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Growth State and Cell Cycle Dependent Phosphorylation of DNA Topoisomerase II in Swiss 3T3 Cells†

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ABSTRACT: We have investigated the amount of DNA topoisomerase II and phosphorylation of the enzyme in Swiss 3T3 cells during the transition from cell quiescence to proliferation. A relatively high level of phosphorylation was observed with proliferating cells while no or a very low level of phosphorylation was observed with quiescent cells. Phosphoamino acid analysis of the phosphorylated topoisomerase II revealed that the phosphorylated aminoacyl residue was serine. When quiescent cells were stimulated to grow by the addition of serum, DNA synthesis began to increase at 9 h after serum addition, reaching a maximum at 15 h and then declining. The amount of topoisomerase II began to increase at 6 h and reached a maximum at 22–27 h, corresponding to the G2 phase. The phosphorylation of topoisomerase II measured by pulse-labeling gradually increased from 6 to 18 h and reached a maximum at 22 h when the amount of the enzyme was maximum. The level of phosphorylation measured by continuous-labeling increased gradually up to 12 h and markedly up to 28 h, and then declined. The increase in the rate of phosphorylation in the G2 phase was affected by inhibiting DNA synthesis, but the increase in the amount of the enzyme was not. Thus, it was suggested that the regulation of phosphorylation of topoisomerase II differs from that of the amount of the enzyme.

DNA topoisomerase II is an enzyme that catalyzes the decatenation and the unknotting of topologically linked DNA circles and the relaxation of supercoiled DNA (Gellert, 1981; Wang, 1985; Vosberg, 1985). The enzyme is essential for the viability of eukaryotic cells because it is involved in chromosome segregation (Yanagida & Wang, 1987). It has been demonstrated that topoisomerase II is a major component of nuclear matrix (Berrios et al., 1985) and mitotic chromosome scaffold (Earnshaw et al., 1985) and that the enzyme is localized at the bases of the radial loop domains of mitotic chromosomes (Earnshaw & Heck, 1985; Gasser et al., 1986). Thus, the enzyme appears to play important roles in the construction of chromosome structure and in the organization of the nuclear matrix.

The activity and levels of topoisomerase II in proliferating cells are higher than those in quiescent cells (Miskimins et al., 1983; Duguet et al., 1983; Taudou et al., 1984; Sullivan et al., 1986; Heck & Earnshaw, 1986; Zwelling et al., 1987; Markovits et al., 1987; Nelson et al., 1987; Chow & Ross, 1987; Hsiang et al., 1988). During the development of *Drosophila*, the expression of topoisomerase II is high at the stages with increased mitotic activity (Fairman & Brutlag, 1988). Furthermore, it has been observed that both the amount and the stability of topoisomerase II are altered during the cell cycle (Heck et al., 1988).

Besides the fluctuation of enzyme levels, little is known about the mechanism to modulate the intracellular activity of topoisomerase II. In our previous study, we purified an unidentified protein kinase from mouse FM3A cells that phosphorylated purified topoisomerase II and observed that the phosphorylation of topoisomerase II by the protein kinase greatly stimulated the enzyme activity (Saijo et al., 1990). In addition, it has been reported that topoisomerase II is phosphorylated by casein kinase II or protein kinase C and that the modification by either kinase stimulates enzyme activity (Ackerman et al., 1985; Sahyoun et al., 1986). Thus, phosphorylation should be one of possible mechanisms that regulate intracellular topoisomerase II activity. Indeed, the enzyme appears to exist in eukaryotic cells as a phosphoprotein (Rottmann et al., 1987; Ackerman et al., 1988; Heck et al., 1989; Saijo et al., 1990).

In order to confirm the physiological meaning of phosphorylation of topoisomerase II, the changes in the level of phosphorylation of topoisomerase II as well as those in its amount during the transition of growth state were studied in the present work.

