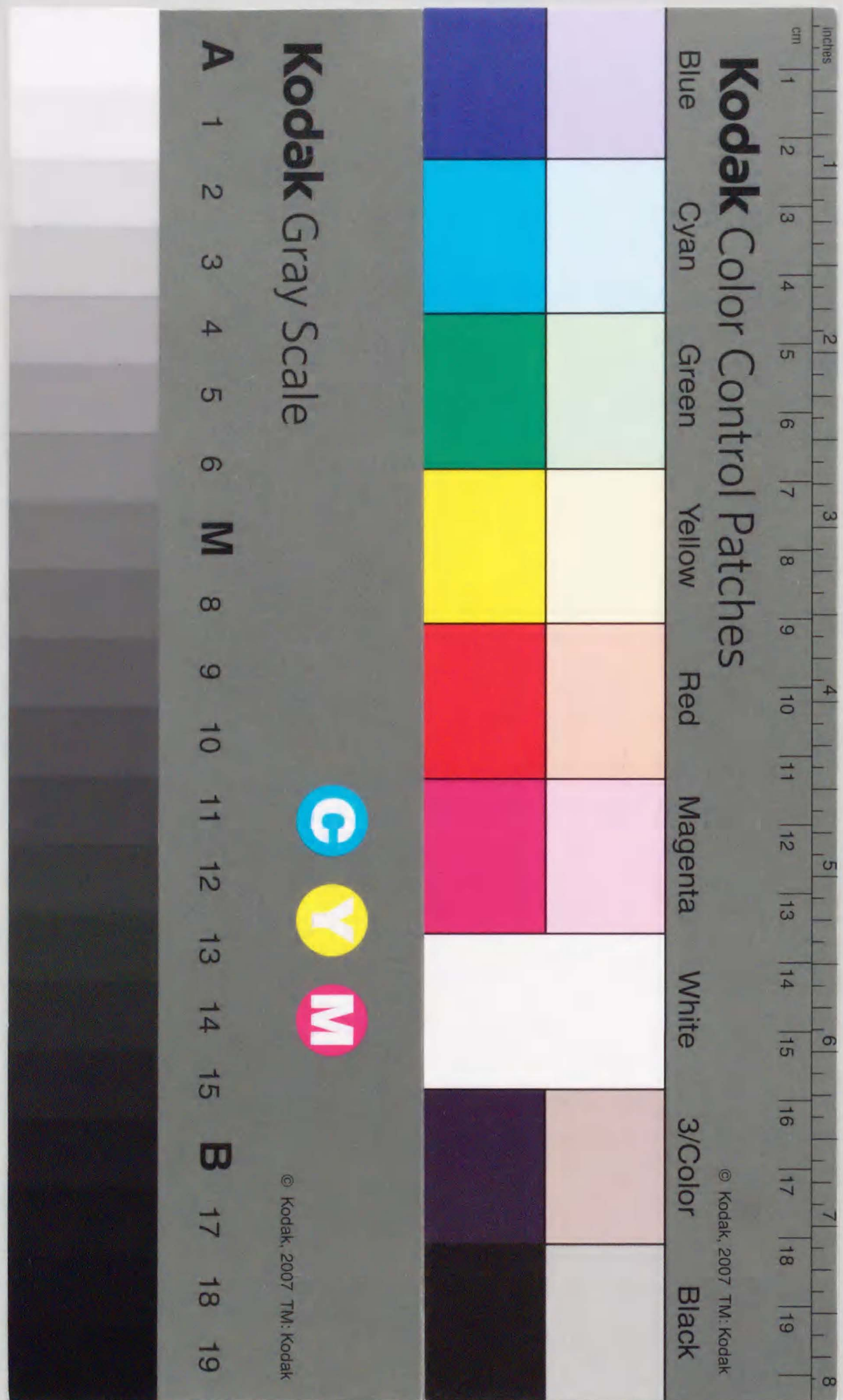

Studies on Mitochondrial DNA Topoisomerase II of *Dictyostelium discoideum*

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Abbreviations:

ATP	adenosine triphosphate
BPB	bromophenol blue
BSA	bovine serum albumin
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EtdBr	ethidium bromide
FITC	fluorescein isothiocyanate
GST	glutathione-S-transferase
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilodalton(s)
NLS	nuclear localization signal
NP40	Nonidet P-40
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
SSC	0.15M NaCl / 0.015M sodium·citrate, pH 7.6
SDS	sodium dodecyl sulfate

I. Abstract

In order to obtain insights into the functions of DNA topoisomerase II during development, I have initiated studies on the enzyme of *D. discoideum*.

First, using oligonucleotide probes designed from sequences of eukaryotic DNA topoisomerase IIs, I cloned a single copy gene for DNA topoisomerase II from *Dictyostelium discoideum* nuclear DNA. The gene, designated *topA* encodes a polypeptide of 1282 amino acids with an molecular mass of about 146 kDa. The deduced amino acid sequence shares similarity to other eukaryotic DNA topoisomerase IIs with identity between 32 and 46%, in which functional domains for the ATPase and for the cleavage and religation of DNA are conserved. The protein is 260-300 amino acids shorter in the C-terminal region and 50-80 longer in the N-terminal region than those of other eukaryotes. Four independent polyclonal antibodies against GST fusion proteins, which contained four regions of the polypeptide detected a single band at about 135 kDa on Western blots. In addition, mRNA and protein levels of *topA* were examined during *D. discoideum* development with maximal expression during the initial growth phase.

Interestingly, an additional N-terminal region of the protein (TopA) contains a putative mitochondrial targeting signal presequence. Western blot analysis of subcellular fractions and immunofluorescence microscopy revealed that this protein is located in mitochondria and not in nuclei. I constructed mutants which overexpressed the wild-type or the N-terminally deleted TopA, and examined the localization of TopAs by immunofluorescence microscopy and proteinase K digestion experiment. These experiments revealed that TopA is located in the mitochondria by virtue of the additional N-terminal region. Furthermore, in the cell extract immunodepleted of TopA, nuclear DNA topoisomerase II activity was not decreased. These results confirmed that TopA is located in the mitochondria, even though its amino acid

sequence is highly similar to those of nuclear type DNA topoisomerase II of other organisms. Thus, this study is the first to establish the location of the mitochondria targeting signal presequence in DNA topoisomerase II, and proteins in *D. discoideum*.

II. General Introduction

Topological changes of cellular DNA are necessary for the faithful transcription, recombination, replication and segregation of chromosomes in organisms. DNA topoisomerase is a ubiquitous enzyme which plays an essential role in regulating topological state of DNA by transient breakage and rejoining (for reviews see Hsieh, 1992; Wang, 1991, 1996; Watt and Hickson, 1994). There are two major classes of DNA topoisomerase, distinguished by their mechanism of action. DNA topoisomerase I introduces a transient single strand nick into DNA and subsequently transfers the complementary strand of the duplex through the nick (Maxwell and Gellert, 1986; Osheroff, 1989). DNA topoisomerase II introduces a transient double strand break, then translocates a stretch of duplex DNA through it (Osheroff, 1989; Vosberg, 1985). DNA topoisomerase II also catalyzes the ATP-dependent relaxation of negatively and positively supercoiled DNA (Osheroff *et al.*, 1983; Schomberg and Grosse, 1986), catenation-decatenation (Goto and Wang, 1982; Hsieh and Brutlag, 1980) and knotting-unknotting of circular DNA (Hsieh, 1983; Liu *et al.*, 1980). In bacteria, the enzyme is called DNA gyrase and is a tetramer consisting of a pair of each of the two subunits (A_2B_2) with an molecular mass of about 95 kDa (B subunit) and about 105 kDa (A subunit) (Higgins *et al.*, 1978; Klevan and Wang, 1980). On the other hand, the eukaryotic enzyme is a homodimer, consisting of a single polypeptide with an molecular mass of about 130-180 kDa (Lynn *et al.*, 1986).

DNA topoisomerase II is charged with the task of resolving topological problems which arise during various processes of DNA metabolism, including transcription (Gartenberg and Wang, 1992; Schultz *et al.*, 1992), recombination (Wang *et al.*, 1990), replication (Brill *et al.*, 1987), chromosome condensation (Adachi *et al.*, 1991; Rose and Holm, 1993) and chromosome segregation during cell division (Rose *et al.*, 1990; Uemura

et al., 1987). As a result of all vital role, the enzyme is essential for the viability of all organisms from bacteria to human. In addition, structural roles of the enzyme as a major protein component of nuclear matrix and chromosome scaffold structure are also argued (Poljak and Käs, 1995).

DNA topoisomerase II has been thoroughly investigated in the terms of functions in proliferative processes, but little is known about its role in DNA metabolism during development and differentiation. From this reason, I had interest in the functions of the enzyme during developmental process, and thought that the cellular slime mold was a suitable organism for studying this problem.

The cellular slime mold *Dictyostelium discoideum* grows as a single-cell amoeba. Upon starvation, cells initiate a multicellular developmental program and finally form fruiting bodies consisting stalk and spore cells (Firtel, 1995; Kimmel, 1988; Loomis, 1996). *D. discoideum* is a useful organism for studying developmental events from two perspectives; the temporal separation between the growth and developmental phases and the ease of a genetic engineering approach including gene tagging (Kuspa and Loomis, 1992) and homologous recombination (De Lozanne and Spudich, 1987). To gain insights into the functions of DNA topoisomerase II during development, I have initiated a study on the enzyme from *D. discoideum*. For this purpose, using oligonucleotide probes designed from sequences of eukaryotic DNA topoisomerase IIs, I cloned and characterized a DNA topoisomerase II gene, which I named *topA*, of *D. discoideum*. Surprisingly, the gene product was actually localized in mitochondria, although the deduced amino acid sequence of TopA showed a significant degree of homology with that of nuclear type DNA topoisomerase IIs from other eukaryotes. Information on topoisomerase IIs is mostly restricted to nuclear enzymes, and little is known about the mitochondrial enzymes to date. Genetic information of the mitochondrial enzyme is only available for the trypanosomatid

topoisomerase II (Fragoso and Goldenberg, 1992; Pasion *et al.*, 1992; Strauss and Wang, 1990), however the mitochondrion of the organism is called kinetoplast and its DNA has a unique network structure formed by two circular DNA species, mimicircles and maxicircles (Ryan *et al.*, 1988). In addition, the mitochondrial targeting signal presequence of the mitochondrial enzyme was not reported.

In section 1 of this thesis, I describe the cloning and characterization of the DNA topoisomerase II gene of *D. discoideum*. In section 2, I demonstrate the mitochondrial localization and the existence of signal presequence for the localization of the protein by immunofluorescence microscopy and proteinase K digestion experiment.

III. Section 1

Cloning and characterization of the gene encoding a mitochondrially localized DNA topoisomerase II in *Dictyostelium discoideum*

Introduction

In eukaryotes, DNA topoisomerase II catalyzes the ATP-dependent relaxation of negatively and positively supercoiled DNA, catenation-decatenation and knotting-unknotting of circular DNA (for reviews see Hsieh, 1992; Wang, 1991; 1996; Watt and Hickson, 1994). The eukaryotic enzyme is a homodimer, consisting of a single polypeptide with molecular mass of about 130-180 kDa. The N-terminal quarter of the enzyme possesses ATPase activity, whereas the central part contains a tyrosine residue that participates in breaking and rejoining of DNA (Lynn *et al.*, 1986). Although the C-terminal one-third of the enzyme has diverse amino acid sequences among eukaryotes, the region has remained very hydrophilic and charged. The overall structure, consisting of three subdomains, is evolutionarily conserved among the eukaryotic enzymes characterized to date.

DNA topoisomerase II is essential for the viability of all organisms and it participates in fundamental cellular functions such as transcription (Gartenberg and Wang, 1992; Schultz *et al.*, 1992), recombination (Wang *et al.*, 1990), replication (Brill *et al.*, 1987), chromosome condensation (Adachi *et al.*, 1991; Rose and Holm, 1993) and chromosome segregation (Rose *et al.*, 1990; Uemura *et al.*, 1987). In addition, structural roles of the enzyme as a major protein component of nuclear matrix and chromosome scaffold structure have also been proposed (Poljak and Käs, 1995). Eukaryotic cells undergo proliferation and development. DNA topoisomerase II has been thoroughly investigated in the terms of functions in proliferative processes, however little is known about its role in development and differentiation.

The cellular slime mold *Dictyostelium discoideum* grows as single-celled amoeba. Upon starvation, cells initiate a multicellular developmental program and finally form fruiting bodies consisting of stalk and spore cells (Firtel, 1995; Kimmel, 1988; Loomis, 1996).

D. discoideum is a useful organism for studying developmental events for two main reasons; the temporal separation between the growth and developmental phases, and the availability of genetic engineering approaches, such as gene tagging (Kuspa and Loomis, 1992) and homologous recombination (De Lozanne and Spudich, 1987). To gain insights into the functions of DNA topoisomerase II during development, I have initiated study on the enzyme from *D. discoideum* as a model organism. In this section, I describe the cloning and characterization of the DNA topoisomerase II gene of *D. discoideum*.

Materials and Methods

Growth and development of Dictyostelium discoideum

D. discoideum strain AX3 (Loomis, 1971) was grown axenically in HL-5 medium (Cocucci and Sussman, 1970) at 22°C on a reciprocal shaker (120 strokes/min). To initiate multicellular development, these axenically grown cells were harvested and washed 3 times with 20 mM K-phosphate buffer (pH 6.4). The cells were then suspended in LPS (40 mM K-phosphate, pH 6.4, 20 mM KCl) at a density of $1x10^8$ cells/ml and plated on 47-mm Millipore filters over an LPS saturated pad in a plate (6 cm) and incubated at 22°C (Cocucci and Sussman, 1970).

Isolation of nuclei, mitochondria and nucleic acid

Axenically grown cells were harvested by centrifugation and washed once in BSS (Bonner, 1947), then resuspended in cold NP40 lysis buffer (10 mM Mg·acetate, 10 mM NaCl, 30 mM HEPES, pH 7.5, 10% sucrose, 2% Nonidet P-40) and lysed on ice. Nuclei were collected by centrifugation at 4,000*xg* for 10 min at 4°C and washed twice in cold –NP40 buffer (Nonidet P-40 omitted from the NP40 lysis buffer). For preparation of mitochondria, axenically grown cells were lysed by the same method as described above. After removing nuclei, supernatant was centrifuged at 10,000*xg* for 15 min at 4°C. The precipitated mitochondria were washed twice in cold –NP40 buffer.

Nuclear DNA was prepared from the nuclei of AX3 cells as described (Richardson *et al.*, 1991). Total RNA was isolated from the cells as described (Chomczynski and Sacchi, 1986) and poly(A)$^+$RNA was prepared from total RNA by Oligotex-dT30 (Nippon Roche) according to the instructions of the supplier.

Cloning and sequencing of the gene for DNA topoisomerase II

To prepare oligonucleotide probes with which to clone the gene for DNA topoisomerase II, five regions were selected from the highly conserved amino acid sequences among the enzymes from *Saccharomyces cerevisiae* (Giaever *et al.*, 1986), *Schizosaccharomyces pombe* (Uemura *et al.*, 1986), *Drosophila melanogaster* (Wyckoff *et al.*, 1989) and human (Tsai-Pflugfelder *et al.*, 1988). The sequences were 1) TGGRNGYGAK; 2) MIMTDQD; 3) KYYKGLGTS; 4) KPGQRKV; 5) NGAEGIGTG and the corresponding oligonucleotide probes were designed as follows, according to the biased codon usage of *D. discoideum* (Warrick and Spudich, 1988); Probe 1) 5'-TTTI GCACC(A/G)TAACC(A/G)TTICIACCACCIGT-3'; probe 2) 5'-ATCTTGATCIGTCA T(A/G)ATCAT-3'; probe 3) 5'-GAIGTACCTA(A/G)ACCTTT(A/G)TA(A/G)TATTT-3'; probe 4) 5'-CATTTICITTGACCTGGTTT-3'; probe 5) 5'-ACCIGTACC(A/G)ATACC TTCIGCACC(A/G)TT-3' (I, inosine). These oligonucleotide were labeled with ^{32}P by T4 polynucleotide kinase and used for Southern blotting (Fig. 1).

Genomic DNA digested with *Eco*RI and *Hin*dIII was separated by electrophoresis on a 0.8% agarose gel. The DNA fraction containing 4.0 kb fragments hybridized with probe 2 were isolated, ligated into pUC18 and transformed into *E. coli* JM109. This subgenomic library was screened by colony hybridization with probe 2 (Hanahan and Meselson, 1983). A clone containing the pTOPA-1 (Fig. 2A) was obtained. As the clone lacked the 5' end, overlapping pTOPA-2 and pTOPA-3 regions (Fig. 2A) were successively cloned using fragments consisting of the 5' end regions of pTOPA-1 and pTOPA-2, respectively, as probes. Three genomic clones thus obtained were sequenced on both strands by the dideoxy chain termination method (Sanger *et al.*, 1977) using Sequenase II (U.S. Biochemical Corp., USA). Sequences were analyzed using the software GENETYX-MAC version 7.0 and the database GENETYX-CD version 31 (Software Development Corp.).

The sequence data in this paper have been deposited in DDBJ/EMBL/GeneBank Data Libraries under the accession number D82024.

Southern and Northern blots

The genomic DNA digested with various restriction enzymes was separated by electrophoresis on 0.8% agarose gels, transferred onto GeneScreen Plus membranes (DuPont NEN, UK), and hybridized with the ^{32}P labeled oligonucleotide probes in hybridization buffer without formamide (Sambrook *et al.*, 1989) at 32°C for 20 hr. The membranes were washed twice with 2x SSC at room temperature and twice with 2 x SSC/1% SDS at 42°C, then exposed to X-ray film. When the cloned DNA fragments were used as probes after labeling by using ^{32}P with a Random Primed DNA Labeling Kit (Boehringer Mannheim, Germany), the membrane was hybridized in another buffer containing 50% formamide as described (Sambrook *et al.*, 1989). The size of each DNA fragment was estimated based on the mobility of EtdBr-stained λ/*Hin*dIII fragments.

For Northern blots, total RNA and poly(A)$^+$RNA were separated on agarose gels containing 1% formaldehyde and transferred onto GeneScreen Plus membranes. The hybridization procedures were the same as those used for Southern blotting as described above.

