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University of Alberta

Studies of the topoisomerase and cofactor requirements for nucleosome reconstitution in a yeast chromatin assembly system

by 

Wendy Irene Garinther

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Biochemistry

Edmonton, Alberta

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Wendy Garinther

211-8604 103 St.
Edmonton, Alberta
T6E 4B6

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Studies of the topoisomerase and cofactor requirements for nucleosome reconstitution in a yeast chromatin assembly system** submitted by **Wendy Irene Garinther** in partial fulfillment of the requirements for the degree of Master of Science.

Dr. M. Schultz (Supervisor)

Dr. M. Ellison

Dr. R. Wozniak

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I, Dr. Michael C. Schultz give Wendy Garinther permission to use the data from the paper entitled "Topoisomerase Function during Replication-Independent Chromatin Assembly in Yeast" for a chapter in her thesis.

M.C. Schultz

Dr. M.C. Schultz

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ABSTRACT

The packaging of DNA into chromatin is a vital cellular activity that can influence many metabolic processes that involve DNA. The detailed characterization of this process is therefore likely to provide new insights into the mechanisms that coordinate DNA-dependent nuclear functions. This thesis describes experiments using biochemical, genetic, and pharmacological methods to characterize the involvement of the DNA topoisomerases and ATP during chromatin assembly in yeast. Chromatin assembly is severely impaired in extracts from a yeast mutant with no active form of topoisomerase I or II (strain top1-top2). Expression of wild-type topoisomerase II in strain top1-top2 fully restored chromatin assembly, and assembly was equally efficient in extracts from strains expressing either topoisomerase I or II. The topoisomerase II poison VP-16 was used to show that topoisomerase II activity during chromatin assembly is the same in the presence and absence of topoisomerase I. Furthermore, topoisomerases I and II co-purify with the chromatin assembly machinery during the initial phases of fractionation of the crude assembly extract. The results indicate that both topoisomerase I and II can function interchangeably during chromatin assembly. ATP was found to be required for chromatin assembly in the yeast extract. The requirement for ATP is partly due to the role of ATP as a cofactor for DNA ligase and topoisomerase II.

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ABBREVIATIONS

AA	Amino acid
ATP	Adenosine triphosphate
AMP-PNP	Adenosine 5'-[β, γ-imido]triphosphate
BHI	Brain Heart Infusion
bp	base pair
BSA	Bovine serum albumin
CAC	Chromatin assembly complex
CAF-I	Chromatin assembly factor-I
cdc	cell division cycle
DNA	Deoxynucleic acid
DEAE	Diethylaminoethyl
DTT	Dithiothreotol
E. coli	Escherichia coli
EDTA	Etylenediaminetetraacetic acid
EGTA	Etyleneglycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
GTE	Glucose-Tris-EDTA
h	hour
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
kb	kilobase
kDa	Kilodaltons
KDNA	Kinetoplast DNA
min	minute
MSI1	Multicopy suppressor of IRA
NAP-I	Nucleosome assembly protein I
OD	Optical density

70p	70% ammonium sulfate pellet
PEG	Polyethylene glycol
PMSF	Phenylmethylsulfonyl fluoride
Rap1p	Repressor/activator protein 1
rDNA	ribosomal DNA
Rlf2p	Rap1p localization factor protein 2
RNA	Ribonucleic acid
SDS	Sodium dodecylsulphate
SV	Simian virus
SV8	Staphylococcus aureus strain V8
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TPCK	L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone
Tris	Tris-(hydroxymethyl) aminomethane
tRNA	transfer ribonucleic acid
ts	temperature sensitive
UV	Ultraviolet
VM-26	4'-demethylepipodophyllotoxin-9-[4,6-(0-thenylidene)-β-D-glycopyranoside
VP-16	4'-demethylepipodophyllotoxin-9-[4,6-(0-ethylidene)-β-D-glycopyranoside
v/v	volume per unit volume
w/v	weight per unit volume
YDBI	Yeast dialysis buffer with inhibitors
YEB	Yeast extract buffer
YPD	Yeast-Peptone-Dextrose

Chapter 1

Introduction

1.1 Overview

The DNA in each human chromosome is extremely long and if it were unraveled, it would span the nucleus of a cell thousands of times. Thus, in order for DNA to reside in the nucleus, which is on average only 10 micrometers in diameter, DNA must be packaged into ordered structures (van Holde, 1988; Wolffe, 1992). The fundamental packaging unit of these ordered structures is the nucleosome. DNA is incorporated into nucleosomes during a process referred to as chromatin assembly (van Holde, 1988; Wolffe, 1992).

The packaging of DNA in chromatin can influence many metabolic processes that involve DNA, for example, transcription, replication, and repair (van Holde, 1988; Wolffe, 1992; Gaillard et al., 1996). It is therefore important to have a clear understanding of all aspects of chromatin assembly. The objectives of this thesis were to study the involvement of the DNA topoisomerases during chromatin assembly, investigate the requirement for ATP during assembly, and lastly, begin the search for novel chromatin assembly factors.

1.2 DNA Topoisomerases

The double-helical structure of DNA presents topological problems that a cell must solve for many of its vital cellular processes to proceed. The DNA topoisomerases have evolved to solve these topological problems. The topoisomerases are nuclear enzymes that transiently break and religate DNA, and are required for replication, transcription, and recombination. These enzymes also play key roles in chromosome structure, and chromosome condensation and decondensation (Wang, 1985; D'Arpa and Liu, 1989; Wang, 1991; Chen and Liu, 1994; Froelich-Ammon and Osheroff, 1995). For example a topoisomerase is required during the elongation step of transcription where the rotation of the transcription machinery relative to the DNA template

generates positive supercoiling ahead of the transcription machinery and negative supercoiling behind it, creating torsional strain. This strain must be relieved by a topoisomerase for the polymerase to move rapidly along its template (Wang, 1985; Wang, 1991; D'Arpa and Liu, 1989; Drolet et al., 1994). Torsional strain is generated during replication in the same manner when the replication fork advances along the DNA. This torsional strain can stop the movement of the replication fork and therefore must be relieved by a topoisomerase (Wang, 1985; Brill et al., 1987; Wang, 1991; D'Arpa and Liu, 1989).

There are two major types of topoisomerases in eukaryotes, type I and type II, and the topoisomerases are classified as such based on their mechanism of action on DNA. Type I topoisomerases transiently break one strand of DNA to form a gate for the passage of another intact strand. Type II topoisomerases make double-strand breaks and allow the passage of another double helix (Rose, 1988; D'Arpa and Liu, 1989; Wang, 1994). The breakage/rejoining activity of the topoisomerases occurs via two transesterification reactions. During the first reaction, a phosphodiester bond in the DNA is broken when the topoisomerase covalently attaches to the DNA by forming a phosphotyrosine linkage between the hydroxyl group of the active site tyrosine and a DNA phosphate at the cleavage site. In the second reaction, the hydroxyl group of the broken DNA attacks the phosphotyrosine linkage, reforming the phosphodiester bond and releasing the topoisomerase. These actions allow the enzymes to catalyze the interconversion of DNA topoisomers (Wang, 1985; D'Arpa and Liu, 1989; Wang, 1994).

The eukaryotic topoisomerases catalyze the relaxation of both positively and negatively supercoiled DNA. However, they are not able to supercoil relaxed DNA (Wang, 1994, Chen and Liu, 1994). The eukaryotic type I

topoisomerase is called DNA topoisomerase I. It exists as a monomer and does not require ATP or Mg^{2+} as cofactors for its catalytic activity. The eukaryotic type II topoisomerase is DNA topoisomerase II. It is a homodimer that requires both ATP and Mg^{2+} for its catalytic activity (Wang, 1994; Froelich-Ammon and Osheroff, 1995; Sinha, 1995).

Because topoisomerase II can introduce double-strand breaks in the DNA, it is able to knot/unknot a single circle of DNA and catenate/decatenate DNA (the reversible separation of interlocked circles of DNA, [Fig. 1.1]) (Wang, 1985). It is the decatenation activity of topoisomerase II that gives this enzyme the unique ability (compared to topoisomerase I) to separate daughter chromosomes at the end of replication (DiNardo et al., 1984). This unique function of topoisomerase II is the reason that topoisomerase II is essential for cell viability in all organisms. In contrast, topoisomerase I is not essential in all organisms (Lee et al., 1993). Topoisomerase I is essential for *Drosophila* development (Lee et al., 1993), but is not essential for viability in eubacteria and yeast (Thrash et al, 1985; Goto and Wang, 1985; Lee et al, 1993).

Besides transcription and replication, chromatin assembly also produces torsional strain in DNA (Leonard and Patient, 1991; Wolffe, 1992). This torsional strain must be relieved in order for chromatin assembly to proceed efficiently. The topoisomerases are known to substitute for each other during many cellular processes including replication and transcription (Brill et al., 1987; Wang, 1987; D'Arpa and Liu, 1989). However, both topoisomerase I and II may not be involved in chromatin assembly. As discussed below, the role of topoisomerase II during chromatin assembly is controversial and has not been clearly elucidated. The main objective of my work was to determine the role of topoisomerase II during chromatin assembly in yeast. Therefore, the focus of the introduction is the properties of topoisomerase II.

1.3 Structural and Functional Domains of Topoisomerase II

Extensive amino acid sequence analysis has revealed that all type II topoisomerases are evolutionarily and structurally conserved (Wang, 1987). Proteolysis studies on purified eukaryotic topoisomerase II from a number of organisms suggest that topoisomerase II is composed of three major structural domains (Lindsley and Wang, 1991; Watt and Hickson, 1994; Jensen et al., 1996). A schematic diagram of the basic structure of yeast topoisomerase II is shown in Fig. 1.2. There are three preferred protease cleavage sites (A, B, and C, Fig. 1.2). Site A is only sensitive to SV8 protease in the absence of AMP-PNP (a non-hydrolyzable ATP analog), while site B is only sensitive to SV8 protease upon binding of AMP-PNP to topoisomerase II (Lindsley and Wang, 1991; Watt and Hickson, 1994; Wang, 1994). These changes in sites of protease sensitivity observed with AMP-PNP reflect conformational changes that occur in topoisomerase II following ATP binding (Lindsley and Wang, 1991).

The N-terminal and central parts of topoisomerase II are conserved, whereas the C-terminal region is highly divergent. The N-terminal domain of the enzyme is known as the ATPase domain and is homologous to the B subunit of DNA gyrase (DNA gyrase consists of an A_2B_2 tetramer of A and B subunits [Wang, 1994]). This domain contains a consensus sequence for an ATP-binding site and it hydrolyzes ATP in vitro (Watt and Hickson, 1994). The central region of topoisomerase II shows homology to the A subunit of DNA gyrase, which functions as the catalytic subunit of DNA gyrase. The active site tyrosine (Y* in Fig. 1.2) that is involved in the cleavage and religation of DNA, is located in this central catalytic domain (Watt and Hickson, 1994; Jensen et al., 1996). The C-terminal domain of topoisomerase II is highly divergent and does not have a DNA gyrase equivalent (Jensen et al., 1996). Deletion analysis of

the N-terminal and central part of eukaryotic topoisomerase II showed that these conserved domains are essential for the *in vivo* and *in vitro* activity of topoisomerase II (Jensen et al., 1996).

Deletion of a large portion of the C-terminal domain has no effect on cell viability or the catalytic activity of topoisomerase II. The C-terminal domain does contain a putative nuclear localization signal that is essential for the *in vivo* activity of topoisomerase II (Caron et al., 1994; Jensen et al., 1996). Topoisomerase II lacking the nuclear localization signal is however, catalytically active *in vitro*. Therefore, inhibition of the *in vivo* activity of topoisomerase II may be due to the decreased amount of enzyme in the nucleus and not due to inhibition of the catalytic activity of the enzyme (Caron et al., 1994; Jensen et al., 1996). The C-terminal region also contains several phosphorylation sites. The cell-cycle specific phosphorylation of topoisomerase II has been suggested to regulate its activity. The deletion of this region containing the phosphorylation sites does not affect topoisomerase II activity *in vitro*. However, the dephosphorylation of the full-size protein inhibits its catalytic activity and also reduces its affinity for DNA. It has therefore been proposed that the C-terminus is a negative regulatory domain and phosphorylation of this region neutralizes this regulation (Gasser et al., 1992; Cardenas et al., 1992; Cardenas and Gasser, 1993).

1.4 Mechanism of Topoisomerase II Activity

A general model for the catalytic activity of topoisomerase II was initially proposed based on the crystal structure of a complex between the ATPase domain of *E. coli* DNA gyrase and AMP-PNP, and the analysis of complexes formed between eukaryotic topoisomerase II and DNA, following addition of AMP-PNP. The model proposed that topoisomerase II acts as a molecular

clamp, that is open in the absence of ATP and is closed when ATP is bound (Roca and Wang, 1992; Wang, 1994; Roca, 1995).

The current two-gate mechanism for the catalytic activity of topoisomerase II is based on this initial model. This mechanism was proposed following the resolution of the crystal structure of a large portion of the yeast enzyme (Berger et al., 1996). This structure led to a model of topoisomerase II as an ATP-modulated clamp with two set of jaws at opposite ends that are connected by multiple joints. Briefly, an enzyme with bound DNA allows the entry of a second DNA duplex through one set of jaws. Once ATP binds, the second DNA duplex is transported through the DNA gate of the first DNA duplex and is then released through the other set of jaws. This mechanism is described in detail below. Experimental evidence has confirmed this two-gate mechanism of topoisomerase II activity (Roca et al., 1996).

The 92 kDa fragment of yeast topoisomerase II that was crystallized contains residues 410-1202. It does not contain the ATPase domain (residues 1-409) or a dispensable portion of the C-terminus (residues 1203-1429). The two major subfragments of the polypeptide chain are denoted B' and A' in reference to their homology to DNA gyrase. The crystal structure of the ATPase domain of *E. coli* DNA gyrase was used in combination with this structure to arrive at the two-gate model (Berger et al., 1996).

The two gate mechanism for the catalytic reaction of topoisomerase II is shown in Fig. 1.3. In the absence of ATP, topoisomerase II is in an open conformation with the two halves of the clamp held together by dimer contacts near the C-terminus of each A' subfragment (1 in Fig. 1.3). The first DNA segment to bind the enzyme is the one that will be cleaved and this segment is therefore termed the gate segment or "G-segment". The binding of the G-segment induces a conformational change that brings the A' subfragments

together and the active site tyrosines into position (2 in Fig. 1.3). The binding of ATP (shown by asterisks) causes the dimerization of the ATPase domains. As the ATPase domains dimerize they capture a second DNA duplex, which is termed the "T-segment", for transport (3 in Fig. 1.3). The binding of ATP and a T-segment cause conformational changes whereby the G-segment is split apart as the A' subfragments separate from each other (in brackets, Fig. 1.3). The T-segment is transported through the DNA gate in the G-segment and into the interior of the enzyme (4 in Fig. 1.3). The T-segment is then released through the dimer interface in the C-terminus of the enzyme and the G-segment is religated (5 in Fig. 1.3). ATP is hydrolyzed and released to regenerate the starting state of the enzyme (Berger et al., 1996; Roca et al., 1996).

1.5 Targeting of Topoisomerases by Anticancer Drugs

Interest in the topoisomerases has greatly expanded over the last decade from basic research into clinical research. The reason for this increased interest is that the topoisomerases are the primary cellular targets of some of the most widely used antibiotics and anticancer drugs available at the present time (Froelich-Ammon and Osheroff, 1995).

Topoisomerase II is the target of a number of structurally diverse compounds. Several topoisomerase II drugs are routinely used in the treatment of human cancers such as testicular and breast cancer, lymphomas, and certain leukaemias. Drugs that target topoisomerase I are presently in clinical trials (Froelich-Ammon and Osheroff, 1995; Watt and Hickson, 1995).

The known topoisomerase drugs are divided in two classes. Class I drugs function by trapping the topoisomerase on the DNA after it has created a single- or double-strand break in the DNA. The topoisomerase-DNA-drug complex is termed the cleavable complex because when it is treated with a protein denaturant such as SDS, the DNA breaks are revealed (Fig. 1.4). Class

I drugs are referred to as topoisomerase poisons (Smith and Souès, 1994; Wang, 1994; Froelich-Ammon and Osheroff, 1995). VP-16 (etoposide), which is used in the present study, is an example of a topoisomerase II-specific topoisomerase poison. Class II drugs function by interfering with the catalytic activity of the topoisomerases. These drugs do not form a cleavable complex and do not directly produce DNA damage. For example, novobiocin, a topoisomerase-II specific drug, inhibits the activity of the enzyme by disrupting the turnover of ATP. These drugs are referred to as topoisomerase inhibitors (Smith and Souès, 1994; Wang, 1994; Froelich-Ammon and Osheroff, 1995).

The cytotoxicity of the topoisomerase poisons is due to the conversion of the transient single- and double-strand DNA breaks into irreversible double-stand breaks. In the cell, the cleavable complex presents a physical block to the progression of such factors as components of the transcription and replication machinery. When for example the replication fork attempts to cross the cleavable complex, the complex is disrupted and the transient DNA breaks become permanent DNA breaks (Zhang et al., 1990; Kaufmann et al., 1991; Vassetzky et al., 1995; Froelich-Ammon and Osheroff, 1995). The permanent DNA breaks become the targets for recombination and repair pathways, which stimulates sister chromatid exchange, generation of insertions and deletions, and chromosomal aberrations. The accumulation of DNA breaks ultimately leads to cell death by necrosis or apoptosis (Froelich-Ammon and Osheroff, 1995). Evidence to support this model comes from studies in yeast that show that *rad52* yeast mutants are hypersensitive to topoisomerase I poisons. RAD52 is involved in the repair of double-strand DNA breaks (Chen and Liu, 1994; Wang, 1994).

Cell killing by the class II topoisomerase inhibitors, which do not induce DNA strand cleavage, is not well understood. One family of topoisomerase II

inhibitors, the bisdioxopiperazines, block the activity of topoisomerase II by trapping the enzyme in a closed clamp conformation after it has bound to DNA (Roca et al., 1994). This mechanism of drug action reflects the molecular clamp model of topoisomerase II described above, where the enzyme is in an open conformation in the absence of ATP and is closed when ATP is present (Roca and Wang, 1992). These inhibitors bind topoisomerase II in the closed clamp form and prevent the enzyme from converting to the open clamp form by inhibiting its ATPase activity (Roca et al., 1994).

The most effective inhibitor of this family, ICRF-193, inhibits the late stages of chromosome condensation and segregation in mammalian cells, causing polyploidy and loss of viability as the cells continue through further rounds of the cell cycle without cell division (Downes et al., 1994; Ishida et al., 1994). Because the topoisomerase inhibitors function by interfering with the catalytic activity of the topoisomerases, it is essential to identify all the cellular processes that the topoisomerases are involved in, in order to then elucidate the various mechanisms by which this class of drug kills cells.

The level of topoisomerases in a cell affects the cell's sensitivity to the two classes of drugs differently. This is because of the different modes of action of the two classes of drugs: the topoisomerase poisons act by stabilizing the covalent DNA cleavage complex, while the topoisomerase inhibitors block the catalytic activity of the enzyme (Nitiss, 1994; Smith and Souès, 1994; Wang, 1994; Froelich-Ammon and Osheroff, 1995). For example, increased topoisomerase levels cause cells to be hypersensitive to topoisomerase poisons and resistant to topoisomerase inhibitors. On the other hand, decreased levels of the topoisomerases cause cells to be resistant to topoisomerase poisons and hypersensitive to topoisomerase inhibitors (Froelich-Ammon and Osheroff, 1995). The sensitivity of cells to topoisomerase

levels may explain the effectiveness of topoisomerase poisons. The level of topoisomerase II increases dramatically in proliferating cells, compared with resting cells. High levels of topoisomerase II are observed in tumour cells, which is consistent with the rapid proliferation potential of tumour cells (Zhang et al., 1990; Froelich-Ammon and Osheroff, 1995; Vassetzky et al., 1995).

1.6 The Process of Chromatin Assembly

Chromatin assembly is the cellular process whereby DNA is packaged into structural units known as nucleosomes. Nucleosomes are the building blocks of chromatin and give decondensed chromatin the "beads-on-a-string" appearance when observed by electron microscopy (van Holde, 1988; Wolffe, 1992). A nucleosome consists of an octamer of histones together with one repeat length of DNA. The repeat length of DNA is defined by the common size of DNA product generated early in the digestion of chromatin by micrococcal nuclease. (Micrococcal nuclease digests the unprotected DNA in the linker regions between nucleosomes). The nucleosome repeat length varies among species from 160 bp to 260 bp (van Holde, 1988). The nucleosome is progressively trimmed to a nucleosome core particle as micrococcal nuclease digestion continues. The nucleosome core particle consists of a histone octamer and the length of DNA that has the strongest contacts with the octamer, making it resistant to extensive micrococcal nuclease digestion. The length of DNA that is part of the nucleosome core particle is approximately 146 bp for all species and is wrapped twice around the histone octamer (Fig. 1.5). The histone octamer consists of two copies each of histones H2A, H2B, H3, and H4 (van Holde, 1988; Wolffe, 1992).

The histones are small basic proteins that range in molecular weight from 11-16 kDa. Approximately 20% of the amino acids of the histones are lysines and arginines. The histones are composed of a charged amino-terminal tail

which contains the bulk of basic residues and a globular domain that facilitates histone-histone and histone-DNA interactions. The major interactions between the DNA and the positively charged histones are electrostatic interactions (van Holde, 1988; Wolffe, 1992).