Materials and Methods

Materials. [^{32}P]Orthophosphate was purchased from NEN; [^{3}H]thymidine from ICN; protein A–Sepharose CL-4B from Pharmacia; anti-rabbit IgG from Medical & Biological Labs;

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rabbit peroxidase anti-peroxidase from Jackson; horseradish peroxidase conjugated anti-rabbit IgG from Seikagaku Kogyo; 3,3′-diaminobenzidine and aphidicolin from Wako; nitrocellulose membrane from Schleicher & Schuell.

Cell Culture. Swiss 3T3 cells were cultured at 37 °C in Dulbecco's modified Eagle's minimum essential medium (DMEM)[1] supplemented with 5% fetal bovine serum (FBS), 100 μg/mL streptomycin sulfate, and 100 units/mL penicillin G potassium. To induce serum deprivation, 2×10^5 cells were inoculated into a dish (inner diameter 100 mm) with the growth medium and incubated for 2 days. Then the medium was changed to the growth medium containing 0.2% FBS, and cells were incubated another 3 days.

Preparation of Anti-Topoisomerase II Antibody. Purified topoisomerase II (100 μg) was electrophoresed on SDS–polyacrylamide gels. The bands of topoisomerase II were excised from the gel. The gel slices were crushed with a Dounce homogenizer and mixed with an equal volume of Freund's complete adjuvant. The emulsified sample was injected subcutaneously into a New Zealand White rabbit. The rabbit was booster-injected at intervals of 1–2 months, and blood was collected from the ear vein 1 week after boosting. Sera were prepared and fractionated by ammonium sulfate precipitation.

Continuous-Labeling. The culture medium was replaced with DMEM containing 10-fold less concentration of phosphate, 0.2% dialyzed FBS, and 0.8 MBq/mL [^{32}P]orthophosphate at 8 h before the termination of serum starvation. Then dialyzed FBS was added at a final concentration of 5%, and cells were harvested at the indicated times.

Pulse-Labeling. Cells were rendered quiescent as described above, and the culture medium was changed to DMEM containing 5% dialyzed FBS. Two hours before the indicated times, the medium was replaced with DMEM containing 100-fold less concentration of phosphate and 5% dialyzed FBS, and the culture was incubated for 1 h. [^{32}P]Orthophosphate was added at a final concentration of 1.2 MBq/mL, and cells were labeled for 2 h. As to the zero-time sample, the treatments were almost the same as described above except that the medium was replaced with DMEM containing 100-fold less concentration of phosphate and no dialyzed FBS.

Measurement of DNA Synthesis. Cells were labeled with 37 kBq/mL [^{3}H]thymidine (1110 GBq/mmol) for 60 min. The labeling was terminated by adding $^1/_{25}$th volume of 5 mg/mL NaN_3 to the culture. The cells were harvested and lysed with 1 mL of 0.8% SDS at room temperature for 30 min, and then an equal volume of 10% trichloroacetic acid containing 0.1 M sodium pyrophosphate was added to the lysate. After being kept at room temperature at least for 20 min, acid-insoluble materials were collected on a Whatman GF/C glass-fiber filter. The radioactivity was measured with a liquid scintillation counter.

Immunoprecipitation. ^{32}P-Labeled cells (2×10^6) were washed once with buffer A [10 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$, 1 mM EDTA, 0.5 mM sodium vanadate, 10 mM *p*-nitrophenyl phosphate, and 0.25 mM PMSF] and suspended in buffer A containing 0.05% Triton X-100 at a concentration of 2×10^6/mL, and stood at 0 °C for 20 min to be lysed. Nuclei were separated from cytosol by centrifugation for 5 min at 1000*g* and washed with buffer A. Nuclear extracts were prepared by adding 250 μL of buffer B [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM EDTA, 2 mM 2-mercaptoethanol, 10 mg/mL pepstatin, 10 mg/mL leupeptin, and 1 mM PMSF] containing 0.4% SDS and heating in boiling water for 3 min. The extracts were centrifuged for 10 min at 13000*g*, and the supernatant was recovered. An equal volume of buffer B containing 2% Triton X-100 was added to the supernatant. Nuclear extracts were treated with 40 μL of 50% (v/v) protein A–Sepharose for 30 min at 4 °C in order to remove proteins binding nonspecifically to protein A–Sepharose. Then 400 μL of the nuclear extracts was incubated with 10–15 μL of anti-mouse topoisomerase II antiserum for 2–4 h at 4 °C, and subsequently mixed with 5 mg of protein A–Sepharose. The mixture stood at 4 °C for 1 h, and the beads were washed 6 times with buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, and 1 mM PMSF and once with water. The proteins adsorbed to the beads were extracted with 40 μL of buffer containing 125 mM Tris-HCl (pH 6.8), 2% SDS, 2% 2-mercaptoethanol, 20% glycerol, and 0.02% bromphenol blue by heating at 90 °C for 3 min, and beads were removed by centrifugation. The supernatant was subjected to electrophoresis on a 7% polyacrylamide gel containing 0.1% SDS. The gel was dried and subjected to autoradiography.