Fusion protein constructs and antibody production

A 1.8 kb *Eco*RV fragment encoding the region from 340 to 964 inclusive of TopA was ligated with *Eco*RI linker (Takara Shuzo, Japan) and inserted into pGEX-2T (Pharmacia Biotech., Sweden) digested with *Eco*RI to construct pN340. A 2.0 kb *Hin*cII fragment encoding the region from 955 to 1282, a 2.4 kb *Ban*III fragment corresponding to the region from 814 to 1282 and a 0.9 kb *Dra*I fragment encoding the region from 28 to 320 were

blunted with Klenow and inserted into pGEX-2T digested with *Sma*I to generate plasmids pC955, pC814 and pN28, respectively (Fig. 7A). The nucleotide sequences of all constructs were confirmed to be connected in frame with the glutatione-S-transferase (GST) coding sequence. These plasmids were transformed into *E. coli* JM109 to produce the fusion proteins.

Expression of the fusion genes were initiated by addition of 0.1 mM IPTG. These fusion proteins in inclusion bodies were purified from crude cell lysate with 1% Triton X-100 and 5 M urea, then separated by SDS-polyacrylamide gel electrophoresis (PAGE) (Sambrook *et al.*, 1989). The products were detected as major bands of the predicted sizes (pN340, 97 kDa; pC955, 65 kDa; pC814, 83 kDa; pN28, 60 kDa) on gels stained with Coomassie Blue (data not shown). The gels were excised and dialyzed against PBS (10 mM Na-phosphate, pH 7.2, 150 mM NaCl). Rabbits were immunized five or six times every two weeks with the crushed gel containing 50 μg of the purified fusion protein. Antisera were prepared from blood obtained from the ear vein of the rabbits. Antibodies were purified from antisera as described (Talian *et al.*, 1983).

Western blots

Extracts obtained from whole cells ($1x10^6$ cells), nuclei (6 μg) or mitochondria (6 μg) were separated by 6% SDS-PAGE, transferred onto nitrocellurose membranes (BA85, Schleicher and Schuell, USA), and reacted with each antiserum. These blots were analyzed, using the enhanced chemiluminescence system (ECL, Amersham, UK) incorporating Protein A-linked horseradish peroxidase (Amersham, UK) following the instructions provided by the supplier.

Results

The structure of a DNA topoisomerase II gene from D. discoideum

To isolate the gene encoding DNA topoisomerase II of *D. discoideum*, five oligonucleotide probes were synthesized (see Materials and Methods). These probes were hybridized with *Eco*RI-, *Hin*dIII- or *Eco*RI/*Hin*dIII-digested genomic DNA prepared from AX3 cells. Probes 2 and 3 hybridized with the same bands (Fig. 1). Using strongly hybridizing probe 2, a clone pTOPA-1 was obtained as a 4.0 kb fragment from an *Eco*RI/*Hin*dIII subgenomic library (Fig. 2A). Overlapping clones pTOPA-2 and pTOPA-3 were isolated from a series of subgenomic DNA libraries. From the nucleotides sequence of the cloned DNA, a long ORF of 3846 bp, encoding a polypéptide of 1282 amino acids with a predicted molecular mass of 145,864 Da and pI 9.3 was identified in this region (Figs. 2A and 3). Using the program GENETYX-MAC ver. 7.0, the following percentages of identity were obtained by pairwise comparison of the deduced amino acid sequence of the ORF and several known topoisomerases; *Saccharomyces cerevisiae*, 44.6%; *Schizosaccharomyces pombe*, 43.2%; *Drosophila melanogaster*, 46.1%; human, 45.5%; *Trypanosoma brucei*, 32.8%. As the homology among these proteins was significant, I concluded that the ORF was DNA topoisomerase II and named it *topA* following the Demerec nomenclature which is recommended for cellular slime molds (Demerec *et al.*, 1966). The nucleotide sequences of probes 2 and 3 corresponded to the regions at nucleotides 1921-1941 and 2152-2178, respectively (Figs. 2A, 3 and accession no. D82024). Southern hybridization of *Eco*RI-, *Hin*dIII- or *Xba*I-digested genomic DNA showed that *topA* is a single copy gene (Fig. 2B). Loomis *et al.* showed that the gene resides on chromosome III of *D. discoideum* (Loomis *et al.*, 1995).

The schematic structure of the putative DNA topoisomerase II (TopA) is shown in

Figure 4A. In the multiple alignment, a number of amino acids are constant. Conservation of these residues in all the sequences suggests that they are critical for the function of the enzyme. The invariant GXXGXG motif at positions 253-258 in the multiple alignment (Figs. 4B and 5) forms a part of the ATP-binding site (Lindsley and Wang, 1991; Tamura and Gellert, 1990). The invariant residues R-Y at positions 898-899 (Figs. 4B and 5) are also involved in the formation of the transient covalent bond with DNA during the strand passage reaction (Horowitz and Wang 1986). Highly conserved regions (Watt and Hickson, 1994), stretches of EGDSA and PLRGK, were also found in TopA (Fig. 5). However, TopA differs from the other eukaryotic enzymes in three respects: a shorter C-terminal segment of about 260-300 amino acids, an extended N-terminal segment of about 50-80 amino acids, and the presence of the additional sequence with 34 amino acid long in the N-terminal region (Figs. 4 and 5).

Expression of topA

Expression of *topA* was examined by Northern blotting using a ^{32}P-labeled 0.8 kb *Acc*I fragment of pTOPA-1 for total RNA or poly(A)$^{+}$RNA prepared from cells in the growth phase. A hybridized band was detected only in the poly(A)$^{+}$RNA and *topA* was expressed as a 4.5 kb polyadenylated RNA (Fig. 2C). I then examined the expression of the gene during development. Poly(A)$^{+}$RNA was isolated from the cells at several stages and hybridized to a ^{32}P-labeled 2.0 kb *Eco*RV fragment from pTOPA-1. The 4.5 kb *topA* mRNA was present at all stages, although the amount declined as development proceeded (Fig. 6A).

Western blots during development

Four independent polyclonal antisera were raised against bacterially expressed GST fusion proteins containing various portions of TopA (Fig. 7A). Each antibody recognized its

respective fusion protein extracted from *E. coli* but not GST (data not shown). I therefore concluded that these antisera recognize the TopA epitopes in the fusion proteins. Three antisera, αC955, αC814 and αN340, recognized a single band at about 135 kDa (Fig. 7B). The antiserum αN28 detected several weak bands in the cell lysate from growth phase. However, after purifying the antibody from the crude antiserum αN28, only a single band was detected at about 135 kDa (Fig. 7B). Figure 7 also shows that none of the four preimmune sera reacted with proteins.

I examined the producton profile of TopA during development. Cell lysates prepared at several stages were analyzed with the purified αN340. The results showed that TopA of about 135 kDa was present at all stages, although as its amount decreased gradually with the developmental progress (Fig. 6B).

Localization of TopA in purified mitochondria

By analyses of the extra N-terminal 70 amino acids of TopA, I found that a region of residue 1-35 contains a putative mitochondrial targeting signal presequence that may fold into an amphiphilic α-helix (Hartl *et al.*, 1989). The helical-wheel illustration in Figure 4C indicates that one face of the helix is nonpolar, while the other is positively charged. Western blotting analysis was performed to determine whether TopA was enriched in nuclei or in mitochondria in *D. discoideum* cells. Significant difference of the TopA level was shown between fractions from nuclei and mitochondria by Western blotting analysis. The density calculation of the fluorogram showed that the content in mitochondria was eightfold higher than that of nuclei (Fig. 8).

Discussion

I cloned and sequenced a DNA fragment of about 5.8 kb containing a gene encoding a polypeptide with highly homologous sequence with other eukaryotic DNA topoisomerase II from *Dictyostelium discoideum*. The gene, designated *topA*, encoded a polypeptide of 1282 amino acids with no introns, and was located on chromosome III (Loomis *et al.*, 1995) as a single copy. Northern blots and Western blots revealed a transcript of about 4.5 kb and a product of about 135 kDa, respectively, from *topA* gene. Although it is interesting to examine the function of TopA during development, the present results did not suggest the obvious involvement of TopA in development of *D. discoideum*.

The amino acid sequence of TopA differs from those of other eukaryotic enzymes in three respects. Firstly, TopA was 260-300 amino acids shorter in the C-terminal region (Fig. 5). The C-terminal segments in most of DNA topoisomerase II homologs are not well conserved among various species but are characterized by the presence of stretches of positively and negatively charged amino acids. The segment also may be required for nuclear localization and the regulation of enzymatic activity by phosphorylation (Cardenas and Gasser, 1993; Caron *et al.*, 1994; Shiozaki and Yanagida, 1992). Proteolytic analysis and a series of deletion mutants of the enzyme have shown that the mutant protein with a truncated C-terminus retained enzymatic activity in vitro but not in vivo (Caron *et al.*, 1994; Shiozaki and Yanagida, 1991). However, DNA topoisomerase IIs of *Trypanosoma* and African swine fever virus also lack this region (García-Beato *et al.*, 1992; Strauss and Wang, 1990). Secondly, TopA possess an extra N-terminal segment of about 70 amino acids (Figs. 4 and 5). Like other mitochondrial proteins of *D. discoideum* (Birney *et al.*, 1995; Troll *et al.*, 1993), positions 1-35 of TopA contains a putative mitochondrial targeting signal presequence that may form an amphiphilic α-helix. In fact, Western blotting analysis of

subcellular fractions showed that the enzyme is localized to mitochondria (Fig. 8). To my knowledge, there is no previous report that describes the localization of DNA topoisomerase II in mitochondria, except for that of trypanosome kinetoplasts (Fragoso and Goldenberg, 1992; Melendy *et al.*, 1988; Pasion *et al.*, 1992; Strauss and Wang, 1990). In *S. pombe,* the N-terminal segment of DNA topoisomerase II has stretches of a nuclear localization sequence and several phosphorylation sites (Shiozaki and Yanagida, 1991; 1992). The segment has a function similar to that of the C-terminal regions of the homologs in other organisms. The function of the N-terminal region of TopA may thus complement the absent C-terminal function required for protein localization. Finally, the predicted amino acid sequence of *topA* contains an insertion of hydrophilic and charged amino acids from positions 134 to 168 (Figs. 4 and 5) and it shows no significant homology with other protein sequences in the SWISS-PROT database. The results of cDNA-directed PCR excludes the possibility that this region is an intron (data not shown).

I attempted to disrupt *topA* gene by homologous recombination. The disruption technique has been established in AX3 strain and usually results in at least 5-10% (some cases, 50%) of disruptants in transformants, however my attempts never succeeded (data not shown), and hence I presume that *topA* would be essential for viability in *D. discoideum* as in other eukaryotes.

In conclusion, the cloned *topA* of *D. discoideum* contained a putative mitochondrial targeting presequence and the gene product TopA was actually localized to mitochondria, although its sequence shows a significant degree of homology with those nuclear type DNA topoisomerase II from other eukaryotes. In this study, I could not find another DNA topoisomerase II which prefers to localize to nuclei from the observation of Southern and Western blots. Southern blotting at reduced stringency again showed a single band (data not shown). Western blot analysis showed a single band with molecular mass of 135 kDa

corresponding to TopA and the protein was enriched in mitochondria (Fig. 8). The nuclear type DNA topoisomerase II may also be encoded by topA and expressed as an different transcribed form from TopA. However, Northern blot analysis showed only one band for the *topA* transcript (Fig. 2C). It is more likely that the nuclear type DNA topoisomerase II is encoded by some gene which is divergent from *topA* in *D. discoideum* genome. To clarify the physiological roles of TopA, I examined the intracellular localization of the enzyme, using a N-terminus deletion mutants of TopA in the next section.

IV. Section 2

Localization of a DNA topoisomerase II to mitochondria in *Dictyostelium discoideum*: deletion mutant analysis and mitochondrial targeting signal presequence

Introduction

DNA topoisomerases play an essential role in regulating the topological state of DNA by transient breakage and rejoining (for reviews see Hsieh, 1992; Wang, 1991; 1996; Watt and Hickson, 1994). DNA topoisomerase II is essential for viability of all organisms because the enzyme has an important role in fundamental cellular functions such as transcription (Gartenberg and Wang, 1992; Schultz *et al.*, 1992), recombination (Wang *et al.*, 1990), replication (Brill *et al.*, 1987), chromosome condensation (Adachi *et al.*1991; Rose and Holm 1993) and chromosome segregation (Rose *et al.*, 1990; Uemura *et al.*, 1987). In addition, structural roles of the enzyme have been proposed, e.g. as a major protein component of nuclear matrix and chromosome scaffold structure (for review; Poljak and Käs, 1995).

Mitochondrial DNAs in many organisms are circular molecules. Replication and transcription of such closed circular DNA will introduce topological stress which must be relieved to allow these processes to continue. The swivel reaction can be fulfilled by DNA topoisomerases capable of relaxing positively supercoiled DNA. However, in eukaryotes, information regarding topoisomerases is mostly restricted to nuclear enzymes, with little known about the mitochondrial enzymes. The mitochondrial topoisomerases from mammals (Castora *et al.*, 1983; 1985; Kosovsky and Soslau, 1993; Lin and Castora, 1991; Lin *et al.*, 1992) and yeast (Ezekiel *et al.*, 1994; Murthy and Pasupathy, 1994; Wang *et al.*, 1995) have been partly characterized, but their low abundance has prevented their extensive purification and biochemical characterization. Genetic information of the enzyme is available only for the trypanosomatid topoisomerase II (Fragoso and Goldenberg, 1992; Pasion *et al.*, 1992; Strauss and Wang, 1990).

I cloned the gene encoding DNA topoisomerase II (*topA*) of *Dictyostelium discoideum*

and determined its nucleotide sequence (see Section 1). The predicted amino acid sequence of the gene had an additional N-terminal region which was expected to be a mitochondrial targeting signal presequence thought to be folded into an amphiphilic α-helix (Hartl *et al.*, 1989). I showed the mitochondrial localization of the enzyme by Western blotting analysis (Fig. 8, see Section 1). In this section, I examined the localization of this protein by immunofluorescence microscopy and proteinase K digestion experiment using a wild-type strain and N-terminal deletion mutants.

Materials and Methods

Strain and cell culture

Dictyostelium discoideum strain AX3 (Loomis, 1971) was grown axenically in HL-5 medium (Cocucci and Sussman, 1970) at 22°C on a reciprocal shaker (120 strokes/min). For transformant selection, AX3 cells grown in HL-5 medium containing 10 mg/ml G418 (Wako Ltd., Japan) were used.

Isolation of nuclei, mitochondria and nucleic acids

Axenically grown cells were harvested by low-speed centrifugation and washed once in BSS (Bonner, 1947), then resuspended in cold NP40 lysis buffer (10 mM Mg-acetate, 10 mM NaCl, 30 mM HEPES, pH 7.5, 10% sucrose, 2% NP40) and lysed on ice. Nuclei were collected by centrifugation at 4,000*xg* for 10 min at 4°C and washed twice in cold –NP40 buffer (NP40 omitted from the NP40 lysis buffer).

For preparation of mitochondria, axenically grown cells were lysed by the same method as described above. After removing nuclei, the supernatant was centrifuged at 10,000*xg* for 15 min at 4°C. The precipitated mitochondria were washed twice in cold –NP40 buffer, and then used for immunofluoresence microscopy. Purified mitochondria were obtained as described (Angata *et al.*, 1995) and the precipitated mitochondrial fraction was washed further twice in cold –NP40 buffer, and used for proteinase K digestion experiments.

Genomic DNA was isolated from the nuclei of the cells as described (Hughes and Welker, 1988). Total RNA was isolated by ISOGEN (Wako Ltd., Japan) according to the manufacturer's instructions.

Vector construction and transformation

Wild-type (WT) and a mutant (ΔN246 lacking the first 82 amino acids) of TopA were fused with the c-Myc tag sequence for *in vivo* immunofluorescence microscopy. Two PCR fragments corresponding to the N-terminus of TopA were prepared. Upstream primers 5'-GGAGATCTATGTCAAAATTATTAAATAATA-3' containing the first 22 nucleotides (underlined) of *topA* from the translational initiation site and 5'-GGAGATCTATGACCA CAAGAAAGATAGAAGA-3' containing an ATG initiation codon (double lines) followed by the sequence from 247-266 (underlined) of *topA* were synthesized for WT and ΔN246, respectively (Fig. 10C). Both primers had a *Bgl*II site to facilitate insertion of the PCR product into the expression vector pBS18 (Kumagai *et al.*, 1989) in frame. The downstream primer used was 5'-TTGACCATCTTTGGAAATAC-3' (nucleotides 843-862). The PCR fragments were connected to the 5'-end of pTOPA-1 at an internal *Eco*RI site of the structural gene (Fig. 10A and B). To fuse the *c-myc* tag sequence to the C-termini of the TopA genes, *Xho*I and *Bam*HI sites were introduced at the end of the *topA* coding region by replacing the 3'-end of the gene in pTOPA-1 with a PCR fragment amplified with 5'-CAATGAATTGGATGATCC-3' (2733-2750) as the upstream primer and 5'-CTGGATCCTCGAGTTTTGATTTAATTTTATCAG-3' (3827-3846, underlined; *Xho*I site, double lines) as the downstream primer using an internal *Xba*I site (Fig. 10B and C). The resultant plasmid was designated pTOPA-11. Then, the fragment corresponding to the c-Myc epitope sequence (EQKLISEEDL) with a stop codon was inserted into the *Xho*I site of pTOPA-11 (Fig. 10C) . All of the connected region and junction sites were confirmed by sequencing. The resultant *topA* genes fused with *c-myc* were digested out with *Bam*HI and *Bgl*II and then inserted into the unique *Bgl*II site between the actin 15 promoter and the 2H3 terminator of the expression vector pBS18. The resultant plasmids, pWT and pΔN246, were introduced into AX3 cells by electroporation as described previously (Yamaguchi *et al.*,

1996), and neomycin-resistant clones, WT and ΔN246, respectively, were isolated. pBS18 was also transformed into AX3 cells according to the same protocol as a control .

Southern and Northern blotting

The genomic DNA fragments (2.5 μg) digested with *Eco*RI were separated by electrophoresis on a 0.8% agarose gel and hybridized as described (see Section 1, Materials and Methods) using probes for *CAR1* (Abe and Maeda 1994) provided by Dr. Maeda of Tohoku University, and *neo*.