The assembly of nucleosomes on DNA occurs in a two step process. The first step involves the deposition of a tetramer of histones H3 and H4. This is followed by the incorporation of two dimers of histones H2A and H2B (van Holde, 1988; Wolffe, 1992). Protein factors have been identified which are required for the formation of the histone octamer. Proteins such as N1/N2 and chromatin assembly factor-I associate with histones H3 and H4 and facilitate their deposition on DNA, while nucleoplasmin and nucleosome assembly protein-I assist with the deposition of histones H2A and H2B on DNA (Dilworth et al., 1987; Ishimi and Kikuchi, 1991; Smith and Stillman, 1991b).

1.7 Chromatin Assembly Factors

To gain a better understanding of the mechanism of chromatin assembly, it is necessary to identify the factors that are involved in this process. The biochemical analysis of an extract from human cell nuclei led to the identification of a multisubunit protein complex that is required for nucleosome assembly. It was named chromatin assembly factor I (CAF-I; Smith and Stillman, 1989; Smith and Stillman, 1991a; Kaufman et al., 1995). CAF-I has also been identified in *Drosophila* extracts and a CAF-I related activity has been detected in *Xenopus* extracts (Bulger et al., 1995; Kamakaka et al., 1996). In addition the purification of CAF-I from yeast has been accomplished (Kaufman et al, 1997).

The chromatin assembly activity of human CAF-I (hCAF-I) is coupled to DNA replication. hCAF-I promotes nucleosome assembly of newly replicated DNA during T-antigen mediated simian virus 40 (SV40) DNA replication, in the

presence of a cytosolic extract. hCAF-I is comprised of three subunits of 150, 60, and 48 kDa (p150, p60, and p48, respectively) and is localized to the nucleus of human cells (Smith and Stillman, 1991a).

The *Drosophila* CAF-I (dCAF-I) consists of four subunits of 180, 105, 75, and 55 kDa (Kamakaka et al., 1996). Chromatin assembly with dCAF-I also occurs preferentially onto newly replicated DNA. However, dCAF-I can assemble nucleosomes on unreplicated DNA. The requirement for ongoing replication appears to be more stringent for hCAF-I than dCAF-I (Kamakaka et al., 1996).

Yeast CAF-I (yCAF-I) also assembles nucleosomes preferentially onto newly replicated DNA (Kaufman et al., 1997). Yeast CAF-I is composed of three subunits, p150, p90, and p50. All three subunits have been conserved from yeast to humans. The genes that encode the p150, p90, and p50 subunits are *CAC1*, *CAC2*, and *CAC3* (chromatin assembly complex), respectively. The *CAC3* gene was previously isolated and termed *MSI1* (Kaufman et al., 1997). *MSI1* is a member of a highly conserved subfamily of WD repeat proteins that has been implicated in histone binding and modification. The p48 and p55 subunits of human and *Drosophila* CAF-I are also encoded by members of this subfamily of WD repeat proteins (Tyler et al., 1996; Kaufman et al., 1997). The *CAC1* gene is identical to *RLF2* (Rap1p localization factor 2), which is required for the proper localization of Rap1p (Enomoto et al., 1997). Rap1p (repressor/activator protein 1) is a key component of telomeric heterochromatin and is involved in the function of telomeres, specifically in telomere length control and telomeric silencing. Consistent with the functions of Rap1p, the results of Enomoto et al. (1997) indicate that Rlf2p/yCAF-I-p90 is required for the function and organization of telomeric chromatin.

Interesting, yeast cells that lack CAF-I are viable, show no growth defects, and presumably assemble normal chromatin at most loci in the genome (Kaufman et al., 1997). These observations imply that there must be other proteins involved in chromatin assembly that have yet to be identified.

1.8 Topoisomerase Function in Chromatin Assembly

Besides replication and transcription, chromatin assembly also produces torsional strain in DNA that must be relieved. Relaxation during chromatin assembly is favored because DNA is twisted when it wraps around a histone octamer to form a nucleosome (van Holde, 1988; Wolffe, 1992). This twisted conformation of DNA is energetically unstable (Sapp and Worcel, 1990). Theoretically, DNA relaxation during chromatin assembly could be provided by separate nicking and ligating enzymes or by a topoisomerase. Biochemical and pharmacological methods were used to identify the activity that relaxes DNA during chromatin assembly. Experiments using *Xenopus* oocyte and egg extracts clearly demonstrate that the relaxing activity required during assembly is provided by topoisomerase I, rather than topoisomerase II or separate nicking and closing activities (Almouzni and Méchali, 1988). Topoisomerase I is also responsible for DNA relaxation during assembly in *Drosophila* extracts (Becker and Wu, 1992). This use of topoisomerase I in preference to topoisomerase II was also observed when chromatin assembly was examined by microinjecting plasmids into oocyte nuclei (Almouzni and Méchali, 1988). Consistent with these results, topoisomerase I also supports chromatin assembly in reconstitution experiments using a fractionated *Xenopus* extract (Sapp and Worcel, 1990). Further evidence to support a dominant role of topoisomerase I during chromatin assembly is provided by a study where topoisomerase II was immunodepleted from a *Xenopus* egg extract. The depletion of topoisomerase II had no effect on chromatin assembly (Hirano and Mitchison, 1993).

Clearly the evidence in the literature suggests that topoisomerase I plays a dominant role during chromatin assembly. The extent of topoisomerase II activity during chromatin assembly in the *Xenopus* system has been addressed by several groups. The results of these studies differ significantly. The experiments used drugs to specifically inhibit either topoisomerase I or II. Wolffe et al. (1987) found that neither camptothecin, a topoisomerase I drug, nor VM-26, a topoisomerase II drug, when used alone, inhibited chromatin assembly. However, when the two drugs were used together, chromatin assembly was inhibited, suggesting that both topoisomerase I and II are involved in assembly. In contrast, Almouzni and Méchali (1988) report that one of the drugs used by Wolffe et al. (1987), camptothecin, does inhibit chromatin assembly. Almouzni and Méchali also found that novobiocin and VP-16, both topoisomerase II-specific drugs, do not inhibit assembly. These results indicate that topoisomerase I is required for assembly and that topoisomerase II is not required for chromatin assembly. Neither of these two studies observed an effect on chromatin assembly when topoisomerase II was inhibited alone. However, a study by Dunaway (1990) showed that in the presence of the topoisomerase II drug, VM-26, the time required for chromatin assembly doubles. This result suggests that topoisomerase II is involved in assembly.

Although there is some evidence that suggests that topoisomerase II is involved in chromatin assembly, it is still widely believed that topoisomerase I plays a dominant role during assembly and that topoisomerase II plays only a minor role, if any, during assembly (reviewed in Almouzni and Wolffe, 1993).

1.9 Objectives of Research

The process of chromatin assembly requires the cooperation of many different proteins. The roles of some of the proteins involved in assembly are well established, while the roles of other proteins are not well defined. For example, as discussed above the involvement of topoisomerase II during chromatin assembly is controversial, based on contradictory findings of several investigators. The focus of my work was to re-investigate the topoisomerase requirement during chromatin assembly, with the goal of clearly demonstrating whether or not topoisomerase II plays a role during assembly.

Another somewhat complex issue regarding chromatin assembly is the requirement for ATP during this process. Because I was studying the role of topoisomerase II, which is an ATP-dependent enzyme, the present analysis was extended to include an investigation of the requirement for ATP during chromatin assembly.

The last objective of my work was in regard to the identification of novel chromatin assembly proteins. Chromatin assembly factor-I is a well characterized protein complex that assembles chromatin, however the function of CAF-I appears to be redundant with proteins that have not been identified (see above). The initial steps in the fractionation of a yeast extract were therefore performed with the long term goal of identifying novel chromatin assembly factors, possibly including functional counterparts of the known subunits of CAF-1.

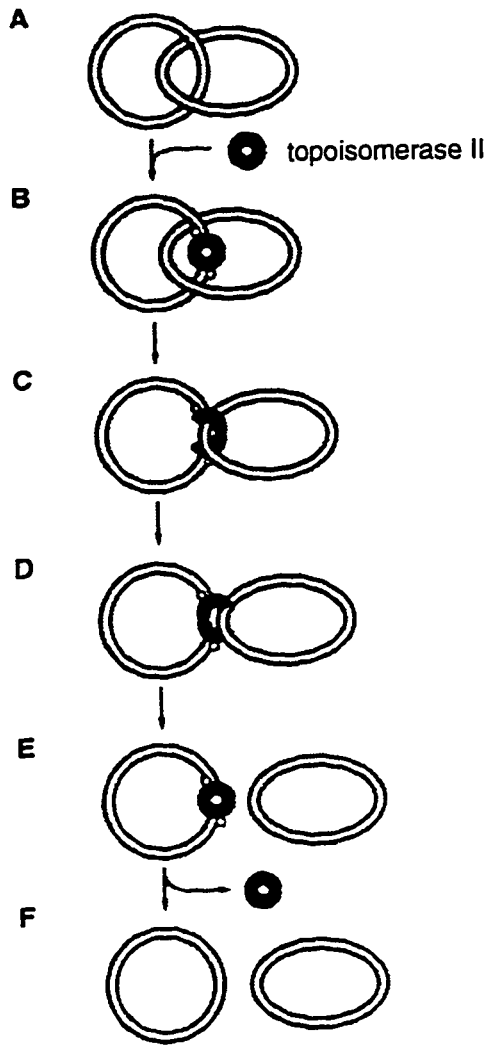


Fig. 1.1 Topoisomerase II can separate two interlocked DNA circles

A) Two interlocked circles of DNA. **B**) Topoisomerase II covalently attaches to both DNA strands of one circle, after it has made a break in both strands, forming a protein gate. **C,D**) The topoisomerase gate opens and closes, allowing the passage of the second DNA circle. **E**) The two DNA circles are now separated. **F**) The covalent attachment of topoisomerase II is reversed, restoring the intact double helix of the DNA circle, and releasing topoisomerase II. (Adapted from Alberts et al., 1994)

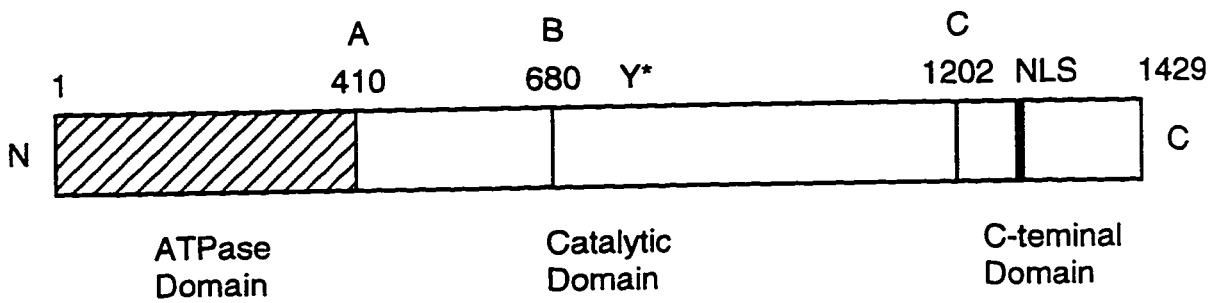
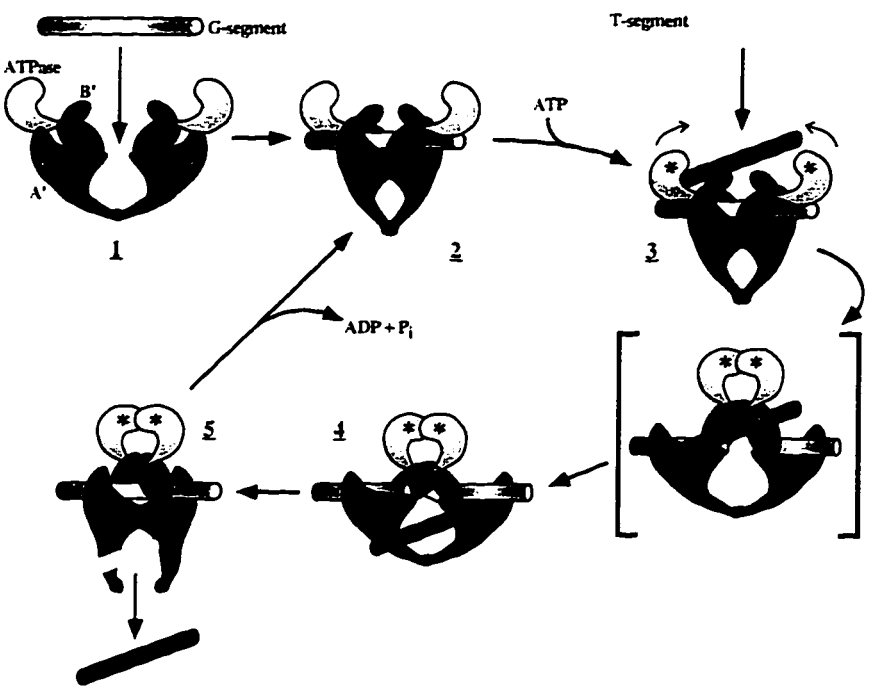


Fig. 1.2 Structural domains of yeast topoisomerase II

The three major structural domains of topoisomerase II are defined by SV8 protease-sensitive sites, labeled A and C. The third cleavage site (B) is only sensitive to protease upon ATP binding to topoisomerase II. The N-terminal ATPase domain is shown by the box with diagonal hatch marks. The central catalytic domain is the open box. The active site tyrosine is labeled Y*. The C-terminal domain is shown by the grey box. The putative nuclear localization signal is denoted by the thick vertical line. (Adapted from Watt and Hickson, 1994)

Fig. 1.3 Two-gate mechanism for the catalytic reaction of topoisomerase II

The A' and B' subfragments, and the ATPase domain are coloured blue, red, and yellow, respectively. The G-segment DNA is grey and the T-segment is green. Topoisomerase II is in the form of an open clamp (1), which binds the first DNA duplex (G-segment), inducing a conformational change in the enzyme (2). Binding of ATP (shown by asterisks) and a second DNA duplex (T-segment) (3) causes a series of conformation changes whereby the G-segment is split apart as the A' subfragments separate from each other. At the same time the ATPase domains dimerize and the T-segment is transported through the DNA gate and released through the dimer interface between the A' subfragments (4, 5). The G-segment is then religated, ATP is hydrolyzed and released, and topoisomerase II returns to it's open conformation (2). (Taken from Berger et al., 1996).


G-segment
T-segment
ATPase
B'
A'
ATP
1
2
3
ADP + P_i
5
4

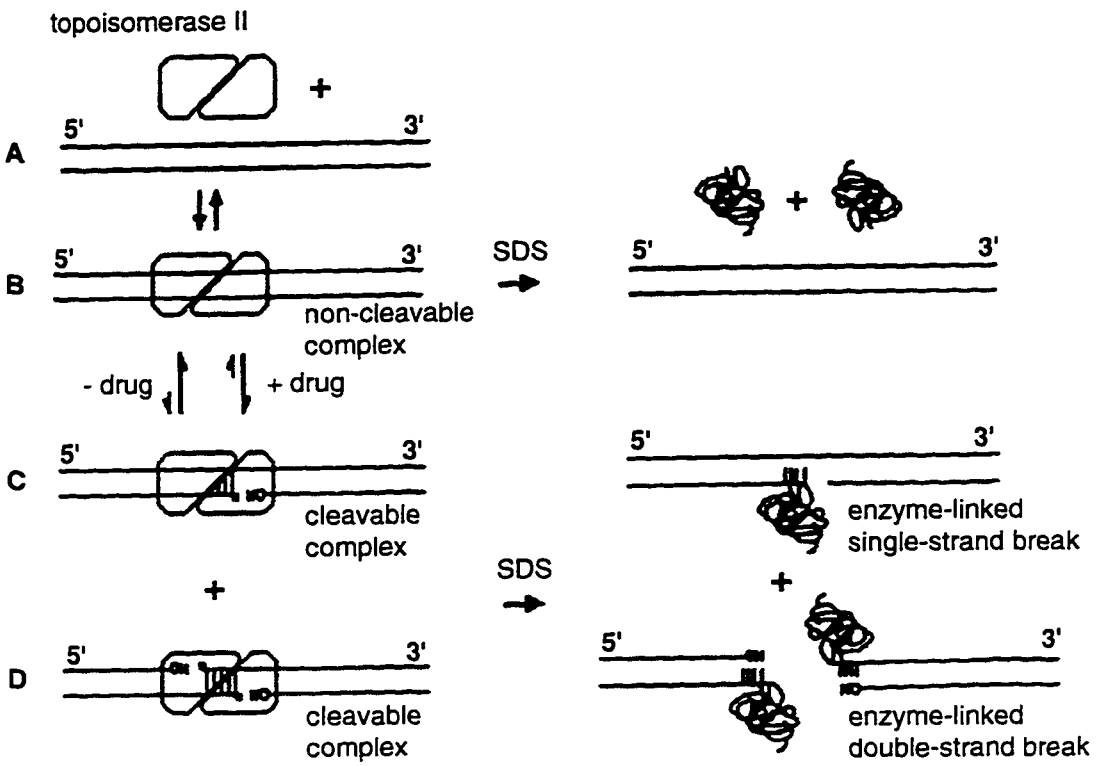


Fig. 1.4 A model for the action of topoisomerase II poisons

A) Topoisomerase II and substrate DNA. During the strand passage reaction, topoisomerase II can form two types of reaction intermediates, the noncleavable complex (**B**), and the cleavable complex (**C** and **D**). Treatment of the noncleavable complex with SDS generates intact DNA and free topoisomerase II. In contrast, SDS treatment of the cleavable complex generates single- and double-strand DNA breaks, with each 5' end of broken DNA covalently linked to a topoisomerase II molecule. (Adapted from D'Arpa and Liu, 1989).

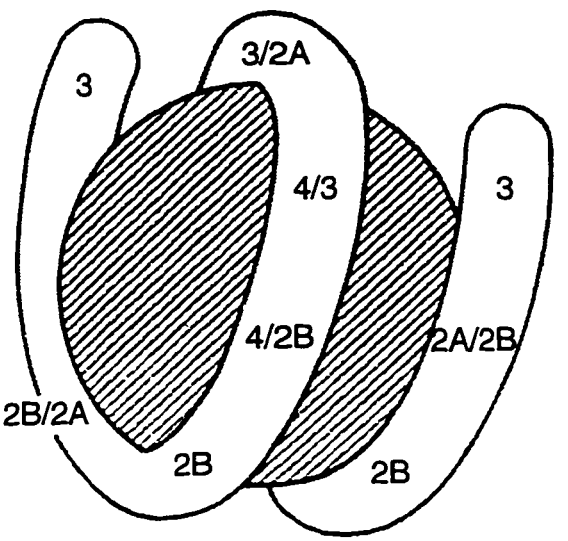


Fig. 1.5 The nucleosome core particle is the basic unit of chromatin

The nucleosome core particle consists of DNA wrapped twice around a histone octamer. This diagram shows the positions of histone-DNA contacts within the core particle. Two numbers together indicate where two histones contact the same region of DNA. (Adapted from Wolffe, 1992).

Chapter 2

Materials and Methods

2.1 Yeast Strains

Four closely related strains of *Saccharomyces cerevisiae* were used in this study. Strains TOP+ (same as W303-1a, originally from R. Rothstein, Columbia University: MAT**a** *ade2-1 trp1-1 can 1-100 leu2-3,112 his3-11,15 ura3-1*), top1 (W303-1a *top1*-8::LEU2), top2 (MAT**a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 top2-1*) and top1-top2 (top2 *top1-8*::LEU2) were kindly provided by Rolf Sternglanz (Brill and Sternglanz, 1988). For clarity the four strains are described as follows:

TOP+	wild-type	both topoisomerase I and II active
top1	Δtopo1	only topoisomerase II active
top2	topo2^{ts}	only topoisomerase I active
top1-top2	Δtopo1,topo2^{ts}	both topoisomerase I and II inactive

A364A (a *ade1 ade2 ura1 his7 lys2 tyr1 gal1* CDC9) and STX435-1-3B (α *ade1 ade2 lys2 ura1 his7 leu1 gal1 cdc9-1*) (Hartwell, 1967) are from the Yeast Genetic Stock Center. These two strains were used to analyze the function of DNA ligase I during chromatin assembly. A364A was also used in the drug inhibition study.

Strain top1-top2/pTOP2 was obtained by transforming the top1-top2 strain with the plasmid YEpTOP2-PGAL1 (Giaever et al., 1988; Worland and Wang, 1989, gift from J. Wang). This plasmid was designed for the overexpression of yeast topoisomerase II under the control of the inducible *GAL1* promoter.

2.2 Media and Growth in Liquid Culture

The yeast strains were grown in one litre of Yeast-Peptone-Dextrose (YPD, 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) at 22°C. In some cases, one half of the culture was shifted to 37°C for one hour prior to harvesting (see figure legends). The cultures were harvested at an of $O.D._{600}$ =

3-3.5, which corresponds to late log phase. The $O.D._{600}$ was determined by diluting 200 µl of yeast culture in 800 µl YPD and taking the O.D. at 600 nm using a spectrophotometer (Ausubel et al., 1993)

Strain top1-top2/pTOP2 was grown in complete minimal dropout medium (0.17% (w/v) yeast nitrogen base, 0.5% (w/v) ammonium sulfate, 0.13% (w/v) dropout powder, without uracil (Ausubel et al., 1993) supplemented with 2% (w/v) glucose, 3% (v/v) glycerol, and 2% (w/v) lactic acid. When the cells reached an $O.D._{600}$ = 3, the culture was split in two. One aliquot of cells were left in glucose medium. The other aliquot was spun down at 4000 rpm for 4 min, the cells were washed with water and then resuspended in complete minimal dropout medium (no uracil) supplemented with 2% (w/v) galactose. Both aliquots were further incubated for 4 h at room temperature, then shifted to 37°C for 1 h prior to harvesting.

2.3 Transformation of Yeast with Plasmid DNA

The top1-top2 strain was transformed with the plasmid YEpTOP2-PGAL1 using an adaptation of two transformation procedures (Elble, 1992; Gietz et al., 1992).

