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Casein kinase II phosphorylates the eukaryote-specific C-terminal domain of topoisomerase II *in vivo*

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The decatenation activity of DNA topoisomerase II is essential for viability as eukaryotic cells traverse mitosis. Phosphorylation has been shown to stimulate topoisomerase II activity *in vitro*. Here we show that topoisomerase II is a phosphoprotein in yeast and that the level of incorporated phosphate is significantly higher at mitosis than in G_1. Comparison of tryptic phosphopeptide maps reveals that the major phosphorylation sites *in vivo* are targets for casein kinase II. Incorporation of phosphate into topoisomerase II is nearly undetectable at the non-permissive temperature in a conditional casein kinase II mutant. The sites modified by casein kinase II are located in the extreme C-terminal domain of topoisomerase II. This domain is absent in prokaryotic and highly divergent among eukaryotic type II topoisomerases, and may serve to regulate functions of topoisomerase II that are unique to eukaryotic cells.

Key words: casein kinase II/cell cycle/phosphorylation/topoisomerase II/yeast

Introduction

Eukaryotic chromosomes undergo major structural changes that reflect their varied functions through the cell cycle. These changes are mediated in part by DNA topoisomerases, and in eukaryotic organisms two types of DNA topoisomerases have been described. Type I topoisomerases alter the DNA linking number in steps of one by transiently introducing a nick in a single DNA strand and allowing the two strands to swivel around each other before religation. In contrast, topoisomerase II creates a double-stranded break, and can pass the same or another DNA molecule through the transient break, changing the linking number by steps of two (reviewed by Wang, 1985). Because of their capacity to relax supercoiled DNA, topoisomerases I and II can substitute for each other in various biological functions. Studies of yeast mutants suggest that topoisomerases I and II are both able to relieve the DNA supercoiling introduced by chain elongation during transcription (Brill and Sternglanz, 1988) and DNA replication (Kim and Wang, 1989a). Similarly, studies with topoisomerase I–topoisomerase II double mutants demonstrated that either enzyme can suppress high levels of mitotic recombination among the ribosomal DNA repeated units (Christman *et al.*, 1988; Kim and Wang, 1989b).

In addition to relaxing supercoiled DNA, topoisomerase II decatenates covalently closed DNA circles. This unique enzymatic activity renders topoisomerase II essential for the proper disjunction of sister chromatids at mitosis (Dinardo *et al.*, 1984; Holm *et al.*, 1985; Uemura and Yanagida, 1986) and for chromosome segregation in meiosis (Rose *et al.*, 1990). Genetic studies of temperature-sensitive *top2* mutants suggest a role for topoisomerase II in chromosome condensation in yeast (Uemura *et al.*, 1987). Similarly, the conversion of nuclei into condensed chromosomes in *Xenopus* egg extracts requires the presence of topoisomerase II, either as a component of the substrate nuclei or of the egg extract (Adachi *et al.*, 1991). This requirement may reflect a structural role for the enzyme, which is suggested by its abundance and presence in the metaphase chromosomal scaffold (Earnshaw *et al.*, 1985; Gasser *et al.*, 1986).

Although the crucial roles of topoisomerase II in mitosis and meiosis suggest that the activity of the enzyme is regulated through the cell cycle, little is known about its regulation. *In vitro* phosphorylation experiments have shown that topoisomerase II is a substrate for casein kinase II (CKII, Ackerman *et al.*, 1985) and protein kinase C (Sahyoun *et al.*, 1986; Rottman *et al.*, 1987). In both cases phosphorylation enhanced the ATP-dependent decatenation activity of topoisomerase II. Topoisomerase II is a phosphoprotein *in vivo* (Ackerman *et al.*, 1988; Rottman *et al.*, 1987; Heck *et al.*, 1989; Saijo *et al.*, 1990) and cell cycle phosphorylation studies in chicken lymphoblastoid cells have shown that topoisomerase II phosphorylation increases as cells enter mitosis (Heck *et al.*, 1989). Soluble extracts from mitotic HeLa cells contain more DNA decatenation activity than extracts obtained from S-phase cells (Estey *et al.*, 1987), suggesting that *in vivo* the activity of topoisomerase II is modulated, at least in part, by phosphorylation. To date, however, neither the sites of modification nor the kinase(s) responsible for them have been identified.

The present study was designed to (i) identify the kinase(s) that phosphorylate topoisomerase II in intact cells, (ii) identify the residues in topoisomerase II that are modified and (iii) investigate whether the sites of phosphorylation vary through the cell cycle. Incorporation of [^{32}P]phosphate in intact, cell cycle-arrested cells shows that topoisomerase II is more highly phosphorylated at mitosis than in G_1 phase. By comparing two-dimensional (2D) phosphopeptide maps, we find that in both G_1 and at mitosis the major sites of modification coincide with sites modified by CKII, and that these sites map to the C-terminal 350 amino acids (aa) of the protein. The biochemical evidence implicating CKII is corroborated by studies of a temperature-sensitive CKII

mutant, allowing us to conclude definitively that CKII is the major kinase phosphorylating topoisomerase II in yeast. Evidence presented elsewhere (M.E.Cardenas, Q.Dang, R.Walter and S.M.Gasser, submitted) demonstrates that CKII copurifies with topoisomerase II over celite and phosphocellulose columns, and that both the decatenation and DNA binding activities of topoisomerase II are stimulated *in vitro* by this kinase. The divergent, phosphate-accepting C-terminal domain of topoisomerase II thus appears to constitute a regulatory domain of the enzyme.