Total RNA was hybridized using the 2.0 kb *Eco*RV fragment of pTOPA-1 as a probe.

Western blotting

Extracts obtained from whole cells (3 x 10^5 cells) were separated by 6% SDS-PAGE, transferred onto BA85 membranes (Schleicher and Schuell, USA), and reacted with anti-TopA antiserum which was raised against the amino acid positions 955 to 1282 (αC955 antiserum, Fig. 7, see Section 1) or anti-c-Myc antibody (anti-9E10 antibody, BAbCO, USA). These blots were analyzed using an enhanced chemiluminescence system (ECL, Amersham, UK) incorporating Protein A-linked or goat anti-mouse IgG-linked horseradish peroxidase (Amersham, UK) according to the method of the supplier.

Indirect Immunofluorescence Microscopy

Axenically grown cells were harvested by centrifugation, and washed twice in K-phosphate buffer (20 mM K-phosphate, pH 6.4, 2 mM EDTA). The cells were fixed, blocked and stained as described by Kobayashi *et al.* (1996), except that then were prefixed in cold methanol (–20°C) for 5 min and incubated with first antibodies (see below) overnight at 4°C. The organelle fractions were treated as described above except that the

paraformaldehyde fixation step was omitted. Affinity-purified αC955 antibody (anti-TopA antibody; Fig. 7, see Section 1) was not diluted and αC955 preimmune serum and anti-c-Myc antibody were diluted to 1:200 and 1:1000 with BAT [10% BlockAce (Dainihonseiyaku Inc., Japan), 0.1% Triton X-100], respectively. The secondary antibodies, FITC-conjugated goat anti-rabbit IgG (Cappel, USA), Texas Red-conjugated rabbit anti-mouse IgG (Cappel, USA) and donkey anti-rabbit IgG (Amersham, UK), were diluted to 1:100, 1:50 and 1:50 with BAT, respectively. Immunostained cells and organelles were examined using a Bio-Rad MRC-500 confocal microscope. Anti-calf mitochondrial complex I antiserum was kindly supplied by Dr. J. Hayashi of our Institute

Proteinase K digestion experiment

Isolated mitochondria were washed once with –NP40 buffer, then mixed with –NP40 lysis buffer at the mitochondrial concentration of 12.5 mg/ml containing proteinase K at various concentrations, and incubated for 1 hr on ice either in the absence or presence of 1% Triton X-100. After incubation, proteinase K was inactivated by addition of PMSF to a final concentration of 1 mM. The mitochondria were washed twice with –NP40 lysis buffer containing 1mM PMSF, and then used for Western blotting.

Immunodepletion experiment

Protein A-Sepharose CL4-B (Sigma Chemical, USA) was swollen and washed 3 times with PBS (10 mM Na-phosphate, pH 7.2, 0.15 M NaCl), then the resin (100 μl) was mixed with each one twentieth diluted preimmune sera or antisera, αC814 and αN340 which were raised against the amino acid positions 814 to 1282 and 340 to 964, respectively (200 μl). The mixtures were incubated at 4°C overnight on a rotatory shaker, then the resin was washed twice with PBS and twice with topo II buffer (50 mM Tris-HCl, pH 8.0,

100 mM NaCl, 50 mM KCl, 0.5 mM EDTA, 10 mM $MgCl_2$). Cell extracts were prepared by centrifugation of ruptured NP40 cells at 10,000xg, and dissolved in extraction buffer [20 mM Tris-HCl, pH 7.5, 0.1 M KCl, 1 mM EDTA, 0.1 mM DTT, 45% (v/v) glycerol, 1 mg/ml BSA, 0.35 M NaCl, 1 mM PMSF, 10 μg/ml leupeptin]. The resins were mixed with an equal volume of undiluted cell extract (about 100 μl) and incubated at 4°C for 1 hr on a rotatory shaker. The supernatant as the depleted extract was collected by centrifugation and used for DNA topoisomerase II assay and Western blotting. For Western blotting, the supernatant was mixed with an equal volume of 2x sample buffer [0.25 M Tris-HCl, pH 6.8, 2% SDS, 20% (v/v) glycerol, 28 mM 2-mercaptoethanol, 0.02% BPB] and boiled for 5 min. The immunoprecipitated resins were washed twice with PBS, mixed with 200 μl of 1x sample buffer and boiled for 5 min. The solution was centrifuged and the supernatant was used.

DNA topoisomerase II assay

The immunodepleted extract (2 μl) was mixed with 18 μl of topo II buffer containing 30 μg/ml BSA, 1 mM ATP and 0.25 μg of kinetoplast DNA and incubated at 22°C for 30 min. Four μl of stop solution [35% (w/v) sucrose, 5% SDS, 40 mM EDTA, 0.25% BPB] was added to each mixture followed by separation on a 0.8% agarose gel, and staining with 0.1 μg/ml ethidium bromide.

Results

Intracellular localization of TopA

The intracellular localization of TopA in *D. discoideum* AX3 cells was analyzed by immunofluorecence microscopy using anti-TopA antibody, αC955. The nucleus showed intense Hoechst staining with large circular boundaries, and mitochondria showed weakly stained as many small granules (Fig. 9A, panels a-c). When the cells were incubated with αC955 and FITC-labeled secondary antibody, only mitochondria showed specific labeling (Fig. 9A, panel f). Preimmune serum (Fig. 9A, panels e) and secondary antibody alone (Fig. 9A, panel d) showed only background staining.

To confirm the above results further, isolated nuclei and mitochondria were also examined (Fig. 9B). The isolated mitochondria were labeled with αC955 as well as by Hoechst staining (Fig. 9B, panels b and e). Secondary antibody alone showed only background staining (Fig. 9B, panel d). Isolated nuclei were stained with anti-TopA antibody; bright granules were seen around the nucleus due to contaminating mitochondria but not within the nucleus itself (Fig. 9B, panels c and f). Staining with anti-calf mitochondrial complex I antiserum (Tanaka *et al.*, 1988) indicated that these granules around the nuclei were mitochondria and not any specific nuclear structures (data not shown). These results indicated that TopA of *D. discoideum* is primarily located in mitochondria.

Construction and characterization of N-terminal deletion mutants

Next, I analyzed the additional N-terminal region of TopA to determine its function in mitochondrial localization. Expression plasmids containing the full-length *topA* (pWT) or the 5'-terminal deletion mutant (pΔN246), both of which were fused with the *c-myc* tag sequence, were constructed (Fig. 10; see Materials and Method) and introduced into AX3

cells. In total, 6 transformants were obtained with wild-type (WT) and 15 were obtained with the mutant (ΔN246) gene. As a control, nine transformants (BS18) with parental pBS18 were obtained.

Both WT and ΔN246 transformants grew nearly as fast as the control cells (BS18) in HL-5 containing 10 μg/ml of G418 on a reciprocal shaker. In addition, all transfomants developed normally on agar plates. The plasmid copy numbers of all transformants were equivalent as determined from the density of the *neo* gene band normalized using that of a single copy gene, *CAR1* (Fig. 11A). The levels of *topA* expression from pWT and pΔN246 were determined by Northern blotting analysis for total RNA using 2.0 kb *Eco*RV fragment of the pTOPA-1 (Fig. 10A) as a probe. The endogenous *topA* gene was expressed at such low levels that the mRNA could not be detected in total RNA (Fig. 2C, see Section 1). In agreement with previous results, total RNA from BS18 transformants did not show any bands, even on long exposure (Fig. 11B, top and middle). All WT and ΔN246 transformants produced the plasmid-derived *topA* mRNA, which was longer than the endogenous mRNA because of the different transcriptional initiation and termination sites. On the other hand, each ΔN246 transformant expressed truncated *topA* mRNA at levels about tenfold those of the WT transformants (Fig. 11B, middle). The level of production of truncated TopA in ΔN246 cells was about 60-fold higher than the wild-type enzyme in WT cells (Fig. 11C, bottom). WT transformants produced TopA only about one and a half-fold greater than that of the endogenous enzyme in BS18 cells (Fig. 11C, top). The control BS18 cells did not show any bands on Western blotting with anti-c-Myc antibody (Fig. 11C, bottom).

Intracellular localization of the N-terminal deletion mutant proteins

To determin directly the intracellular localization of the protein, transformed cells were

examined by immunofluorescence microscopy using anti-TopA or anti-c-Myc antibody. It should be noted that anti-TopA antibody can bind with both endogenous and c-Myc-tagged protein derived from the introduced plasmid, while anti-c-Myc antibody can detect only the c-Myc-tagged protein (Fig. 11C, top). As shown in Figure 12A (panels a, b, d and e), each mitochondrion of both BS18 and WT cells was stained by Hoechst and also by anti-TopA antibody with a one-to-one correspondence. ΔN246 cells were stained so intensely that specific regions could not be detected by the anti-TopA antibody (Fig. 12A, panel f). Figure 12B (panels d-f) shows the results of anti-c-Myc antibody treatment. Although the control BS18 (Fig. 12B, panel d) did not show any signals, WT transformants clearly showed distinct fluorescence corresponding to each mitochondrion (Fig. 12B, panels b and e). Again, the ΔN246 cells were stained strongly throughout the whole cell (Fig. 12B, panel f). These results indicate that the wild-type TopA is localized in mitochondria, although the localization of the truncated TopA could not be determined.

Localization analysis by proteinase K digestion experiment

As the intracellular localization of the truncated TopA was not clear because of the intense fluorescence in ΔN246 cells as described above, I examined its localization by proteinase K digestion. Firstly, the isolated mitochondria from the WT cells were treated with proteinase K in the absence or presence of 1% Triton X-100. As shown in Figure 13A, TopA was protected from digestion. Permeabilization of the membrane with Triton X-100 gave proteinase K access to the protein and caused digestion of TopA. Isolated mitochondria from WT cells were treated with various concentrations of proteinase K (Fig. 13B, top). The result showed that TopA was protected from digestion even at a proteinase K concentration of 500 μg/ml, indicating that wild-type TopA resides in the mitochondria. In contrast with the WT cells, the band of the truncated TopA in ΔN246 cells

disappeared in the presence of 100 μg/ml proteinase K (Fig. 13B, middle). However, the endogenous TopA in ΔN246 cells was protected from digestion by proteinase K even at a concentration of 500 μg/ml (Fig. 13B, bottom) as in the case of WT cells. The lower band in Figure 13B (bottom) is likely to be a degradation product of TopA. These results indicate that the truncated protein is not localized in mitochondria, while the endogenous TopA is contained within mitochondria, indicating that high-level expression of truncated TopA does not disturb endogenous TopA import into mitochondria in ΔN246 cells.

The relationship between TopA and nuclear topoisomerase II

Finally, whether TopA is associated with nuclear topoisomerase II activity was examined by immunodepletion experiments. The cell extracts were immunodepleted of TopA with anti-TopA antisera, αC814, αC955 and αN340 (Fig. 7, see Section 1). Preimmune sera or buffer (PBS) were used as controls. Depletion of TopA was evaluated by Western blotting. As shown in Figure 14A, TopA in the supernatant was depleted by immune serum αC814 (lane 5) or αC955 (lane 9) and not with PBS (lane 1) or preimmune sera (lanes 3 and 7). αN340 was used as an example of serum which could not deplete TopA (lanes 11 and 12). The reason why immune serum αN340 could not precipitate TopA in the reaction would be attributed to the ability of it to react with TopA on Western blotting (Fig. 7, see Section 1), but not with native TopA. The lower band in each lane is a degradation product of TopA. Topoisomerase II activities in these depleted supernatants were assayed using kinetoplast DNA decatenation reaction. Figure 14B clearly shows that the supernatant depleted with αC814 (lane 5) or αC955 (lane 7) retained the same level of the topoisomerase II activity as that depleted with PBS or preimmune sera (Fig. 14B, lanes 3, 4 and 6). When each supernatant was sequentially diluted, all of the activity disappeared at the same dilution. These results indicate that the two immune sera (αC814 and αC955)

do not react with nuclear DNA topoisomerase II. Thus, TopA is not associated with nuclear topoisomerase II activity.

Discussion

In the section 1, I found that *Dictyostelium discoideum* DNA topoisomerase II (TopA) is localized to mitochondria by Western blotting analysis of subcellular fractions. The present results from immunofluorecence microscopy experiments also revealed the localization of TopA to mitochondria but not to the nucleus *in vivo* (Fig. 9). Furthermore, immunodepletion experiments showed that the TopA enzyme is not associated with nuclear topoisomerase II activity (Fig. 14). These results indicate that TopA is a mitochondrion-specific DNA topoisomerase II in *D. discoideum*.

Eukaryotic DNA topoisomerase II have a common structure (Watt and Hickson, 1994); the N-terminal quarter of these proteins has an ATPase activity and the center part contains a tyrosine residue that participates in catalysis of breaking and rejoining of DNA. However, the C-terminal one-third of the sequences are diversed among organisms. Most eukaryotic DNA topoisomerase II have a C-terminal region containing many hydrophilic and charged residues, which is thought to be a nuclear localization sequence. TopA of *D. discoideum*, however, lacked this C-terminal region, but had a long N-terminus containing a sequence structurally analogous to the mitochondrial targeting presequence seen in other nuclear-encoded mitochondrial proteins in *D. discoideum* (Birney and Klein, 1995; Troll *et al.*, 1993). N-terminal deletion mutant analyses in this study also revealed that TopA is localized to the mitochondria by virtue of this additional N-terminal region (Figs. 12 and 13), indicating that this sequence acts as a mitochondrial targeting signal presequence in the TopA of *D. discoideum*. The enzyme of *Schizosaccharomyces pombe* also has an additional N-terminal segment, but this region is known to contain a nuclear localization sequence and phosphorylation sites (Shiozaki and Yanagida, 1991; 1992). To identify the cleavage site of the signal peptide, I tried to determine the N-terminus of the mature protein. However, this

was unsuccessful because the amounts of the protein in *D. discoideum* mitochondria were too low to allow purification.

The mRNA (Fig. 11B, middle) and protein (Fig. 11C, bottom) levels of truncated *topA* in ΔN246 cells were 10-fold and 60-fold greater compared to those of WT. Although reasons which caused this difference between the truncated *topA* and the native *topA* are difficult to explain at present, several possibilities exist: (i) the difference of the context sequence around the translational initiation site may affect the transcriptional and translational efficiency; (ii) the deleted 246 nucleotide region may contain *cis*-acting, negative element or destabilize its own mRNA; (iii) feedback mechanisms by the mRNA and/or protein (also signal sequence) may regulate the activity of its own transcription and/or translation in WT cells.

The requirement for DNA topoisomerase II in the replication and segregation of chromosomal DNA as elucidated in *S. pombe* (Uemura *et al.*, 1987) suggests that a nuclear DNA topoisomerase II must also exist in *D. discoideum*. Indeed, topoisomerase II activity was present in the the cell extract after treatment with anti-TopA antibody (Fig. 14). If the amino acid sequence of the nuclear enzyme is similar to the mitochondrial enzyme, another band corresponding to the nuclear enzyme might be observed on Western and Southern blots. However, Western blotting of whole-cell lysates showed just a single band, the 135 kDa band characteristic of the mitochondrial enzyme. Southern blotting at reduced stringency again showed a single band (data not shown). From these results, I favor the view that the nuclear type enzyme is encoded by another gene which is divergent from the *topA* gene.

Previously, the trypanosomatid topoisomerase II genes were reported as mitochondrial topoisomerase II genes, but the mitochondrial targeting signal presequence was not elucidated (Fragoso and Goldenberg, 1992; Melendy *et al.*, 1988; Pasion *et al.*, 1992;

Strauss and Wang, 1990). In this study, the mitochondrial targeting signal presequence of DNA topoisomerase II was revealed and this is the first to be elucidated in a topoisomerase II and in *D. discoideum* by deletion mutant experiments. In a preliminary experiment, I attempted unsuccessfully to detect the mitochondrial topoisomerase II activity in the crude mitochondrial extract (data not shown). It is likely that the enzyme is present at low levels or has low activity in *D. discoideum* mitochondria. To detect the mitochondrial topoisomerasae II activity, further purification will be required.

V. General Conclusion

In this study, I cloned *topA* as a DNA topoisomerase II gene from *Dictyostelium discoideum* and characterized it. Results of this study showed that, although the amino acid sequence of the protein (TopA) encoded by *topA* is highly homologous to that of other eukaryotic DNA topoisomerase IIs, it had some interesting characters different from other eukaryotic enzymes.

Eukaryotic DNA topoisomerase IIs are divided into three structural domains consisting of the ATPase domain, the catalytic domain (breaking and rejoining of DNA) and the C-terminal domain. The C-terminal domain contains many charged amino acid residues and is required for nuclear localization (Caron *et al.*, 1994;,Shiozaki and Yanagida, 1992). TopA is consist of 260-300 amino acids which is shorter in the C-terminal region than those of other eukaryotes. Interestingly, Western blot analysis of subcellular fractions and immunofluorescence microscopy showed that TopA is localized in mitochondria and not in nuclei as is seen in other eukaryotic enzymes. Moreover, DNA topoisomease IIs of *Trypanosoma* and African swine fever virus also lacked the C-terminal domain and the Trypanosomatid enzyme is also localized in mitochondria (Fragoso and Goldenberg, 1992; García-Beato *et al.*, 1992; Melendy *et al.*, 1988; Pasion *et al.*, 1992; Strauss and Wang, 1990). These suggest that mitochondrial enzymes of eukaryotes may not have the C-terminal domain because the role of this domain is not necessary for enzyme localization. In addition, this domain is noticed only in the eukaryotic enzyme, but not in bacterial one (Adachi *et al.*, 1987; Moriya *et al.*, 1985; Swanberg and Wang, 1987). Taking these together, it is possible that the nuclear type DNA topoisomerase IIs of eukaryotes acquired the ability of nuclear localization by adding the C-terminal sequence to the ancestral enzyme during evolution.

TopA is 50-80 longer in the N-terminal region than those of other eukaryotes and an extended N-terminus contains a sequence structurally analogous to mitochondrial targeting presequence, showing the helical-wheel structure with an amphiphilic α-helix. Using wild-type and N-terminal deletion mutants, I examined the localization of TopA by immunofluorescence microscopy and proteinase K experiments. The results clearly showed that TopA is localized in mitochondria by virtue of an extended N-terminus with 82 amino acids containing a sequence structurally analogous to the mitochondrial targeting presequence. The trypanosomatid enzyme is also localized to mitochondria, however it has not been elucidated a mitochondrial targeting presequence experimentally (Fragoso and Goldenberg, 1992; Melendy *et al.*, 1988; Pasion *et al.*, 1992; Strauss and Wang, 1990). Thus, this study is the first to establish the location of the mitochondrial targeting signal presequence in DNA topoisomerase II.

