complex that was not reversed in these reactions. It is possible

Figure 3. Effects of ATP and ATP γ S on cleavage reaction and its reversion. The DNA substrate and its cleavage reaction was the same as described in Fig. 1. There was no ATP co-factor in reactions shown in lanes 1 and 2, while those in lanes 3 and 4 contained 1.25 mM ATP γ S and lanes 5 and 6 contained 1.25 mM ATP. Lane 7 shows the minus enzyme control. Heating reversion was carried out at 65°C (lanes 2, 4, and 6). Samples were analyzed on a 1.5% agarose gel in the presence of 0.1% of SDS. The arrow indicates the position of the substrate DNA.

that either the reversion is less efficient for the negatively supercoiled DNA substrate, or perhaps detection of double strand DNA breaks in the circular DNA is more sensitive than in the linear DNA. There is also some covalently closed, circular DNA released after proteinase digestion (Fig. 2B, lanes 2 and 4), which was produced from the complete reversion of the cleavable complex to yield the free, intact DNA. Similar reversion products were also obtained from these circular DNA substrates using the heat treatment instead (data not shown).

Characterization of the cleavage reversion reaction

We have demonstrated that VM26 stimulated double strand DNA cleavage by topoisomerase II can be reversed to single strand DNA cleavage with either heat or EDTA/salt treatment. To further analyze the cleavage reversion, the effects of temperature on the heat reversion efficiency were investigated. As the temperature increased from 45°C to 65°C, reversion of the cleavable complex gradually increased and reached a maximum by 65°C (data not shown). The midpoint of this reversion curve is around 55°C. The temperature dependence of the reversion of *Drosophila* topoisomerase II cleavable complex is very similar to that of the mammalian enzyme (21). Chelation of Mg⁺⁺ in the reaction mixture by the addition of EDTA and an increase in the ionic strength by adding NaCl to 0.5 M can also reverse some of the cleavable complex into the protein-linked complex. Either salt or EDTA alone cannot induce a reversion quite as efficiently as the combination of them. The efficiency of reversion is not affected by VM26 over a wide range of concentrations. For the DNA cleavage mediated by *Drosophila* topoisomerase II, significant stimulation can be observed in a reaction containing 0.3 μ g/ml VM26 and the amount of DNA cleavage increases with higher VM26 concentrations up to the maximal cleavage at a VM26 concentration of 100 μ g/ml. Both the heat and EDTA/salt reversion steps can efficiently reverse the cleavage reactions carried out in the range of 0.3 to 100 μ g/ml VM26.

In the absence of VM26, the presence of the cofactor ATP in the reaction has only a modest effect on the topoisomerase II mediated DNA cleavage and results in about a 2-fold enhancement in the double strand DNA cleavage (15). However, in the presence of VM26, ATP can markedly increase the cleavage efficiency, while its analog ATP γ S exerts a lesser effect (compare lanes 3 and 5 with lane 1 in Fig. 3). The extent of these

Figure 4. Kinetics of reversion of the cleavable complex by EDTA/CsCl. The same DNA substrate as described in Fig. 1 was used to form topoisomerase II/DNA complexes under similar reaction conditions, except that DNA and enzyme concentrations were 10-fold higher (2.1 nM DNA and 25 nM topoisomerase II). An aliquot was taken out and salt was diluted with 10 volumes of TE, followed by the addition of SDS to 1% to induce DNA cleavage (lane 2). Reversion was initiated by the addition of EDTA to 12 mM and followed by CsCl to 5 M. Aliquots were withdrawn at various timepoints after the addition of EDTA/CsCl: 10 sec. (lane 3), 10 min. (lane 4), 3 h. (lane 5), and 20 h. (lane 6). CsCl salt was diluted out with 10 volumes of TE and followed by the addition of SDS to 1%. Lane 1 is the substrate DNA and the arrow marks the position of the substrate DNA.

stimulatory effects was quantified by titrating the amount of enzyme necessary to give rise to the same level of DNA cleavage. It appears that ATP and its analog ATP γ S can stimulate the cleavage about 10- and 2-fold, respectively (data not shown). Heat reversal of the cleavable complexes is equally effective either in the absence or presence of ATP cofactors (Fig. 3, lanes 2, 4, and 6), and these protein-linked reversion products can be converted into full-length DNA after protease digestion (data not shown).