A 25 ml culture of top1-top2 in YPD was grown to an $O.D._{600}$ of 0.2-1.0. The cells were harvested by centrifugation at 4000 rpm for 5 min, 4°C. The pellet was resuspended in 1 ml of YPD per transformation being done, and 1 ml aliquots were transferred to eppendorf tubes. The cells were pelleted by briefly centrifuging the tubes in a microfuge at 14,000 rpm. The pellet was washed with 750 µl of TE/LiAc (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM lithium acetate, pH 7.5) and then the pellet was resuspended in 50 µl of TE/LiAc. 5 µl of denatured herring sperm DNA (10 mg/ml) was added, followed by 5 µl of plasmid DNA. Next, 500 µl of PLATE media (45% PEG 3350 (Sigma), 100 mM lithium acetate, pH 7.5, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, sterile filtered)

was added and the tubes left overnight at room temperature. The tubes were then centrifuged briefly in a microfuge at 14,000 rpm, and the pellet resuspended in 100 µl of sterile milliQ water. The cells were plated on selectible plates for plasmid selection. In this case, complete minimal dropout medium plates without uracil (2% [w/v] agar added to medium) were used.

2.4 Plasmid Isolation from Yeast

Plasmid DNA was isolated from yeast transformants by the procedure of Baker and Schatz (1987). A scoop of cells taken from a selectible plate was resuspended in 200 µl of lysis buffer (1 M Sorbitol, 0.1 M NaH_2PO_4, pH 7, 60 mM EDTA) with 3 mg/ml Zymolyase (Seikagaku, Japan) and incubated at 37°C for 1 h. 400 µl of 0.2 M NaOH/1% SDS was then added and the sample put on ice for 5 min. Next, 300 µl of 3 M sodium acetate, pH 4.8 was added and the tube incubated for 5 min on ice, followed by centrifugation at 14,000 rpm for 10 min in a microfuge. The supernatant was transferred to a new tube and mixed with one volume of isopropanol. After 10 min on ice, the tube was centrifuged for 15 min in a microfuge and the pellet resuspended in 50 µl of TE and stored at -20°C.

2.5 Transformation of *E. coli* with Plasmid DNA

E. coli (JM109) cells made competent by the $CaCl_2$ method (Maniatis, 1989), were transformed with the isolated plasmid DNA. 200 µl of frozen *E. coli* cells were thawed on ice and mixed with 5-10 µl of DNA in a 5 ml tube and incubated for 30 min on ice. The cells were then incubated at 37°C for 5 min, followed by 2 min on ice. 800 µl of Brain Heart Infusion (BHI) broth was added and the tubes were shaken at 37°C for at least 1 h. The cells were pelleted and resuspended in 100 µl of BHI broth, then plated on BHI plates (2% [w/v] agar added to BHI broth) containing ampicillin (100 µg/ml). The plates were left at 37°C, overnight. The next day a colony was picked from a plate and used to

inoculate 10 ml of BHI broth containing ampicillin (100 µg/ml), which was shaken at 37°C, overnight. This culture was used to isolate the plasmid DNA.

2.6 Plasmid Isolation from *E. coli*

The plasmid DNA was isolated from the transformed *E. coli* using a modification of the alkaline lysis mini-prep method described in Maniatis et al. (1989). The 10 ml bacterial culture was centrifuged at 4500 rpm for 10 min, 4°C (Beckman Avanti 30, F0650 rotor). The pellet was resuspended in 200 µl of cold Glucose-Tris-EDTA buffer (GTE, 50 mM glucose, 25 mM Tris-HCl, pH 8, 10 mM EDTA) by vortexing, followed by addition of 400 µl of 0.2 M NaOH/1% SDS. The tubes were mixed by inversion and then 300 µl of cold potassium acetate solution (5 M acetate) was added, vortexed gently, and then put on ice for 5 min. This was followed by centrifugation at 14,000 rpm for 5 min, 4°C in a microfuge. The supernatant was transferred to a new tube for phenol/chloroform extraction. One volume of isopropanol was added and the tubes left on ice for 10 min. The DNA was pelleted by centrifugation at 14,000 rpm for 15 min and the pellet resuspended in 50 µl of TE containing DNAase-free RNAase (20 µg/ml). The DNA was stored at -20°C.

2.7 Restriction Enzyme Digestion of Plasmid DNA

The plasmid DNA isolated from *E. coli* was analyzed by comparing it's restriction digest pattern with the pattern of the original DNA used to transform the yeast strain. The reactions were set up as follows: 1 µl of DNA, 1 µl of 10X NEBuffer 2, 1 µl of Hind III (NEB), and 7 µl of sterile water. The reactions were incubated for 1 h at 37°C and were stopped by the addition of 2 µl of loading buffer. The DNA was resolved on a 0.8% agarose gel, which was stained with 0.5 µg/ml ethidium bromide following electrophoresis. The DNA was visualized using a UV light box.

2.8 Cell Harvesting for Extract Preparation

To harvest the cells (Schultz et al., 1991), the culture was poured through crushed ice packed in a filter funnel and collected in a 500 ml centrifuge bottle. Once collected, the cells were spun at 4000 rpm for 4 min, 4°C (Jouan CR 412). The cells were then resuspended in 12 ml cold distilled water and transferred into a 50 ml tube which had been previously weighed and cooled. The tube was spun at 4000 rpm for 4 min, 4°C. Next, the supernatant was decanted off and the weight of the cells determined by weighing the cells in the tube and subtracting the tube weight. The cells were resuspended in 1.3 volumes (with respect to weight of cells) of yeast extraction buffer (YEB, 100 mM Hepes-KOH, pH 7.9, 245 mM KCl, 5 mM EGTA, 1 mM EDTA) and spun at 4000 rpm for 4 min, 4°C. The cell pellet was then resuspended in 1.3 volumes of YEB with protease inhibitors (2.5 mM dithiothreitol (DTT), 0.2 mM PMSF, 10 mM Benzamidine-HCl, 25 µg/ml TPCK, 3.5 µg/ml Pepstatin A, 5 µg/ml Leupeptin, 10 µg/ml Aprotinin) and spun at 4000 rpm for 4 min, 4°C. The supernatant was decanted and the cell paste scraped into a 5cc syringe using a metal spatula. The paste was squirted into a 50 ml tube filled with liquid nitrogen. The frozen cells were stored at -70°C.

2.9 Extract Preparation

A yeast whole cell extract was prepared using a modification of the procedure by Schultz et al. (1991). 2.5 g of frozen cells were broken in a home coffee mill using dry ice as the coolant. The mill was chilled first by covering the blades with nuggets of dry ice and running it until the dry ice was a powder. The frozen cells were added and the mill run for 5 min which reduces the cells to a powder mixed in with the dry ice. The frozen powder was transferred to a cold beaker. When the powder just starts to thaw, 3.25 ml of YEB with protease inhibitors was added to resuspend the powder. The suspension was

transferred to a cooled centrifuge tube and spun at 27 000 rpm for 2 h, 4°C (Beckman L7-65 Ultracentrifuge, SW 55 Ti rotor). The supernatant, which is the extract, was collected using a needle and syringe to puncture the side of the tube. The extract was dialyzed for 4 hours against 50 volumes of yeast dialysis buffer I (YDBI, 20 mM Hepes-KOH, pH 7.9, 50 mM KCl, 5 mM EGTA, 0.05 mM EDTA, 20 % glycerol, 2.5 mM DTT, 0.2 mM PMSF, 0.5 µg/ml Leupeptin). Following dialysis, the extract was aliquoted into eppendorf tubes and quickly frozen using liquid nitrogen, then stored at -70°C. Protein concentration of the extract was determined using the Bio-Rad Protein Assay Kit, which is based on the Bradford assay (Bradford, 1976). Bovine serum albumin was used as the reference.

2.10 Plasmid Supercoiling Assay

Nucleosome assembly was monitored by the plasmid supercoiling assay (Germond et al., 1975). Reactions were performed using 100 µg of yeast extract, unless otherwise stated, in a final volume of 20 µl, for 1 h at 30°C. The stock solutions used in the reactions are as follows: 10x Chromatin Assembly Buffer (75 mM $MgCl_2$, 10 mM DTT, 0.5 mM EDTA) - 2 µl, ATP (60 mM) - 1 µl, creatine phosphokinase (2 µg/ml) - 1 µl, creatine phosphate (400 mM) - 1 µl, template (10 ng/µl) - 2 µl. The template was pBluescript KS II (Stratagene) internally labeled at the *Hind* III site, (Razvi et al., 1983, described below) mixed 1:4 with unlabeled pBluescript KS II relaxed with calf thymus topoisomerase I (Life Technologies). The extract was added in 13 µl, obtained by adding YDBI to the appropriate volume of extract. The reactions were stopped by adding 200 µl of stop buffer (0.3 M sodium acetate, 0.5% SDS, 10 mM EDTA), then extracted (phenol/chloroform, chloroform). The DNA was precipitated using 3 volumes of 95% ethanol and placed at -70°C for at least one hour. The DNA was recovered by spinning the tubes at 14,000 rpm for 15 min. The pellet was

washed using 70% ethanol and when the pellet was dry it was resuspended in 12 µl of TE (10 mM Tris-HCl, pH 8, 1 mM EDTA). 3 µl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) was added and the DNA was resolved on a 0.8% agarose gel using TAE (40 mM Tris-acetate, 1 mM EDTA) running buffer. Assembly of nucleosomes was observed by the incorporation of negative supercoils into the DNA, as detected by autoradiography.

2.11 Internal Labeling of pBluescript

The labeling of pBluescript (Razvi et al., 1983) for use in the chromatin assembly reactions was done as follows. 2 µl of pBluescript KS II (0.25 µg/µl), that had been linearized by *Hind* III digestion and treated with calf intestinal alkaline phosphatase, was mixed with 1.2 µl of 10X T4 polynucleotide kinase buffer (NEB), 7.5 µl [γ-^{32}P]dATP (Amersham, 10 µCi/µl), and 1 µl T4 polynucleotide kinase (NEB, 10 U/µl) and incubated at 37°C for 3 h. Then 100 µl of 5X T4 DNA ligase buffer (Gibco BRL), 3 µl T4 DNA ligase (Gibco BRL, 1 U/µl), and 385 µl of sterile milliQ water was added to the kinase reaction and incubated at 4°C, overnight. To separate the unincorporated nucleotides, the reaction was run over a 3 ml G-50 Sephadex (Sigma) spin column (Maniatis et al., 1989). Glass wool was used to plug the outlet of a 3cc syringe and the G-50 Sephadex was added and spun at 4000 rpm for 30 sec. One column volume of STE (0.1 M NaCl, 10 mM Tris-Cl, pH 8, 1 mM EDTA) was used to wash the column and then the sample was loaded and spun at 4000 rpm for 30 sec. The sample was then extracted (phenol/chloroform, chloroform) and 1/7 volume of 5M ammonium acetate added. The sample was split into 2 eppendorf tubes and 3 volumes of 95% ethanol added to precipitate the DNA. The DNA was resuspended in 25 µl of TE, with a final concentration of 20 ng/µl.

2.12 *In vitro* Add-back Experiments using Purified Topoisomerases and DNA Ligase

The chromatin assembly reactions were set up as previously described with the addition of either 1 µl of calf thymus topoisomerase I (Life Technologies, 15U/µl) or 2 µl of human topoisomerase II (TopoGEN, Inc., 2 U/µl) in a final volume of 20 µl. 2 µl of *E. coli* T4 ligase (Life Technologies, 1U/µl) was used for the ligase experiment.

2.13 Micrococcal Nuclease Analysis

The various micrococcal nuclease experiments were all conducted as follows. Modifications to the protocol for some experiments are stated after the general procedure. A 120 µl chromatin assembly reaction (900 µg protein and 120 ng of template) was set up as previously described, except that only unlabeled relaxed pBluescript KS II was used. After the 1 h assembly reaction, 20 µl of the reaction mix was removed for the 0 time point, and 6 µl of 0.1 M $CaCl_2$ and 4.4 µl of micrococcal nuclease (Sigma, 0.5 U/µl) was added to the remaining 100 µl reaction and digested at 37°C. 20 µl aliquots were stopped at various time intervals by adding 1.5 µl of 0.5 M EDTA and processed according to the Sarkosyl method of Becker and Wu (1992). Specifically, each sample received 5 µl of 2.5g/100 ml Sarkosyl, 100 mM EDTA, and then was digested with 200 µg/ml RNase A at 37°C for 15 min, followed by 1 mg/ml proteinase K at 37°C for 30 min. The samples were then extracted (phenol/chloroform, chloroform) and the DNA precipitated as described previously. The products were resolved by 1.5% agarose gel electrophoresis in Tris-glycine running buffer. Reaction products were detected by Southern blotting using Genescreen Plus (NEN) and ^{32}P-labeled pBluescript KS II probe prepared by random primed synthesis (oligonucleotides were from the DNA Core Facility, Biochemistry Department, University of Alberta).

2.14 Southern Blot Analysis

After the DNA products were separated on the agarose gel, the DNA was transferred to a Genescreen Plus (NEN) membrane. The transfer buffer was 0.4 M NaOH (Haniford et al., 1991). Following transfer, the DNA was crosslinked to the membrane using a Stratagene UV Stratalinker according to the manufacturers instructions. The membrane was then transferred to a hybridization tube and soaked in pre-hybridization solution (0.5 M NaH_2PO_4, pH 7.2, 5% SDS, 1 mM EDTA) at 65°C for 3 h using a hybridization oven. The membrane was probed with ^{32}P-labeled pBluescript KS II that was prepared by the random oligonucleotide primer method (Feinberg and Vogelstein, 1984).

The labeling reaction of pBluescript was set up as follows: 10 µl ABC mix (0.25 M Tris-HCl, pH 8, 25 mM $MgCl_2$, 50 mM 2-mercaptoethanol, 0.1 mM each of dATP, dGTP, dTTP, 1 M Hepes, pH 6.6, 54 nM oligonucleotide in 3 mM Tris-HCl, pH 7, 0.2 mM EDTA), 2 µl BSA, 12.5 µl pBluescript (2 ng/µl, digested with *Pvu* I and *Pvu* II) , 5 µl [α-^{32}P]dCTP (Amersham, 10 µCi/µl), 1 µl DNA Polymerase I Large Fragment (Klenow) (NEB , 5 U/µl), and 19.5 µl of sterile milliQ water. The reaction was incubated at 37°C for 3 h, then stopped by adding 2.5 µl of bromophenol blue (1 mg/ml) and 60 µl blue dextran (10 mg/ml). To separate out the unincorporated nucleotides, the reaction was chromatographed on a 5 ml G-50 Sephadex (Sigma) column equilibrated in DNA buffer (10 mM Tris-HCl, pH 7.4, 5 mM NaCl, 1 mM EDTA). The labeled DNA was collected and 1/10 volume of 2M NaOH was added to the DNA and left for 2 min. This was followed by the addition of 1/10 volume of 2M HCl. The labeled DNA was added to the pre-hybridization solution and incubated at 65°C, overnight. The membrane was then treated with wash buffer (40 mM $Na_2H_2PO_4$, pH 7.2, 1% SDS) at 55°C for 30 min; the wash was repeated once. The DNA was detected by autoradiography.

2.15 Modifications in the Micrococcal Nuclease Analysis Procedure

The amount of micrococcal nuclease was reduced by 6-fold for the digestions of naked DNA. In one set of experiments, 12 µl of poly-L-glutamate (ICN, 5 µg/µl), was included in the 120 µl reaction, to inhibit an activity that appears to destabilize chromatin in the extract. In these reactions, 1 mg of protein and 12 µl of micrococcal nuclease (0.5 U/µl) was used and the time points of digestion were reduced. The experiment with poly-L-glutamate without extract contained 3 µl of purified yeast histones (0.5 µg/ml, Schultz et al., 1997), 3 µl of calf thymus topoisomerase I (Life Technologies, 8 U/µl), and 0.75 µl of poly-L-glutamate (5 µg/µl). Carrier tRNA was added to these samples and the naked DNA reactions during precipitation.

2.16 Labeling of 123 bp Ladder

The 123 bp ladder (Gibco BRL) used as a marker for the micrococcal nuclease analysis was labeled with ^{32}P after dephosphorylation with alkaline phosphatase (Maniatis et al., 1989). For dephosphorylation, 10 µl of the 123 bp ladder (1 µg/µl) was mixed with 1 µl of calf intestinal alkaline phosphatase (NEB, 10 U/µl), and 9 µl NEBuffer 2 and incubated at 37°C for 1 h. The reaction was stopped by adding 0.2 µl of 0.5 M EDTA to the reaction and incubating it at 75°C for 10 min. The tube was then placed on ice for 2 min. 100 µl of stop buffer was added and the DNA extracted and precipitated. The DNA was resuspended in 20 µl of water.

1 µl of this DNA was then mixed with 7.5 µl [γ-^{32}P]dATP (Amersham, 10 µCi/µl), 1 µl T4 polynucleotide kinase (NEB, 10 U/µl), and 1 µl of 10X T4 polynuceotide kinase buffer (NEB). The reaction was incubated at 37°C for 1 h. 490 µl of stop buffer was added and the unincorporated nucleotides separated by G-50 Sephadex spin column and the sample processed as

described in the section "Internal labeling of pBluescript". The DNA was resuspended in 100 µl of water.

2.17 Drug Inhibition Study

Chromatin assembly reactions were performed as described above, in the presence of increasing concentrations of VP-16 ([etoposide] Bristol-Meyers Squibb). The drug was provided as a 34 mM solution in 20 mg/ml benzyl alcohol and 30.5% ethanol. Each 20 µl assembly reaction received 1.2 µl of solvent (20 mg/ml benzyl alcohol, 30.5% ethanol) containing the amount of VP-16 to give the final VP-16 concentrations indicated in the figure legends. The reaction components were mixed on ice, VP-16 being added last. The reactions were stopped by adding 5 µl of 2.5 g/100 ml Sarkosyl, 100 mM EDTA, and the resulting mixture was then treated with 1 mg/ml proteinase K for 30 min at 37°C. 200 µl of stop buffer was added, the protein extracted and DNA precipitated. The products were run on a 0.8% agarose gel in TAE running buffer and the DNA detected by autoradiography.

2.18 ATP Experiments

Chromatin assembly reactions were performed as described, in the presence or absence of ATP (see figure legends). In some reactions, 0.6 µl of 100 mM ATP-γ-S or 100 mM AMP-PNP, which are non-hydrolizable ATP analogs, was added in place of the ATP. ATP was depleted in the extract that contained endogenous ATP with 1 µl of apyrase (Sigma, 3 U/µl) (Cande, 1982).

Partial Purification of the Chromatin Assembly Machinery

2.19 Ammonium Sulfate Precipitation

In pilot experiments, a step-wise series of precipitations involving the addition of increasing amounts of ammonium sulfate to the TOP+ extract was performed (Englard and Seifter, 1990). Solid ammonium sulfate was slowly added to the extract to 35% saturation and stirred on ice until dissolved. The

precipitated proteins were pelleted by centrifugation at 14,000 rpm for 15 min, 4°C. 100 µl of the supernatant was removed for later experiments, the remaining supernatant was adjusted to 45% saturation by adding more ammonium sulfate, and the precipitated protein was pelleted as before. This procedure was repeated two more times, with the supernatant adjusted to 55% and then 70% saturation. Each pellet was resuspended in YDBI. All samples were dialyzed against 200 volumes of YDBI (no KCl) for 4 h and then against 200 volumes of regular YDBI (50 mM KCl), overnight. Conductivity was measured to ensure that dialysis had gone to completion. The samples were stored at -70°C.

The final ammonium sulfate precipitation method involved the addition of ammonium sulfate to the extract to 50% saturation in one step. The precipitated proteins were pelleted as above and the supernatant was adjusted to 70% saturation. The 70% saturated supernatant was centrifuged and the ammonium sulfate pellet (referred to as 70p), which contains the proteins of interest, was resuspended in 400 µl of YDBI. The sample was then dialyzed, and used as the starting material for the column chromatography.

2.20 Analysis of Ammonium Sulfate Precipitation Samples

100 µg of protein from each supernatant and pellet collected during the ammonium sulfated precipitations was used in the chromatin assembly reaction. To determine if plasmid supercoiling was dependent on histones, 5 µg of the 70% pellet (70p) was used in chromatin assembly reactions, in the presence or absence of 1 µg of purified yeast histones (Schultz et al., 1997). The 70p was also analyzed using micrococcal nuclease digestion. 600 µg of protein was used in the 120 µl chromatin assembly reaction. The sample was digested with 12 µl of micrococcal nuclease (0.5 U/µl).

2.21 DEAE Sepharose Fast Flow Chromatography

The column was first prepared by washing it with 10 bed volumes of YDBI (0.1 M KCl), followed by 5 bed volumes of YDBI (1 M KCl). The column was then equilibrated with YDBI (0.1 M KCl). As a pilot experiment, 1 mg of the ammonium sulfate pellet (diluted to 1 mg/ml) was loaded onto a 1.3 ml DEAE Sepharose Fast Flow (Pharmacia Biotech) column. Once the protein had entered the resin, the column was washed with YDBI (0.1 M KCl) and fractions collected. Proteins that bound to the resin were then eluted with YDBI (1 M KCl). The fractions were dialyzed against YDBI (50 mM KCl) for 4-6 h.

The final chromatography procedure involved a series of column elutions using increasing amounts of KCl. 2 mg of the ammonium sulfate pellet (diluted to 5 mg/ml) was loaded onto the 1.3 ml DEAE Sepharose Fast Flow column. The column was washed with YDBI (0.1 M KCl) as before and fractions collected. This was followed by washes with YDBI (0.3 M KCl) and YDBI (0.6 M KCl). The fractions were dialyzed and their protein concentrations determined using the Bio-Rad Protein Assay Kit.