In conclusion, TopA is actually restricted to mitochondria, although its amino acid sequence shows a significant degree of homology with that of nuclear type DNA topoisomerase II from other eukaryotes. The requirement for DNA topoisomerase II in the replication and segregation of chromosomal DNA as elucidated in *S. pombe* (Uemura *et al.*, 1987) suggests that a nuclear DNA topoisomerase II must also exist in *D. discoideum*. Indeed, topoisomerase II activity was present in the nuclear fraction. If the amino acid sequence of the nuclear enzyme is similar to the mitochondrial enzyme, another bands corresponding to the nuclear enzyme might be observed on Western and/or Southern blots. However, in these experiments, I could not find another DNA topoisomerase II band which is localized in nuclei. In addition, immunodepletion of TopA from cell extract did not decrease nuclear DNA topoisomerase II activity, indicating that TopA is not associated with nuclear DNA topoisomerase II activity. In *D. discoideum*, it is likely that the nuclear type enzyme is encoded by a different gene which is divergent from *topA* and from common

DNA topoisomerase II.

I attempted to disrupt *topA* using homologous recombination, however the attempt was unsuccessful. TopA would be essential for viability in *D. discoideum*, and play an essential role in mitochondrial DNA function. Further studies of TopA will provide many important clues for solving the molecular mechanisms of transcription and replication in mitochondria.

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VIII. Figures and Figure legends

Fig. 1. Southern blots of *D. discoideum* genomic DNA by oligonucleotide probes. The DNA from AX3 cells was digested with *Eco*RI (E), *Hin*dIII (H),*Eco*RI/*Hin*dIII (E/H), fractionated on a 0.8% agarose gel, and hybridized with the indicated probes. Positions of DNA size markers are indicated on the right.

Fig. 2. Restriction map of the DNA topoisomerase II gene (*topA*) and Southern and Northern blot analysis. A) Restriction map of the DNA topoisomerase II gene (*topA*) of *D. discoideum*. The arrow indicates the ORF and the direction of transcription. pTOPA-1, pTOPA-2 and pTOPA-3 indicate the regions of cloned and sequenced genomic DNA. The symbols used for restriction enzymes are: A, *Acc*I; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; Ha, *Hae*III; Hf, *Hin*fI; X, *Xba*I. The positions corresponding to oligonucleotide probes 2 and 3 are marked with corresponding numbers. (B) Southern blots of *D. discoideum* genomic DNA. The DNA from AX3 cells was digested with *Eco*RI (lane 1), *Hin*dIII (lane 2), *Xba*I (lane 3), *Eco*RI/*Hin*dIII (lane 4), *Eco*RI/*Xba*I (lane 5), then fractionated on a 0.8% agarose gel and hybridized with labeled pTOPA-1. Positions of DNA size markers are indicated on the left. (C) Northern blots of *topA* transcript. Total RNA (30 μg, lane 1) and poly(A)$^+$RNA (10 μg, lane 2) were fractionated on a 1.0% agarose gel containing formaldehyde. The positions of small and large rRNAs are indicated on the left. The arrow on the right indicates the position of the 4.5 kb *topA* transcript.

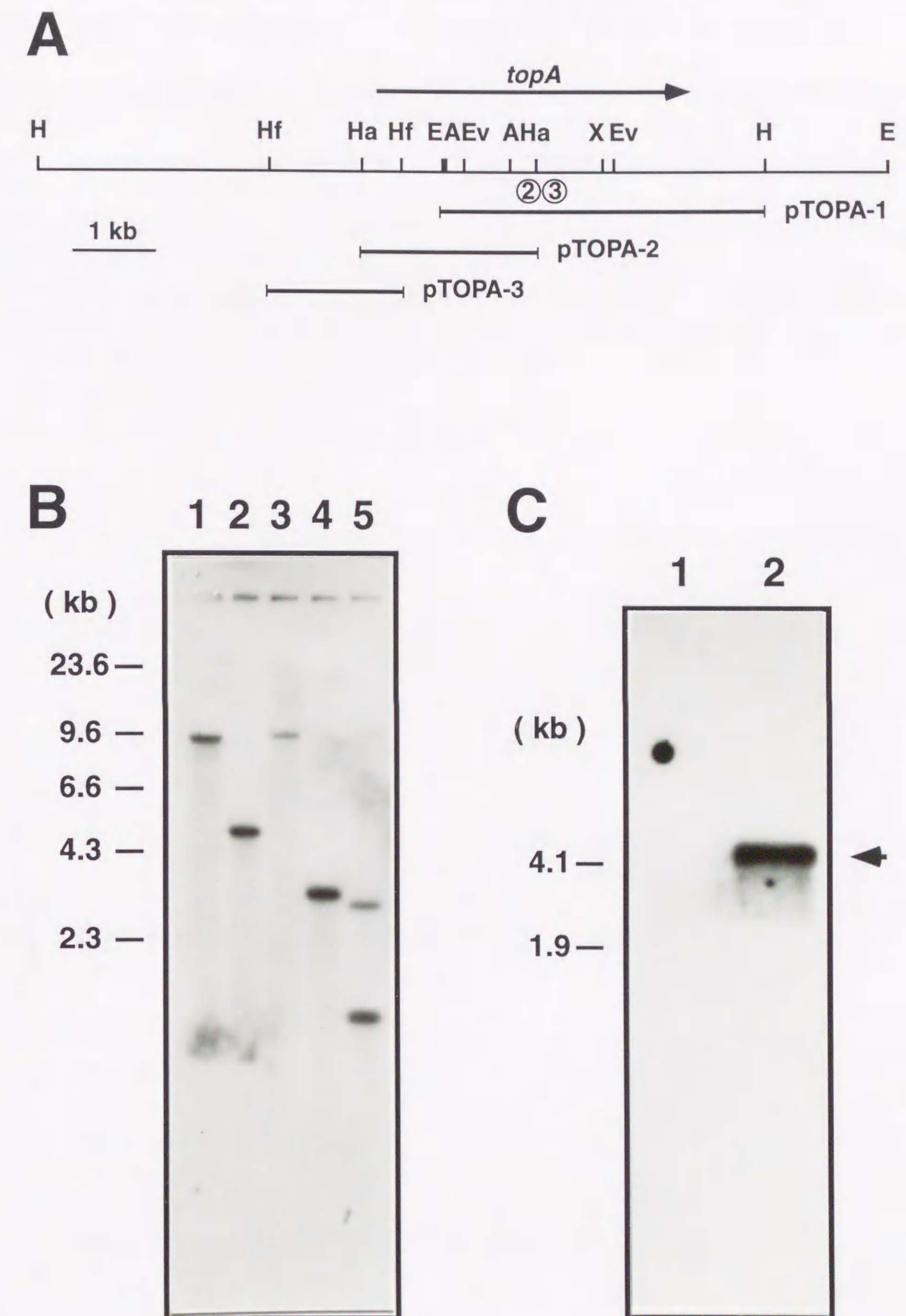

Fig. 3. Nucleotide and deduced amino acid sequences of *topA*. The complete nucleotide sequence of *topA* is shown with the flanking regions. The deduced amino acid sequence is shown below the nucleotide sequence with single letter code. The asterisk indicates a stop codon TAA. The positions corresponding to oligonucleotide probes 2 and 3 are marked with underline. The active site tyrosine residue is marked with "@".


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GTTCATCTTT GAAATTTCTC GGAGAAAAAT TATAAATTCC ATTAATAGGA AAATGATTTT -742
GACAAAACCA AAATCCACCA TCATAAAGTT TAACTAAATG AAAATCTAAT TGGTTTGATA -682
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TTTTTTTTTT TTTTCATATT TTCAAAACGA ATAAAAACGA ATTATTGTTT TTATGATTAT -22

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AATTCAAAAA TTCAAAAATT AGAATCTGTT GAAGAAATCA TTGATCAATT CTATAAAGTT 3339
N  S  K  I   Q  K  L   E  S  V   E  E  I  I   D  Q  F   Y  K  V  1113

CGTTTACAAT TCTATGGAAA ACGTAGAGAA TACCTCTTGA AATCATTGGA TAATCAAATT 3399
R  L  Q  F   Y  G  K   R  R  E   Y  L  L  K   S  L  D   N  Q  I  1133

AAACGTTTAA CAACTACAAT ACAATTCCTT GAAGTTATTG CAAGTGGTAA ATTAAAAATT 3459
K  R  L  T   T  T  I   Q  F  L   E  V  I  A   S  G  K   L  K  I  1153

CAAGGTAGAT CAAAACAAGA TTTAATCAAA GAGTTGGAAA GTGGTGAAAT TGTTGGTTTC 3519
Q  G  R  S   K  Q  D   L  I  K   E  L  E  S   G  E  I   V  G  F  1173
```

```
GAAAATTTTG GAACTCATCC ACCAGAGGTT TATCAACATC TTTTCTCTTT ATCAATTTTA 3579
E  N  F  G   T  H  P   P  E  V   Y  Q  H  L   F  S  L   S  I  L   1193

GATATTACAA AAGAAAGAAT TGATAATTTA ACTAATCAAT TAACAAAAAG AAAATCTGAA 3639
D  I  T  K   E  R  I   D  N  L   T  N  Q  L   T  K  R   K  S  E   1213

CATCAATCAA TTTCATCTTC TGATCCAAAA TCACTTTGGA CTGCTGATTT ACAACAATTA 3699
H  Q  S  I   S  S  S   D  P  K   S  L  W  T   A  D  L   Q  Q  L   1233

AAAGAATATT TAGAAAAAAG TGATAAAGAA TTTCAAAAAA AACCTTTAAA AACTTCCTCT 3759
K  E  Y  L   E  K  S   D  K  E   F  Q  K  K   P  L  K   T  S  S   1253

TCTTCATCAT TTGATGTTTC TTCTTCTTCT GAATCTGCAA AATTATCTTC AACTAGAAAA 3819
S  S  S  F   D  V  S   S  S  S   E  S  A  K   L  S  S   T  R  K   1273

TCAAAAACTG ATAAAATTAA ATCAAAATAA AAAAACTATT TTAATATTTA AATACTTAAT 3879
S  K  T  D   K  I  K   S  K  *
                                                                  1282

TAAAAAATAT ATATATATAT ATTTATTTAA TATAATAGTT TTTTTTATTA AATCTTTTTA 3939
TCTTTATTAT AATTACTTAA TATTATATTA TTACACGTTG ATAATTTTTA TTATTTTTTA 3999
AAATCATTAA AATTATCATT AATATATTTT TCAATTTTTA ATATTTCGGT TGTATTTGAT 4059
TTATTAATCC AATCAGTAAC CAATGGGACA ATCTTTAAGA TTTCAGCTTT TGATAATTCA 4119
GGTACAACTC TTTGAATGAT TGGATATGGT TGAATCTTTT CTTTCTTTGG TGCTTTTTGA 4179
ATTTCATATG ATAATTTACC ACACTCTACA ATTTCACCGC TTAAAGTTAA ACCATTTGTT 4239
TCATATAAAT CTAATGGTAA AGTTACAAAA CAAACACTTG GTTCTTTTAA AGTGTTATTA 4299
GTATCAGCTC CACTCTTTGA TTTTTTGTTT TTTCCAGTGG AGAGATTTCC TTTTGAAGGT 4359
GAGTCAAGTT TAACTTGATC TTGTTTTTCA ATTAATTCTT TATAGATTGA TAGTTCTCTT 4419
TCATCATAAG AGTTTAAGCA AACTCTTTCG CCCAATTTCA AAGTATTAAT TGGATACCAA 4479
TTTCTAATTG AATTACTATC TGATGCTTTA CCGTTTGGAT GAATTGCAAC TGTACAATGA 4539
GGAATTACAT TAGCAGTTGG AATACCTTGA ACCTTAAAGG CTAAAGCATT TTGACTGAAA 4599
CCAATTGCTT CAATTTTAGC TGTCCACTTT GTTCCAACCT TATAAATATT TTCAAAATCA 4659
TTTGATGTTG AAGTTGATAC AGTTGTTGAA GCTGTAGTTG TGGTGGTTGA AGAACAAGCT 4719
GTTTGAGTTG AAACATCATC AACAACAATT GTTGTTGTTG TTGTTGTTGT TTTAGTTGAA 4779
GATTCAGAGA TTGATAAATT TTTAATTTCA TTTAAAACAT TTGTTTCATC AGTTGAATCT 4839
GAAGATTTTT CAATTGGTTT CCAAAGCTT//                                 4868
```

Fig. 4. Domain structure of TopA of *D. discoideum*. (A) A schematic representation of the domain structure of *D. discoideum, S. cerevisiae* and *T. brucei* DNA topoisomerase II is shown. The N-terminal, ATP binding domain is shown as a diagonal hatched box. The central, DNA breakage/rejoining domain is an open box. The C-terminal domain is shaded. The N-terminal extension in *D. discoideum* is shown in black. The insert in *D. discoideum* is a vertical hatched box. The horizontally hatched box is the region containing the two amino acid sequence motifs (EGDSA and PLRGK) that is conserved in all DNA topoisomerase II. The active site tyrosine residue is shown as a vertical line labeled with Y. The putative nuclear location sequence (NLS) is denoted by vertical lines labeled NLS in *S. cerevisiae*. (B) Multiple alignment comparing DNA topoisomerase II sequences of *D. discoideum* (Ddisc) with *S. cerevisiae* (Schce), *S. pombe* (Schpo) and *T. brucei* (Trybr). The putative mitochondrial targeting signal presequence in *D. discoideum* is underlined. The NLS in *S. pombe* is shown as a dotted line. The conserved residues at positions 253, 256 and 258 in the "ATPase domain" are marked with circles, and that at position 899 in the "catalytic domain" is marked with an asterisk. Dashes show gaps inserted for optimal alignment. Dots are used for residues that are identical to those of *D. discoideum*. (C) A putative mitochondrial targeting signal presequence from residue 1-35 is folded as an α-helix. The positively charged residues are denoted by a plus mark and the nonpolar residues are shaded. The first residue is positively charged by an amino group.

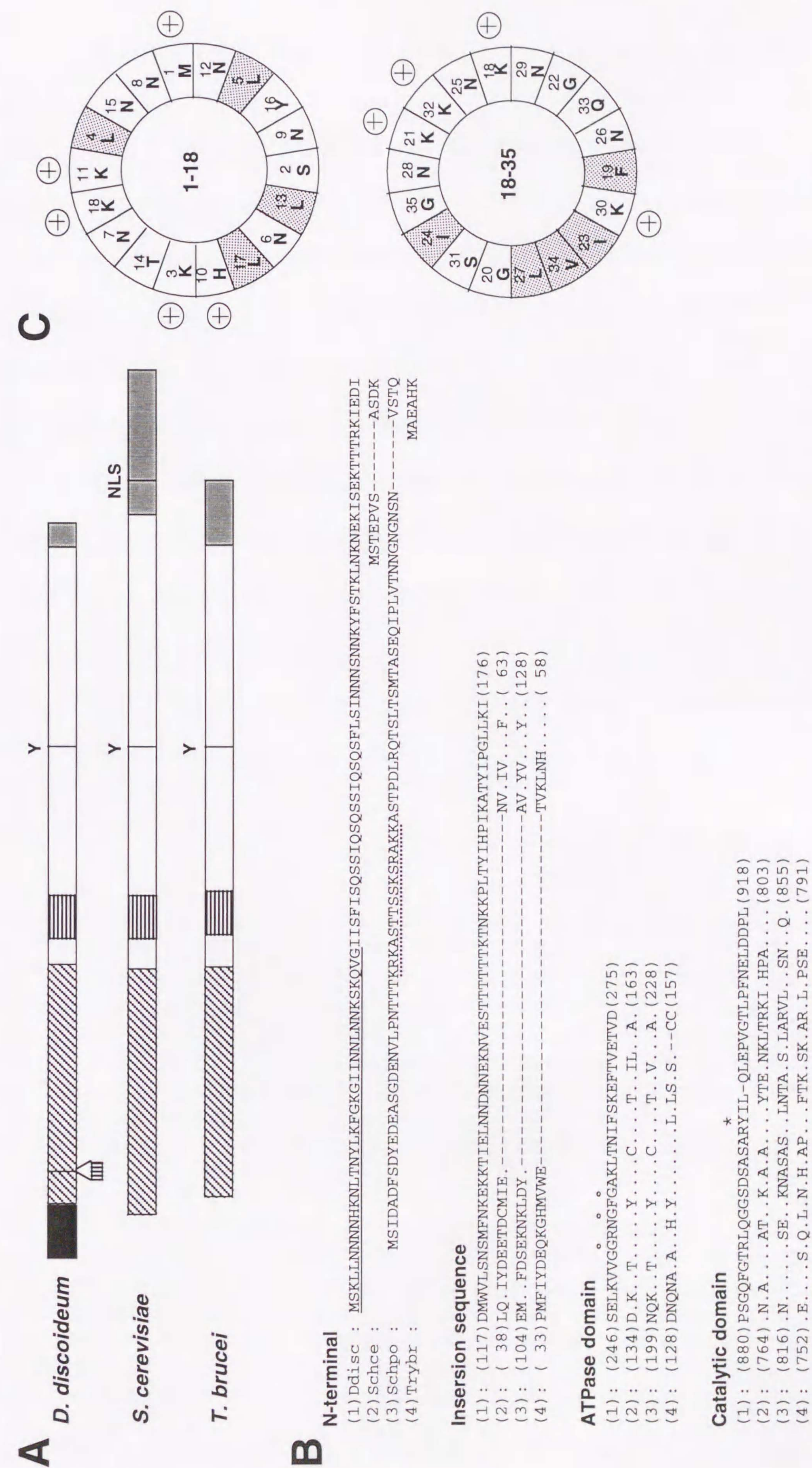


Fig. 4. Amino acid alignments of TopA with that of DNA topoisomerases II of other organisms. Conserved amino acid residues are indicated with asterisks. Dashes are introduced to maximize the matches between amino acid sequences. In the consensus [illegible] sequence, amino acids in upper case are identical among all DNA topoisomerase II, and amino acids in lower case represent those found in more than [illegible] of the seven sequences. The conserved active site tyrosine residue is marked with "@". The [illegible] region in the E. coli gyrase B protein indicates a region of 172 amino acids that has been omitted, since this region is not homologous to other DNA topoisomerase II sequences. The abbreviations are as follows. EcGyrA, E. coli DNA gyrase subunit A [illegible]; E. coli DNA gyrase subunit B [illegible], [illegible] [illegible]; [illegible] [illegible], [illegible] [illegible], [illegible] [illegible], [illegible] [illegible] [illegible].

Fig. 5. Amino acid alignments of TopA with that of DNA topoisomerase II of other organisms. Conserved amino acid residues are indicated with asterisks. Dashes are introduced to maximize the matches between amino-acid sequences. In the consensus (CONS) sequence, amino acids in upper case are identical among all DNA topoisomerase IIs, and amino acids in lower case represent conservation in more than four of the seven sequences. The conserved active site tyrosine residue is marked with "@". The arrow (at position 765) in the *E. coli* gyrase B protein indicates a region of 172 amino acids that has been omitted, since this region is not homologous to other DNA topoisomerase II sequences. The abbreviations are as follows: EcoliA, *E. coli* DNA gyrase subunit A; EcoliB, *E. coli* DNA gyrase subunit B; Human, human; Schpo, *Schizosaccharomyces pombe* ; Schce, *Saccharomyces cerevisiae;* Trybr, *Trypanosoma brucei*; ASFV, African swine fever virus; Ddisc, *D. discoideum.*


```
         1                                                          60
EcoliB :
 Human :                                                   MEVSPLQPVN
 Schpo :       MSIDADFSDYEDEASGDENVLPNTTTKRKASTTSSKSRAKKASTPDLRQTSLTS
 Schce :
 Trybr :
  ASFV :
 Ddisc : MSKLLNNNNHKNLTNYLKFGKGIINNLNNKSKQVGIISFISQSSIQSQSSIQSQSFLSIN

  CONS : ............................................................