Immunoblotting. Harvested cells (2×10^5) were washed once with buffer A and lysed with 40 μL of buffer containing 125 mM Tris-HCl (pH 6.8), 2% SDS, 2% 2-mercaptoethanol, 20% glycerol, and 0.02% bromphenol blue by boiling for 3 min. Lysates were separated in a 7% polyacrylamide gel containing 0.1% SDS. After electrophoresis, gels were soaked in Tris/glycine buffer [25 mM Tris/192 mM glycine (pH 8.2)] containing 0.2% SDS and 20% methanol for 20 min. Proteins were transferred from the gels to nitrocellulose membranes at 10 V/cm for 8 h at 4 °C in the above buffer. After transfer, the membranes were soaked in TBS [50 mM Tris-HCl (pH 7.4)/150 mM NaCl] containing 0.1% Triton X-100 and 1% skim milk for 1 h and then incubated with 1000-fold-diluted anti-topoisomerase II antiserum in TBS containing 0.1% Triton X-100 and 1% skim milk overnight. The membranes were washed 4 times with TBS containing 0.1% Triton X-100 and 1% skim milk and incubated with 200-fold-diluted anti-rabbit IgG in TBS containing 1% skim milk for 1 h. Then the membranes were washed with TBS containing 0.1% Triton X-100 and 1% skim milk and incubated with 10000-fold-diluted peroxidase anti-peroxidase in TBS containing 1% skim milk for 1 h. After being washed, the membranes were incubated in TBS containing 0.5 mg/mL 3,3′-diaminobenzidine and 0.05% H_2O_2. Alternatively, 500-fold-diluted peroxidase-conjugated anti-rabbit IgG was used as secondary antibody, and topoisomerase II was detected using the ECL Western blotting detection system (Amersham).

Phosphoamino Acid Analysis. Phosphoamino acid analysis was done as described previously (Cooper et al., 1983).

Results

Amounts of Topoisomerase II and Levels of Its Phosphorylation in Quiescent and Proliferating Cells. In order to investigate whether the amount of topoisomerase II and the level of its phosphorylation vary in relation to growth states, we compared these parameters of serum-starved quiescent Swiss 3T3 cells with those of proliferating cells.

The amount of topoisomerase II was measured by immunoblotting with a rabbit anti-mouse topoisomerase II antiserum. The relative amount of topoisomerase II in proliferating cells was much higher than that in quiescent cells (Figure 1A).

To detect phosphorylated topoisomerase II, cells in each growth state were labeled with [^{32}P]orthophosphate, and to-

[1] Abbreviations: DMEM, Dulbecco's modified Eagle's minimal essential medium; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