The kinetics of reversion is rapid. A significant amount of reversion takes place within 1 min. after shifting the incubation temperature to 65°C or upon the addition of EDTA/NaCl (data not shown). Efficient reversion was also observed when the reversion was induced by adding EDTA to 12 mM and CsCl to 5 M (Fig. 4). A slower reversion rate was observed here because of the much higher concentration of enzyme and DNA used in this experiment (see Fig. 4 legend). It is interesting to note that even after a prolonged incubation of 22 hours in EDTA/CsCl, a substantial amount of protein-linked reversion complex still persists (Fig. 4, lane 6).

Analysis of the reversion complexes by CsCl density gradient

Since the reversed topoisomerase II/DNA complex was stable in EDTA and 5 M CsCl, this prompted us to analyze and isolate these complexes by CsCl density gradient equilibrium centrifugation. In such a gradient, radiolabeled linear pBR322 DNA forms a single band with a measured density of 1.72 g/cm³ (Fig. 5A). For pBR322 DNA with a base composition of 54% guanosine and cytosine, this measured buoyant density is close to the calculated value based upon the known relation between buoyant density of DNA and its base composition (27). Only one peak, at the density expected for free DNA, is apparent when topoisomerase II/DNA complexes are formed in the absence of VM26 (Fig. 5B), indicating that the majority of topoisomerase II/DNA complex formed in the absence of VM26 is not stable in a high concentration of CsCl. Most of the non-covalent protein/DNA complexes are expected to dissociate in such a high salt solution. When topoisomerase II/DNA complexes

Figure 5. CsCl density gradient centrifugation studies of topoisomerase II/DNA complexes. The banding patterns of linear *Bam* HI-pBR322 DNA alone, complexes of pBR322 DNA with topoisomerase II formed without VM26, and the complexes formed in the presence of VM26 are shown in panels A, B, and C, respectively. The insets in panels A and B magnify the same region from the gradient profiles and they highlight the presence of a small fraction of the stable complex formed in the absence of VM26. The open squares are the measured density across the gradient.

formed in the presence of VM26 are analyzed by the CsCl density gradient, multiple peaks are apparent (Fig. 5C). The first peak from the bottom of the gradient (fraction 15 in Fig. 5C) bands at a position with the density expected for free DNA. The generation of discrete species of lower buoyant densities indicates that the topoisomerase II/DNA complexes formed in the presence of VM26 are stable in a concentrated salt solution (~ 4.5 M CsCl) for at least 44 h during ultracentrifugation. The multiplicity in buoyancy shift results from the binding of different numbers of topoisomerase II molecules to each DNA molecule. We can resolve up to 5 distinct species corresponding to DNA bound with 1 to 5 molecules of topoisomerase II protomer (homodimer) per DNA molecule (Fig. 5C). This conclusion was confirmed by using an equation that quantitatively relates the buoyancy shifts to the number of protein molecules linked to the DNA (28). Detection of this reversed topoisomerase II/DNA complex is not

Figure 6. Analysis of the stable complex isolated from CsCl density gradient. A. Complexes were formed in the presence of VM26 between topoisomerase II and a 5'-end labeled, 1.6 Kb DNA fragment isolated from *Hinf* I digestion of 6/122b plasmid DNA. They were analyzed on a CsCl density gradient. DNA and complexes were assigned based on their densities as follows: free DNA (Fraction 1), complex of one topoisomerase II protomer bound per DNA (Fraction 8, denoted as complex 1), complex of two topoisomerase II protomers bound to DNA (Fraction 12, complex 2), and complex of three topoisomerase II protomers bound to DNA (Fraction 15, complex 3). B. Both DNA and complexes were isolated from a CsCl density gradient, the profile of which is shown in Fig. 6A. Samples were diluted with 10 volumes of TE and SDS was added to 1%. They were incubated at 45°C for 30 min. either in the absence of proteinase K (lanes 1 to 4) or in the presence of 0.2 mg/ml proteinase K (lanes 5 to 8), followed by electrophoresis in a 1.5% of agarose gel containing 0.1% of SDS. Samples containing DNA, complex 1, complex 2, and complex 3 were analyzed in lanes 1 and 5, 2 and 6, 3 and 7, 4 and 8, respectively.

limited to *Drosophila* enzyme; similar density gradient banding profiles were observed for the mammalian enzyme under these conditions (data not shown).