         61                                                        120
EcoliB :               MSNSY-------DSSSIKVLKGLDAVRKRPGMYIGDTDDGT-----
 Human : ENMQVNKIKKNEDAKKRLS-------VERIYQKKTQLEHILLRPDTYIGSVE--LVTQQM
 Schpo : MTASEQIPLVTNNGNGNSN-------VSTQYQRLTPREHVLRRPDTYIGSIE--PTTSEM
 Schce :             MSTEPVS-------ASDKYQKISQLEHILKRPDTYIGSVE--TQEQLQ
 Trybr :                         MAEAHKYKKLTPIEHVLTRPEMYIGSLD--TTATPM
  ASFV :                             MEAFEISDFKEHAKKKSMWAGALNKVTISGLM
 Ddisc : NNSNNKYFSTKLNKNEKISEKTTTRKIEDIYQKKTPTEHVLLRPDSYIGTIE--KIEDDM
                                                         *
  CONS : ..................s........s..yqkltplehvlkrpdtyiGs.e..t....m

         121                                                       180
EcoliB : -----------------------------------------------------GLHHMVF
 Human : WVYDEDVG-INYR-----------------------------------EVTFVPGLYKIFD
 Schpo : WVFDSEKNKLDYK-----------------------------------AVTYVPGLYKIFD
 Schce : WIYDEETDCMIEK-----------------------------------NVTIVPGLFKIFD
 Trybr : FIYDEQKGHMVWE-----------------------------------TVKLNHGLLKIVD
  ASFV : GVFTEDEDLMALP----------------------------------IHRDHCPALLKIFD
 Ddisc : WVLSNSMFNKEKKTIELNNDNNEKNVESTTTTTTKTNKKPLTYIHPIKATYIPGLLKIYD
                                                               *
  CONS : wvyde....m..k...................................vt.vpgLlkifd

         181                                                       240
EcoliB : EVVDNAIDEALAGHCKE----IIVTIHAD-NSVSVQDDGRGIP------TGIHPEEGVSA
 Human : EILVNAAD---NKQRDPKMSCIRVTMIR-KQLISIWNNGKGIP---VVEHK---VEKMYV
 Schpo : EIIVNAAD---NKVRDPNMNTLKVTLDPEANVISIYNNGKGIP---IEIHD---KEKIYI
 Schce : EILVNAADN--NKVRDPSMKRIDVNIHAEEHTIEVKNDGKGIP---IEIHN---KENIYI
 Trybr : EILLNASDNISN--RSARMTYIRVTI-TDTGEITIENDGAGIP---IVRSR---EHKLYI
  ASFV : ELIVNATDH-ERACHSKTKKVTYIKISFDKGVFSCENDGPGIPIAKHEQASLIAKRDVYV
 Ddisc : EILVNAAD---NKKRDSKMSFIKVEINPNENSISIMNDGKGIP---VVMHQ---TENCYV
         *   ** *                              * ***
  CONS : EilvNAaD...nk.rdp.m..i.vti..d.n.isi.ndGkGIP...ie.h.....ek.yi

         241                                                       300
EcoliB : AEVIMTVLHAGGKFDDNSYKVSGGLHGVGVSVVNALSQKL--ELVIQREGKIHRQIYEHG
 Human : PALIFGQLLTSSNYDDDEKKVTGGRNGYGAKLCNIFSTKFTVETASREYKKMFKQTWMDN
 Schpo : PELIFGNLLTSSNYDDNQKKVTGGRNGYGAKLCNIFSTEFVVETADKERMKKYKQTWYDN
 Schce : PEMIFGHLLTSSNYDDDEKKVTGGRNGYGAKLCNIFSTEFILETADLNVGQKYVQKWENN
 Trybr : PEMVFGHLLTSSNYDDDNQNAVAGRHGYGAKLTNILSLSFSV--CCRTNGREFHMSWQDH
  ASFV : PEVASCFFLAGTNINKAKDCIKGGTNGVGLKLAMVHSQWAILTTA--DGAQKYVQQINQR
 Ddisc : VEMVMGNLMSGSNFNDSELKVVGGRNGFGAKLTNIFSKEFTVETVDKSSGKKYFQRWSNN
                                *  * *
  CONS : pemifg.lltssnydddekkvtgGrnGyGaklcnifStef.vetad...gkky.q.w.dn
```

```
          301                                                        360
 EcoliB : VPQAPLAVTGETEKTG---TMVRFWPSLETFTNVTEFE---------------YEILAKR
  Human : MGRAGEMELKPF--NGEDYTCITFQPDLSKFKM-----QSLDKDIVALMVRRAYD-IAGS
  Schpo : MSRKSEPVITSL-KKPDEYTKITFKPDLAKFGM-----DKIDDDMVSIIKRRIYD-MAGT
  Schce : MSICHPPKITSY-KKGPSYTKVTFKPDLTRFGM-----KELDNDILGVMRRRVYD-INGS
  Trybr : MRKATAPRVSNVGTKEKNVTRVKFLPDYERFGMKE---KKISNDMKRVLYKRIMD-LSAM
   ASFV : LDIIEPPTITP---SREMFTRIELMPVYQELGYAEPLSETEQADLSAWIYLRACQCAAYV
  Ddisc : MGDRSEPIITPIGEGESDYTKITFKPDLEKFKI----KSLWDDNILQLMERRLYD-IAGC
                             *     *
   CONS : m..a.ep.itp..kkg..yTkitfkPdlekfgm.......d.di...m.rr.yd.iag.

          361                                                        420
 EcoliB : LRELS-FLNSGVSIRLRDKRDGKEDHFHYEGGIKAFVEYLNKNKTPIHPNIFYFSTEKDG
  Human : TKDVKVFLN-GNKLPVKGFRSYVDMYLK-----------DKLDETGNSLKVIHEQVNHRW
  Schpo : VRETKVYLN-NERISISGFKKYVEMYLAS-----------DTKPDEEPPRVIYEHVNDRW
  Schce : VRDINVYLN-GKSLKIRNFKNYVELYLKSLEKKRQLDNGEDGAAKSDIPTILYERINNRW
  Trybr : FPNIQITLN-GSSFGFKSFKDYATLY-----------SAMTPKGEKPPPPYVYESKSGC-
   ASFV : GKGTTIYYN-DKPCRTGSVMALAKMYTLLSAPNSTIHTATIKADAKP-------YSLHPL
  Ddisc : NTELMVTLN-GKRLNY-NFQSYVKLYEHHLNNSTNVKIMRHIVKNP---FEFGEIS-PRW
                  *
   CONS : .re..vylN.gksl....fk.yvemyl.......................ye..n.rw

          421                                                        480
 EcoliB : IGVEVALQWNDGFQENIYCFTNNIPQRDGGTHLAGFRAAMTRTLNAYMDKEGYSKKAKVS
  Human : EVCLTMSEKG--FQQI--SFVNSIATSKGGRHV---DYVADQIVTKLVDVVKKKNKGGVA
  Schpo : DVAFAVSDGQ--FKQV--SFVNNISTIRGGTHV---NYVANKIVDAIDEVVKKENKKA-P
  Schce : EVAFAVSDIS--FQQI--SFVNSIATTMGGTHV---NYITDQIVKKISEILKKKKKK--S
  Trybr : -VAFIPSVVP--GVRRMFGVVNGVVTYNGGTH-C---NAAQDILTGCLDGVERELKKENK
   ASFV : QVAAVVSPKF--KKFEHVSIINGVNCVK-GEHVTFLKKTINEMVIKKFQQTIKDKNRKTT
  Ddisc : KIGIGLSETGQ-FTQV--SFVNSINTVKGGTHV---NFLADQIVRYVGEKLKKKH-SDLE
                                *       * *
   CONS : .vaf.vs.....fqq...sfvNsi.t.kgGtHv...ny.adqiv.k..e.vkkkkk....

          481                                                        540
 EcoliB : ATGDDAREGLIAVVSVKVP-DPKFSSQTKDKLVSSEVKSAVEQQMNELLAEYLLENPTDA
  Human : VKAHQVKNHMWIFVNALIE-NPTFDSQTKENMTLQPKSFGSTCQLSEKFIKAAIGCGIVE
  Schpo : VKAFQIKNYVQVFVNCQIE-NPSFDSQTKETLTTKVSAFGSQCTLSDKFLKAIKKSSVVE
  Schce : VKSFQIKNNMFIFINCLIE-NPAFTSQTKEQLTTRVKDFGSRCEIPLEYINKIMKTDLAT
  Trybr : VMDTNRVLRHFTILVFLVQVQPKFDSQNKARLVSTPTMPRVPRQDVMKYLLRM---PFLE
   ASFV : LRDSC---SNIFVVIVGSIPGIEWTGQRKDELSIAENVFKTHYSIPSSFLTSMTRS-IVD
  Ddisc : IRPMNIKHHLALFVNCLVD-NPSFDSQSKETLTTKPMLFGSTPEIPESLLAQFVKNSKII
                                     * *
   CONS : vk..qikn....fvnclie.np.fdsQtKe..ltt.p..fgs.cqipekfl.......v

          541                                                        600
 EcoliB : KIVVGKIIDAARAREAARRAREMTRRKGALDLAGLPGKLADCQERDPALSELYLVEGDSA
  Human : SILNWVKFKAQVQLNKKCSAVKH----NRIKGIPKLDDANDAGGRNSTECTLILTEGDSA
  Schpo : EVLKFATAKADQQL-SKGDGGLR----SRITGLTKLEDANKAGTKESHKCVLILTEGDSA
  Schce : RMFEIADANEENAL-KKSDGTRK----SRITNYPKLEDANKAGTKEGYKCTLVLTEGDSA
  Trybr : AHVSTITGQLAQELNKEIGTGRRMSSKTLLTSITKLVDATSTRRDPKHTRTLIVTEGDSA
   ASFV : ILLQSISKKDNH---------------KQVDVDKYTRARNAGGKRAQDCMLLAAEGDSA
  Ddisc : ERVAGWALMKQKADLIHSTSGRQSKT-TLIKSISKLDDANWAGGLKSKECTLIITEGDSA
                                                            *   *****
   CONS : e..l.....ka...l.kk...gr......it...kl.dan.aggk.s..ctLiltEGDSA
```

```
         601                                                     660
EcoliB : GGSAKQGRNRKNQ----------AILPLKGKILNVEKA--------------RFDKMLSS
 Human : KTLAVSGLGVVGR-------DKYGVFPLRGKILNVREA--------------SHKQIMEN
 Schpo : KSLAVSGLSVVGR-------DYYGVFPLRGKLLNVREA--------------SHSQILNN
 Schce : LSLAVAGLAVVGR-------DYYGCYPLRGKMLNVREA--------------SADQILKN
 Trybr : KALAQNSLSSDQK-------RYTGVFPLRGKLLNVRNK--------------NLKRLRNC
  ASFV : LSLLRTGLTLGKSNPSGPSFDFCGMISLGGVIMNACKKVTNITTDSGETIMVRNEQLTNN
 Ddisc : KSLALAGLSVVGR-------NSYGVFPLRGKLLNVRDV--------------ASKQLLSN
                                    * *   *
  CONS : kslav.gl.vvgr.......dyygvfpLrGkllNvrea..............s.kqll.n

         661                                                     720
EcoliB : QEVATLITALGCGIGRDEY----NPDKLRYHSIIIMTDADVDGS-HIRTLLLTFFYRQMP
 Human : AEINNIIKIVGLQY-KKNYEDEDSLKTLRYGKIMIMTDQDQDGS-HIKGLLINFIHHNWP
 Schpo : KEIQAIKKIMGFTH-KKTY---TDVKGLRYGHLMIMTDQDHDGS-HIKGLIINYLESSYP
 Schce : AEIQAIKKIMGLQH-RKKY---EDTKSLRYGHLMIMTDQDHDGS-HIKGLIINFLESSFL
 Trybr : KELQELFCALGLEL-DKDYTDADE---LRYQRILIMTDQDADGS-HIKGLVINAFESLWP
  ASFV : KVLQGIVQVLGLDF-NCHYKTQEERAKLRYGCIVACVDQDLDGCGKILGLLLAYFHLFWP
 Ddisc : EEINNLTTILGLSHKNS-YDTDESMEDLRYGRVMIMADQDHDGS-HIKGLVMNFIHYFWP
                   *       *        ***       * * **   *  *
  CONS : .eiq.i.kilGl.h..k.Y...e..k.LRYg.imimtDqDhDGs.hIkgLlinffes.wp

         721                                       ↓             780
EcoliB : EIVERGHVYIAQ--PPLYKVKKGKQEQYIKDDEAMDQYQISI--------RGLSIQRYKG
 Human : SLL-RHR-FLEEFITPIVKVS---KNKQEMAFYSLPEFEEWKSSTPNH--KKWKVKYYKG
 Schpo : SLL-QIPGFLIQFITPIIKCT---RGNQVQAFYTLPEYEYWKEANNNG--RGWKIKYYKG
 Schce : GLL-DIQGFLLEFITPIIKVSITKPTKNTIAFYNMPDYEKWREEESHK--FTWKQKYYKG
 Trybr : SLLVRNPGFISIFSTPIVKARL--RDKSVVSFFSMKEFHKWQRSNANT--P-YTCKYYKG
  ASFV : QLI--IHGFVKRLLTPLIRVYEKGKTMP-VEFYYEQEFDAWAKKQTSL--VNHTVKYYKG
 Ddisc : NLL-K-RGFLVEFVTPIIKAT--KSSTQKKSFFTIKDYEKWRETISSDQLKQYTIKYYKG
                        *                                         ***
  CONS : sll.ri.gfl.efitPiikv...k..kq..afy.mpeyekw.e...n.....wt.kyYKG

         781                                                     840
EcoliB : LGEMNPEQLWETTMDPESRRMLRVTVKDAIAADQLFTTLMGDAVEPRRAFIEENALKAAN
 Human : LGTSTSKEAKEYFADMK-RHRIQFKYSGPEDDAAISLAFSKKQIDDRKEWLTNFMEDRRQ
 Schpo : LGTSDHDDMKSYFSDLD-RHMKYFHAMQEKDAELIEMAFAKKKADVRKEWLRTYRPGI--
 Schce : LGTSLAQEVREYFSNLD-RHLKIFHSLQGNDKDYIDLAFSKKKADDRKEWLRQYEPGT--
 Trybr : LGTSTTAEGKEYFKDME-KHTMRL-LVDRSDHKLLDNVFDSQEVEWRKDWMT--------
  ASFV : LAAHDTHEVKSMFKHFD-NMVYTFTL-DDSAKELFHIYFGGESELRKRELCTGVVPLT--
 Ddisc : LGTSTSAEAKEYFSNLD-KHVIKFIWGDEADD-LIKMAFAKDLSSLRQRWIKETDMSQG-
         *
  CONS : Lgtst..e.keyfsdld.rh...f...d..d..li..af.kk..d.rkewlt...p....