Results

Topoisomerase II is hyperphosphorylated at mitosis

As a first step to examine the phosphorylation of topoisomerase II in living cells, we raised a polyclonal antibody against purified yeast topoisomerase II. We conclude that this antibody is specific for topoisomerase II based on the following criteria: the antibody (i) reacts very efficiently in Western blots with a single 170 kDa polypeptide that fractionates with both nuclear extracts and nuclear scaffolds and which comigrates with purified yeast topoisomerase II (Cardenas *et al.*, 1990; Verdier *et al.*, 1990), (ii) reacts with fusion proteins of the predicted sizes made in *Escherichia coli* from *trpE* fusions with the yeast *TOP2* gene (data not shown) and (iii) immunoprecipitates a single 170 kDa yeast phosphoprotein (Figure 1). The anti-topoisomerase II antibody yields exclusively nuclear staining in yeast spheroplasts fixed for immunofluorescence (Figure 2), and both the amount of protein immunoprecipitated and the immunofluorescence signal are enhanced when topoisomerase II is overexpressed in cells transformed with the plasmid YEpTOP2-PGAL1.

As has been described for topoisomerase II from the sponge *Geodia* (Rottman *et al.*, 1987), *Drosophila* (Ackerman *et al.*, 1988), chicken (Heck *et al.*, 1989) and mouse (Saijo *et al.*, 1990), we find that yeast topoisomerase II is a phosphoprotein *in vivo* (Figure 1). To investigate whether topoisomerase II phosphorylation changes throughout the cell cycle, we determined the amount of topoisomerase II protein and measured the extent of phosphate incorporated into this protein in cells arrested at specific phases of the cell cycle. For estimating protein levels of topoisomerase II, cells were metabolically radiolabelled with [^{35}S]sulphate and arrested by adding α-factor (G_1 arrest) or nocodazole (metaphase arrest) to the radioactive medium. For phosphate incorporation, cell cycle arrested cells were labelled with [^{32}P]inorganic phosphate in low-phosphate media (see Materials and methods). In low-phosphate media yeast cells have a longer generation time (2.5 h) than in normal media (2 h). Because our aim is to detect phosphorylation occurring at the cell cycle arrest points, cells were incubated in α-factor or nocodazole for one generation time. Following cell lysis, topoisomerase II was immunoprecipitated and analysed by SDS–gel electrophoresis.

While the total amount of topoisomerase II precipitated is the same in both G_1 and metaphase, the incorporation of ^{32}P in three different arrest-labelling experiments was 6- to 10-fold higher in metaphase cells (Figure 1). Our results are in agreement with the 4.5-fold increase in phosphorylation observed in chick lymphoblastoid cells as they reached mitosis (Heck *et al.*, 1989). The simplest interpretation of these results is that either a kinase activity that modifies

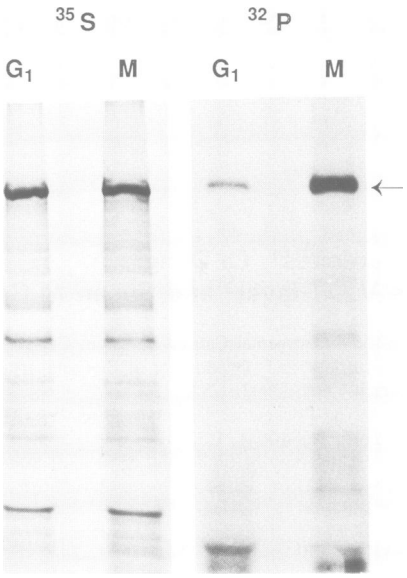

Fig. 1. Topoisomerase II phosphorylation in G_1 or mitosis. Cells were arrested in G_1 phase with α-factor or at mitosis (M) with nocodazole, labelled with [^{35}S]sulphate (^{35}S) or [^{32}P]orthophosphate (^{32}P) and topoisomerase II was immunoprecipitated from cell lysates. Radiolabelled topoisomerase II was visualized by SDS–PAGE and autoradiography. Topoisomerase II migrates at the position marked by the arrow.

topoisomerase II is enhanced or a phosphatase activity is reduced, resulting in a more highly phosphorylated enzyme at the mitotic arrest point. This does not appear to be a non-specific hyperphosphorylation due to nocodazole arrest, since another nuclear phosphoprotein (RAP-1) does not show mitosis-specific hyperphosphorylation (data not shown). Analysis of the phosphorylation state of topoisomerase II at other points in the cell cycle using cell division cycle (cdc) mutants has proven difficult, since even at the permissive temperature in selective low-phosphate medium, the mutants tested grew very poorly. For this reason we limited our study to the G_1 and mitotic arrests presented above.

Interphase and metaphase phosphorylation patterns of topoisomerase II reveal common acceptor sites

To determine whether sites of phosphorylation vary through the cell cycle and to identify the kinase(s) that phosphorylates topoisomerase II *in vivo*, it was necessary to obtain 2D tryptic phosphopeptide maps from *in vivo* ^{32}P-labelled topoisomerase II. After immunoprecipitation with anti-topoisomerase II serum and SDS gel electrophoresis, at most ~20% of the 170 kDa phosphoprotein could be recovered by elution from gel slices. To overcome this limitation, it was necessary to overexpress topoisomerase II to a low extent. Cells transformed with the plasmid YEpTOP2-PGAL1, which encodes the *Saccharomyces cerevisiae* topoisomerase II gene under the control of the *GAL1* promoter (Giaever *et al.*, 1988; Worland and Wang, 1989), were shifted to galactose medium for 2 h (see Materials and methods) prior to cell cycle arrest and phosphate incorporation. This period of galactose induction led to an 8- to 10-fold increase in topoisomerase II above the level expressed from the chromosomal *TOP2* gene (not shown), and again about a fifth of the radioactive topoisomerase II could be eluted from the gel. The intracellular distribution of this overexpressed topoisomerase