The requirement of VM26 in the formation of stable topoisomerase II/DNA complexes in CsCl gradient is probably not absolute. Upon careful inspection of the CsCl density gradient for the complexes formed without VM26, one can detect a small but reproducible peak at a position corresponding to the complex with one protomer of enzyme bound to DNA (inset of Fig. 5B). Analysis in the comparable region of the DNA alone gradient shows no detectable counts above background (inset in Fig. 5A). Therefore the reversion complex can form, albeit with a poor efficiency, in the absence of any drug.

Figure 7. A DNA nick is associated with the CsCl stable complex. A. A 1.6 Kb, *Sal* I digested DNA fragment from of 6/122b, was 5' end labeled and circularized in the presence of ethidium bromide to prepare radioactively labeled, negatively supercoiled DNA substrate. Circular DNA was isolated from an agarose gel and purified through an NACS-52 column. Complexes formed between topoisomerase II and circular DNA in the presence of VM26 were analyzed by the CsCl density gradient. DNA and complexes are assigned based on their densities: DNA (Fr 7), complex of one topoisomerase II protomer bound to DNA (Fr 13, denoted as complex 1), and complex of two topoisomerase II protomers bound to DNA (Fr 16, denoted as complex 2). The radioactivity in Fr 7 (free DNA) is 1.6×10^4 cpm per μ l, which is beyond the scale shown here. B. DNA and the complex of one topoisomerase II bound per DNA were isolated, treated with proteinase K, and analyzed by electrophoresis in a 3% agarose gel containing 0.5 μ g/ml ethidium bromide. The marker lanes indicate the mobilities of the following species: NC (nicked circular DNA prepared by partial DNase I digestion, lane 1); RC (relaxed circular DNA prepared from relaxation of supercoiled DNA by topoisomerase, lane 2). SC (supercoiled circular substrate DNA, lane 3); L (linear DNA prepared from restriction digestion, lane 4). DNA and complex 1 are shown in lanes 5 and 6, respectively.

We have taken advantage of equilibrium centrifugation in a CsCl density gradient to isolate stable topoisomerase II/DNA complexes and to test the stability of their linkage in a number of denaturing environments. The complexes formed in the presence of VM26 between topoisomerase II and a 1.6 Kb linear DNA were fractionated in a CsCl density gradient and resulted in four major peaks (Fig. 6A). The measured densities suggest that they correspond to free DNA (1st peak from the bottom of the gradient, Fig. 6A), 1 topoisomerase II promoter bound per 1.6 Kb DNA substrate (2nd peak), 2 promoters per DNA (3rd

peak), and 3 promoters per DNA (4th peak). Both the free DNA and the complexes were isolated from the CsCl gradient and analyzed by electrophoresis in an agarose gel containing SDS. All of the complexes have slower mobilities than the free DNA (lanes 1–4, Fig. 6B). The retarded mobilities are due to protein molecules bound to DNA in the complexes, since they comigrate with free DNA after protease digestion (lanes 5–8, Fig. 6B). Generation of the intact DNA substrate after protease digestion indicates that there are no double strand DNA breaks associated with the CsCl stable complexes. Not only is the topoisomerase/DNA complex isolated from the CsCl gradient stable in the presence of 0.1% SDS during agarose gel electrophoresis, but we also noticed that it is stable in 1% SDS or 1% SDS at an elevated temperature up to 90°C (data not shown). Furthermore, the label transfer experiment, similar to one carried out for the DNA cleavage complex of *Drosophila* topoisomerase II (15), also demonstrated a tight linkage between the enzyme and DNA in this CsCl stable complex. In this experiment, CsCl stable complex was isolated using a uniformly radiolabeled DNA as the substrate. Subsequent nuclease digestion of the purified complex and analysis by SDS/polyacrylamide gel electrophoresis indicated that the radiolabel from the residual oligonucleotides was linked to topoisomerase II (data not shown). All these data on the stability of the CsCl stable complex strongly suggest that a covalent bond is likely involved in linking enzyme/DNA in this complex.