         841                                                     900
EcoliB : IDI/    MSDLAREITPVNIEEELKSSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAM
 Human : RKLLGLPEDYLYGQTTTYLTYNDFINKELILFSN-SDNERSIPSMVDGLKPGQRKVLFTC
 Schpo : ----------YMDYTQPQIPIDDFINRELIQFSM-ADNIRSIPSVVDGLKPGQRKVVYYC
 Schce : ----------VLDPTLKEIPISDFINKELILFSL-ADNIRSIPNVLDGFKPGQRKVLYGC
 Trybr : -KANAFTGEVDIDRSKKMLTVTDFVHKEMVHFAL-VGNARALAHSVDGLKPSQRKIIWAL
  ASFV : ------ETQTQSIHSVRRIPCSLHLQVDTKAYKLDAIE-RQIPNFLDGMTRARRKILAGG
 Ddisc : -----------IDHSIKEITYPDFINKELIHYSW-AANLRSIPSLIDGLKPGQRKILFAS
                                                *      **     *
  CONS : ...........d.s.k.ipi.dfinkeli.fsl.adn.RsipsvvDGlkpgqRkvl..c
```

```
         901                                                       960
EcoliB : NVLGNDWNKAYKKSARVVGDVIG-KYHPHGDSAVYDTIVRMAQPF--S-LRYMLVDGQGN
 Human : F---KRNDKREVKVAQLAGSVAEMSSYHHGEMSLMMTIINLAQNFVGSNNL-NLLQPIGQ
 Schpo : F---KRNLVHETKVSRLAGYVASETAYHHGEVSMEQTIVNLAQNFVGSNNI-NLLMPNGQ
 Schce : F---KKNLKSELKVAQLAPYVSECTAYHHGEQSLAQTIIGLAQNFVGSNNI-YLLLPNGA
 Trybr : M---RRSGNEAAKVAQLSGYISEASAFHHGETSLQETMIKMAQSFTGGNNV-NLLVPEGQ
  ASFV : VKCFASN-NRERKVFQFGGYVADHMFYHHGDMSLNTSIIKAAQYYPGSSHLYPVFIGIGS
 Ddisc : F---KRRLTNEIKVSQLSGYVAEQTSYHHGEQSLNSTIVKMAHNFVGSNNL-PLLTPSGQ
                     *               **           *                *
  CONS : f...krnlk.e.Kvaqlagyvae.tayhHGe.sl..tiikmAqnfvgsnnl.nll.p.Gq

         961            @                                         1020
EcoliA : FGS--IDGDSAAAMRYTEIRL-AKIAHELMADLEKETVDFVDNYDGTEKIPDVM-PTKIP
 Human : FGTRLHGGKDSASPRYIFTML-SSLARLLFPPKDDHTLKFLYD-DNQRVEPEWYIP-IIP
 Schpo : FGTRSEGGKNASASRYLNTAL-SPLARVLFNSNDDQLLNYQND-EGQWIEPEYYVP-ILP
 Schce : FGTRATGGKDAAAARYIYTEL-NKLTRKIFHPADDPLYKYIQE-DEKTVEPEWYLP-ILP
 Trybr : FGSRQQLGNDHAAPRYIFTKL-SKVARLLFPSEDDPLLDYIVE-EGQQVEPNHYVP-ILP
  ASFV : FGSRHLGGKDAGSPRYISVQLASEFIKTMFPAEDSWLLPYVFE-DGQRAEPEYYVP-VLP
 Ddisc : FGTRLQGGSDSASARYIL-QLE-PVGTLPFNELDDPLLNYLEE-EGESIQPDYIIP-IIP
         **     *      **    *                             *    *   *
  CONS : FGtr..gGkdaaapRYi.t.L.s.larllfp..ddpll.y..e.dgq.vePeyyvP.ilP

         1021                                                     1080
EcoliB : NLLVN-GSSGIAVGMATNIPPHNLTEVINGCLAYIDDEDISIEGLMEHIPGPDFPTAAII
 Human : MVLIN-GAEGIGTGWSCKIPNFDVREIVNNIRRLMDGEEP----------------LP--
 Schpo : MVLVN-GAEGIGTGWSTFIPNYNPKDITANLRHMLNGEPL----------------EI--
 Schce : MILVN-GAEGIGTGRSTYIPPFNPLEIIKNIRHLMNDEEL----------------EQ--
 Trybr : LLLCN-GSVGIGFGFSSNIPPFHRLDVSAAVRAMISGERA----------------KSVV
  ASFV : LAIMEYGANP-SEGWKYTTWARQLEDILALVRAYVDKDNPKHELLHYAI-----KHKITI
 Ddisc : MLLVN-GSEGIGVGMSTSIPLFSPIDIIDQLMLRLNNQVA----------------LK--
               *      *
  CONS : mllvn.GaegigtGwst.ippfnp.diian.r...nge.....................

         1081                                                     1140
EcoliA : NGRRGIEEAYRTGRGKVYIRARAEVEVDAKTGRETIIVHEIPYQVNKARLIEKIAELV--
 Human : --MLPSYKNFKGTIEELAPNQYVISGEVAILNSTTIEISELPVRTWTQTYKEQVLEPMLN
 Schpo : --MTPWYRGFRGSITKVAPDRYKISGIINQIGENKVEITELPIRFWTQDMKEYLEAGLV-
 Schce : --MHPWFRGWTGTIEEIEPLRYRMYGRIEQIGDNVLEITELPARTWTSTIKEYLLLGLS-
 Trybr : RRLVPWAVGFQGEIRRGPEGEFIAVGTYTYCKGGRVHVTELPWTCSVEAFREHI------
  ASFV : LPLRPSNYNFKGHLKRFGQYYYSYGTYDISEQRNIITITELPLRVPTVAYIESI-----K
 Ddisc : -KLIPWYRGFKGTIS-PDRHTYRTNGVIKLVGRN-LEITELPIGRWTSDYKE-VLNDLID
                                                * *         *
  CONS : ..m.pw.rgfkgti....p..y...g.i...grn.ieitElP.r.wt..ykE.il..l..

        1141                                                      1200
EcoliB : -KEKRVEGISALRDESDKDGMRIVIEVKRDAVG--------EVVLNNLYSQTQLQVSFGI
 Human : GTEKTPPLITDYREYHTDTTVKFVVKMTEEKLA--------EAERVGLHKVFKLQTSLTC
 Schpo : GTEKIRKFIVDYESHHGEGIVHFNVTLTEAGMK--------EALNESLEVKFKLSRTQAT
 Schce : GNDKIKPWIKDMEEQHDDNI-KFIITLSPEEMA--------KTRKIGFYERFKLISPISL
 Trybr : SYLATKDIVNRIADYSGANHVDIDVEVAQGAVNTY------AECESEL----GLTQRIHI
  ASFV : KSSNRMTFIEEIIDYSSSETIEILVKLKPNSLNRIVEEFKETEEQDSIENFLRLRNCLH-
 Ddisc : -----KDVIKSFQESNTENSVHFTILLNNNQLEQ-----MEDLTENELIKLFKLSASLNF
                                                              *
  CONS : g.ek.k..i.d..dys..n.v.f.v.l...l.........e.....l...fkL..sl..
```

```
          1201                                                       1260
EcoliA : NMVALHH-GQPKIMNLKDIIAAFVRHRREVVTRRTIFELRKARDRAHILEALAVALANID
 Human : NSMVLFD-HVGCLKKYDTVLDILRDL-FEL--RLKYYGLRKEWLLGMLGAESAKLNNQAR
 Schpo : SNMIAFD-ASGRIKKYDSVEDILTEF-YEV--RLRTYQRRKEHMVNELEKRFDRFSNQAR
 Schce : MNMVAFD-PHGKIKKYNSVNEILSEF-YYV--RLEYYQKRKDHMSERLQWEVEKYSFQVK
 Trybr : NGTV-FS-PNGTLSPLESDLTPVLQWHYDR--RLDLYKKRRQRNLTLLEQELAREKSTLK
  ASFV : SHLN-FVKPKGGIIEFNSYYEILYAW-LPY--RRELYQKRLMREHAVLKLRIIMETAIVR
 Ddisc : HLTC-FD-ENSKIQKLESVEEIIDQF-YKV--RLQFYGKRREYLLKSLDNQIKRLTTTIQ
                                    *       *
  CONS : n.mv.fd.p.gkikky.sv.eil..f.yev..Rl..yqkRker.l..l..e..r...q.r

          1261                                                       1320
EcoliB : -PIIELIR-HAPTPAEAKTALVANPWQLGNVAAMLERAGDDAARPEWLEPEFGVRDGLYY
 Human : FILEKIDGKIIIENKPKKELIKVL-IQRGYDSDPVKAWK------EAQQKVPDEEENEES
 Schpo : FIHMIIEGELVVSKKKKKDLIVEL-KEKKFQP---ISKPKKGHLVDLEVENALAEEEQSG
 Schce : FIKMIIEKELTVTNKPRNAIIQEL-ENLGFPR---FNKEGKPYYGSPNDEIAEQINDVKG
 Trybr : FV-QHFGAGHIDFANATEATLEKVCSKLGLVR----------------------------
  ASFV : Y-INESAELNLS-HYEDEKEASRILSEHGFPP----------------------------
 Ddisc : FLEVIASGKLKIQGRSKQDLIKEL------------------------------------

  CONS : fi..ii.g.l....k..k.li.el...lg..............................

          1321                                                       1380
EcoliA : LTEQQAQAILDLRLQKLTGLEHEKLLDEYKELLDQIAELLRILGSADRLMEVIREELELV
 Human : ----------DNEKETEKSDSVTDSGPTFNYLLDMPLWYL---TK-EKKDELCRLRNEKE
 Schpo : DVSQDEDSD------------------AYNYLLSMPLWSL---TY-ERYVELLKKKDEVM
 Schce : ATSDEEDEESSHEDTENVINGPEELYGTYEYLLGMRIWSL---TK-ERYQKLLKQKQEKE
 Trybr : ------------------------VDDSFDYILRKPITFY---TK-TSFENLLKKIAETE
  ASFV : ---LNHTLIISPEFASIEELNQKALQGCYTYILSLQARELLIAAK-TRRVEKIKKMQARL
 Ddisc : ----------ESGEIVGFENFGTHPPEVYQHLFSLSILDI---TK-ERIDNLTNQLTKRK

  CONS : ............e...........l...y.yllsmpiw.l...tk.er..ellkk..e.e

          1381                                                       1440
EcoliB : REQFGDKRRTEITANSADINLEDLITQEDVVVTLSH----------QGYVKYQPLSEYEA
 Human : QEL--DTLKRKSP---SDLWKEDLATFIEELEAVEAKEKQDEQV------GLPG-KGGKA
 Schpo : AEL--DALIKKTP---KELWLHDLDAFEHAWNKVMDDIQRE---MLEEEQSSRDFVNRTK
 Schce : TEL--ENLLKLSA---KDIWNTDLKAFEVGY--------QE---FLQRDAEARG-GNVPN
 Trybr : RRI--EALKKTTP---VQLWLGELDQFDRFFQDHE-----------------KKMVEAIL
  ASFV : DKV--EQLLQESPFPGASVWLEEIDAVEKAIIKGRNTQWKFH/
 Ddisc : SEH--QSISSSDP---KSLWTADLQQLKEYLEK-------------------------SD

  CONS : .el..e.l.k..p...kdlwledldafe....k...........................

          1441                                                       1500
EcoliA : QRRGGKGKSAARIKEEDFIDRLLVANTHDHILCFSSRGRVYSMKVYQLPEATRGARGRPI
 Human : KGKK-------TQMAEVLPSPRGQRVIPRITIEMKAEAEKKNKKKIKNENTEGSPQEDGV
 Schpo : KKPRGKSTGTRKPRAIAGSSSSTAVKKEASSESKPSTTNRK--QQTLLEFAASKEPEKSS
 Schce : KGSKTKGKGKRKLVDDEDYDPSKKNKKSTARKGKKIKLEDKNFERILLE---QKLVTKSK
 Trybr : KERRQRS-PPSDLLPGLQQPR------------------------LEVEE-AKGGKKFEM
 Ddisc : KEFQKKPLKTSSSSSFDVSSSSESAKLSSTRKSKTDKIKSK/

  CONS : k...k..............s.s...k.......k......k......le...........
```

```
        1501                                                       1560
EcoliB : VNLLPLEQDERITAILPVTEFEEGVKVFMATANGTVKKTVLTEFNRLRTAGKVAIKLV--
 Human : ELEGLKQRLEKKQKREPGTKTKKQTTLAFKPIKKGKKRNPWPDSESDRSSDESNFDVPPR
 Schpo : DINIVKTEDNSHGLSVEENRISKSPGLDSSDSGKSRKRSQSVDSEDAGSKKPVK-KIAAS
 Schce : APTKIKKEKTP---SVSETKTEEEENAPSSTSSSSIFDIKKEDKDEGELSKISN-KFKKI
 Trybr : RVQVRKY-VPPPTKRGAGGRSDGD---GGATAAGAAAAVGGRGEKKGPGRAGGVRRMVL-

  CONS : .....k........................t.....k.....d.................

        1561                                                       1620
EcoliA : ------DGDELIGVDLTSGEDEVMLFSAEGKVVRFKESSVRAMGCNTTGVRGIRLGEGDK
 Human : ETEPRRAATKTKFTMDLDSDEDFSDFDEKTDDEDFVPSDASPPKTKTSPKLSNKELKPQK
 Schpo : ASGRGRKTNKPVATTIFSSDDE---------DDLLPSSLKPSTITSTKAS-AKNKGKKAS
 Schce : STIFDKMGSTSATSKENTPEQD---------DVATKKNQTTAKKTAVKPKLAKKPVRKQQ
 Trybr : -------DALAKRVTRLLPRLLF/

  CONS : ...............................d.....s........t.............

        1621                                                       1680
EcoliB : VVSLIVPRGDGAILTATQNGYGKRTAVAEYPTKSRATKGVISIKVTERNGLVVGAVQVDD
 Human : SVVSDLEADDVKGSVPLSSSPPATHFPDETEITNPVPKKNVTVKKTAAKSQSSTSTTGAK
 Schpo : SVKKQSPEDDDDDFIIPGSSSTP--------------------KASSTNAEPPEDSDSPI
 Schce : KVVELSGESDLEIL----DSYTD--------------------REDSNKDEDDAIPQRSR

  CONS : .v.......d.........s.......................k................

        1681                                                       1740
EcoliA : CDQIMMITDAGTLVRTRVSEISIVGRNTQGVILIRTAEDENVVGLQRVAEPVDEEDLDTID
 Human : KRAAPKGTKRDPALNSGVSQKPDPAKTKNRRKRKPSTSDDSDSNFEKIVSKAVTSKKSKGE
 Schpo : RKRPTRRAAATVKTPIYVDPSFDSMDEPSMQDDSFIVDNDEDVD---------D----YDE
 Schce : RQRSSR--AASVPKKSYVET-------LELSDDSFIEDDEEENQ---------GSDVSFNE