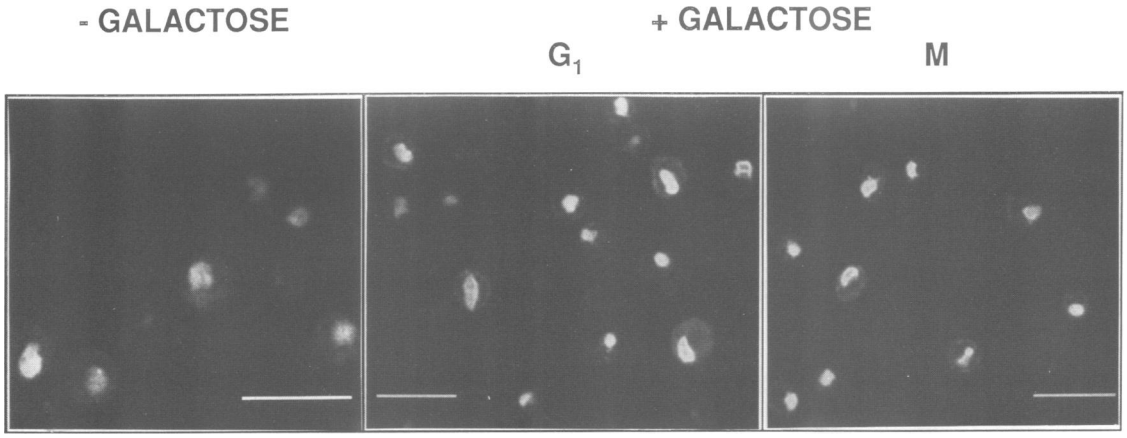

Fig. 2. Overexpressed topoisomerase II localizes to the nucleus. Cells transformed with YEpTOP2-PGAL encoding the topoisomerase II gene under control of the *GAL*1 promoter were grown in synthetic low phosphate medium supplemented with 3% glycerol and 2% lactic acid. In an exponential phase of growth, cells were arrested with α-factor (G_1) or nocodazole (M). 1.5 h after addition of cell cycle inhibitors, the cells were concentrated to 3×10^7 cells/ml in conditioned medium containing 2% galactose. Following 2 h in galactose medium, the cells were fixed, prepared for immunofluorescence and stained with topoisomerase II antibody. The figure shows the confocal microscopy of topoisomerase II immunostaining of control non-induced (- GALACTOSE) and galactose-induced cells (+ GALACTOSE) arrested in G_1 or mitosis (M). The bar indicates 5 μm.

II was followed by indirect immunofluorescence. While the immunofluorescence staining was stronger in induced than in uninduced cells, topoisomerase II was still exclusively localized to the nucleus in non-synchronized cells and in those arrested in G_1 or in metaphase (Figure 2). After a 2 h induction, cytosolic fluorescence staining was observed in $<5\%$ of the total population, ruling out the possibility that the *in vivo* phosphorylation state of the overproduced protein is an artefact due to mislocalization from the nucleus to the cytoplasm.

Topoisomerase II was immunoprecipitated from ^{32}P-labelled cells arrested in G_1 with α-factor or at mitosis with nocodazole. In each case $>85\%$ of the cells showed a uniform phenotype either as unbudded cells (G_1) or as equally sized attached cells (mitosis). Two-dimensional phosphopeptide mapping reveals remarkably similar peptide migration patterns in both populations of cell cycle arrested cells, as shown in Figure 3. The arrowed spots appear to have identical migration but are significantly more intense at the mitotic arrest point, while two major G_1 phosphopeptides (Figure 3, arrowheaded spots) are greatly reduced in the corresponding map at mitosis.

Due to the lower phosphate labelling of topoisomerase II at interphase (6- to 10-fold lower than in mitosis), the interphase analyses are exposed for much longer times. Thus the presence of weak spots in the G_1 samples may reflect contamination by a small population of cells in G_2 or mitosis, since even a small proportion of highly phosphorylated mitotic topoisomerase II would emit a significant signal. Similarly, the most highly modified phosphopeptide in G_1 (indicated by the larger arrowhead in Figure 3) may be an interphase-specific modification, which is present in the cell population blocked in mitosis as a low level contaminant. The significance of these sites and relative intensities in G_1 and M phases will be discussed in detail below, once the phosphopeptides are identified with respect to the primary protein structure.

Phosphoamino acid analyses of the labelled proteins shows that in both cases the major phosphate-accepting amino acid is serine, although a significant level of phosphothreonine was detected (Figure 3). The proportion of phosphothreonine was reproducibly enhanced at mitosis.