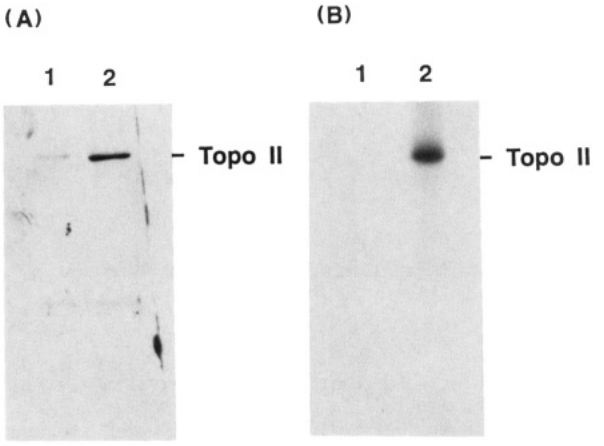

FIGURE 1: Amounts of topoisomerase II and levels of its phosphorylation in quiescent and proliferating cells. (A) Immunoblotting using anti-topoisomerase II antiserum was performed as described under Materials and Methods. Lane 1, total cellular proteins prepared from cells incubated for 72 h under serum starvation; lane 2, proteins from exponentially growing cells. (B) ^{32}P-Labeled nuclear proteins were precipitated with anti-topoisomerase II antiserum and subjected to SDS–polyacrylamide gel electrophoresis as described under Materials and Methods. Lane 1, nuclear extract from cells labeled for 12 h after 60-h serum starvation; lane 2, nuclear extract from exponentially growing cells labeled for 12 h.

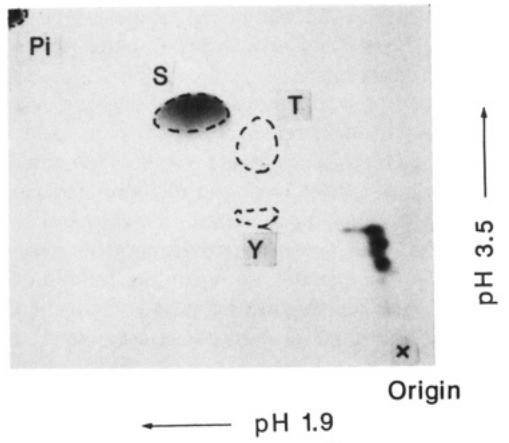

FIGURE 2: Phosphoamino acid analysis of ^{32}P-labeled topoisomerase II. Phosphorylated topoisomerase II was hydrolyzed in 6 N HCl at 110 °C for 2 h. The hydrolysate was mixed with phosphorylated amino acid standards and analyzed by two-dimensional thin-layer electrophoresis. Autoradiogram of the thin-layer plate is shown. Dashed circles indicate the positions of standards. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine; Pi, inorganic phosphate.

poisomerase II was immunoprecipitated with the anti-topoisomerase II antiserum. As shown in Figure 1B, a relatively high level of phosphorylation of topoisomerase II was observed with proliferating cells while no or, if any, a very low level of phosphorylation of the enzyme was observed with quiescent cells although topoisomerase II existed in the quiescent cells as shown in Figure 1A.

The aminoacyl residue of topoisomerase II that was phosphorylated in proliferating Swiss 3T3 cells was identified by acid hydrolysis and two-dimensional electrophoresis. The phosphorylated aminoacyl residue was serine (Figure 2), but phosphothreonine was detected upon overexposure of the autoradiogram (data not shown).

Changes in the Amount of Topoisomerase II and in the Level of Phosphorylation during Serum-Stimulated Cell Growth. Quiescent Swiss 3T3 cells were stimulated to grow by the addition of 5% serum. DNA synthesis began to increase at 9 h after serum addition and reached a maximum at 15 h and then declined. The amount of topoisomerase II was