  CONS : .........a.......v....................d....................e

        1741
EcoliB : GSAAEGDDEIAPEVDVDDEPEEE/
 Human : SDDFHMDFDSAVAPRAKSVRAKKPIKYLEESDEDDLF/
 Schpo : SD/
 Schce : ED/

  CONS : .d...................................
```

Fig. 6. Developmental Northern and Western blots analysis. (A) Developmental Northern blots. Poly(A)$^+$RNA (3 μg) was isolated from AX3 cells at the indicated intervals (hr after onset of development). The morphology observed at each time point is indicated below. (B) Developmental Western blots. Cell lysate (3 x 10^6 cells) was prepared from cells at the indicated intervals. After separation by SDS-PAGE, TopA was detected using the purified antibody, αN340. The lane marked 20 hr was less intensely stained with amido black than the other lanes.

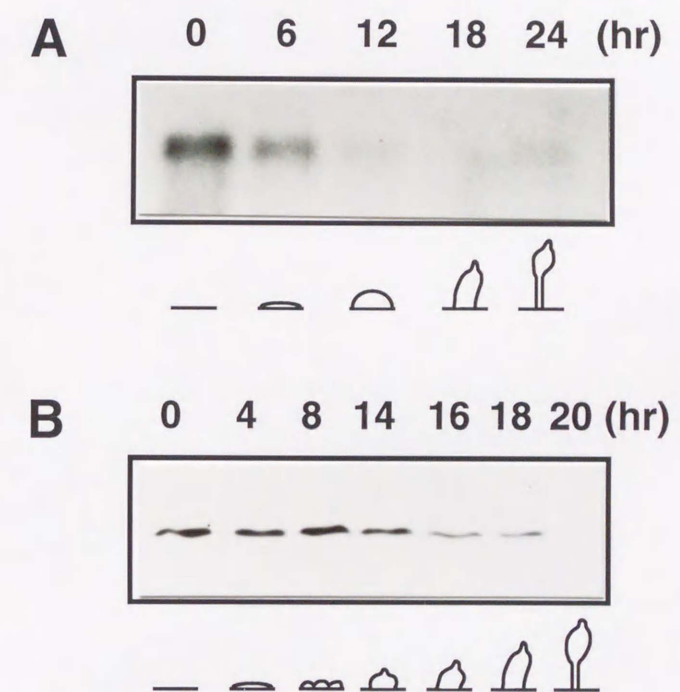


Fig. 7. Western blots of growth phase cell using four independent antibodies. (A) The schematic representation of TopA is the same as that shown in Figure 2A. N28, N340, C955 and C814 indicate the portion of TopA used for the fusion protein. (B) Whole cell lysates ($1x10^6$ cells) were separated by 6% SDS-PAGE, then Western blotted with preimmune sera (lanes 1, 4, 7 and 10), immune sera (lanes 2, 5, 8 and 11) or purified antibodies (lanes 3, 6, 9 and 12). The antibodies were αC955 (lanes 1-3), αC814 (lanes 4-6), αN340 (lanes 7-9) and αN28 (lanes 10-12). The arrow indicates the position of TopA at about 135 kDa.

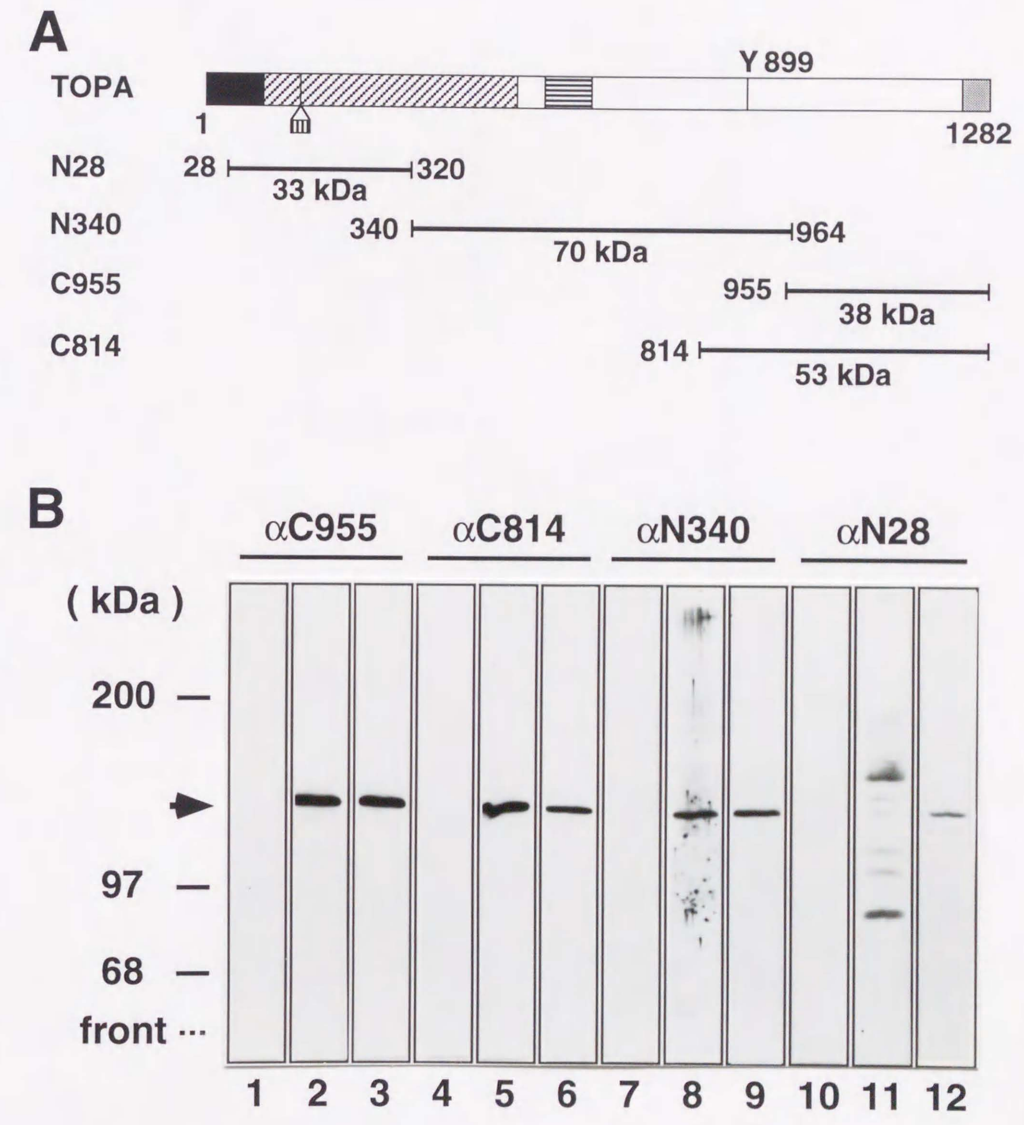


Fig. 8. Western blot analysis of subcellular fractions. Nuclear (lane 1) and mitochondrial (lane 2) extracts (6 μg) were separated by 6 % SDS-PAGE and analyzed using αC955 antiserum. The arrow indicates the 135 kDa of TopA polypeptide.

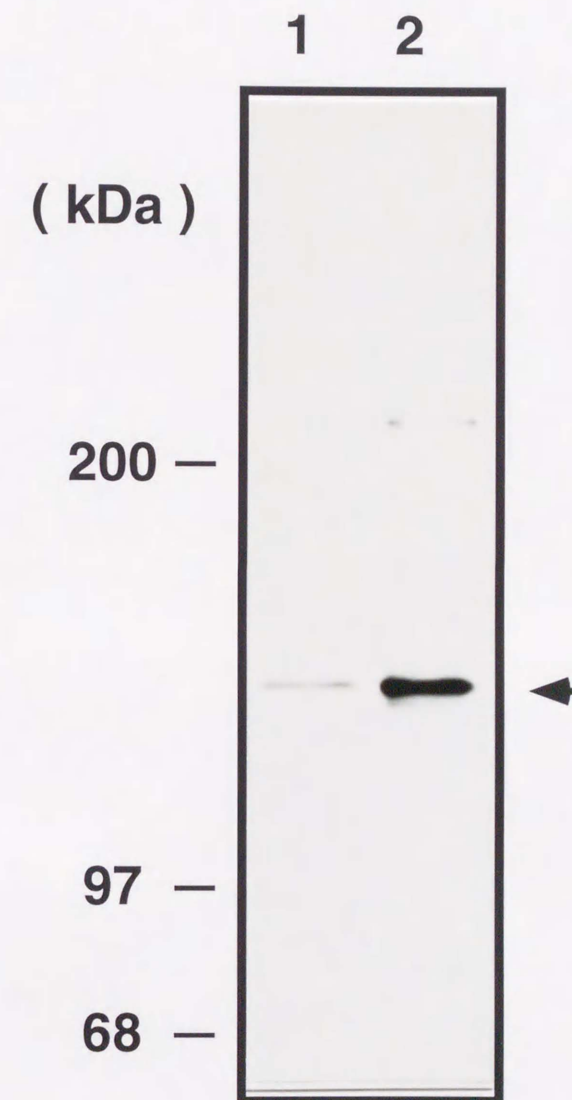
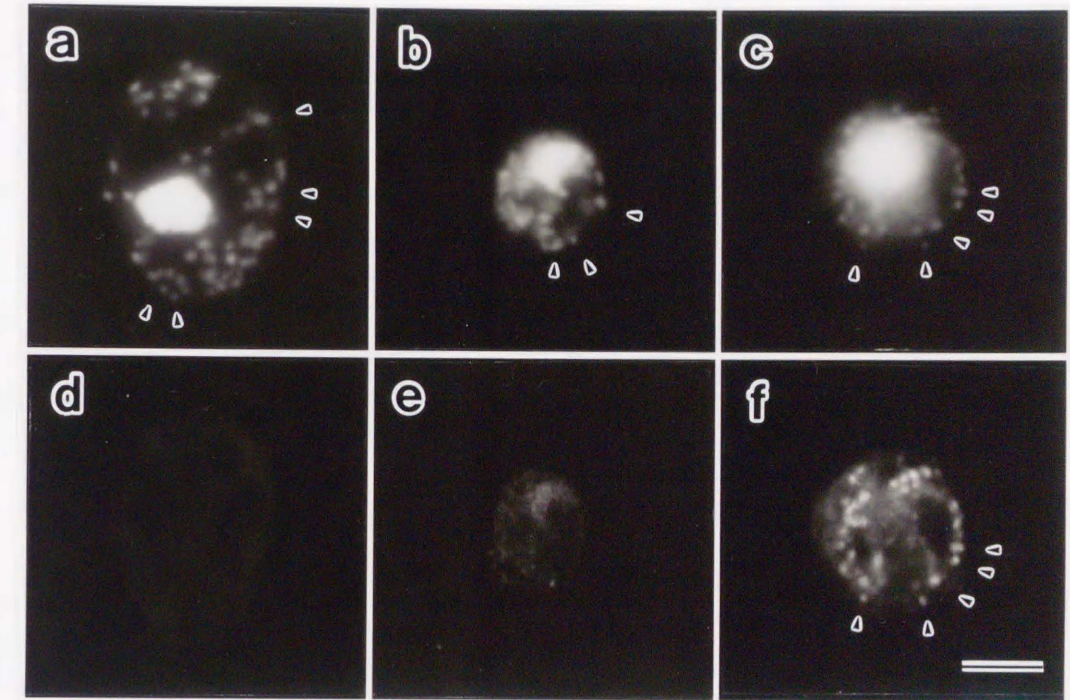


Fig. 9. Intracellular localization of TopA. (A) Whole cells were fixed and stained with second antibody alone (a and d), with preimmune serum (b and e) or with a purified anti-TopA antibody, αC955 (c and f) as first antibody. (B) Isolated mitochondria (a, b, d and e) and nuclei (c and f) were fixed and stained with the purified antibody (b and e, mitochondria; c and f, nucleus) or second antibody alone (a and d, mitochondria). In both A and B, a, b and c show Hoechst staining and d, e and f show FITC staining. Arrowheads mark several mitochondria. Bar, 5 μm.

A



B

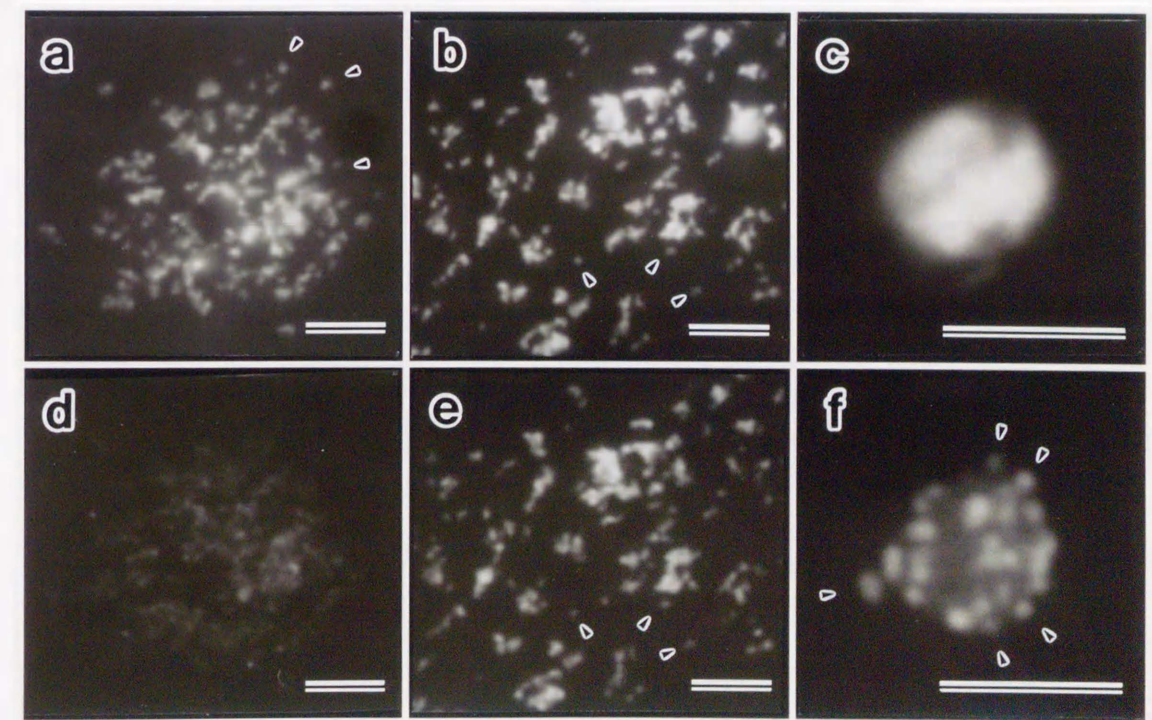
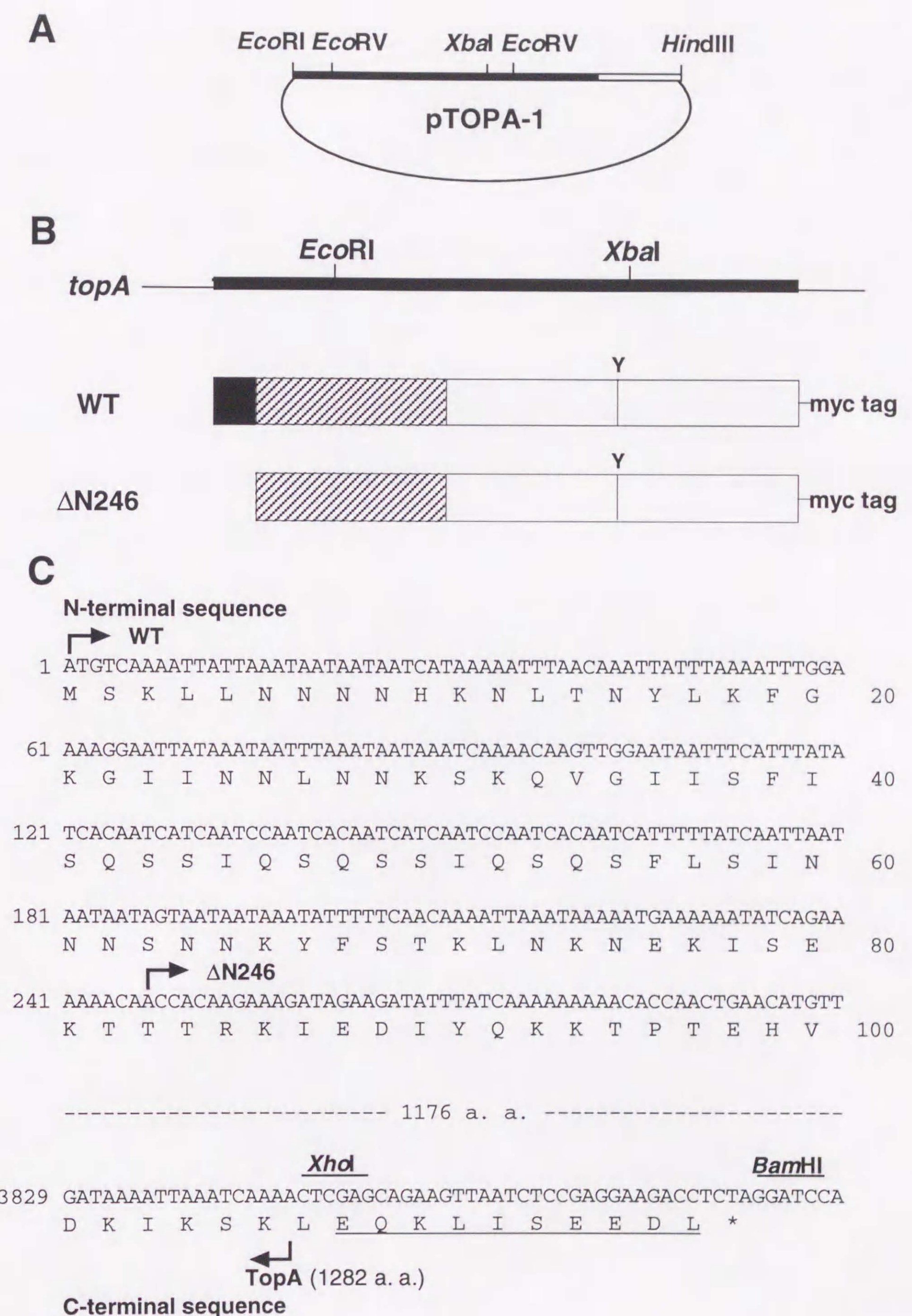


Fig. 10. Construction of the *myc*-tagged wild-type (WT) and 5'-terminal deletion mutant (ΔN246) of *topA*. (A) The plasmid pTOPA-1 contained a part of the structural gene of TopA (black) and its 3'-flanking region (white). Indicated restriction sites were used for construction of the *myc*-tagged mutants and for preparation of the probe used for Northern blotting as described in Materials and Methods. (B) The domain structures of wild-type and truncated TopA are shown. The additional N-terminal region is shown in black, the ATP-binding domain is shown as a diagonal hatched box, the DNA breakage/rejoining domain is an open box, and the tyrosine residue in the active-site is shown as a vertical line marked with a "Y". Restriction sites used for vector construction are shown in the top figure (see Materials and Methods). (C) Amino acid sequences of the N-terminal and C-terminal regions of engineered TopA. In ΔN246, an ATG initiation codon was added. The right-word arrows indicate the start site of each construct and the left-word arrow indicates the end of TopA. Underlined residues correspond to the c-Myc epitope tag. *Xho*I and *Bam*HI sites used for insertion of the c-*myc* epitope sequence are indicated.