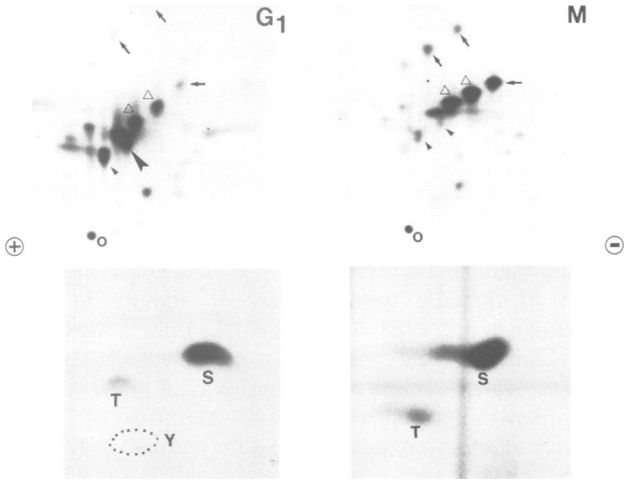

Fig. 3. Phosphopeptide and phosphoamino acid analysis of topoisomerase II immunoprecipitated from cells arrested in G_1 or at mitosis. G_1 or metaphase arrested cells expressing topoisomerase II from the *GAL*1 promoter were labelled with [^{32}P]orthophosphate. Following immunoprecipitation and SDS–PAGE, topoisomerase II was eluted from the gels and subjected to trypsin digestion for phosphopeptide analysis or to acid hydrolysis for phosphoamino acid analysis. Upper panels show the phosphopeptide maps from the G_1 or metaphase (M) arrested cells. Arrows indicate three major mitotic phosphopeptides that correspond to weakly phosphorylated G_1 peptides and arrowheaded spots are major G_1 phosphopeptides that are minor phosphorylation sites in metaphase. The open triangles represent phosphopeptides that are present in both maps at approximately equal intensities. Six-fold less radioactivity was incorporated in topoisomerase II for the G_1 map than for the mitotic map, although both derived from an equal number of cells. For visualization purposes, the autoradiographic exposures were compensated. The symbols + and − indicate electrode positions for migration in the first dimension. The phosphoamino acid analysis from the corresponding samples are shown in the lower panels. S, T and Y indicate the positions of phosphoserine, phosphothreonine and phosphotyrosine as detected by ninhydrin staining of phosphoamino acid standards. o indicates the origin of migration.

Topoisomerase II is phosphorylated in vivo at the same peptides modified by casein kinase II in vitro

Topoisomerase II from both vertebrate and invertebrate organisms has been shown to be a substrate for several kinases *in vitro*. Phosphorylation by CKII (Ackerman *et al.*,

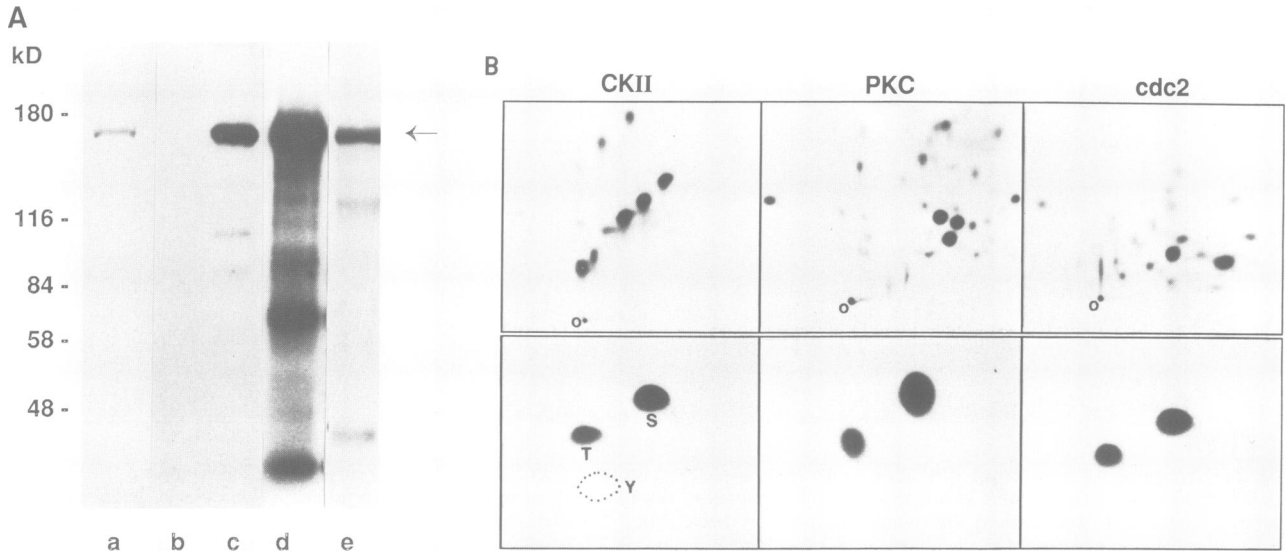

Fig. 4. **(A)** Phosphorylation of yeast topoisomerase II *in vitro*. Glycerol-gradient purified topoisomerase II was incubated with [γ-^{32}P]ATP in the absence (lane b) or presence of casein kinase II (lane c), protein kinase C (lane d) or cdc2 kinase (lane e). Lane a shows the Coomassie blue staining of the glycerol-gradient purified topoisomerase II. Lanes b–e show the autoradiograph of the SDS–PAGE of the phosphorylation products. Low molecular weight phosphorylated polypeptides correspond to autophosphorylation by the kinase preparations. The arrow indicates the position of topoisomerase II. **(B)** Phosphopeptide maps and phosphoamino acid analysis of topoisomerase II following *in vitro* phosphorylation. Topoisomerase II was phosphorylated by casein kinase II (CKII), protein kinase C (PKC) or cdc2 kinase (cdc2), and the ^{32}P-labelled phosphorylation products were separated by SDS–PAGE. Following elution from the gel, topoisomerase II was digested with trypsin and the phosphopeptides were resolved on cellulose TLC plates. Autoradiographs of the resolved peptides are shown in the upper panels; electrophoresis was in the horizontal direction with the anode to the right of the origin (labelled o) and chromatography was from bottom to top. A sample of phosphorylated topoisomerase II eluted from the gel was hydrolysed in 6 M HCl at 110°C for 1 h. Phosphoamino acids were separated on TLC plates by two-dimensional electrophoresis and detected by autoradiography (Cooper *et al.*, 1983). S, T and Y denote the positions of phosphoserine, phosphothreonine and phosphotyrosine, respectively, as determined by ninhydrin stained standards.

1985), protein kinase C and p34^{cdc2} protein kinase has been shown to increase topoisomerase II activity *in vitro* (Ackerman *et al.*, 1985; Sahyoun *et al.*, 1986; Saijo *et al.*, 1990; Cardenas *et al.*, submitted). As part of the strategy to identify which kinases are of physiological relevance, the two-dimensional phosphopeptide patterns of yeast topoisomerase II were determined after phosphorylation by kinases known to stimulate topoisomerase II *in vitro*. These maps were then compared with the tryptic phosphopeptide maps obtained from topoisomerase II labelled in intact cells.