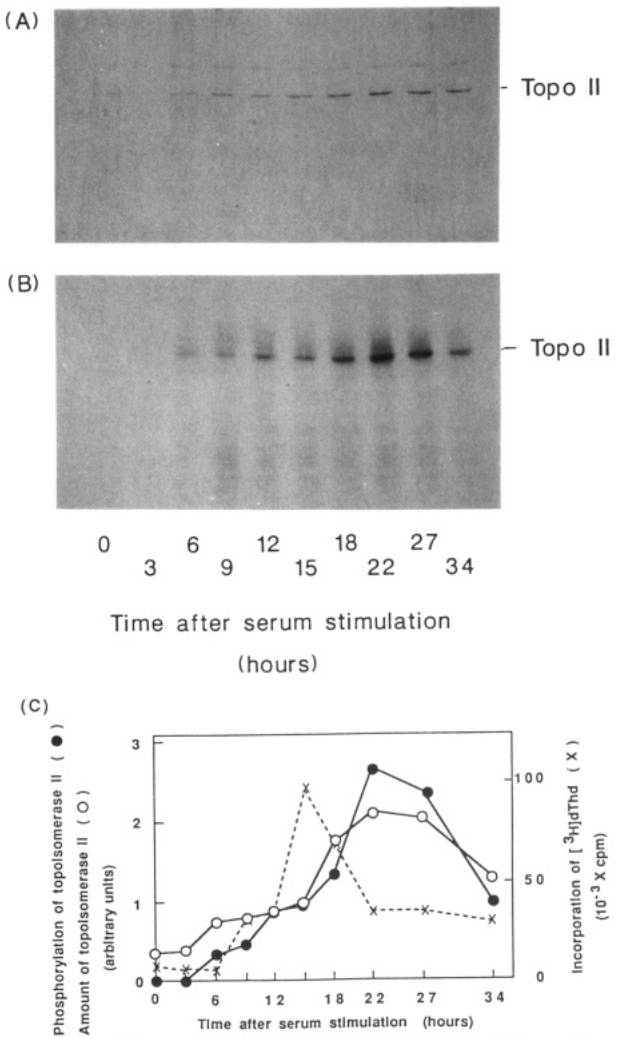

FIGURE 3: Changes in the amount and phosphorylation of topoisomerase II after serum stimulation. (A) Immunoblotting with anti-topoisomerase II antiserum. Quiescent cells were stimulated to grow by the addition of serum, and cells were harvested at the indicated times. Immunoblotting was performed as described under Materials and Methods. (B) Immunoprecipitation of ^{32}P-labeled nuclear proteins with anti-topoisomerase II antiserum. Cells were pulse-labeled for 2 h with [^{32}P]orthophosphate and immunoprecipitated as described under Materials and Methods. The midpoint of pulse-labeling is indicated in the figure. (C) The bands in panels A and B were quantified by densitometry. (○) Amount of topoisomerase II; (●) phosphorylation of topoisomerase II. The dashed line indicates the incorporation of [^{3}H]thymidine measured as described under Materials and Methods.

measured by immunoblotting with the cells harvested at the indicated times. The amount of topoisomerase II increased at 6 h after serum addition and maintained a fairly constant level up to 12 h and then increased again at 15 h (Figure 3A). The maximal level was attained at 22–27 h after serum addition, corresponding to the G2 phase (Figure 3A,C).

Phosphorylation of topoisomerase II after serum stimulation was examined by pulse-labeling with ^{32}P for 2 h at the indicated times (Figure 3B). The incorporation of ^{32}P into topoisomerase II was detected at 6 h after serum addition and increased gradually up to 18 h. A maximal incorporation of ^{32}P was observed at 22 h when the amount of topoisomerase II reached the maximum (Figure 3B,C).

When the level of phosphorylation of topoisomerase II was examined by continuous-labeling, [^{32}P]orthophosphate was added 8 h before serum addition, and the cells were harvested at the indicated times after serum addition. The level of phosphorylation of topoisomerase II increased gradually up