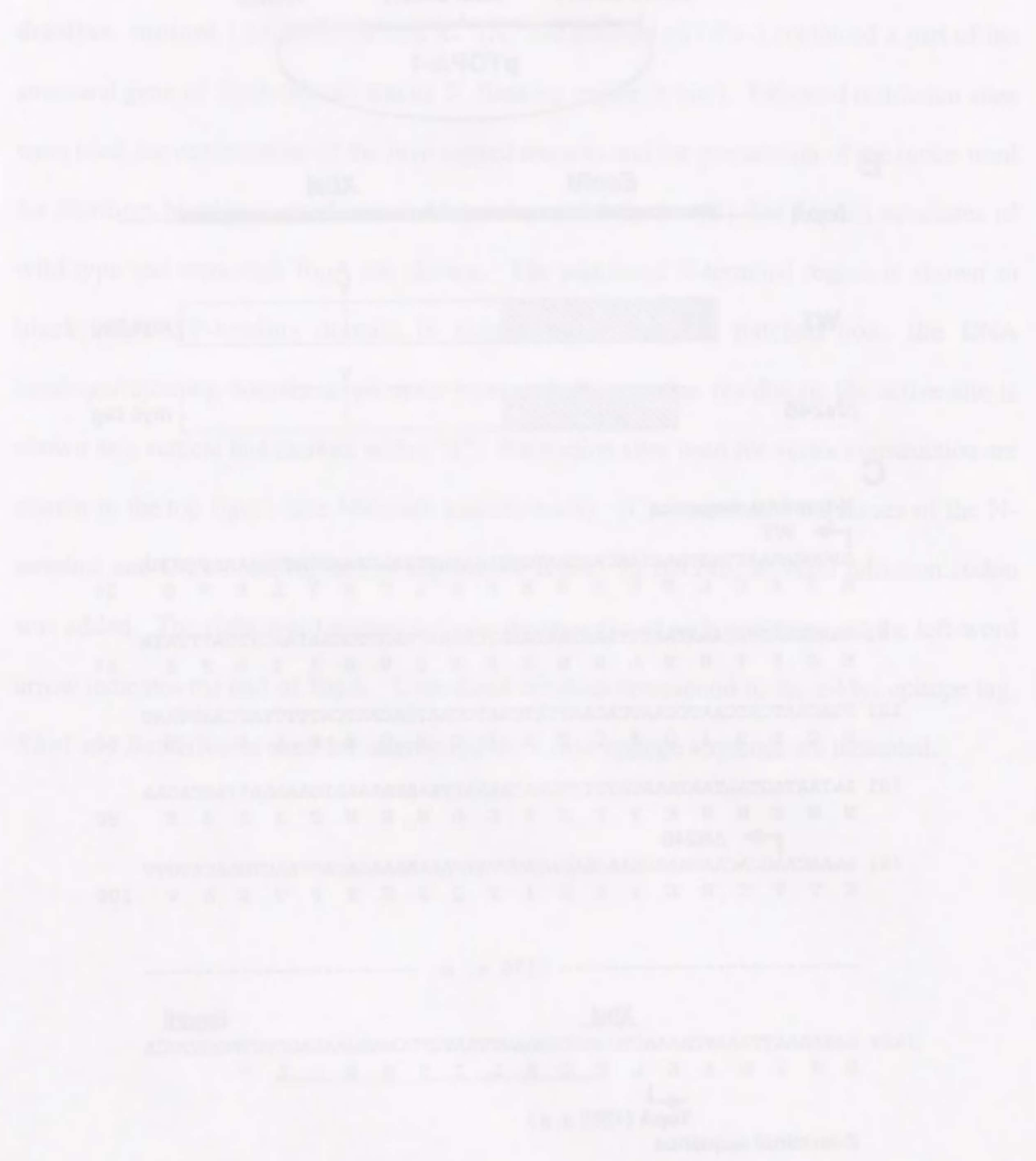


Fig. 11. Expression of the wild-type (WT) and the N-terminal deletion mutant (ΔN246) *topA*. (A) Southern blotting analysis. DNA (2.5 μg) from independent transformants was digested with *Eco*RI, fractionated on a 0.8% agarose gel and hybridized with the ^{32}P-labeled *neo* gene or *CAR1* gene. (B) Northern blotting analysis. Total RNA (20 μg) from each transformant or poly(A)$^{+}$RNA (5 μg) from AX3 cells (lane A) was fractionated on a 1.0% agarose gel containing formaldehyde and hybridized with the ^{32}P-labeled 2.0 kb *Eco*RV fragment from pTOPA-1 or the actin gene. (C) Western blotting analysis. Whole-cell extract (3 x 10^{5} cells) was fractionated by 6% SDS-PAGE and treated with anti-TopA antibody, αC955 (αTopA) or anti-c-Myc antibody (αMyc).

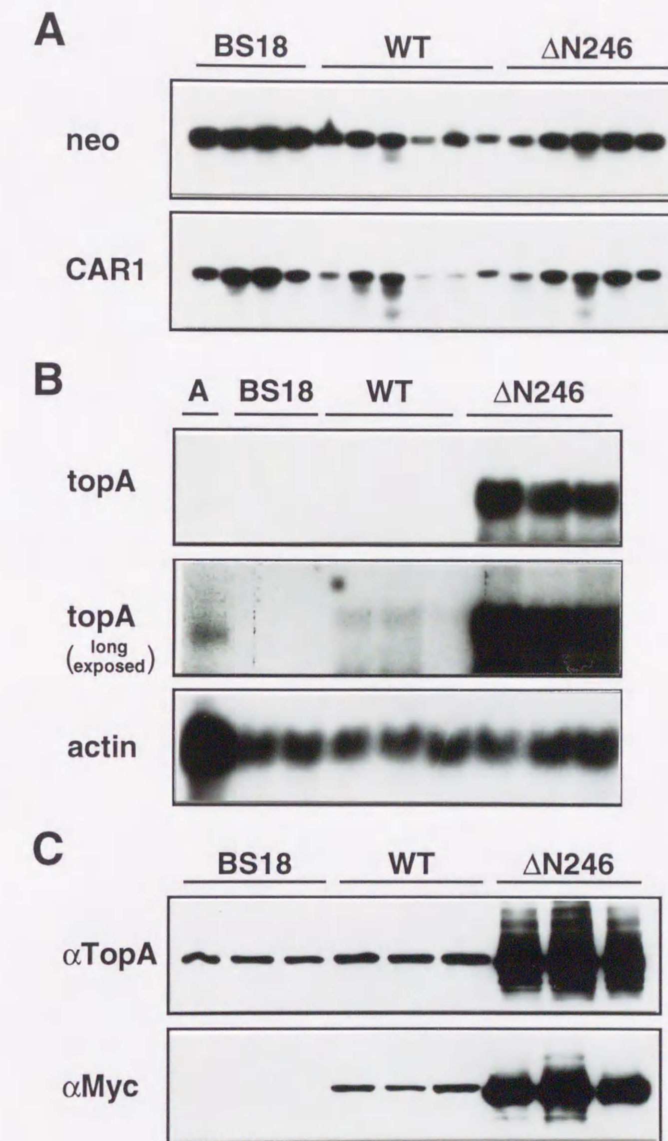
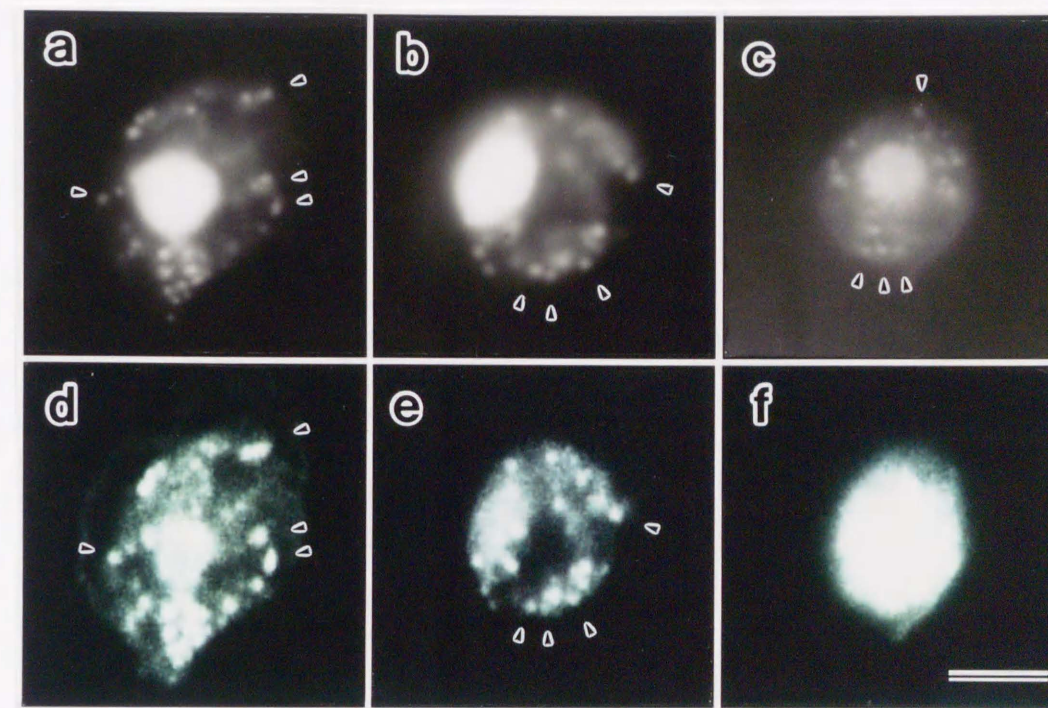


Fig. 12. Intracellular localization of tagged TopA. Whole cells of each transformant were fixed and stained with a purified anti-TopA antibody, αC955 (A) and anti-c-Myc antibody (B) as the first antibody, then both were stained with a Texas-Red-linked second antibody. Panels a and d, BS18; panels b and e, WT; panels c and f, ΔN246. a–c, Hoechst staining; d–f, Texas-Red staining. Arrowheads mark several mitochondria. Bar, 5 μm.

A



B

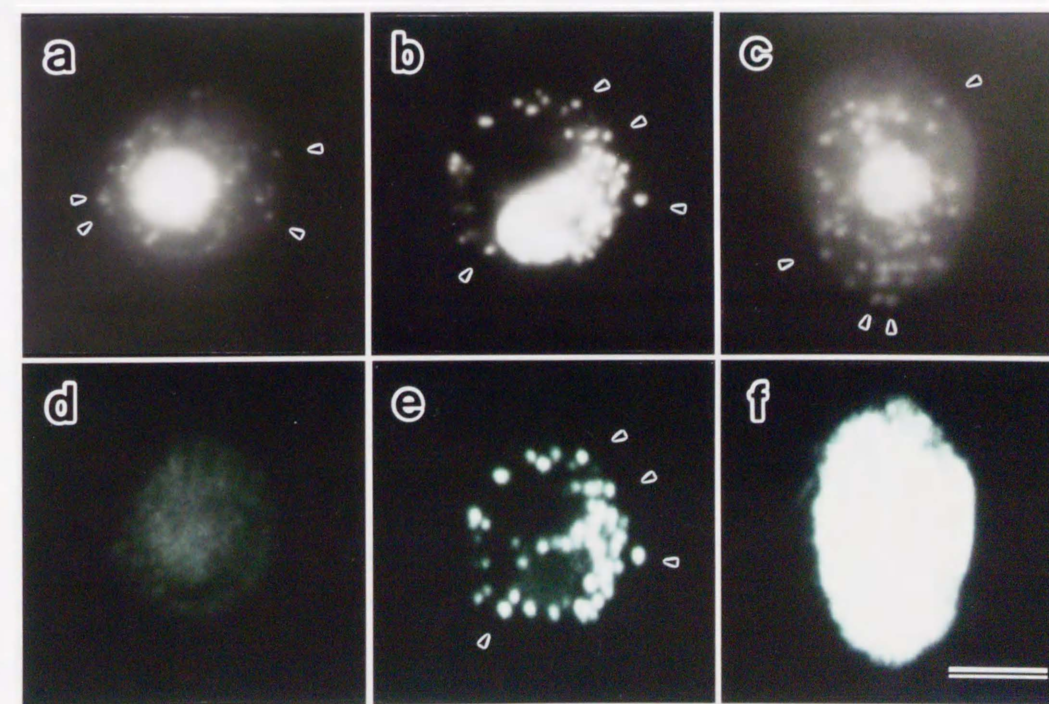
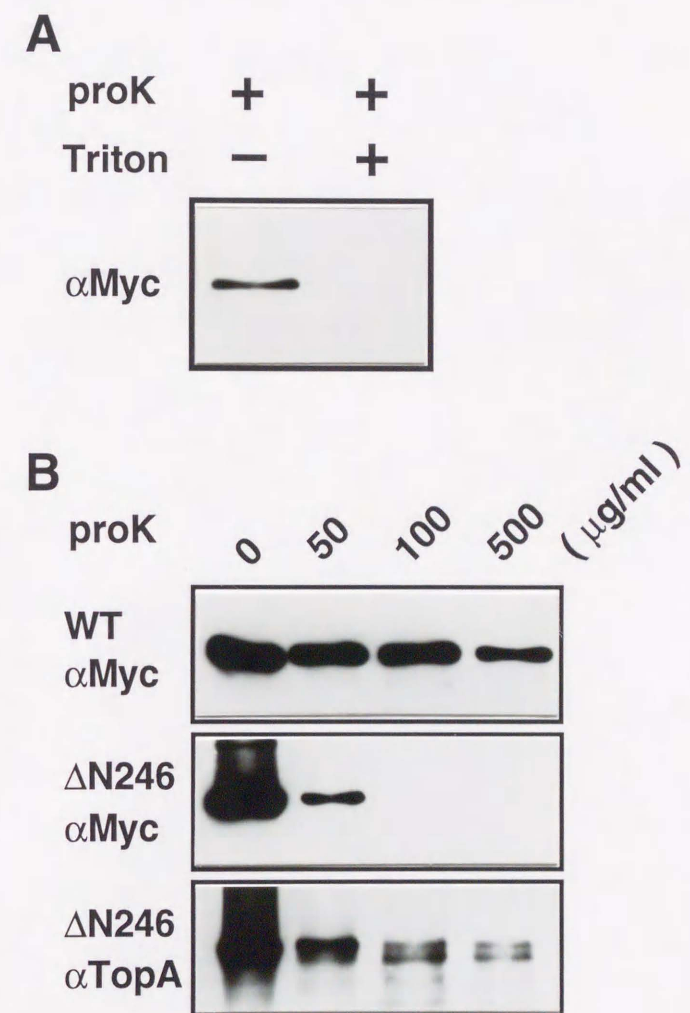


Fig. 13. Proteinase K treatment of tagged wild-type TopA and truncated TopA. (A) Isolated mitochondria from WT cells were incubated for 1 hr on ice with 5 mg/ml proteinase K (proK) in the absence or presence of 1% Triton X-100 (Triton). The mitochondrial extracts (20 μg) were analyzed by 6% SDS-PAGE and Western blotting with anti-c-Myc antibody (αMyc). (B) Mitochondria were treated for 1 hr on ice with proteinase K at the indicated concentrations and were analyzed using anti-c-Myc antibody (αMyc) or anti-TopA antiserum, αC955 (αTopA). The transformants used were WT and ΔN246.



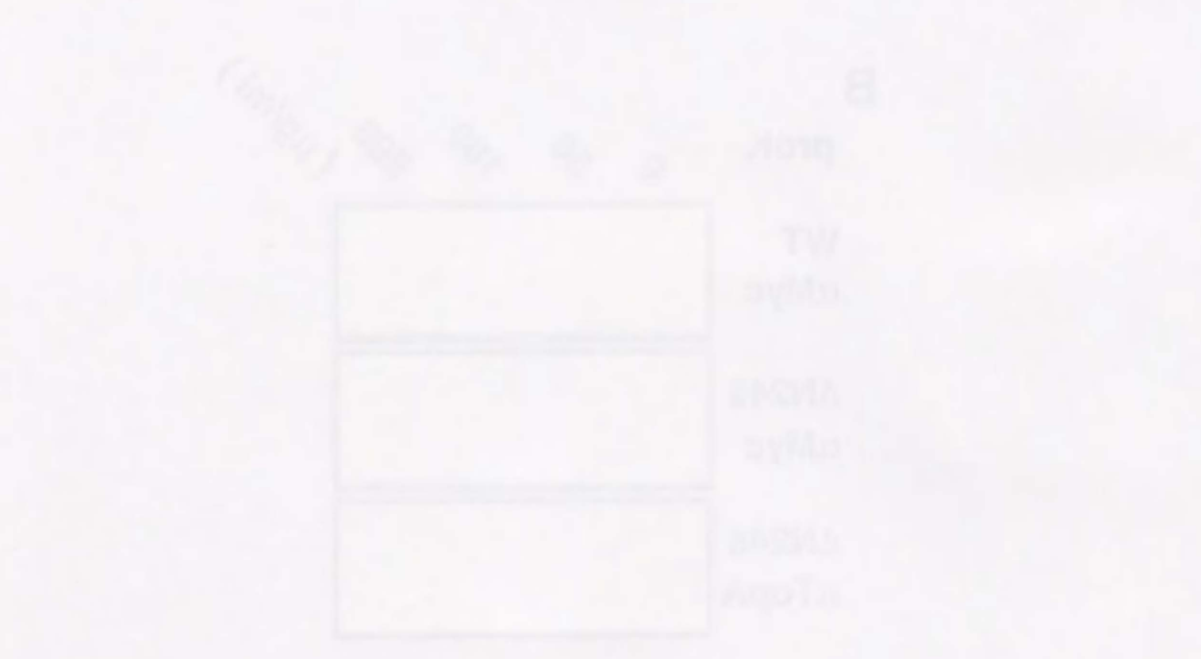
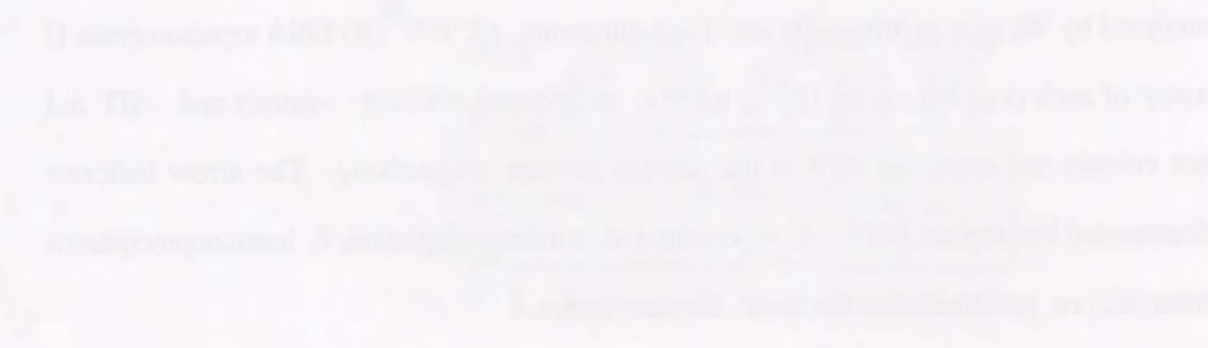
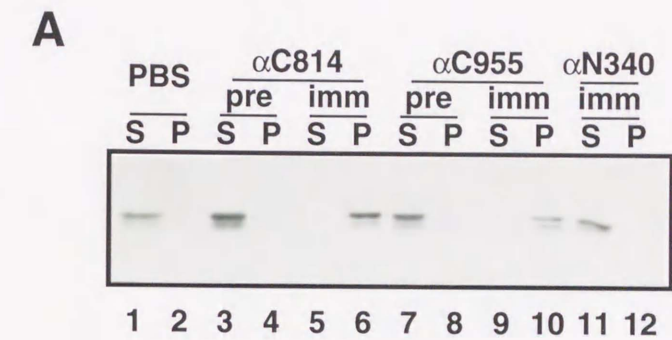
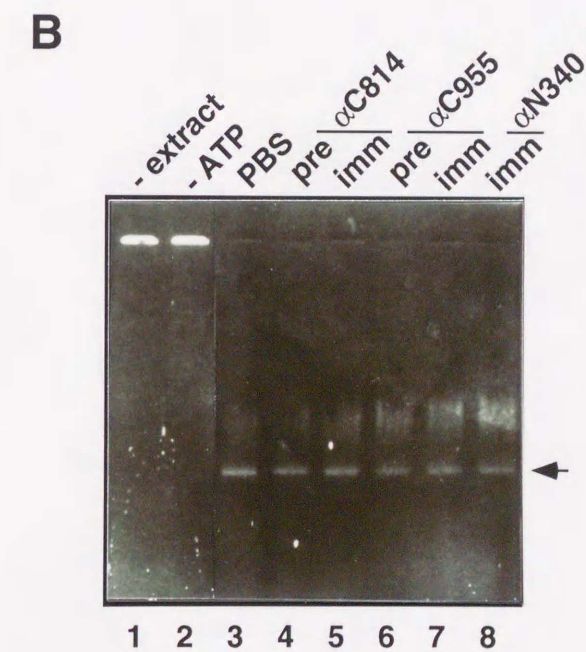



Fig. 14. Immunodepletion analysis. (A) Immunodepletion of TopA from cell extract by each anti-TopA antiserum (αC814, αC955 and αN340) or PBS as a control. The same volume of supernatant or pelleted fraction was separated by 6% SDS-PAGE and then analyzed by Western blotting with anti-TopA antiserum, αC955. (B) DNA topoisomerase II assay of each depleted extract (PBS, αC814, αC955 and αN340). –extract and –ATP did not contain cell extract or ATP in the reaction mixture, respectively. The arrow indicates decatenated kinetoplast DNA. S, supernatant after immunodepletion; P, immunoprecipitated material; pre, preimmune serum; imm, immune serum.





of *Dictyostelium discoideum*