For the *in vitro* mapping topoisomerase II was purified from *S.cerevisiae* as previously described (Worland and Wang, 1989) and was subsequently subjected to glycerol gradient centrifugation to separate the enzyme from a copurifying kinase. This procedure yields a topoisomerase II fraction, free of the copurifying kinase, that has no Coomassie blue stainable contaminants. In yeast the copurifying kinase has been identified definitively as CKII (Cardenas *et al.*, submitted).

The kinase-free topoisomerase II fraction was tested as a substrate for protein kinase C (Marais and Parker, 1989), p34^{cdc2} kinase (Labbé *et al.*, 1989) and CKII (Nakagawa *et al.*, 1989). As shown in Figure 4A, topoisomerase II is a good substrate for all three kinases *in vitro* (Figure 4A, lanes c–e). Each kinase phosphorylates topoisomerase II at both serine and threonine residues, in a ratio of 70:30% (Figure 4B). Phosphorylation of *Drosophila* or calf thymus topoisomerase II by CKII (Ackerman *et al.*, 1988) and protein kinase C (Sahyoun *et al.*, 1986) has been previously reported to occur exclusively on serine residues. This difference may reflect variation between species in either the kinase or in topoisomerase II preparations.

From the phosphopeptide analysis we find that CKII phosphorylates seven major tryptic peptides (Figure 4B). Based on relative mobility in the 2D system, these peptides appear to be unrelated to either the three prominent peptides produced by protein kinase C or the two peptides strongly modified by p34^{cdc2} kinase (Figure 4B). On the other hand, inspection of the *in vivo* topoisomerase II phosphopeptide maps revealed a remarkable similarity to the patterns obtained from topoisomerase II phosphorylated by CKII *in vitro* (compare phosphopeptide maps in Figures 3 and 4B). To assess this similarity more closely, equivalent amounts of trypsin-digested, ^{32}P-labelled topoisomerase II phosphorylated *in vivo* or with CKII *in vitro* were mixed and analysed on TLC plates. With the exception of three strong and two minor peptides (Figure 5, large and small arrows, respectively), the major phosphopeptides obtained from *in vivo* phosphorylation comigrate with the major phosphopeptides that result from phosphorylation with CKII *in vitro*. Similar results were obtained with cells that were arrested in G_1 or metaphase (Figure 5), confirming that the G_1 and M patterns of phosphorylation are closely related, reflecting modification by CKII or a kinase with very similar specificity in both mitosis and in G_1.

Phosphorylation of topoisomerase II is temperature sensitive in a conditional CKII mutant

In order to show that topoisomerase II is modified by CKII itself and not a related kinase of similar specificity, we have tested the incorporation of [^{32}P]phosphate into topoisomerase II in a temperature-sensitive (ts) CKII mutant. CKII from yeast has been purified (Rigobello *et al.*, 1982; Padmanabha and Glover, 1987), partially sequenced

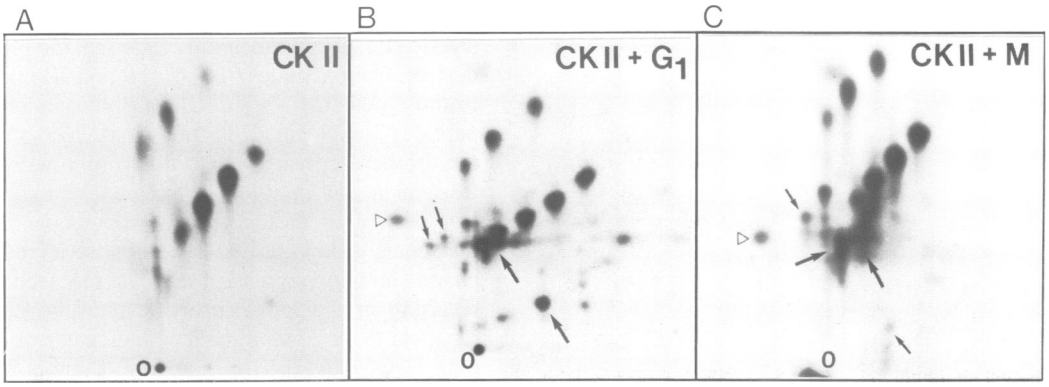

Fig. 5. Comparison of the phosphopeptide maps of topoisomerase II phosphorylated *in vitro* or *in vivo*. Phosphopeptide analysis were carried out as described in Materials and methods. Panel **A** shows the phosphopeptide map of topoisomerase II phosphorylated by CKII *in vitro*. Radioequivalent amounts (by Cerenkov counting) of tryptic digest of topoisomerase II phosphorylated *in vitro* with CKII were mixed with tryptic digests from G_1 (panel **B**) or mitosis (panel **C**) phosphorylated topoisomerase II. Large and small arrows point to major and minor peptides, respectively, that are not phosphorylated by CKII. The open triangle indicates a spot of variable intensity that appears to be free or modified [^{32}P]phosphate. o marks the origin where the sample was applied on the cellulose plate, and migration was as described for Figures 3 and 4.

(Padmanabha and Glover, 1987) and the genes for two distinct α subunits, CKA1 and CKA2, have been cloned. Disruption of either the *CKA1* or *CKA2* gene does not confer any obvious phenotype (Chen-Wu *et al.*, 1988; Padmanabha *et al.*, 1990). However, cells carrying the *cka1 cka2* double disruption are inviable and arrest growth primarily as large budded cells (Padmanabha *et al.*, 1990). Recently, *cka1 cka2* ts mutants were constructed that are lethal at the restrictive temperature (D.E.Hanna and C.V.C.Glover, unpublished) and show very low levels of CKII activity *in vitro* at both permissive and non-permissive temperatures when either topoisomerase II or casein is used as substrate (Cardenas *et al.*, submitted).