2.22 Analysis of Column Fractions

13 µl of each column fraction (0.5-4 µg of protein), was used in the chromatin assembly reaction. Some reactions were supplemented with 0.35 µg of purified yeast histones (Schultz et al, 1997). Radiolabeled pBluescript KS II was used alone in the reactions containing the final chromatography samples (equivalent to 4 ng of DNA per reaction). The chromatin assembly reactions for the pilot experiment samples contain the mix of labeled and unlabeled pBluescript KS II (20 ng per reaction). Carrier tRNA was added during the precipitation of DNA.

For the add-back experiments involving fractions collected during the 0.1 and 0.6 M KCl washes, 2 µl of human topoisomerase II (TopoGEN, Inc., 2 U/µl)

and 0.35 µg of purified yeast histones (Schultz et al., 1997) were added to the chromatin assembly reactions.

One of the peak protein fractions collected during the 0.3 M KCl wash was analyzed by micrococcal nuclease digestion. Approximately 36 µg of protein and 30 ng of DNA was used in the 120 µl chromatin assembly reaction, followed by micrococcal nuclease digestion as described above.

2.23 Topoisomerase II Assay

The presence of topoisomerase II in the various chromatography fractions was assayed by the decatenation of kinetoplast DNA (Marini et al., 1980) and the concomitant appearance of a monomer DNA of 2.5 kilobases (kb). A modification of the original assay was used as follows. All reactions contained 0.2 µg of kinetoplast DNA (KDNA, TopoGEN, Inc.) in a final volume of 20 µl. The first control contained KDNA incubated in the absence of enzyme. The positive control contained 2 µl of 10X topoisomerase II buffer (TopoGEN, Inc.) and 2 µl of human topoisomerase II (TopoGEN, Inc., 2U/ µl). The negative control contained 2 µl of 10X topoisomerase I buffer (TopoGEN, Inc.) and 2 µl of human topoisomerase I (TopoGEN, Inc., 2U/µl). The reactions were incubated at 37°C for 30 min and stopped by addition of 3 µl of 5% Sarkosyl and 5 µl of loading buffer.

Reactions using yeast proteins contained 0.2 µg of KDNA, 3 µl of 10X topoisomerase II buffer, and 25.5 µl of each column fraction. These reactions were incubated at 37°C for 1 h and stopped by addition of 5 µl of 5% Sarkosyl. The samples were digested using 1 mg/ml proteinase K at 37°C for 15 min. 200 µl of stop buffer was then added, followed by phenol/chloroform extraction and DNA precipitation. The DNA was resolved on a 1% agarose gel, which was stained with 0.5 µg/ml ethidium bromide following electrophoresis. The DNA was visualized using a UV light box.

Chapter 3

The Function of Topoisomerase II during Chromatin Assembly[1]

[1] A version of this chapter has been published. Garinther, W.I. and Schultz, M.C. (1997). Mol. Cell. Biol. 17: 3520-3526.

3.1 Introduction

While it has been established that both topoisomerase I and II are involved in replication and transcription (Wang, 1987; D'Arpa and Liu, 1989), the role of topoisomerase II during chromatin assembly is controversial and has not been clearly elucidated. The evidence in the literature suggests that topoisomerase I plays a dominant role during assembly. The functional dominance of topoisomerase I during chromatin assembly is unusual, considering the functional redundancy of the topoisomerases during such processes as replication and transcription, and the fact that yeast cells remain viable when topoisomerase I is deleted (Thrash et al., 1985; Goto and Wang, 1985).

In view of the contradictory results regarding the role of topoisomerase II during chromatin assembly, we decided to re-investigate the topoisomerase requirement during chromatin assembly. This chapter describes the results of experiments performed in a yeast *in vitro* system that contains all the components necessary for nucleosome assembly onto naked DNA.

3.2 Chromatin Assembly in Extracts that lack Topoisomerase Activity

These experiments were performed using a whole cell extract from the yeast *Saccharomyces cerevisiae*. This yeast extract supports transcription by RNA polymerase I, II, and III (Schultz et al., 1991). It has also been shown to support chromatin assembly (Schultz et al., 1997). The chromatin assembly reaction occurs under physiological salt conditions, is ATP-dependent, uses soluble histones, and generates physiologically spaced nucleosomes. Yeast as the model system provides an opportunity to use both biochemical and genetic methods to study the process of chromatin assembly.

The goal of my work was to analyze the topoisomerase requirement during chromatin assembly. For this purpose previously described mutants of topoisomerase I and II were used (Brill and Sternglanz, 1988).

The initial experiments were performed to determine if a topoisomerase is required to provide the relaxing activity necessary for chromatin assembly onto closed circular DNA. Extracts were prepared from a wild-type strain, designated TOP+, and an isogenic mutant strain, top1-top2. The top1-top2 strain has the topoisomerase I gene deleted and the topoisomerase II gene carries a temperature sensitive (*ts*) conditional lethal mutation. These cells grow at the permissive temperature (22°C) but die when the cells are shifted to the restrictive temperature (37°C). Extracts from the top1-top2 strain have been shown to contain no detectable topoisomerase activity. This was demonstrated using a plasmid relaxation assay, where the input supercoiled DNA remained supercoiled in the presence of the top-top2 extract. The TOP+ extract, on the other hand, was readily able to relax the supercoiled DNA (Schultz et al., 1992). Of note, the top1-top2 extract used for the relaxation assay was prepared from cells that were grown at the permissive temperature. This indicates that the *top2*^{ts} allele is inactive for relaxation *in vitro* whether or not the cells were shifted to the restrictive temperature. The *ts* allele of topoisomerase II therefore behaves like many other mutant proteins (e.g. Johnston and Nasmyth, 1978; Hockman and Schultz, 1996); it confers a *ts* growth phenotype and is inactive when assayed in extracts prepared from cells grown at the permissive temperature.

Chromatin assembly reactions were performed using the TOP+ and top1-top2 extracts. Relaxed closed-circular DNA was used as the template for nucleosome assembly in the reactions. Chromatin assembly was assessed using a plasmid supercoiling assay which involves the resolution of DNA

products after protein removal on an agarose gel, followed by autoradiography. The assembly of nucleosomes onto closed circular DNA results in the appearance of one negative supercoil per nucleosome after removal of protein from the DNA. Therefore, the level of negative supercoiling of the DNA is a measure of the number of nucleosomes that were assembled onto the template (Germond et al, 1975). Plasmid supercoiling was efficient in the TOP+ extract, but was severely impaired in the top1-top2 extract (Fig. 3.1A). The lack of supercoiling in the top1-top2 extract was seen over a wide range of protein concentrations (Fig. 3.1B).

To show that the lack of supercoiling observed with the top1-top2 extract was not due to the accumulation of nicked template, the products of assembly reactions with these two extracts were resolved on an agarose gel run in the presence of ethidium bromide. Nicked template is resolved from closed-circular template when electrophoresed under these conditions. Fig. 3.1C shows that the proportion of nicked template is the same for both samples. Therefore, the failure to observe supercoiling in the top1-top2 extract was not due to nicking of the template.

A mixing experiment was carried out next to determine if an inhibitor of chromatin assembly was present in the top1-top2 extract. The TOP+ and top1-top2 extracts were mixed together and used in an assembly reaction. Assembly was compared in the TOP+ extract alone, the top1-top2 extract alone, and in a mixture of both extracts. The amount of supercoiling in the mixture of the TOP+ and top1-top2 extracts was the same as with the TOP+ extract alone (Fig. 3.1D). This result demonstrates that the top1-top2 extract does not contain an excess of an inhibitor that affects chromatin assembly.

The top1-top2 extract contains all cellular proteins, except active topoisomerase I and II. We can therefore conclude from this set of experiments

that a topoisomerase is required for the relaxation of the DNA template that accompanies supercoiling due to chromatin assembly.

The assembly of nucleosomes in the TOP+ and top1-top2 extracts was also analyzed by micrococcal nuclease digestion. Micrococcal nuclease digests the unprotected DNA in the linker regions between nucleosomes. When the yeast extract was absent during the assembly reaction, the DNA template was rapidly digested by micrococcal nuclease (Fig. 3.2A). The amount of micrococcal nuclease used in this experiment had to be reduced by 6-fold in order to observe the smear of DNA before it was fully digested and not retained in the gel. Bands of 165 bp and 330 bp, corresponding to a mononucleosome (monosome) and dinucleosome (disome), respectively, were generated in the TOP+ extract (Fig. 3.2B). This digestion pattern corresponds to the physiological repeat length of nucleosomes observed *in vivo* in yeast (van Holde, 1988). A monosome and faint disome was also observed for the top1-top2 extract (Fig. 3.2C). This result indicates that a nucleosome can form on the DNA in the absence of topoisomerase activity. However, as shown in Fig. 3.1A, not enough nucleosomes were assembled to supercoil the DNA.

The absence of a well-defined micrococcal nuclease ladder of the assembled chromatin was of some concern. This could be due to such phenomena as nucleosome sliding and chromatin remodeling (Varga-Weisz et al., 1995; Kingston et al., 1996). For example, there are a number of activities in yeast that have been implicated in the disruption of chromatin structure during transcriptional activation (Kingston et al, 1996; Cairns et al., 1996). The failure to obtain an extensive micrococcal nuclease ladder may also reflect the presence of templates that were not fully packed with nucleosomes at the time of digestion. In an attempt to stabilize the assembled nucleosomes, poly-L-glutamate was added to the reactions. Poly-L-glutamate has been shown to

stabilize nucleosomes assembled in human cell extracts (Banerjee and Cantor, 1990). In the presence of poly-L-glutamate, a trisome was observed and four bands could be detected upon longer exposure of the film, for both extracts (Fig. 3.3). Poly-L-glutamate cannot assemble nucleosomes on its own. This was shown by adding poly-L-glutamate to an assembly reaction containing purified yeast histones and topoisomerase I, but no yeast extract (Fig. 3.4). Under these conditions the DNA was very quickly digested with a reduced amount of micrococcal nuclease, as seen with naked DNA, and no nucleosome-sized fragments were generated.

3.3 Demonstration of Topoisomerase I and II Function in Chromatin Assembly by in vivo Manipulations of Topoisomerase Activity

Using yeast strains with mutations in either *TOP1* or *TOP2* alone, we were able to determine the level of assembly-driven supercoiling in the presence of an individual topoisomerase. The two strains used in these experiments are isogenic to the TOP+ and top1-top2 strains. In the top1 strain only topoisomerase II is active, since the topoisomerase I gene has been deleted. The top2 strain has the wild-type *TOP1* gene, and the temperature sensitive allele of the *TOP2* gene.

As shown above, supercoiling was readily detected in the TOP+ extract (Fig. 3.5, lanes 1 and 2), and was not observed in the top1-top2 extract, whether or not the cells were shifted to the restrictive temperature prior to harvesting (lanes 7 and 8). Supercoiling was also observed in the top2 extract (lanes 5 and 6), which was expected, since topoisomerase I is already known to play an important role in assembly in higher eukaryotic systems. However, supercoiling was just as efficient in the top1 extract, indicating that topoisomerase II can also provide the relaxing activity required for chromatin assembly (lanes 3 and 4). This result was unexpected because of the overwhelming support in the

literature for the idea that topoisomerase II does not play a significant role in chromatin assembly (see chapter 1).

We decided to analyze the function of topoisomerase II in more detail by testing if the kinetics of supercoiling was affected when assembly was performed with topoisomerase II alone, rather than with both topoisomerase I and II. Aliquots from parallel TOP+ and top1 assembly reactions were stopped at various times and the products were resolved by agarose gel electrophoresis (Fig. 3.6A). The level of supercoiling in this experiment was quantitated by phosphoimager analysis. The amount of supercoiled DNA in the highly supercoiled form was expressed as a percentage of the total DNA present in a lane. For quantitation we arbitrarily designated any topoisomers that migrated faster than the top five bands in the input DNA as "highly supercoiled". Fig. 3.6A shows that the time course of assembly was similar between the TOP+ and top1-top2 extracts. The quantitation of the reaction products in Fig. 3.6B confirms that the accumulation of highly supercoiled DNA occurred at the same rate in both extracts. Also, the final level of supercoiling achieved in the presence of topoisomerase II alone was the same as in the presence of both topoisomerase I and II. This result demonstrates that in yeast the kinetics of supercoiling in the presence of topoisomerase II alone is the same as in the presence of both topoisomerase I and II.

We then tested if we could restore chromatin assembly in the top1-top2 strain by the *in vivo* add-back of wild-type topoisomerase II. This was accomplished by transforming the top1-top2 strain with a plasmid that expresses yeast topoisomerase II under control of the inducible *GAL1* promoter (Giaever et al, 1988). The transformed strain was designated top1-top2/pTOP2. Galactose induction of this strain was expected to promote assembly-driven supercoiling, if topoisomerase II can function during chromatin assembly. As

expected, no supercoiling was observed in the top1-top2 extract even under inducing conditions (Fig. 3.7, lanes 1 and 2). There was leaky expression of topoisomerase II, shown by the appearance of some supercoiling before the promoter was induced by galactose (lane 3). However, when topoisomerase II was fully expressed in the top1-top2/pTOP2 strain, supercoiling was completely restored (lane 4). This experiment further supports our conclusion that topoisomerase II can function during chromatin assembly in yeast.

The assembly of nucleosomes in the top1-top2/pTOP2 extract prepared from cells after galactose induction was analyzed by micrococcal nuclease digestion. Clear monosomes and disomes were observed over the time course of digestion (Fig. 3.8). Therefore, the expression of topoisomerase II in the top1-top2 cells results in an cellular extract that supports the efficient formation of normal nucleosomes.

3.4 Demonstration of Topoisomerase I and II Function in Chromatin Assembly by in vitro Manipulations of Topoisomerase Activity

To confirm our *in vivo* observations that topoisomerase I or II could provide the relaxing activity required for chromatin assembly, we tested whether the addition of purified topoisomerase I or II would rescue plasmid supercoiling in the top1-top2 extract.

Purified calf thymus topoisomerase I or human topoisomerase II was added to the top1-top2 extract and assembly reactions were performed under standard conditions. Fig. 3.9A shows that supercoiling was rescued with topoisomerase I, as expected (lane 3). Similarly, topoisomerase II also rescued the capacity of the top1-top2 extract to supercoil the template (lane 4). This result reinforces the conclusion drawn from the previous experiments, that both topoisomerase I and II can provide the relaxing activity required for nucleosomes to assemble on the DNA.

Even though we have demonstrated that the nicking-closing activity required for chromatin assembly is provided by a topoisomerase, we did attempt to set up conditions under which separate nicking and closing activities might substitute for topoisomerase activity.

We have observed, by resolving the DNA in a gel in the presence of ethidium bromide, that approximately one-third of the template recovered from a standard assembly reaction is nicked (Fig. 3.1C). More detailed analysis has revealed that extensive nicking and religation of the template occurs during the assembly reaction. In extracts that are deficient in DNA ligase I, which repairs nicked DNA, large amounts of nicked DNA accumulate (Fig. 4.1C, chapter 4). We therefore tested if excess DNA ligase in combination with the endogenous nicking of the template could substitute for topoisomerase activity in the assembly reaction. We added T4 DNA ligase to the chromatin assembly reaction with the top1-top2 extract. Fig. 3.9B shows that the addition of DNA ligase was not able to rescue supercoiling in the top1-top2 extract. This experiment demonstrates that only the coupled nicking-closing activity of a topoisomerase can support chromatin assembly.

3.5 Assessment of Topoisomerase II Activity during Chromatin Assembly in the Presence of Topoisomerase I

In *Xenopus* eggs and oocytes, chromatin assembly occurs in the presence of both topoisomerase I and II, however the involvement of topoisomerase II is limited (Almouzni and Méchali, 1988; Hirano and Mitchison, 1993). The failure to detect a significant role for topoisomerase II is not due to low content of the enzyme in the cell, in fact, oocytes contain a large stockpile of topoisomerase II in their nuclei (Almouzni and Wolffe, 1993). The reason why topoisomerase II does not play an equivalent role in the relaxation of torsionally strained DNA during chromatin assembly in the *Xenopus* system is not clear.

To this point we have established that topoisomerase II can function during assembly in yeast in the absence of topoisomerase I. We wished to determine if topoisomerase II can function during chromatin assembly when topoisomerase I is present, as in a wild-type cell. To determine if topoisomerase II had access to the DNA in the presence of topoisomerase I, we used the anticancer drug VP-16 (etoposide). VP-16 is a specific inhibitor of topoisomerase II (Rose, 1988; D'Arpa and Liu, 1989; Zhang et al., 1990). As described in chapter 1, VP-16 inhibits topoisomerase II during the breakage-rejoining reaction. The drug traps the enzyme on the DNA after it has nicked or linearized the DNA, forming a cleavable complex. Treatment with a protein denaturant such as SDS reveals the protein-linked DNA breaks. Therefore, the inhibition of topoisomerase II by VP-16 directly results in the accumulation of linear DNA and to a lesser extent nicked DNA following protein removal (D'Arpa and Liu, 1989; Zhang et al, 1990).

The standard control in VP-16 experiments is to perform the reactions with just the solvent used to dissolve the drug (Almouzni and Méchali, 1988). However, this control does not assess possible inhibitory effects of the drug that are independent of its activity against the topoisomerase. We were able to test for any non-specific effects by observing the effect of drug added to assembly reactions using the top2 extract, which contains no topoisomerase II.

Assembly reactions were performed in the presence of increasing amounts of VP-16, with the top1 and top2 extracts. As mentioned above, the accumulation of linear DNA is expected only when topoisomerase II is present, that is, only in the top1 reactions. Fig. 3.10A shows that linear DNA did appear in the top1 reactions (top panel), indicating that topoisomerase II was inhibited in these reactions. The inhibition of topoisomerase II was also indicated by the decrease in supercoiling observed at the higher concentrations of VP-16.

There was no accumulation of linear molecules in the top2 reactions and the amount supercoiling was not affected at the higher concentrations of VP-16 (bottom panel). We conclude that presence of VP-16 does not affect the assembly reactions in a non-specific manner. In other words, the effects of VP-16 on chromatin assembly are due exclusively to the inhibition of topoisomerase II rather than to non-specific inhibition of other components of the assembly machinery.

We then added VP-16 to assembly reactions with two wild-type extracts. One wild-type strain, TOP+, is isogenic to the top1 and top2 strains, and the other strain, A364A, has different markers. The accumulation of linear DNA was observed in the assembly reactions with both wild-type extracts (Fig. 3.10B). The proportion of linear DNA generated in the presence of topoisomerase I (Fig. 3.10B, lanes 2-4) is the same as in the absence of topoisomerase I (Fig. 3.10A, upper panel, lanes 2-4). This result indicates that during assembly, topoisomerase II has full access to the DNA even in the presence of wild-type amounts of topoisomerase I. Topoisomerase II is therefore catalytically active during chromatin assembly when topoisomerase I is present.

3.6 Discussion

DNA topoisomerases I and II are the major eukaryotic enzymes that can relieve torsional strain in DNA generated during many vital cellular processes. It has been previously established that topoisomerase I and II can function interchangeably to provide the relaxing activity required for replication and transcription (Wang, 1987; D'Arpa and Liu, 1989). We provide evidence that topoisomerase I and II can also substitute for one another to provide the relaxing activity necessary to support chromatin assembly.

We studied the role of both topoisomerase I and II during assembly by a combined biochemical and genetic approach using a yeast *in vitro* system that

contains all the components required for nucleosome assembly onto naked DNA. Plasmid supercoiling observed following the assembly reaction has been shown to be due to chromatin assembly (Schultz et al., 1997). This was demonstrated by three different experiments. The deproteinized template was relaxed by *E.coli* topoisomerase I, which relaxes negative but not positive supercoils (Wang, 1991). This shows that before protein removal the template was positively supercoiled, which is expected when supercoiling is driven by chromatin assembly. As was done in the present study, the protection of DNA by nucleosomes from micrococcal nuclease digestion and the dependence of supercoiling on histones also indicated that plasmid supercoiling was due to chromatin assembly.

Chromatin assembly extracts were prepared from previously described mutant yeast strains of one or both topoisomerases. The topoisomerase I mutant eliminates the enzyme altogether and the topoisomerase II mutation is a *ts* mutation. Chromatin assembly of relaxed plasmid DNA was assessed using a plasmid supercoiling assay and micrococcal nuclease digestion analysis.

Our initial experiment showed that the top1-top2 extract, which contains no topoisomerase activity, was unable to supercoil the plasmid DNA. This inability to supercoil the DNA was not due to excessive nicking of the template, as shown by resolving the reaction products on a gel in the presence of ethidium bromide. The amount of nicked template was the same for both the TOP+ and top1-top2 reactions. By mixing the wild-type TOP+ extract with the top1-top2 extract and performing an assembly reaction, we determined that the presence of the top1-top2 extract did not affect the ability of the TOP+ extract to supercoil the template. Therefore the top1-top2 extract does not contain an excess of an inhibitor of chromatin assembly.

When assembled chromatin in the TOP+ and top1-top2 extracts was analyzed by micrococcal nuclease digestion, we observed that nucleosomes were formed in both extracts. This result indicates that some nucleosomes can form on the DNA in the absence of topoisomerase activity. However, topoisomerase activity is required for full supercoiling of the DNA. It is likely that the topoisomerases do not become involved in assembly until a threshold of torsional strain is reached that precludes the assembly of DNA with the histone octamer. Below this threshold, when there are just a few nucleosomes assembled on the DNA, there is only limited torsional strain in the template. These nucleosomes are readily detected by micrococcal nuclease digestion. The formation of nucleosomes in the absence of topoisomerase activity has been shown previously by Sapp and Worcel (1990). Sapp and Worcel used a fractionated *Xenopus* extract and concluded that topoisomerase activity was required to supercoil the relaxed input DNA, but was not required for the assembly of nucleosomes.