Since the covalent linkage between DNA and topoisomerase II in the CsCl stable complex does not involve double stranded DNA breaks (Fig. 6B), it is likely that the covalent bond is formed between a single stranded DNA break and a subunit of topoisomerase II homodimer. This CsCl stable complex is thus very similar to the reversion complexes generated by either heat or EDTA/salt treatment; in this case the reversion is effected by EDTA and a high concentration of CsCl. To further confirm that the EDTA/CsCl reversion complex also involves protein linkage to a nick in the DNA molecule, we prepared a radiolabeled circular DNA substrate. The complex formed between topoisomerase II and a 1.6 Kb negatively supercoiled DNA was analyzed by a CsCl density gradient (Fig. 7A). Both free DNA and enzyme/DNA complexes (indicated in Fig. 7A) were detected and isolated from the CsCl gradient. After proteinase digestion they were analyzed by electrophoresis in a 3% agarose gel containing 0.5 μ g/ml ethidium bromide. The presence of ethidium in the gel electrophoresis system allows the separation of covalently closed circular DNA from the nicked species. It is clear that DNA in the CsCl stable complex after the removal of protein by protease treatment comigrates with nicked circular DNA (Fig. 7B). The nicked circular DNA structure was also confirmed by an analysis of electrophoresis either in a polyacrylamide gel containing urea or an alkaline agarose gel (data not shown). In these gel electrophoresis systems, most of the DNA isolated from the free DNA region remains as covalently closed species, while the DNA from the CsCl stable complex can be resolved into two species, corresponding to linear and circular single stranded DNAs. Therefore, we have established that the topoisomerase II/DNA complex isolated from the CsCl density gradient has the same characteristics with respect to DNA cleavage and complex stability as the complex reversed from the cleavable complex by either heat or EDTA/salt treatment. The complex contains a single strand DNA break with topoisomerase II covalently linked to the nicked DNA. The chemical bond between topoisomerase II and nicked DNA is

likely the same phospho-tyrosine diester bond present in the cleavable complex.

DISCUSSION

Formation of protein/DNA complexes between DNA and topoisomerase II has been demonstrated by various techniques including nitrocellulose filter binding, gel mobility shift, nuclease protection, and CsCl equilibrium density centrifugation (29, 30, 24, and this paper). The available data suggest the existence of at least three types of DNA/topoisomerase II complexes. The first one is the noncovalent enzyme/DNA complex that is sensitive to dissociation by either high salt or an excess of competitor DNA (see for example ref. 20 and 29; and Fig. 5B in this paper). The second one is the cleavable complex which can be induced to generate double stranded DNA breaks (double strand cleavable complex). The third one is another form of cleavable complex which can lead to the production of single strand cleavage complex (single strand cleavable complex). These three types of complexes are in apparent equilibrium with each other and many factors can affect this equilibrium. For example, the experiments presented here demonstrate that the commonly employed reversion conditions, EDTA/salt or heat treatment, can move the equilibrium away from the double strand cleavable complex and yield both dissociable complex and single strand cleavable complex. Not only is the formation of single strand cleavable complex favored by the reversion treatments, its distribution can also be modulated by the presence of divalent cations. Single strand DNA cleavage of duplex DNA by *Drosophila* topoisomerase II can be enhanced by substituting Mg^{++} with Ca^{++} , Mn^{++} , or Co^{++} in the reaction mixture or by the addition of molar excess of EDTA (23, 31). Even though the single strand DNA cleavage under those conditions is not as efficient as that reported here, it is clear that the formation of single strand cleavable complex is not restricted only to the reversion of topoisomerase II mediated DNA cleavage in the presence of VM26.

The topoisomerase II/DNA complex formed in the presence of VM26 is extremely stable. A hallmark of this stable complex is that it cannot be completely reversed to dissociable complex by the commonly employed reversion conditions like treatment with heat or EDTA/salt. The anti-tumor drug VM26 greatly stimulates the formation of this irreversible complex between topoisomerase II and DNA, although the continuous presence of this drug is not required to maintain this complex. The effective free drug concentration in the experiments monitoring the formation of enzyme/DNA complex, like CsCl gradient ultracentrifugation, is greatly diminished in comparison with the binding reaction mixture. It is interesting to note that the cytotoxic action of VM26 appears to be irreversible as well. The traversal of cell cycle of mammalian cells can be blocked by VM26, and the cell growth cannot resume even after reincubating the cells in a drug-free medium (22). It is possible that the incomplete reversion of topoisomerase II/DNA complex in the presence of VM26 and the formation of single strand cleavable complex may play an important role in the cytotoxic action of teniposide.

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