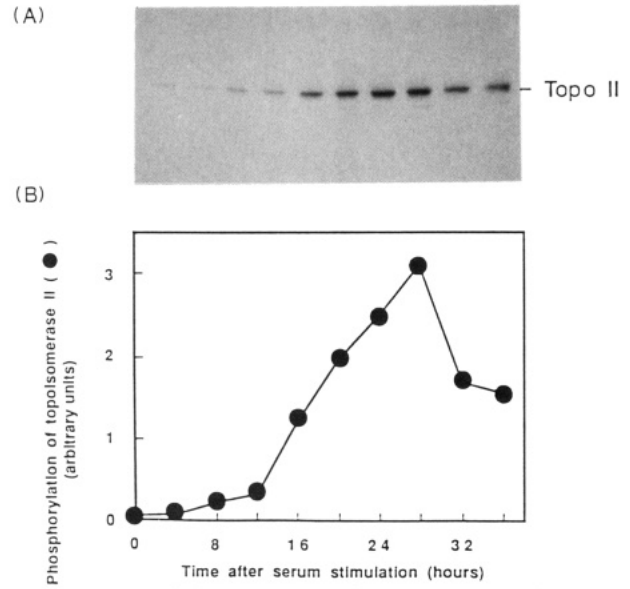

FIGURE 4: Levels of phosphorylation of topoisomerase II after serum stimulation. (A) Quiescent cells were stimulated to grow by the addition of serum and continuously labeled with [^{32}P]orthophosphate as described under Materials and Methods. The cells were harvested at the indicated times, and nuclear extracts were immunoprecipitated with anti-topoisomerase II antiserum. (B) The bands in panel A were quantified by densitometry.

to 12 h and increased markedly up to 28 h, and then declined (Figure 4A,B).

Influence of Inhibition of DNA Synthesis on Increases in the Amount of Topoisomerase II and in the Rate of Phosphorylation. To examine whether DNA synthesis is required for the increases in the amount of topoisomerase II and the rate of phosphorylation, aphidicolin, a specific inhibitor for DNA polymerase α, was added to the culture at 6 h following serum addition. The incorporation of [^{3}H]thymidine was almost completely inhibited by the addition of aphidicolin (Figure 5, stippled bars). The increase in the amount of topoisomerase II was observed at 12 and 22 h regardless of DNA synthesis (Figure 5, open bars).

The "rate" of phosphorylation of topoisomerase II was measured by pulse-labeling with ^{32}P for 2 h, indicating the rate of net phosphorylation. The "rate" of phosphorylation in the absence of DNA synthesis at 12 h after serum addition, corresponding to early S phase, increased as compared to that at 6 h and was the same as the "rate" of phosphorylation at 12 h without the inhibition of DNA synthesis. As compared to the "rates" of phosphorylation at 12 h, however, at 22 h, little increase in the "rate" of phosphorylation was observed in the absence of DNA synthesis, while a considerable increase in the "rate" of phosphorylation was observed with noninhibited cells (Figure 5, closed bars). These results were obtained reproducibly.

Discussion

We have investigated the amount of topoisomerase II and the level of its phosphorylation in Swiss 3T3 cells during the transition from cell quiescence to proliferation. In quiescent cells, only 20-fold less topoisomerase II existed as compared with the amount in log-phase cells, and almost no phosphorylation of topoisomerase II occurred (Figure 1). Since topoisomerase II is a component of the nuclear matrix, the level of topoisomerase II in quiescent cells should be the minimal amount to maintain the nuclear structure supposing that all matrix-associated topoisomerase II is detected by the antibody. However, the following possibilities cannot be excluded at

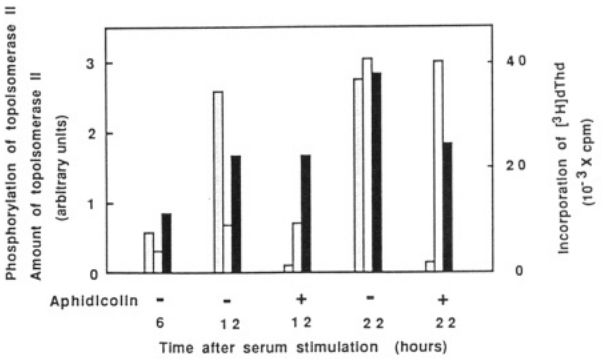

FIGURE 5: Effect of DNA synthesis inhibition on increases in the amount of topoisomerase II and the rate of phosphorylation. Quiescent cells were stimulated to grow by the addition of serum. Aphidicolin was added 6 h after serum stimulation at a final concentration of 5 μg/mL. The amount of topoisomerase II was measured by immunoblotting followed by densitometry with the cells harvested at the indicated times. The rate of phosphorylation was measured by immunoprecipitation of nuclear extracts prepared from the cells pulse-labeled with [^{32}P]orthophosphate for 2 h. Values were determined by densitometry. DNA synthesis was measured by pulse-labeling with [^{3}H]thymidine for 60 min as described under Materials and Methods. Open column, total amount of topoisomerase II; closed column, phosphorylation of topoisomerase II; dotted column, DNA synthesis.

present. The matrix-associated topoisomerase II cannot be extracted by the method used here, and the enzyme is destroyed during extraction.