Because the top1-top2 extract contains all cellular proteins except active topoisomerase I and II we conclude that a topoisomerase is required to resolve the torsional strain during chromatin assembly. Therefore yeast does not contain any significant nicking-closing activity that can replace the topoisomerases during assembly. In support of this conclusion was our observation that added DNA ligase in combination with endogenous nicking of the template does not support plasmid supercoiling. The fact that a separate nicking and closing activity cannot take the place of a topoisomerase during chromatin assembly has also been demonstrated in the *Xenopus* system (Almouzni and Méchali, 1988).

Using topoisomerase mutant strains, we were able to determine which topoisomerase could support chromatin assembly. Assembly reactions were

performed in yeast strains with only one functional topoisomerase, either topoisomerase I or II. Plasmid supercoiling was efficient in the top2 extract, which contains only topoisomerase I. Supercoiling was also efficient in the top1 extract, where topoisomerase II is the only active topoisomerase. This experiment clearly demonstrates that either topoisomerase I or II can provide the relaxing activity required for assembly-driven supercoiling.

A series of add-back experiments was performed to confirm the observation that both topoisomerase I and II can support chromatin assembly. The first experiment was an *in vivo* add-back, where wild-type topoisomerase II was expressed in top1-top2 cells. The presence of topoisomerase II in extracts prepared from these cells fully rescued plasmid supercoiling. Purified topoisomerase I or II was added separately to the assembly reaction with the top1-top2 extract and also resulted in the restoration of plasmid supercoiling.

The kinetics of supercoiling was analyzed for reactions performed in the presence of topoisomerase II alone and with both topoisomerases present. This was done to further evaluate the participation of topoisomerase II during chromatin assembly. The time course of assembly was the same for both the wild-type TOP+ extract and the top1 extract. This result implies that nucleosome assembly in the presence topoisomerase II alone is just as efficient as when both topoisomerase I and II are present.

The collective results using the topoisomerase mutants, the *in vivo* and *in vitro* add-back experiments, and the kinetic study, indicate that topoisomerase II functions as efficiently as topoisomerase I to provide the relaxing activity required for supercoiling of topologically constrained DNA during chromatin assembly.

The topoisomerase II-specific drug, VP-16 was used in assembly reactions to determine the accessibility of the DNA to topoisomerase II in the

presence of topoisomerase I. This experiment was performed to obtain support for the hypothesis that topoisomerase II can function during chromatin assembly when topoisomerase I is also present. The level of topoisomerase II activity can be estimated in the presence or absence of topoisomerase I in these experiments. The maximum level of topoisomerase II activity during chromatin assembly will be observed in the top1 extract which does not contain topoisomerase I. The activity of topoisomerase II is detected by the accumulation of linear DNA, which is a result of the action of VP-16. Therefore, the maximum amount of linear DNA will accumulate in the top1 extract. The amount of linear DNA observed in the top1 extract was the same as that seen in the wild-type extracts. These results clearly shows that topoisomerase II is catalytically active during assembly in the presence of topoisomerase I.

In summary, the results presented here demonstrate that both topoisomerase I and II can provide the relaxation activity required to relieve the torsional strain generated during chromatin assembly *in vitro*. We suggest that both topoisomerases play a role during assembly *in vivo*. We have shown that topoisomerase II activity during chromatin assembly is not inhibited by the presence of topoisomerase I. We believe that in a wild-type cell both topoisomerases participate in assembly. However, the actual contribution of each topoisomerase to assembly in a wild-type cell could be different at particular sites in the genome. This contribution could reflect the relative abundance of the topoisomerases at different locations in the cell. For example, topoisomerase I is highly concentrated in the nucleolus where rDNA is transcribed (Fleischmann et al., 1984; Muller et al., 1985). A primary role for topoisomerase I in rDNA transcription has been suggested for topoisomerase I (Garg et al., 1987; Rose et al., 1988; Zhang et al., 1988). This may also be also be the case for topoisomerase I during chromatin assembly of rDNA. The

inhibition of topoisomerase I has been found to disrupt nucleosome stability in transcribed rDNA. The inhibition of topoisomerase II did not affect nucleosome stability in rDNA (Cavilli et al., 1996). Therefore, it appears that in the nucleolus topoisomerase I may play a dominant role in chromatin assembly of rDNA. On the other hand, topoisomerase II has been found to be distributed uniformly throughout the nucleus during interphase in yeast (Klein et al., 1992). Topoisomerase II may therefore function during assembly at locations other than the nucleolus.

We have shown that in yeast, topoisomerase II is catalytically active during chromatin assembly in the presence of topoisomerase I. This is not the situation in *Xenopus* ooyctes and eggs, where it appears that the presence of topoisomerase I prevents topoisomerase II from acting during chromatin assembly. The reason for the preferential use of topoisomerase I during chromatin assembly in *Xenopus* is unclear.

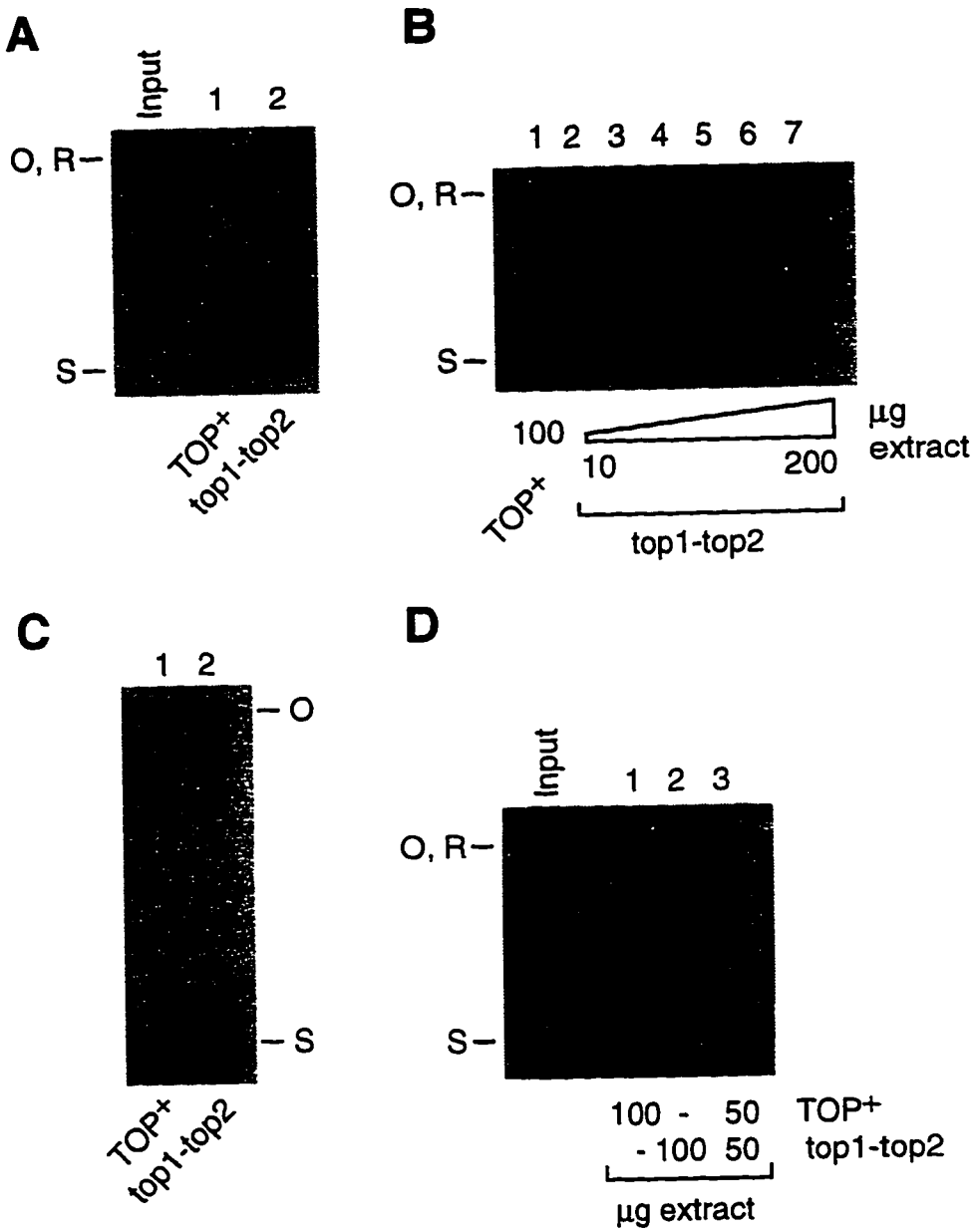
The use of topoisomerases to relax DNA during such vital cellular processes as replication, transcription, and chromatin assembly is likely to be important for the maintenance of a healthy cell. The orderly progression of these processes requires that breaks in the DNA must be made. The generation of such breaks could potentially cause damaging mutations and ultimately lead to cell death, if the DNA breaks persist in the cell (Froelich-Ammon and Osheroff, 1995). It is perhaps because the topoisomerases couple DNA cleavage with repair (and therefore restrict the lifespan of strand breaks), that these enzymes are used during strand transfer reactions in favour of separate nicking and closing activities.

In summary, our results demonstrate that in terms of the ability to provide the relaxing activity required for chromatin assembly, topoisomerase II appears to be completely redundant with topoisomerase I. Therefore, topoisomerases I

and II are functionally interchangeable not only during transcription and replication as previously described but during chromatin assembly as well.

Figure 3.1 A topoisomerase is required for DNA supercoiling driven by chromatin assembly in a yeast whole-cell extract

Open circular (O), relaxed (R), and highly supercoiled products (S) are resolved by native agarose gel electrophoresis and visualized by autoradiography. (**A**) Comparison of assembly-driven supercoiling in the TOP+ and top1-top2 extracts. The input DNA used as the template for assembly is labeled. (**B**) Protein titration of the top1-top2 extract. (**C**) Detection of open circular (nicked) DNA generated during chromatin assembly. The products of the assembly reactions were separated by agarose gel electrophoresis in the presence of 0.25 μg/ml ethidium bromide. (**D**) Mixing experiment to demonstrate that the top1-top2 extract does not contain an excess of an inhibitor of chromatin assembly. Assembly reactions were performed with the TOP+ and top1-top2 extracts individually, and with a mixture of an equal amount of protein from each extract.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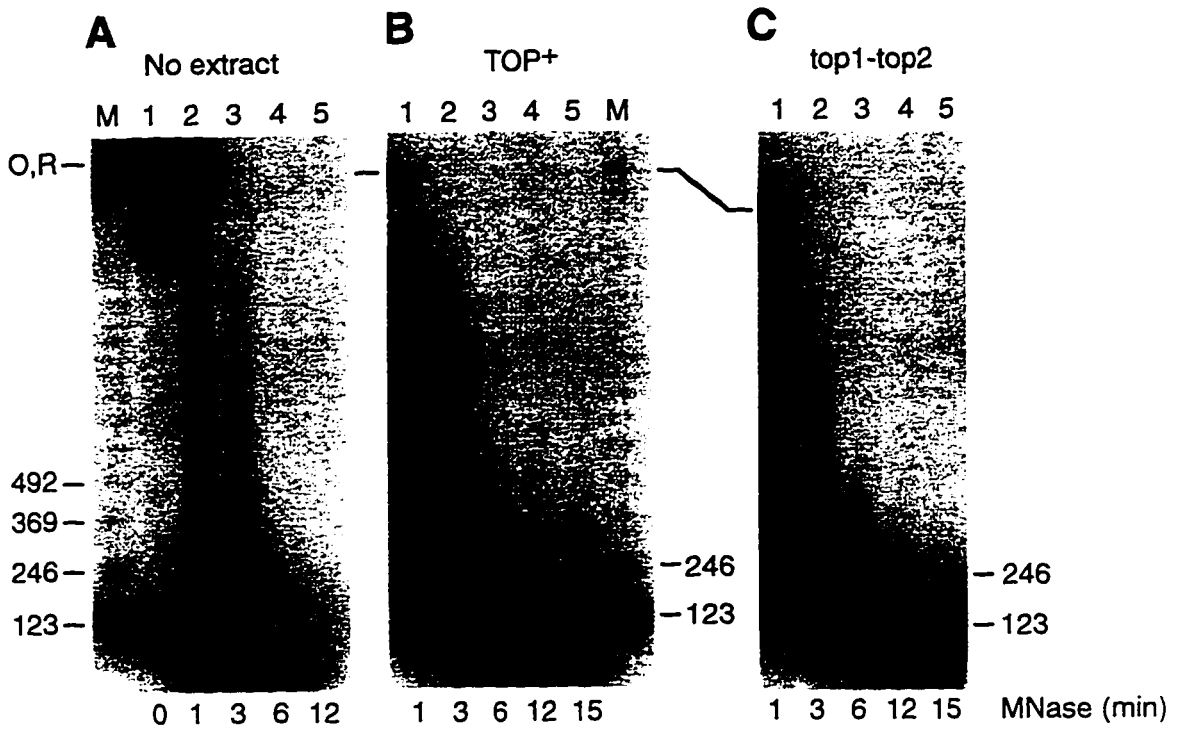


Fig. 3.2 Micrococcal nuclease assay for the formation of nucleosomes in wild-type and topoisomerase-deficient extracts

Assembly reactions were treated with micrococcal nuclease for the indicated times. Products were detected by Southern blotting. No bands corresponding to nucleosomes are observed in the absence of yeast extract (**A**), whereas mononucleosomes and dinucleosomes are detected in the TOP+ (**B**) and the top1-top2 extracts (**C**). Open circular (O), and relaxed (R) products are labeled. The 123 and 246 bp markers migrate in advance of the mono- and dinucleosomes respectively.

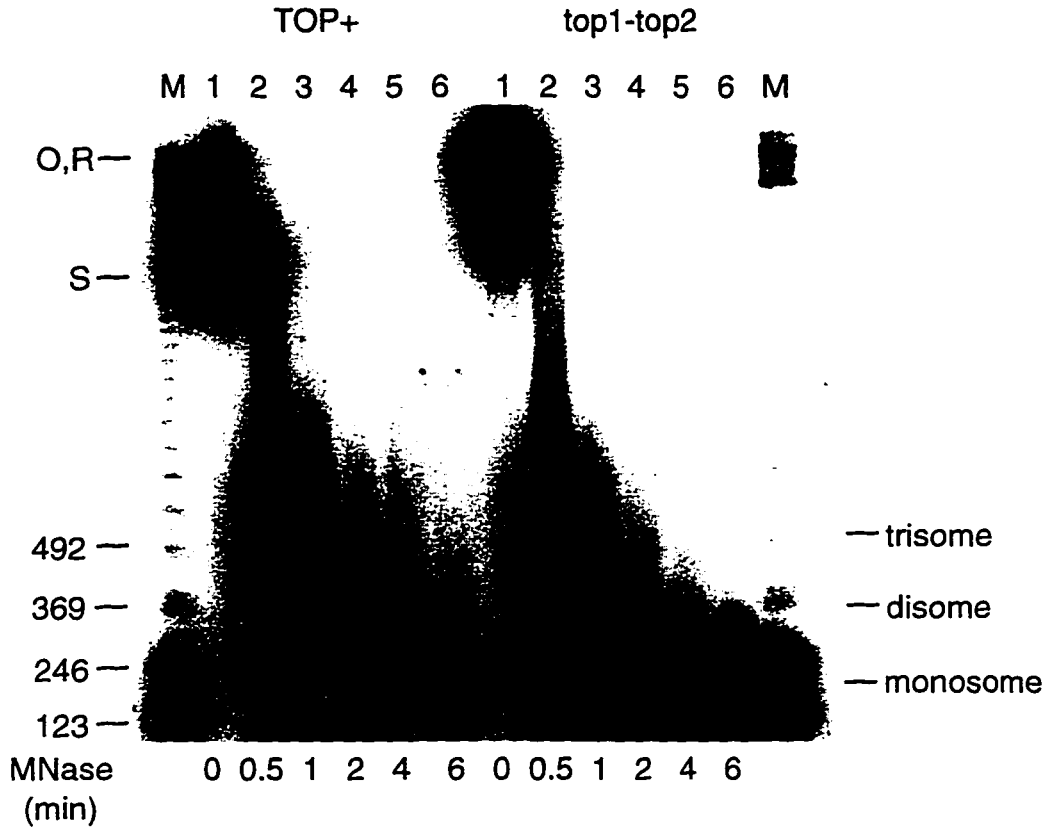


Fig. 3.3 Micrococcal nuclease digestion of chromatin assembled in the presence of poly-L-glutamate

Assembly reactions were performed in the presence of poly-L-glutamate and then treated with micrococcal nuclease for the indicated times. Products were detected by Southern blotting. Bands corresponding to mono-, di-, and trinucleosomes are observed in the TOP+ and top1-top2 extracts when poly-L-glutamate is included in the assembly reactions to stabilize the nucleosomes. Open circular (O), relaxed (R), and supercoiled products (S) are indicated. The 123, 246, and 369 bp markers migrate in advance of the mono-, di-, and trinucleosomes respectively.

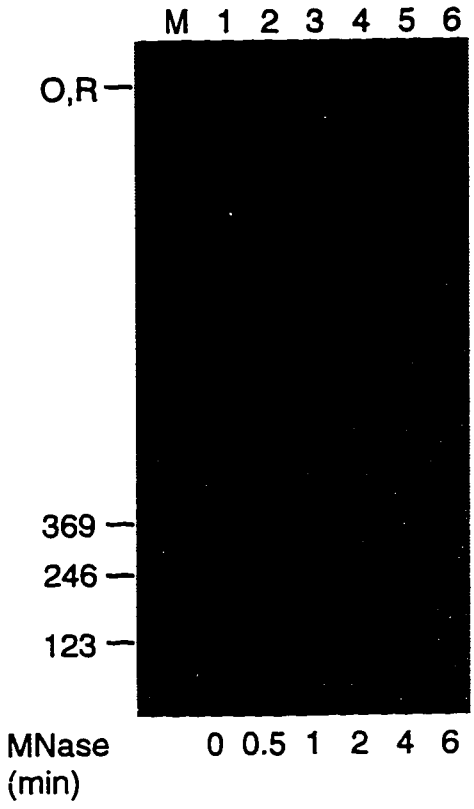


Fig. 3.4 Micrococcal nuclease digestion of chromatin assembled with poly-L-glutamate in the absence of yeast extract

The assembly reaction included poly-L-glutamate, purified yeast histones, and topoisomerase I, but no yeast extract. The assembly reaction was treated with a limiting amount of micrococcal nuclease for the indicated times. The DNA was quickly digested and no nucleosome-sized fragments were generated. Open circular (O), and relaxed (R) products are labeled.

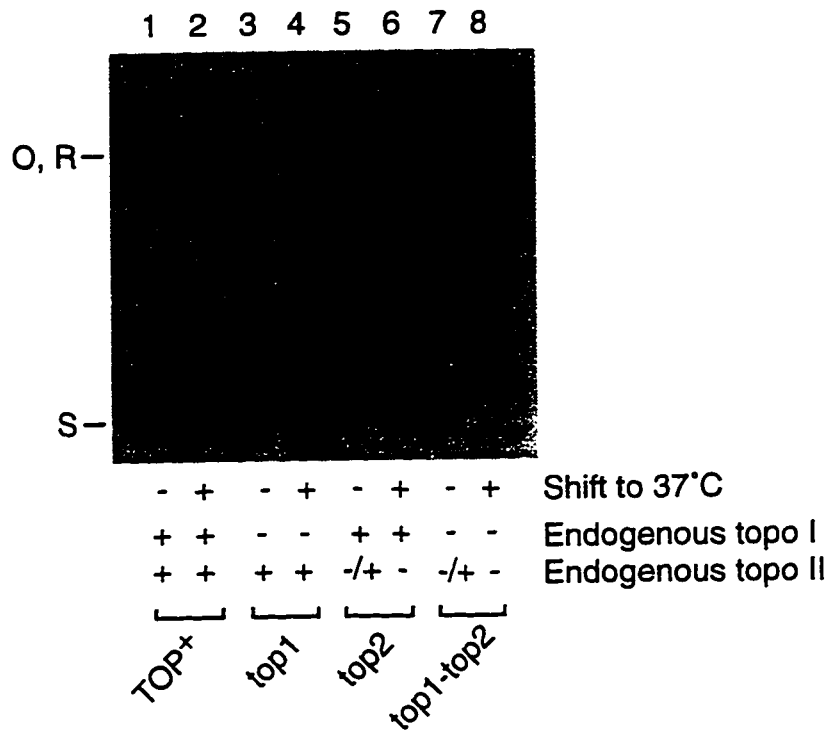
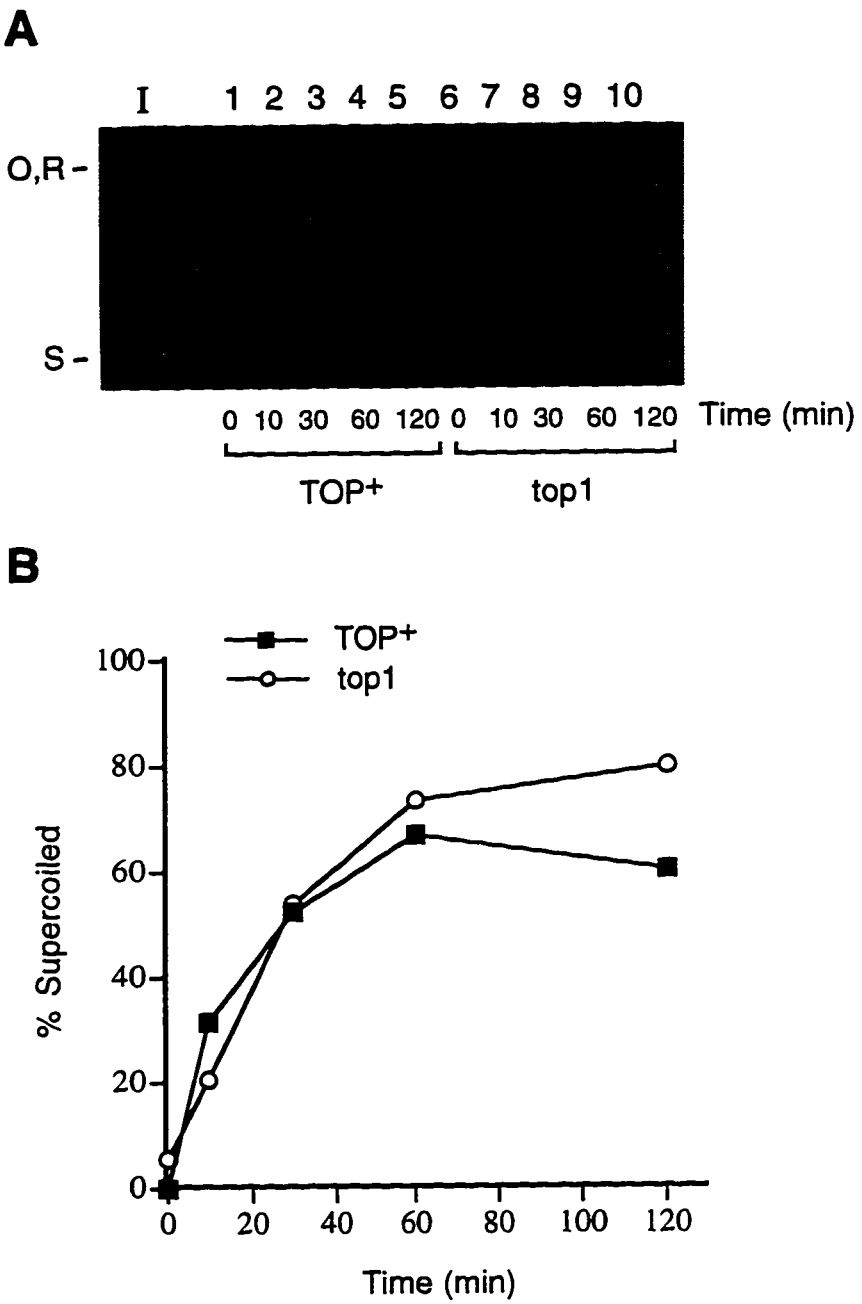


Fig. 3.5 Topoisomerase I or II is sufficient for assembly-driven DNA supercoiling

Yeast strains were grown at room temperature and harvested before or after shifting to 37°C for 1 hour. The active topoisomerases in the various extracts are indicated. Both topoisomerases are active in the TOP+ extract (lanes 1,2). The top1 extract contains only topoisomerase II (lanes 3,4), whereas the top2 extract contains only active topoisomerase I (lanes 5,6). Both topoisomerases are inactive in the top1-top2 extract (lanes 7,8). The products of the assembly reactions were resolved by agarose gel electrophoresis and visualized by autoradiography. Open circular (O), relaxed (R), and highly supercoiled products (S) are indicated.