The phosphorylation of topoisomerase II was examined in the *cka1 cka2* ts strain YDH8 bearing the topoisomerase II expression plasmid. An exponentially growing culture was divided into two, and half was incubated at 25°C, while the other half was shifted to the non-permissive temperature (37°C) for one hour prior to the addition of galactose. After one hour of induction, cells were labelled with [^{32}P]inorganic phosphate for an additional hour and lysed. While topoisomerase II immunoprecipitated from the *cka1 cka2* ts cells grown at 25°C exhibited approximately normal levels of phosphorylation, topoisomerase II immunoprecipitated from the 37°C shifted cells contained only trace amounts of radioactive phosphate (Figure 6). Coomassie blue staining of topoisomerase II precipitated from non-labelled fractions of the two cultures shows similar amounts of protein at both temperatures (Figure 6), indicating that the decreased ^{32}P signal for topoisomerase II at 37°C reflects a decrease in the [^{32}P]phosphate incorporated in topoisomerase II, rather than a decrease in protein level, in the CKII ts mutant at the restrictive temperature.

In control experiments, when the *S.cerevisiae* strain GA-24 (containing wild-type CKII) was subjected to a temperature shift experiment as described above, the topoisomerase II immunoprecipitated from these cells contained essentially identical amounts of incorporated phosphate at 25 and 37°C (data not shown). Thus, the temperature shift protocol itself does not affect topoisomerase II phosphorylation. Parallel with the loss of phosphorylation in the CKII mutant, we find that the topoisomerase II purified from the *cka1 cka2* ts cells after growth at 37°C has a lower specific activity for decatenation than the enzyme isolated from cells maintained at the permissive temperature (data not shown).

CKII phosphorylation sites are in the C-terminal domain of yeast topoisomerase II

CKII is a threonine and serine specific kinase that does not require a cyclic nucleotide or Ca^{2+} for activity. As deduced from analyses of protein and synthetic peptide substrates, the minimal sequence required for phosphorylation by CKII is an acidic amino acid in the third position following the serine or threonine target residue, Ser(Thr)-X-X-Glu(Asp) (see Pinna, 1990, for a recent review). However, optimal substrates often contain either multiple negatively charged residues (including phosphoserine) or β-turn inducing residues towards the C-terminus. Basic residues at positions −1 or +1 and proline at −1 are disfavoured (Pinna, 1990).

A computer search for the consensus CKII substrate motif identified 23 potential phosphorylation sites in topoisomerase II (indicated by vertical arrows in Figure 7). Several of these phosphorylation sites fall within a single peptide after cleavage by either trypsin or arginine-C (tryptic or arginine-C derived peptides are indicated as open boxes below the potential phosphorylation sites in Figure 7). Thus, the tryptic peptides in the C-terminal third, labelled 6, 8, 8.1, 9 and 10, could all be multiply phosphorylated, producing more than one radiolabelled spot on the 2D analysis. In some cases multiple phosphorylations would occur sequentially, because the modification of a given site creates a CKII recognition site immediately upstream of it (see Pinna, 1990, for a discussion). For example, phosphorylation at site Ser1273 creates a CKII consensus for Ser1270. Once phosphorylated, this serine creates a consensus such that Ser1267 becomes a target site as well.

It is possible to calculate the migration of each phosphorylated peptide in the 2D analysis using a recently published algorithm (Boyle *et al.*, 1991). The relative mobility and charge for each tryptic phosphopeptide in topoisomerase II has been calculated (see Table I) and the resulting prediction for the 2D migration of the CKII-phosphorylated, trypsin-digested peptides from topoisomerase II is shown in Figure 8A. The positions of phosphopeptides 1−5 and 7 are indicated by empty circles in Figure 8A, while the rest are coded as indicated. By

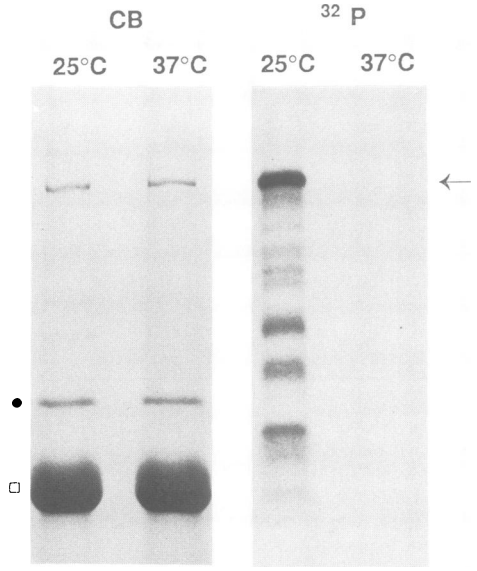

Fig. 6. Phosphorylation of topoisomerase II in a casein kinase II conditional mutant. A culture of YDH8 (*cka1 cka2* ts) bearing the plasmid YEpTOP2-PGAL1 was divided into two halves; one half was incubated at 25°C while the other half was incubated at 37°C. One hour after the temperature shift, 2% galactose was added to induce topoisomerase overexpression and incubation was continued for another hour. The cultures were divided again and [^{32}P]orthophosphate was added to one half, while the other half was not labelled. Topoisomerase II was immunoprecipitated from all cell lysates (each sample derived from approximately the same number of cells) and the Coomassie blue staining (CB; from non-labelled cultures) and the autoradiograph (^{32}P; from ^{32}P-labelled cells) of topoisomerase II immunoprecipitated from the 25°C or 37°C cultures are shown. The position at which topoisomerase II migrates is indicated by an arrow. The major bands below topoisomerase II on the stained gel are bovine serum albumin (●), which is added to saturate the protein A−Sepharose beads, and the heavy chain of the IgG (□). Upon longer exposures of the ^{32}P autoradiograph, background bands of contaminating proteins and a faint band corresponding to topoisomerase II could be seen in the 37°C culture.