The amount of topoisomerase II and the level of its phosphorylation began to increase in late G1 phase and reached maxima in the G2 phase (Figures 3 and 4). A similar fluctuation pattern of phosphorylation of topoisomerase II was observed with chicken lymphoblastoid cells (Heck et al., 1989). The time when the level of phosphorylation reaches the maximum is close to the time when the function of topoisomerase II is required because genetic analysis of topoisomerase II mutants of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* has indicated that the enzyme is essential both for the chromosome condensation and for the disjunction of sister chromatids (Yanagida & Wang, 1987). In addition, we observed that treatment of purified topoisomerase II with calf intestinal alkaline phosphatase abolished the enzyme activity and treatment of the inactivated topoisomerase II with a protein kinase purified from mouse FM3A cells restored the enzyme activity (Saijo et al., 1990). Stimulation of topoisomerase II activity by protein kinases has been also observed with casein kinase II and protein kinase C (Ackerman et al., 1985; Sahyoun et al., 1986). Thus, it seems very likely that phosphorylation is a physiological device for regulating topoisomerase II activity.

The increase in the rate of phosphorylation of topoisomerase II in the G2 phase was affected by treatment of aphidicolin, but the increase in the amount of topoisomerase II was not (Figure 5). Thus, the regulation of phosphorylation of topoisomerase II differs from that of the amount of the enzyme.

Drake et al. (1987) isolated two distinct forms of topoisomerase II with molecular masses of 180 (p180) and 170 kDa (p170) from human and mouse cells, whose V8 protease cleavage patterns differed from each other. Specific antibodies to either polypeptide did not cross-react, and cDNAs encoding each polypeptide were similar but distinct (Chung et al., 1989). The levels of p170 were high during rapid proliferation and declined as the cells reached the plateau phase of growth, while the levels of p180 changed in a reverse manner (Drake et al., 1989). The fluctuation pattern of the amount of topoisomerase II reported here corresponds to that of p170. We have also

detected a faint band migrating slightly slower than p176 that is the topoisomerase II described here (data not shown). Although we were not able to quantify the amount and the level of phosphorylation of the faint band, it seemed that both changed little during the transition of growth states.

The protein kinase that is responsible for the phosphorylation of topoisomerase II in mammalian cells has not been confirmed yet. The fluctuation pattern of phosphorylation of topoisomerase II is similar to that of phosphorylation of histone H1 (Bradbury et al., 1974; Gurley et al., 1981; Ajiro et al., 1981) which is known to be phosphorylated by $p34^{CDC2}$ protein kinase (Labbe et al., 1988, 1989; Arion et al., 1988). At present, however, there is no evidence indicating the involvement of $p34^{CDC2}$ protein kinase in the phosphorylation of topoisomerase II. With sponge cells, it has been shown that topoisomerase II is phosphorylated in response to the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate, which stimulates protein kinase C (Rottmann et al., 1987). DNA topoisomerase II has also been shown to be phosphorylated by casein kinase II in *Drosophila* embryonic cultured cells (Ackerman et al., 1988). In addition, it has been confirmed that there are several parts in the *Drosophila* topoisomerase II gene that fit the minimal consensus sequences for casein kinase II and protein kinase C (Wyckoff et al., 1989). Phosphorylation of purified topoisomerase II by casein kinase II and protein kinase C resulted in the stimulation of the enzyme activity by about 3-fold (Ackerman et al., 1985; Sahyoun et al., 1986). In addition, the protein kinase that we purified from mouse FM3A cells greatly stimulated the activity of topoisomerase II purified from mouse cells (Saijo et al., 1990). Thus, the next aim of our study is to confirm the protein kinase responsible for the phosphorylation of topoisomerase II in mammalian cells by examining the possibility of the involvement of casein kinase II, protein kinase C, and several other protein kinases such as the mouse kinase and $p34^{CDC2}$ protein kinase in the phosphorylation.

Registry No. DNA topoisomerase, 80449-01-0; serine, 56-45-1.

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