Fig. 3.6 Time course of assembly-driven supercoiling in extracts from TOP+ and top1 cells

(**A**) Autoradiograph showing the time course of supercoiling in the TOP+ and top1 extracts. The assembly reactions were stopped at various times as indicated. (**B**) Quantitation of (A) by phosphoimager analysis. Open circular (O), relaxed (R), and highly supercoiled products (S) are resolved by agarose gel electrophoresis. The percent supercoiled DNA was calculated as the ratio of highly supercoiled species (topoisomers migrating faster than DNA in the input, I) to the total DNA per lane.


A
I 1 2 3 4 5 6 7 8 9 10
O,R -
S -
0 10 30 60 120 0 10 30 60 120 Time (min)
TOP+
top1
B
TOP+
top1
% Supercoiled
100
80
60
40
20
0
0 20 40 60 80 100 120
Time (min)

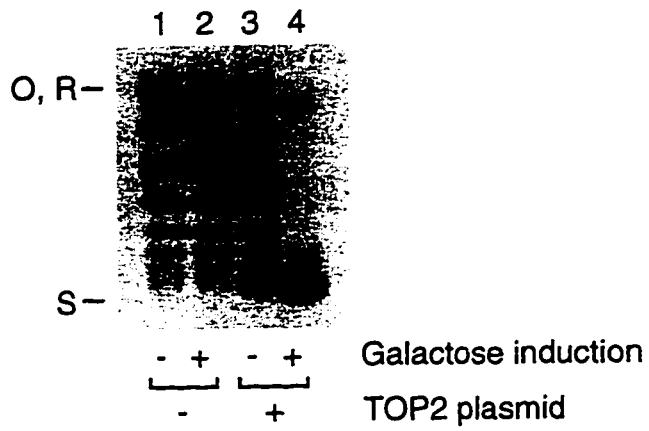


Fig. 3.7 In vivo add-back of wild-type topoisomerase II rescues DNA supercoiling in the top-top2 extract

DNA supercoiling is restored when wild-type topoisomerase II, under control of the *GAL1* promoter, is expressed in strain top1-top2 (lanes 3 and 4). The transformed strain is designated top1-top2/pTOP2. Galactose induction of strain top1-top2 lacking the topoisomerase II overexpression plasmid does not cause any change in supercoiling (lanes 1 and 2). Open circular (O), relaxed (R), and highly supercoiled products (S) are indicated.

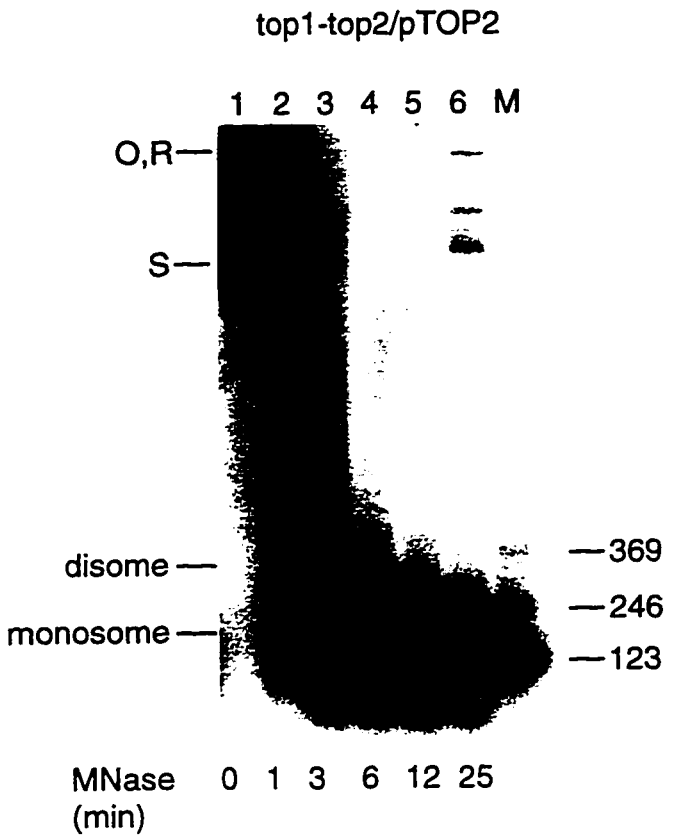


Fig. 3.8 Micrococcal nuclease digestion of chromatin assembled in the top1-top2/pTOP2 extract

The assembly of nucleosomes in the top1-top2/pTOP2 extract, prepared from top1-top2 cells expressing wild-type topoisomerase II, was analyzed by micrococcal nuclease digestion. The assembly reaction was treated with micrococcal nuclease for the indicated times. Products were detected by Southern blotting. Bands corresponding to mono-and dinucleosomes were detected. Open circular (O), relaxed (R), and highly supercoiled products (S) are indicated. The 123 and 246 bp markers migrate in advance of the mono- and dinucleosomes respectively.

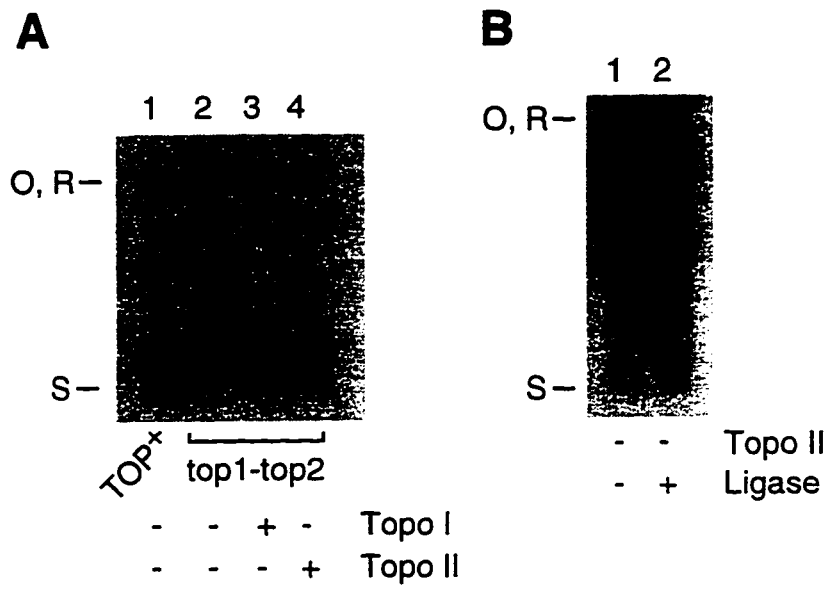


Fig. 3.9 In vitro add-back of purified topoisomerases and DNA ligase to the top1-top2 extract

(**A**) DNA supercoiling driven by chromatin assembly is restored when purified topoisomerase I (lane 3) or topoisomerase II (lane 4) is added to the top1-top2 extract. (**B**) DNA supercoiling is not restored when *E. coli* T4 DNA ligase is added to the top1-top2 extract (lane 2). Open circular (O), relaxed (R), and highly supercoiled products (S) are resolved by agarose gel electrophoresis and visualized by autoradiography.

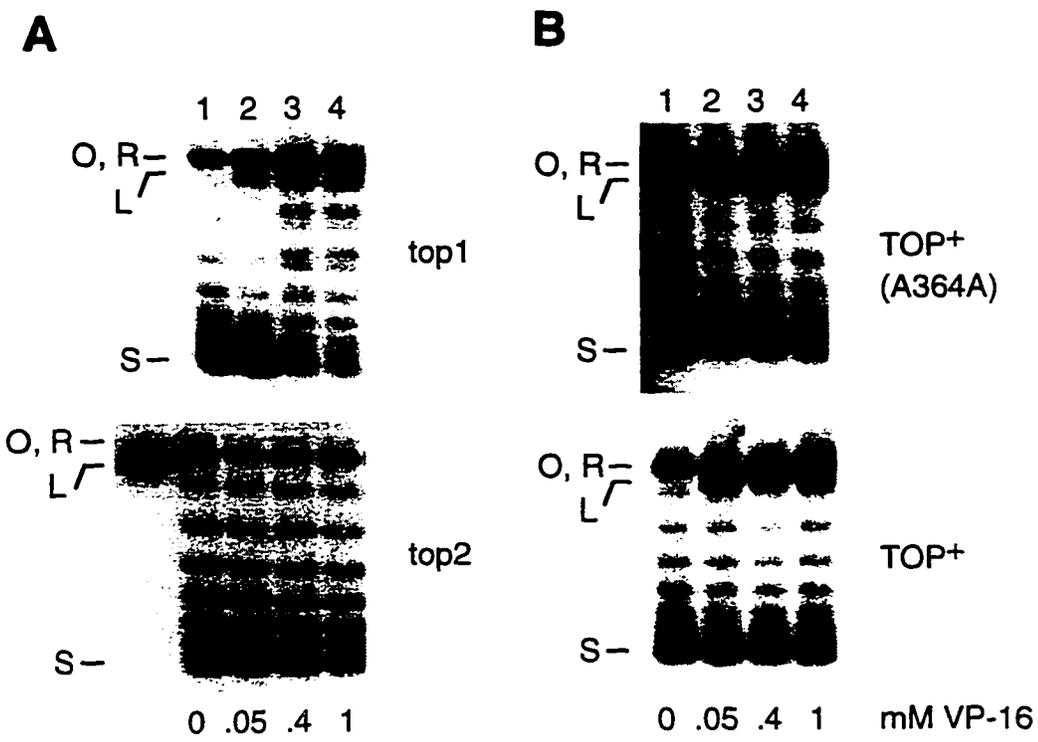


Fig. 3.10 Topoisomerase II has full access to the DNA during chromatin assembly in the presence of topoisomerase I in wild-type extracts

Assembly reactions were performed in the presence of increasing amounts of the topoisomerase II poison, VP-16 (etoposide) as indicated. (**A**) Assembly reactions with the top1 extract (contains only active topoisomerase II) and the top2 extract (contains only active topoisomerase I). The accumulation of linear DNA is only observed when topoisomerase II is active. (**B**) Assembly reactions with two wild-type extracts. Open circular (O), relaxed (R), linear (L), and highly supercoiled products (S) are resolved by agarose gel electrophoresis following protein removal, and visualized by autoradiography.

Chapter 4

The Requirement for ATP during Chromatin Assembly in a Yeast Extract

4.1 Introduction

ATP is required for many biochemical mechanisms in the cell, including many that are localized to the nucleus. There is some evidence that chromatin assembly requires ATP. For example, chromatin assembly in a human cell-free extract requires ATP hydrolysis for the stabilization of chromatin (Banerjee and Cantor, 1990; Banerjee et al., 1991). Two independent studies using *in vitro Drosophila* systems also found that ATP is essential for chromatin assembly (Becker and Wu, 1992; Bulger et al., 1995). This evidence comes from replication-independent systems of chromatin assembly. ATP is also needed in human and *Drosophila* assembly systems where chromatin assembly is coupled to DNA replication (Stillman, 1986; Kamakaka et al, 1996). In the replication-dependent system, ATP is required for replication itself. The fact that assembly is ATP-dependent in the replication-independent system suggests that ATP may also be required during the nucleosome deposition step of replication-dependent assembly.

However, the requirement for ATP is not observed in all chromatin assembly systems. ATP is not required for the assembly of nucleosomes in a *Xenopus* extract (Almouzni and Méchali, 1988).

Previous work has both supported and discounted a role for ATP in chromatin assembly. Our discovery that the ATP-dependent enzyme topoisomerase II can function as an accessory factor for chromatin assembly suggests that ATP is required during assembly. In order to test this possibility, we examined the requirement for ATP during chromatin assembly in a yeast whole cell extract.

4.2 ATP Requirement during Chromatin Assembly

Chromatin assembly reactions were carried out in the presence or absence of ATP, using the TOP+ extract. Fig. 4.1A shows that plasmid

supercoiling in a typical yeast assembly extract requires ATP (compare lanes 1 and 2). When ATP was omitted from the reaction, supercoiling of the relaxed DNA was inhibited, whereas in the presence of ATP, efficient supercoiling occurs.

The non-hydrolyzable analogs of ATP, ATP-γ-S and AMP-PNP, were added to parallel reactions in the place of ATP to test if hydrolysis of the γ–phosphate is required for chromatin assembly. We found that neither ATP analog could substitute for ATP in these reactions (Fig. 4.1A, lanes 3 and 4). This result indicates that ATP hydrolysis is an integral part of the assembly process in the yeast extract.

We discovered that some TOP+ extracts contained a sufficient amount of endogenous ATP to support plasmid supercoiling (Fig. 4.1B, lane 1), as indicated by the fact that the addition of exogenous ATP to the reaction was not necessary to observe efficient supercoiling. However, when apyrase, an enzyme which degrades ATP (Cande, 1982), was added to the reaction, supercoiling was no longer observed, and linear DNA accumulated (Fig. 4.1B, lane 2). If apyrase was heat-denatured (90°C, 10 min) before it was added to the reaction, supercoiling was not affected (not shown). ATP-γ-S was added to this ATP-independent extract in place of ATP, to determine if it would compete with the endogenous ATP and inhibit plasmid supercoiling. The results show that supercoiling in this extract remains efficient in the presence of ATP-γ-S (Fig. 4.1B, lane 3). This implies that there is a sufficient amount of endogenous ATP in the extract to support assembly and not be affected by competition with ATP-γ-S.

4.3 Involvement of ATP-dependent DNA Ligase

In order for supercoiling of the plasmid to be observed, the template must be a closed circle. Therefore, any DNA that has been nicked must first be

repaired before supercoiling can occur. DNA ligase is an enzyme that repairs nicks and double-strand breaks and is dependent on ATP for activity (Alberts et al, 1994). We decided to test whether Cdc9p, the DNA ligase I of yeast (Johnston and Nasmyth, 1978) was involved in repair of nicked template. To do this extracts were made from a *ts cdc9* strain and a wild-type strain (Hartwell et al., 1973).[1] The results of the plasmid supercoiling assay using these two extracts show that linear and open circular/relaxed DNA accumulated in the *cdc9* reaction and very little supercoiling was observed (Fig. 4.1C, lanes 3 and 4). This indicates that template repair by Cdc9p occurs during assembly. Since DNA ligation by Cdc9p is ATP-dependent, linear DNA would be expected to accumulate in reactions depleted of ATP by apyrase. The experiment in Fig. 4.1B confirms this expectation.

The DNA ligase and ATP depletion experiments demonstrate that the requirement for ATP in the yeast extract is due in part to the function of DNA ligase in template repair. It however does not explain the need for ATP entirely. The reaction products from the extract that contained no endogenous ATP were run on a gel containing ethidium bromide, in order to resolve nicked or linearized template from closed circular template. The presence of a supercoiled band in Fig. 4.1D (lane 1) indicates that even in the absence of ATP, a proportion of the template is in a closed circular conformation. This closed circular template does not appear to be efficiently supercoiled in the absence of ATP (Fig. 4.1A compare topoisomer distribution between lanes 1 and 2). Since the template that was not nicked or linearized fails to be fully supercoiled in the absence of ATP, this suggests that ATP has a role besides the one in repair.

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[1] The Cdc9p experiment was performed by Brent Altheim.

4.4 Involvement of Topoisomerase II

Another enzyme that requires ATP as a cofactor is topoisomerase II. A role for topoisomerase II during assembly could also explain the requirement for ATP, and indeed we have found that topoisomerase II can function during chromatin assembly. To investigate whether the requirement for topoisomerase II was the reason for the ATP dependence, the topoisomerase mutants, top1 and top2, were used in the supercoiling assay, in the presence or absence of ATP. If ATP is required solely as a cofactor for topoisomerase II activity, than supercoiling in the top2 extract, which contains only active topoisomerase I, should not be severely affected by the absence of ATP. If this was the case, in the absence of ATP we would also expect to see very little supercoiling in the top1 extract, which contains only topoisomerase II. Fig. 4.2 shows that the omission of ATP inhibits plasmid supercoiling to the same extent in both the top1 and top2 extracts. This result indicates that the requirement for ATP that is independent of DNA ligase in our yeast extract is not because it is a cofactor for topoisomerase II.

4.5 Discussion

This chapter describes the experiments that were carried out to determine if chromatin assembly in a yeast whole cell extract required the presence of ATP.

By simply performing assembly reactions in the presence or absence of ATP, using a wild-type yeast strain, we determined that ATP was required for chromatin assembly to occur in the yeast extract. The addition of exogenous ATP to the reaction was not always necessary for chromatin assembly, indicating that the amount of endogenous ATP present varied from extract to extract. However, the need for ATP was common to all extracts. When the ATP

was depleted with apyrase, in the extract that contained endogenous ATP, supercoiling was severely inhibited.

It was not surprising to find that ATP remained in some extracts even after dialysis had gone to completion, since it has previously been observed that ATP is difficult to remove unless extracts are subjected to column chromatography or enzymatic treatment (for example with apyrase; Klemm et al., 1997).

Through the use of non-hydrolyzable analogs of ATP, we showed that not only does ATP have to be available, but it must also be hydrolyzed for chromatin assembly to occur. This implies that ATP is required as a positive cofactor for assembly, most likely because of its participation in phosphoryl-transfer reactions.

By using a yeast strain that was deficient in DNA ligase, we showed that DNA ligase, which is an ATP-dependent enzyme, plays a role in template repair during assembly. In the absence of DNA ligase, supercoiling is inhibited, while nicked and linear DNA accumulate. Consistent with this result was the accumulation of linear DNA in the reactions performed in the absence of ATP. Therefore, part of the ATP-dependence of the supercoiling reaction reflects the involvement of DNA ligase in template repair during assembly. This repair process maintains the template in a closed circular configuration and therefore allows for supercoiling.

The requirement for ATP is only partially explained by the involvement of DNA ligase during assembly. For instance, not all of the template is nicked or linearized, as shown by the supercoiled DNA band in the ethidium bromide gel for the reaction without endogenous ATP. This closed circular DNA is not efficiently supercoiled in the absence of ATP, suggesting an additional role for ATP besides the one in DNA repair.

We have shown previously that topoisomerase II, another ATP-dependent enzyme, can function during chromatin assembly. However, using the topoisomerase mutants we have demonstrated that in the absence of ATP supercoiling in an extract that contains only topoisomerase I is inhibited to the same degree as in an extract in which only topoisomerase II is present. This result indicates that it is unlikely that assembly is ATP-dependent only because of a requirement for topoisomerase II.

ATP does act as a positive cofactor for DNA ligase and topoisomerase II function during chromatin assembly in yeast. However, the requirement for ATP that is independent of DNA ligase and topoisomerase II has yet to be defined.

In order to speculate on the ATP-dependence of chromatin assembly in the yeast extract that is independent of DNA ligase and topoisomerase II, the results of ATP studies using other chromatin assembly systems are discussed below. It appears that the requirement for ATP during chromatin assembly is complex and that multiple aspects of assembly may involve ATP.