comparing the predicted and the actual migration of the tryptic phosphopeptides, it is clear that the phosphopeptides derived from peptides 6, 8, 8.1, 9 and 10 migrate similarly to those detected after CKII phosphorylation. All of these peptides are located in the final 350 amino acids of topoisomerase II in a region that is highly divergent among the sequenced type II topoisomerases (Wyckoff *et al.*, 1989). For several of these peptides, the sequential addition of phosphate groups results in a stepwise increase in negative charge and lowered migration in the second dimension, resulting in a diagonal array of phosphopeptides. This is identical to the predominant peptide patterns observed after both *in vivo* and *in vitro* labelling (Figures 3, 4B and 8B). The only instance in which the predicted and observed migrations appear to differ, is for the three phosphopeptides derived from peptide 10. Because this is the only potential phosphate-accepting peptide that migrates toward the positive electrode, we can definitively assign the three spots labelled C1−C3 to peptide 10. On the other hand, for reasons that are presently unclear, this peptide has a slower mobility in TLC than predicted, placing its series of phosphopeptides nearer the origin (Figure 8, compare peptide 10 spots in panel A with those labelled C1−C3 in panel B).

Phosphoamino acid determination was made on the seven major spots from the tryptic digestion of topoisomerase II (Table II; for groups A and B see Figure 8B). With the exception of the uppermost phosphopeptide in group B (B1), the other six major CKII-dependent spots in the 2D peptide map all consist of both phosphothreonine and phosphoserine. This is consistent with our prediction that they are multiply phosphorylated peptides, containing both serine and threonine residues, and must therefore represent peptides 6, 8, 9 and/or 10. As predicted in Figure 8A, spot B1 contains only phosphothreonine (Table II) and should represent peptide 8 modified on Thr1259. Addition of phosphate groups on Ser1273, Ser1270 and Ser1267, in that order, would account for the descending series of spots in group B.

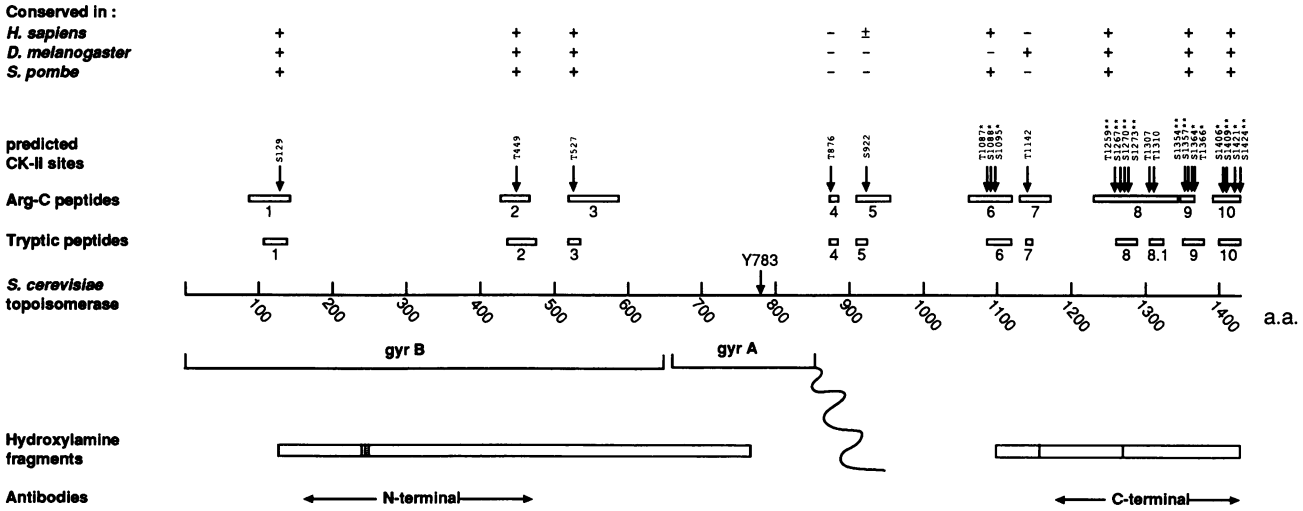

Fig. 7. Schematic representation of yeast topoisomerase II, its phosphopeptides and CKII target sites. Topoisomerase II from *S.cerevisiae* is portrayed linearly as amino acids (aa) 1−1429. The tyrosine at aa 783 contacts DNA during catalysis. Regions of homology to the bacterial gyrase subunits A (*gyr*A) and B (*gyr*B) are shown. The amino acid residues that best fit the CKII consensus for phosphoacceptor sites are indicated by arrows above the map, and the position of the residue corresponding to the target serine or threonine is given. The corresponding tryptic peptides or the arginine-C peptides are indicated as open bars. Single stars indicate sites which are likely to be phosphorylated by CKII *in vitro*; two stars indicate sites that are definitely phosphorylated by CKII. Above the corresponding peptides it is indicated whether or not the target site (or a CKII site in the near vicinity), is conserved (+) or not conserved (−) in topoisomerase II from *Homo sapiens*, *D.melanogaster* or *S.pombe* [based on data from Tsai-Pflugfelder *et al.* (1988) and Wyckoff *et al.* (1989)]. Below the figure are indicated the regions identified by the N-terminal and C-terminal specific antisera, and the possible fragments produced by hydroxylamine cleavage that are recognized by these sera.

While the analyses presented thus far confirm that the modified sites are in the C-terminal third of topoisomerase II, some ambiguity is created by the predicted comigration of phosphopeptides 6 with 9, and phosphopeptides 8 with 8.1 in the tryptic analysis. It seems unlikely that any of the seven major phosphopeptides represents more than a single modified peptide, because we were unable to resolve any of the spots into two phosphopeptides by altering chromatography conditions (data not shown). Nonetheless, to identify the modified peptides positively, we have cleaved CKII phosphorylated topoisomerase II with hydroxylamine and arginine-C, and both SDS gels and 2D peptide analyses were performed on the modified peptides. One-dimensional gel analysis of the arginine-C digestion of CKII-modified topoisomerase II reveals a phosphopeptide at ~13 kDa and a series of peptides of <6 kDa (data not shown). The largest phosphopeptide agrees well with a predicted 12.8 kDa arginine-C peptide (see Figure 7), which contains tryptic peptides 8 and 8.1 and six potential CKII phosphorylation sites. 2D analyses also confirm the observations made from the tryptic maps: multiply phosphorylated forms of peptides 8, 9 and 10 were identified at their predicted sites of migration (data not shown).