The need for ATP during chromatin assembly has been associated with the phosphorylation of histones and assembly factors. In a human cell-free extract, ATP hydrolysis substantially enhanced the stability of the assembled chromatin (Banerjee and Cantor, 1990). The investigators later attributed the requirement for ATP hydrolysis to the phosphorylation of histone H3. They found that the phosphorylation of histone H3 was necessary for the stabilization of chromatin assembled in the cellular extract (Banerjee et al., 1991). The phosphorylation and dephosphorylation of histone H3 was easily manipulated by altering the amount of ATP in the assembly reactions. It appears therefore, that the phosphorylation state of histone H3 would be greatly affected by ATP

levels in the cell and this in turn would affect the stability of assembled chromatin.

Evidence for the requirement of ATP in relation to assembly factors has come from several studies. The *Xenopus* assembly factor nucleoplasmin is one such factor that is affected by ATP. Nucleoplasmin is an acidic protein that binds histones H2A and H2B (Dilworth et al., 1987). Nucleoplasmin is essential for the addition of histones H2A and H2B to form the complete histone octamer. The phosphorylated form of nucleoplasmin has been shown to be more active in chromatin assembly than the unphosphorylated form (Sealy et al., 1986). The role of phosphorylation of nucleoplasmin may explain the observation that immature chromatin, that does not contain histones H2A and H2B, is assembled in the absence of ATP (Almouzni and Wolffe, 1993).

In yeast, the nucleosome assembly protein I (NAP-I), also has a high affinity for histones H2A and H2B (Ishimi and Kikuchi, 1991). NAP-I is able to assemble nucleosomes by first mediating histone octamer formation and then transferring the octamer to the DNA. NAP-I contains two potential phosphorylation sites. Whether phosphorylation of NAP-I affects its activity is not yet known.

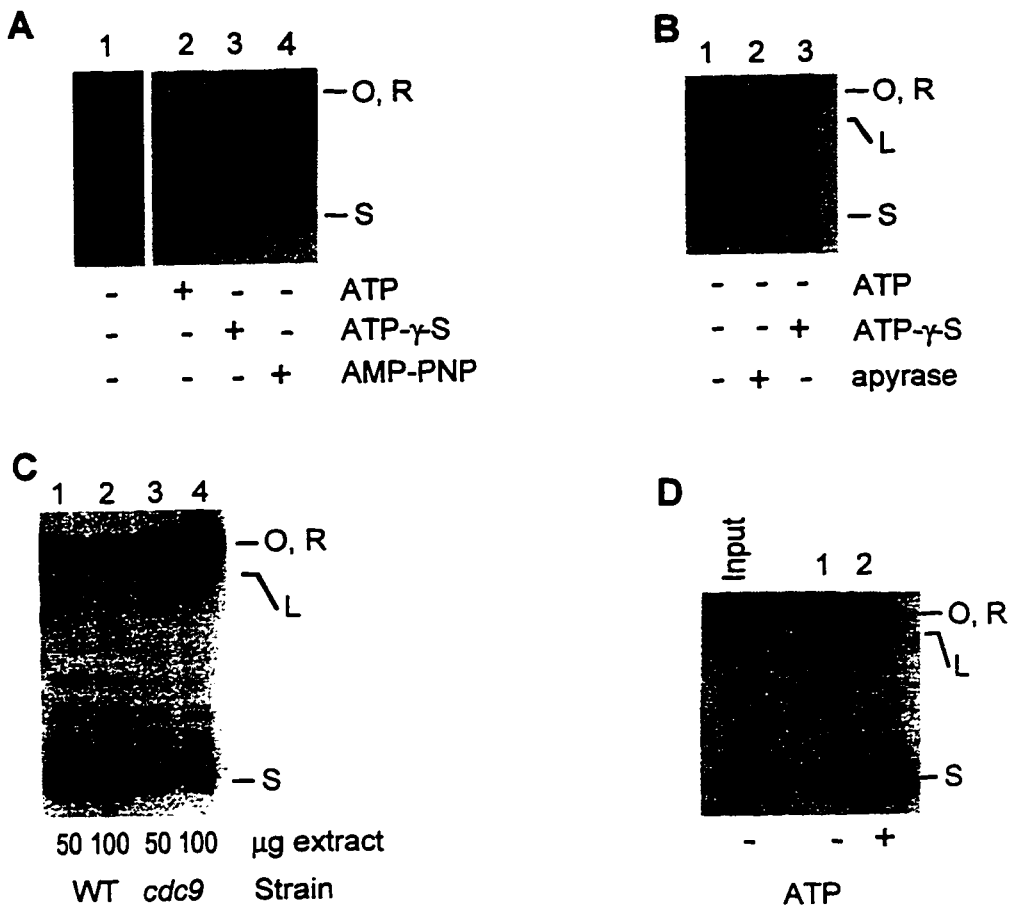
Another assembly factor that requires ATP is the human chromatin assembly factor-I (hCAF-I). The p150 and p60 subunits of hCAF-I are phosphorylated (Smith and Stillman, 1991a). The function of this modification is not yet known. However, the role of phosphorylation of the p150 and p60 subunits has been the subject of some speculation. The phosphorylation of hCAF-1 could occur only during S phase of the cell cycle, when DNA replication occurs. This cell cycle specific modification has been shown previously for a multisubunit replication factor that is specifically phosphorylated during S phase (Din et al., 1990). It has been proposed that phosphorylation of hCAF-I during S

phase could regulate the formation of the hCAF-I complex and control the activity of hCAF-I (Smith and Stillman, 1991a). With respect to the activity of hCAF-I, it does bind histones *in vitro*. Phosphorylation of hCAF-I could affect its binding ability to histones (Smith and Stillman, 1991a). This type of regulation has been shown for the binding of histones by nucleoplasmin, as described above. The assembly of chromatin by hCAF-I occurs preferentially on newly replicated DNA (Smith and Stillman, 1989). Therefore, the phosphorylation of two of the subunits of hCAF-I indicates that ATP is not only required for replication but also for chromatin assembly. Another possibility for the ATP-dependence of chromatin assembly is that ATP may be needed to provide energy for a step in assembly. However, there is no information on this possibility at the present time.

In summary, it is possible that ATP is required during chromatin assembly in the yeast extract for the phosphorylation of histone H3 and/or the phosphorylation of an assembly factor, in addition to the need for ATP by DNA ligase and topoisomerase II. Through the use of biochemical methods such as amino acid substitution, the requirement of histone H3 phosphorylation can be studied at the present time. Studying the phosphorylation of assembly factors in yeast will not be as straightforward, since yeast CAF-I is not essential for cell viability. Therefore, there must be other proteins that function as assembly factors. Until all of the assembly factors have been identified and cloned it will not be possible to determine the entire role of ATP during chromatin assembly.

Fig. 4.1 ATP requirement during assembly-driven supercoiling in a yeast extract

(**A**) An extract dependent on added ATP for supercoiling (lane 1) was supplemented with ATP (lane 2), ATP-γ-S (lane 3), or AMP-PNP (lane 4). (**B**) In an extract with enough endogenous ATP to support supercoiling (lane 1), the reaction is not affected by ATP-γ-S (lane 3) but is strongly inhibited by apyrase (lane 2). (**C**) DNA ligase I (Cdc9p) is required for template repair during chromatin assembly. Assembly reactions were performed using extracts from wild-type (lanes 1 and 2) and *cdc9* (lanes 3 and 4) cells. Open-circular (nicked) DNA and linear DNA accumulate in the absence of active DNA ligase I. (**D**) Accumulation of linear DNA during assembly in the absence of ATP. The products of reactions with the ATP-dependent extract (same as in [A]) are resolved by agarose gel electrophoresis in the presence of 0.25 μg/ml ethidium bromide. The input DNA is labeled. Open circular (O), relaxed (R), linear (L), and supercoiled products (S) are indicated.


A
1 2 3 4
O, R
S
- + - - ATP
- - + - ATP-γ-S
- - - + AMP-PNP
B
1 2 3
O, R
L
S
- - - ATP
- - + ATP-γ-S
- + - apyrase
C
1 2 3 4
O, R
L
S
50 100 50 100 µg extract
WT cdc9 Strain
D
Input
1 2
O, R
L
S
- - + ATP

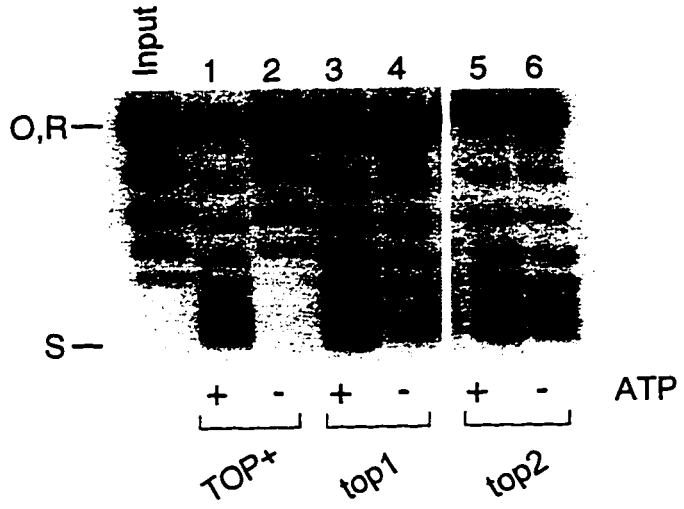


Fig. 4.2 ATP is not required during chromatin assembly because it is a cofactor for topoisomerase II

Assembly reactions were performed in the presence or absence of ATP as indicated, using an ATP-dependent TOP+ extract (lanes 1, 2), the top1 extract (lanes 3, 4), or the top2 extract (lanes 5, 6). The input DNA is labeled. Open circular (O), relaxed (R), and supercoiled products (S) are resolved by agarose gel electrophoresis and visualized by autoradiography.

Chapter 5

Partial Purification of the Chromatin Assembly Machinery in a Yeast Whole Cell Extract

5.1 Introduction

To gain a better understanding of the process of chromatin assembly, it will be necessary to identify the factors that are involved. One way that this can be accomplished is the fractionation of an extract that supports chromatin assembly. By fractionating the extract, it should be possible to purify and then characterize the proteins responsible for this process.

A protein complex involved in chromatin assembly has been identified by such a purification process. This complex was named chromatin assembly factor I (CAF-I). CAF-I has been purified from extracts of human cells, *Drosophila* embryos and recently from yeast (Smith and Stillman, 1989; Kamakaka et al, 1996; Kaufman et al, 1997). Interestingly, yeast cells that lack CAF-I are viable, show no growth defects, and presumably assemble normal chromatin at most loci in the genome (Kaufman et al., 1997). These observations imply that there must be other proteins involved in chromatin assembly that have yet to be identified.

We have begun the fractionation of a yeast whole cell extract with the long term goal of identifying such proteins. The initial steps of this process are described in this chapter. Table 1 outlines the results obtained using the fractionation procedure developed here. Chromatin assembly activity was assessed subjectively in terms of the level of DNA supercoiling supported by the relevant fractions. Thus, high assembly activity corresponds to highly efficient supercoiling, in which all the closed circular products generated by assembly migrate in a single band at the position of the supercoiled plasmid obtained from *E.coli*. Low chromatin assembly activity corresponds to low supercoiling efficiency and refers to fractions that generate topoisomers that migrate slower than the supercoiled plasmid obtained from *E.coli*.

5.2 Ammonium Sulfate Precipitation

Ammonium sulfate precipitation is routinely used as the first step in protein purification, prior to chromatography. It is a highly effective method for the concentration of proteins and removal of contaminating proteins. Ammonium sulfate precipitation has proven to be a valuable step in the purification of a protein kinase and transcription factors, performed in our lab.

A pilot experiment involving a step-wise series of ammonium sulfate precipitations was carried out first (Englard and Seifter, 1990). This experiment was performed to determine how much ammonium sulfate could be added to the TOP+ extract without precipitating out any of the proteins involved in chromatin assembly. Each of the supernatants and resuspended pellets was used in chromatin assembly reactions. Fig. 5.1 shows that the proteins involved in chromatin assembly remained in the supernatant up to 55% saturation. The proteins remaining in the 55% supernatant were precipitated by addition of ammonium sulfate to 70% saturation. The 70% pellet (70p), which contains the proteins that were present in the 55% supernatant, displayed very efficient supercoiling. It appears that as more proteins are removed from the extract, supercoiling becomes more efficient. This is shown by comparing the 35% supernatant (lane 1) which generates many intermediate topoisomers, to the 70% pellet (lane 6), which generates a tight supercoiled band with no intermediates. This result suggests that proteins that interfere with nucleosome assembly are being removed.

Based upon the results of this experiment, we designed a streamlined ammonium sulfate precipitation procedure. As the first step in the ammonium sulfate precipitation procedure, the extract was adjusted to 50% saturation and the precipitated proteins were removed. The proteins in the 50% supernatant were then precipitated by the addition of ammonium sulfate to 70% saturation.

The 70% pellet was expected to contain the proteins of interest. Fig. 5.2A compares chromatin assembly of the 50% and 70% pellets (50p and 70p, respectively). Highly efficient supercoiling was observed only with the 70% pellet, indicating that all of the proteins involved in chromatin assembly are contained in this pellet.

To confirm that the supercoiling observed with the 70% pellet was due to assembly of nucleosomes, we tested if assembly was dependent upon the histones. A limiting amount of the 70% pellet was used in the chromatin assembly reactions (Fig. 5.2B). In the absence of exogenous histones, the limiting amount of the 70% pellet was unable to supercoil the input DNA. However when histones were added to the reaction, supercoiling was restored. This demonstrates that the supercoiling observed with the 70% pellet was histone-dependent and therefore likely due to the assembly of nucleosomes. We further conclude that the supercoiling was not due to the concentration of a factor that could supercoil DNA on its own.

Assembly in the 70% pellet was also analyzed by micrococcal nuclease digestion (Fig. 5.3). A monosome and disome were observed over the time course of digestion, also indicating that nucleosomes were assembled by the proteins contained in the 70% pellet.

5.3 Chromatography

The 70% pellet was used as the starting material for chromatography on DEAE Sepharose Fast Flow resin (Pharmacia Biotech), which is a weak anion exchanger. As a pilot experiment, 1 mg of the ammonium sulfate pellet was loaded onto the column. Fractions were collected from 0.1 M KCl and 1 M KCl washes. Chromatin assembly reactions were performed using several of the fractions, in the absence or presence of purified histones, since we were not certain if a sufficient amount of histones would be present in the fractions for

chromatin assembly. As shown in Fig. 5.4, the proteins involved in chromatin assembly eluted from the column with the 1 M KCl wash (lanes 7 and 8). The efficiency of supercoiling was increased by the addition of histones, indicating histone-dependence. This pilot experiment demonstrated that the assembly machinery could bind to the resin at low salt and be removed in an active form after elution with high salt. In light of this result, a series of salt washes were performed as described below.

2 mg of the ammonium sulfate pellet was loaded onto the same column and fractions were collected from washes using 0.1, 0.3, and 0.6 M KCl. The protein concentration of each fraction was very low, such that the amount of protein used in the chromatin assembly reactions was approximately 100X less than for a reaction using crude whole cell extract. For this reason, the amount of template DNA was decreased from 20 ng per reaction to 4 ng of DNA. Purified histones were also added to some of the reactions as before. Fig. 5.5A shows that the proteins responsible for chromatin assembly eluted from the column with the 0.3 M KCl wash (lanes 6-9). The efficiency of supercoiling following chromatography appeared similar to the input 70% pellet in this experiment (compare lane 1 with lanes 6-9). With the smaller amount of template DNA, the addition of histones was not necessary for efficient supercoiling. However, if the reactions were performed using the standard amount of template DNA, supercoiling became dependent on the addition of histones (Fig. 5.5B). It should be noted that the amount of supercoiling in these fractions is quite remarkable considering the small amount of protein present.

The micrococcal nuclease digestion analysis of one of the 0.3 M KCl fractions revealed a monosome band, indicating the assembly of nucleosomes (Fig. 5.6).

5.4 Topoisomerase II Assay

Because the primary focus of my work was to elucidate the involvement of topoisomerase II in chromatin assembly, I tested if topoisomerase II was enriched in the chromatography fractions that supported chromatin assembly.

The decatenation of kinetoplast DNA (KDNA) provides a specific assay for topoisomerase II (Marini et al., 1980). KDNA is comprised of a network of interlocked circles of DNA, the majority of which are 2.5 kb monomers. In order to resolve this catenated network of DNA into monomers, it is necessary to introduce double-strand breaks in the DNA. Therefore, topoisomerase II, but not topoisomerase I, has the ability to decatenate KDNA and generate monomer DNA. The fractions used in the chromatin assembly reactions were subjected to this assay. (Fig. 5.7, lanes 4-9). Lane 1 shows KDNA incubated in the absence of enzyme. The KDNA remained catenated and therefore was unable to enter the gel because of its large size. Lane 2 was a negative control, which contained purified topoisomerase I in the reaction. Topoisomerase I can only introduce single-strand DNA breaks and so the KDNA remained unresolved. The positive control (lane 3) contained purified topoisomerase II which resolved the KDNA, as shown by the appearance of the 2.5 kb monomer.

The majority of topoisomerase II in the fractions was found in the two peak fractions from the 0.3 M KCl wash (lanes 6 and 7). These are the same fractions that supported chromatin assembly (Fig. 5.5A), indicating that topoisomerase II is present as expected with the other proteins responsible for the assembly of nucleosomes.

From the results in Fig. 5.7, it was possible that the failure to observe supercoiling in the 0.1 M and 0.6 M fractions (Fig. 5.5A) could be due to the separation of topoisomerase II from the rest of the chromatin assembly

machinery. To test this possibility, topoisomerase add-back experiments were performed using the 0.1 and 0.6 M KCl fractions. Purified topoisomerase II was added to a peak fraction from the 0.1 M KCl wash and the 0.6 M KCl wash (Fig. 5.8). The addition of topoisomerase II did not rescue supercoiling (lanes 2 and 5). Topoisomerase II and purified histones were added to another reaction containing a peak fraction from the 0.1 M KCl wash (lane 3). This too had no affect on supercoiling. These results suggest that other important proteins for chromatin assembly were also missing from these fractions. The experiment also demonstrates that topoisomerase II can not supercoil DNA on its own.

The same procedures described above for the TOP+ extract were also performed using the top1 extract, which contains only topoisomerase II. Fig. 5.9 shows the results of chromatin assembly reactions using several chromatography fractions. As with the TOP+ extract, the proteins responsible for chromatin assembly eluted in the 0.3 M KCl wash (lanes 3-6) and supercoiled the input DNA efficiently. Topoisomerase II activity was also observed to be the strongest in these fractions (Fig. 5.10, lanes 5-7).

5.5 Discussion

This chapter describes the initial steps in the fractionation of the chromatin assembly machinery from a yeast whole cell extract. Ammonium sulfate precipitation was chosen as the first step because it is a relatively simple method for concentrating proteins in an extract and at the same time removing unnecessary proteins. As shown in Table 1, 25% of the total protein was removed during this step for the TOP+ extract and 56% for the top1 extract. (This table does not show specific activity and is simply provided as a guide to protein recovery at each step of the purification). The efficiency of supercoiling increased substantially following the ammonium sulfate precipitation. This is demonstrated by the tight supercoiled band observed for the 70% pellet as

compared to the whole cell extract, where in addition to a supercoiled band, intermediate topoisomers are observed following assembly.

The supercoiling supported by the 70% pellet was due to the assembly of nucleosomes, as shown by it's dependence on histones and it's micrococcal nuclease digestion pattern.

Further purification of the assembly factors was achieved by chromatography of the 70% pellet on DEAE Sepharose Fast Flow. This is a weak anion exchange resin. The proteins involved in chromatin assembly eluted in the 0.3 M KCl wash. The efficiency of supercoiling observed with the chromatography fractions was greater than that observed with the input sample, considering that the amount of protein used in the chromatin assembly reactions was 100X less than what was used with the ammonium sulfate sample. This suggests that proteins that interfere with chromatin assembly are being removed. The increased efficiency of assembly may also reflect the increased concentration of the chromatin assembly factors in the extract. This supercoiling was also shown by histone add-back and micrococcal nuclease digestion to be due to the assembly of nucleosomes.

Topoisomerase II was assayed in the chromatography fractions and the majority of topoisomerase II was found in the fractions that supported chromatin assembly. Therefore, topoisomerase II is present with all the other proteins responsible for nucleosome assembly. Topoisomerase II is therefore available to function during chromatin assembly.

The addition of purified topoisomerase II and histones was not sufficient to rescue supercoiling in the fractions where supercoiling was not observed. This indicates that additional proteins that are involved in assembly were also absent from these fractions.

The assembly machinery was also partially purified from the top1 extract. The results obtained with this extract were the same as the TOP+ extract. The absence of topoisomerase I in the top1 extract did not affect the results of the chromatography. Topoisomerase II was also found to be enriched in the fractions that supported assembly. These results show that the absence of topoisomerase I does not change the elution profile of the assembly factors including topoisomerase II.

The initial steps in the purification of the chromatin assembly machinery in yeast have been completed. Since the identification of CAF-I several years ago, a primary focus of the field has been to clone its subunits. However, the recent purification of CAF-I in yeast led to the discovery that CAF-I is not essential for cell viability and that cells have no growth defects without CAF-I (Kaufman et al., 1997). This information suggests that there are other chromatin assembly factors that have yet to be identified. Therefore, although the analysis of CAF-I will no doubt continue, a new focus will be the identification of novel assembly factors. Further purification of the assembly factors in the yeast extract will hopefully lead to the identification of these novel proteins.

Table 5.1 Partial Purification of the Chromatin Assembly Machinery

Fraction	Volume (ml)	Protein (mg)	Protein Recovery (%)*
1. TOP+ extract	3.3	49	100
2. Ammonium sulfate pellet	0.81	36.5	75
3. DEAE Sepharose Fast Flow	2.2	0.30	15
1. top1 extract	1.8	25	100
2. Ammonium sulfate pellet	0.32	11.1	44
3. DEAE Sepharose Fast Flow	2.2	0.48	24

* The protein recovery was calculated as the percent of input protein. The DEAE Sepharose protein recovery is based on 2 mg of input protein.

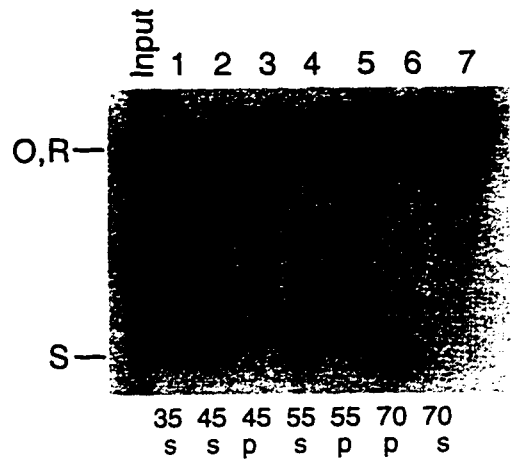


Fig. 5.1 The proteins involved in chromatin assembly remain in solution up to 55% saturation of the yeast extract with ammonium sulfate

Assembly reactions were performed using the supernatants (s) and pellets (p) collected during a step-wise series of ammonium sulfate precipitations of proteins in the TOP+ extract. The number reflects the percent saturation of the extract with ammonium sulfate i.e. 45s is the 45% supernatant and 45p is the 45% pellet. The input DNA is labeled. Open circular (O), relaxed (R), and highly supercoiled products (S) are resolved by agarose gel electrophoresis and visualized by autoradiography.

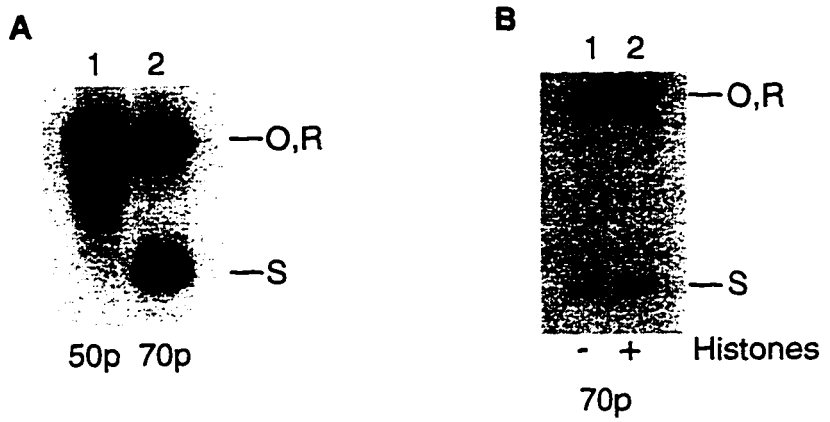


Fig. 5.2 DNA supercoiling by the 70% ammonium sulfate pellet is histone-dependent

(**A**) Comparison of DNA supercoiling in the 50% ammonium sulfate pellet (50p) and the 70% pellet (70p). (**B**) DNA supercoiling in a limiting amount of the 70% pellet was impaired in the absence of purified yeast histones (lane 1) and restored in the presence of histones (lane 2). The reaction products are resolved on an agarose gel and visualized by autoradiography. Open circular (O), relaxed (R), and highly supercoiled products (S) are labeled.

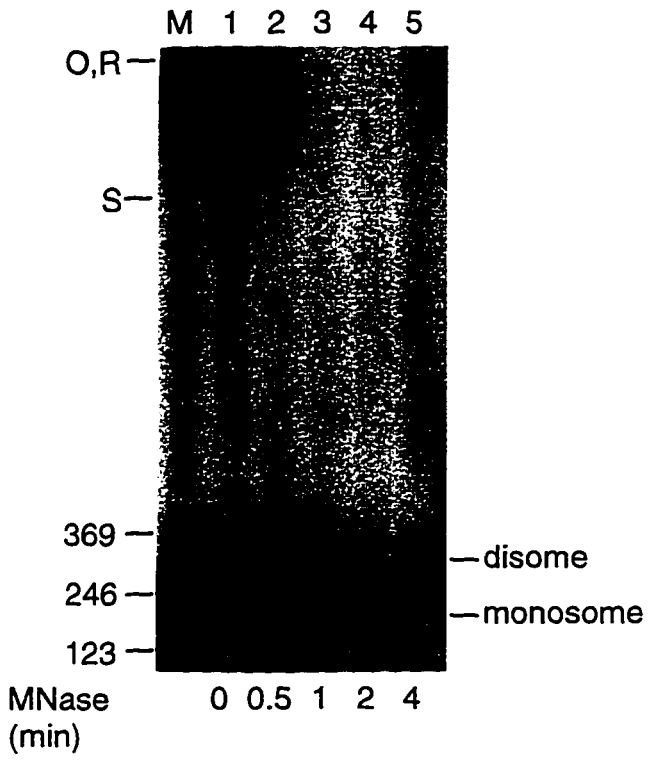


Fig. 5.3 Micrococcal nuclease digestion of chromatin assembled in the 70% ammonium sulfate pellet

Assembly reactions with the 70% pellet were treated with micrococcal nuclease for the indicated times. Products were detected by Southern blotting. Bands corresponding to mono- and dinucleosomes were observed. Open circular (O), relaxed (R), and highly supercoiled products (S) are labeled. The 123 and 246 bp markers migrate ahead of the mono- and dinucleosomes respectively.

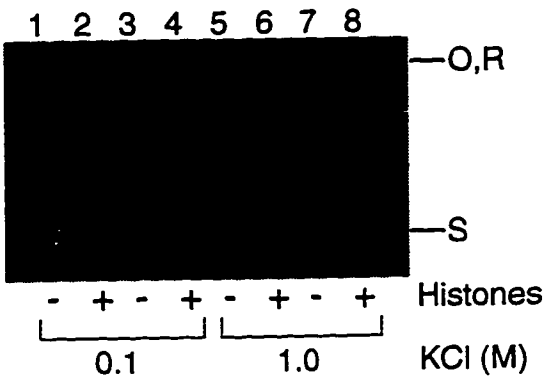


Fig. 5.4 The proteins involved in chromatin assembly remain active following elution from a DEAE Sepharose column

Assembly reactions were performed using the peak fractions collected from 0.1 M KCl (lanes 1-4) and 1 M KCl (lanes 5-8) washes of a DEAE Sepharose column. The reactions were performed in the absence or presence of purified yeast histones as indicated. Open circular (O), relaxed (R), and highly supercoiled products (S) are resolved by agarose gel electrophoresis and visualized by autoradiography.

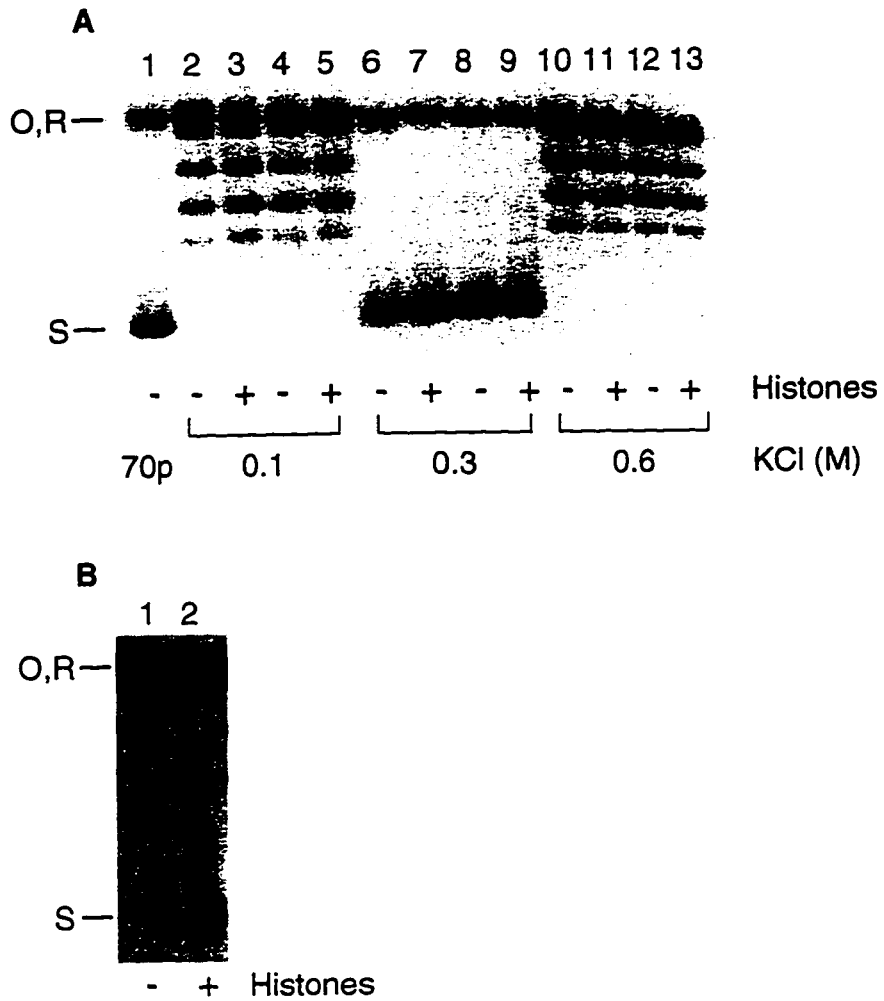


Fig. 5.5 Determination of chromatin assembly activity in the DEAE Sepharose column peak fractions

(**A**) Assembly reactions were performed with the peak fractions from 0.1 M KCl (lanes 2-5), 0.3 M KCl (lanes 6-9), and 0.6 M KCl (10-13) washes of the DEAE Sepharose column. Purified yeast histones were added to some reactions as indicated. An assembly reaction using the input 70% pellet was also included (lane 1). The amount of template DNA used in these reactions was 4 ng. (**B**) An assembly reaction with a peak fraction from the 0.3 M KCl wash was performed with 20 ng of template DNA. The accumulation of a highly supercoiled product required the addition of exogenous histones (lane 2). Open circular (O), relaxed (R), and highly supercoiled products (S) are labeled.

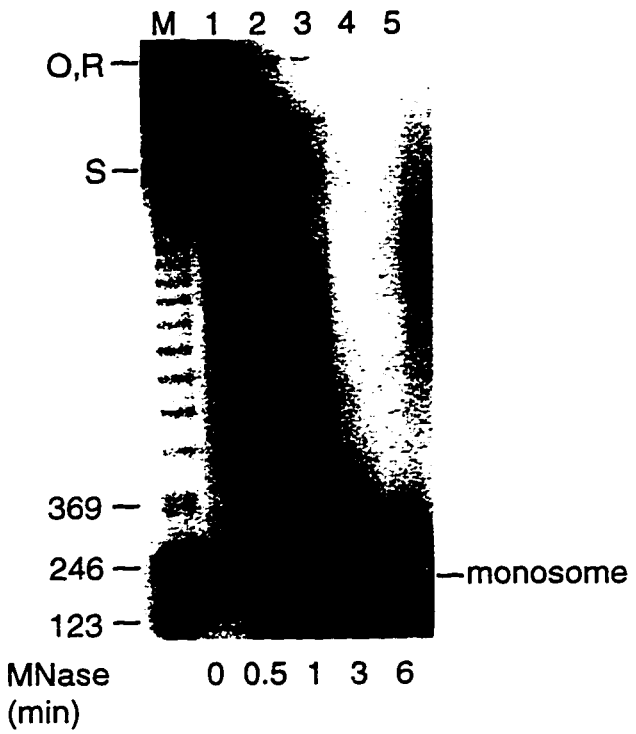


Fig. 5.6 Micrococcal nuclease analysis of chromatin assembled in a peak 0.3 M KCl fraction

An assembly reaction using a peak fraction from the 0.3 M KCl wash of the DEAE Sepharose column was treated with micrococcal nuclease for the indicated times. A band corresponding to a mononucleosome was detected by Southern blotting. Open circular (O), relaxed (R), and highly supercoiled products (S) are indicated. The 123 bp marker migrated in advance of the mononucleosome.

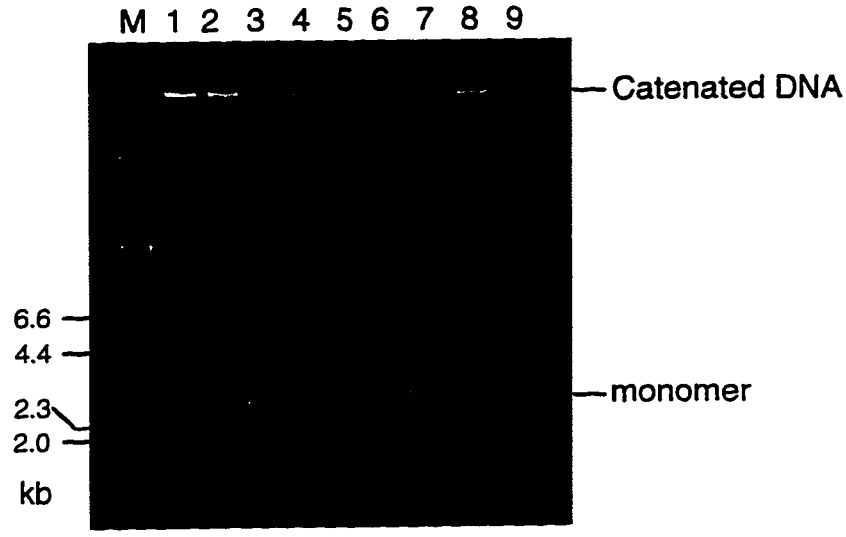


Fig. 5.7 Detection of topoisomerase II activity by the decatenation of kinetoplast DNA in the peak column fractions

Peak fractions from 0.1, 0.3, and 0.6 M KCl washes of the DEAE Sepharose column were analyzed for topoisomerase II activity by the decatenation assay using KDNA. The unresolved KDNA remains in the wells of the gel and is labeled catenated DNA. KDNA that has been resolved by topoisomerase II appears as a 2.5 kb monomer. Lane 1 contains undigested KDNA. KDNA remains unresolved in the presence of purified topoisomerase I (lane 2). Purified topoisomerase II decatenates the KDNA into monomers (lane 3). The remaining lanes contain KDNA with peak fractions from the 0.1 M KCl (lanes 4,5), 0.3 M KCl (lanes 6,7), and 0.6 M KCl (lanes 8,9) washes. The λ DNA/Hind III marker was used (lane M). The decatenation products are analyzed on a 1% agarose gel. Following electrophoresis the gel was stained with ethidium bromide and visualized using a UV light box.

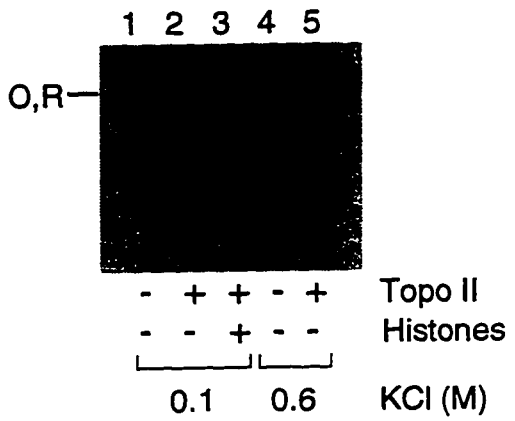


Fig. 5.8 Topoisomerase II and histones are not the only limiting factors in the assembly-deficient peak column fractions

Assembly reactions were performed using two peak fractions from 0.1 M KCl (lanes 1-3) and the 0.6 M KCl (lanes 4 and 5) washes of the DEAE Sepharose column. Purified topoisomerase II was added to some of the reactions (lanes 2, 3, and 5). One reaction received both purified yeast histones and topoisomerase II (lane 3). Open circular (O), and relaxed (R) products are resolved by agarose gel electrophoresis and visualized by autoradiography.

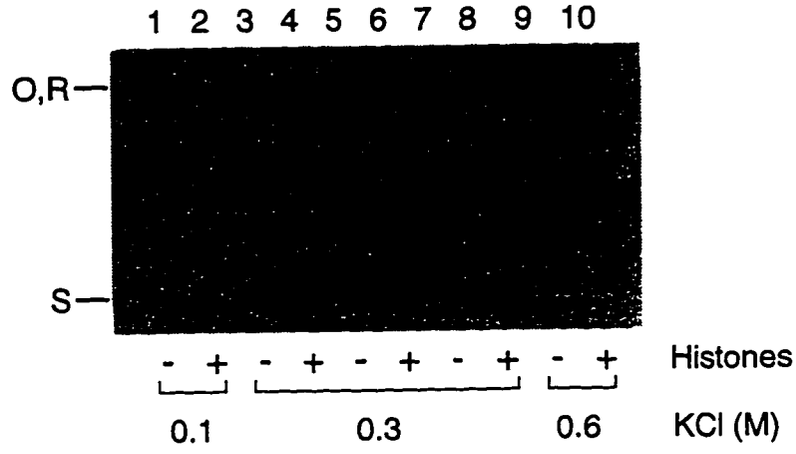


Fig. 5.9 Determination of chromatin assembly activity in the DEAE Sepharose column peak fractions with the top1 extract

Assembly reactions were performed with the peak fractions collected from 0.1 M, 0.3 M, and 0.6 M KCl washes of the DEAE Sepharose column. Purified yeast histones were added to some reactions as indicated. The amount of template DNA used in these reactions was 4 ng. Open circular (O), relaxed (R), and supercoiled products (S) are labeled. The DNA from the reaction in lane 2 was not recovered in this experiment but has been previously observed to remain relaxed.

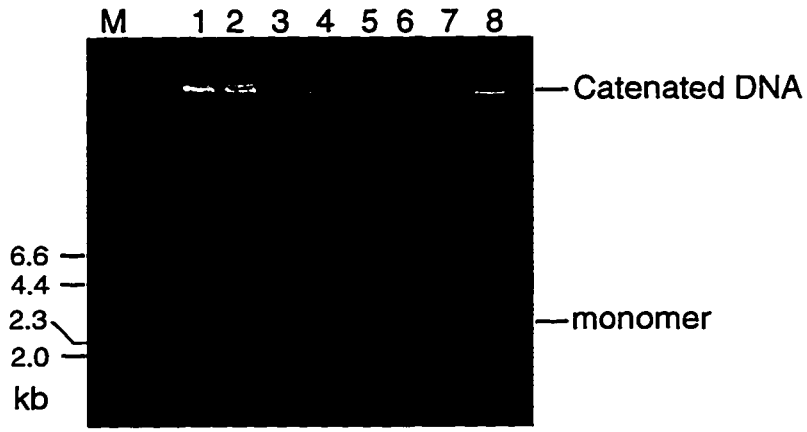


Fig. 5.10 Detection of topoisomerase II activity by the decatenation of kinetoplast DNA in the peak column fractions of the top1 extract

Peak fractions from the 0.1, 0.3, and 0.6 M KCl washes of the DEAE Sepharose column of the top1 extract were analyzed for topoisomerase II activity by the decatenation assay using KDNA. The unresolved KDNA remains in the wells of the gel and is labeled catenated DNA. KDNA that has been resolved by topoisomerase II appears as a 2.5 kb monomer. Lane 1 contains undigested KDNA. KDNA remains unresolved in the presence of purified topoisomerase I (lane 2). Purified topoisomerase II decatenates the KDNA into monomers (lane 3). The remaining lanes contain KDNA with peak fractions from the 0.1 M KCl (lane 4), 0.3 M KCl (lanes 5-7), and 0.6 M KCl (lane 8) washes. The λ DNA/Hind III marker was used (lane M). The decatenation products are analyzed on a 1% agarose gel. Following electrophoresis the gel was stained with ethidium bromide and visualized using a UV light box.

Chapter 6

Conclusions

6.1 Conclusions

The packaging of DNA into chromatin is an important cellular activity, not only because of the requirement to constrain the DNA within a nucleus, but also because of the influence that chromatin has on other nuclear activities. Chromatin assembly requires many proteins in order for the assembly of nucleosomes to proceed to completion. Using a yeast *in vitro* system I was able to use a combined biochemical, genetic, and pharmacological approach to study this process. The main objective of my work was to re-investigate the DNA topoisomerase requirement during chromatin assembly, in particular the role of topoisomerase II during assembly. The focus on topoisomerase II was in light of prior contradictory findings regarding a role for topoisomerase II during assembly.

Using mutant yeast strains of one or both topoisomerase as the basis for my experiments, I found that both topoisomerase I and II can provide the relaxing activity that is required during chromatin assembly, and that topoisomerase II copurifies with the chromatin assembly machinery during the initial steps of biochemical fractionation. Thus, topoisomerase I and II have redundant functions not only during replication and transcription but also during chromatin assembly.

Previous work by several groups has provided evidence that both supports and discounts a role for ATP during chromatin assembly. My finding that topoisomerase II, an ATP-dependent enzyme, does play a role during chromatin assembly, suggests that ATP is required during assembly. This led me to investigate in more detail the ATP requirement during chromatin assembly in a yeast extract.

I found that ATP is required for chromatin assembly to occur in the yeast extract. The reason that ATP is needed during assembly is partially explained

by the involvement of DNA ligase and topoisomerase II during assembly. ATP acts as a positive cofactor for both of these enzymes. The need for ATP during assembly has also been associated with the phosphorylation of histones and chromatin assembly factors. It appears that ATP may be involved in multiple aspects of chromatin assembly, including the initial assembly of nucleosomes, and the stability of the assembled nucleosomes.

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