Final evidence identifying the location of the phosphorylation sites is provided by immunoprecipitation of CKII-labelled and hydroxylamine-cleaved topoisomerase II with domain-specific antibodies. Topoisomerase II was phosphorylated by CKII and cleaved with hydroxylamine, which preferably cleaves at asparagine–glycine dipeptides, but which will also accept leucine, alanine or methionine in the second position. Hydroxylamine frequently yields partial cleavage, producing a highly complex pattern of fragments from a protein as large as topoisomerase II. Therefore we have used antibodies to immunoprecipitate the phosphopeptides derived from topoisomerase II and to map the phosphorylated polypeptides within topoisomerase II. Antibodies were raised against fusion proteins created from the cloning of either the last 336 aa of yeast topoisomerase II or an internal fragment of 332 aa in frame with the *trpE* protein of *E.coli* (see Materials and methods; regions fused are indicated in Figure 7). The antibody directed against the extreme C-terminus precipitates all major CKII-labelled phosphopeptides, while the antibody specific for the N-terminus precipitates no major fragments (Figure 9). A serum raised against total protein precipitates nearly the same pattern as the anti-C-terminal serum. If our prediction for C-terminal phosphorylation were correct, the anti-C-terminal (C-Ter) antiserum should recognize all phosphate-accepting peptides except peptide 6, which after complete hydroxylamine cleavage would migrate as a fragment of ~6.7 kDa. A weakly phosphorylated polypeptide indicated by the arrow in Figure 9, is left in the supernatant after the anti-C-Ter immmunoprecipitation, and would be consistent with phosphorylation of tryptic peptide 6. Thus all CKII modifications appear to map to sites in the C-terminal 350 aa, as indicated with asterisks in Figure 7. Site-directed mutagenesis is under way to evaluate the physiological effects of altering these residues.

Table I. Tryptic peptides containing putative CKII sites

	Amino acids	M_r (kDa)	R_f	CKII sites
1	104–136	3.85	0.656	Ser129
2	444–476	3.45	0.656	Thr449
3	519–536	2.10	0.613	Thr527
4	872–884	1.50	0.660	Thr876
5	908–926	2.14	0.643	Thr912
6	1085–1121	4.18	0.596 (1)	Thr1087*
			0.593 (2)	Ser1088*
			0.589 (3)	Ser1095*
7	1138–1149	1.47	0.629	Thr1142
8	1259–1289	3.42	0.556 (1)	Thr1259**
			0.552 (2)	Ser1267**
			0.547 (3)	Ser1270**
			0.542 (4)	Ser1273**
8.1	1304–1324	2.20	0.553 (1)	Thr1307
			0.547 (2)	Thr1310
9	1350–1382	3.75	0.606 (1)	Ser1357**
			0.602 (2)	Ser1354**
			0.598 (3)	Ser1364*
			0.594 (4)	Thr1366*
10	1398–1429	3.67	0.586 (1)	Ser1406*
			0.582 (2)	Ser1409**
			0.578 (3)	Ser1421*
			0.573 (4)	Ser1424**

The table lists the tryptic peptides derived from yeast topoisomerase II containing putative CKII target sites based on the consensus S/T-x-x-acidic (see text). The peptides are numbered in order from the N-terminus to the C-terminus. We have named one peptide 8.1, since after arginine-C digestion it remains associated with the tryptic peptide 8; through much of our analysis the two could not be distinguished. The boundaries of the peptides are indicated in column 1, their predicted molecular weights in column 2, and the chromatographic mobilities under the conditions used (Peter *et al.*, 1990) are given in column 3, as calculated by the formulae in Boyle *et al.* (1991). Where more than one residue can be modified, the number of assumed phosphate groups is given after the R_f value. In the last column the putative target for modification is indicated. A double asterisk indicates an optimal target site, and a single asterisk indicates a probable *in vivo* site for which there remains an ambiguity.

Discussion

CKII phosphorylates topoisomerase II in vivo

We have presented both biochemical and genetic evidence that CKII is the major kinase that modifies topoisomerase II in intact yeast cells. It has been shown previously that topoisomerase II is an excellent substrate for CKII *in vitro* (Ackerman *et al.*, 1985; Cardenas *et al.*, submitted). Phosphorylation by CKII is able to stimulate the ATP-dependent decatenation activity of topoisomerase II (Ackerman *et al.*, 1985) and following dephosphorylation modification by CKII is sufficient to restore decatenation activity (Cardenas *et al.*, submitted). Thus it is likely that CKII-modification of topoisomerase II serves to regulate topoisomerase II activity *in vivo*. Earlier Ackerman *et al.* (1988) showed by simple SDS–gel electrophoresis that proteolytic fragments of *Drosophila* topoisomerase II labelled in intact cells, have similar sizes to topoisomerase II modified by CKII *in vitro*. While these one-dimensional gels suggested a role for CKII, we now present precise mapping data of the modified sites and provide genetic confirmation of the major role played by CKII in the regulation of DNA topoisomerase II.

The results presented here, as well as a number of other recent studies, suggest that CKII is activated by, and acts upon, a number of proteins that perform pivotal functions in the regulation of cell proliferation. On the one hand a number of growth factors, including insulin (Sommercorn *et al.*, 1987), insulin-like growth factor (Klarlund and Czech,

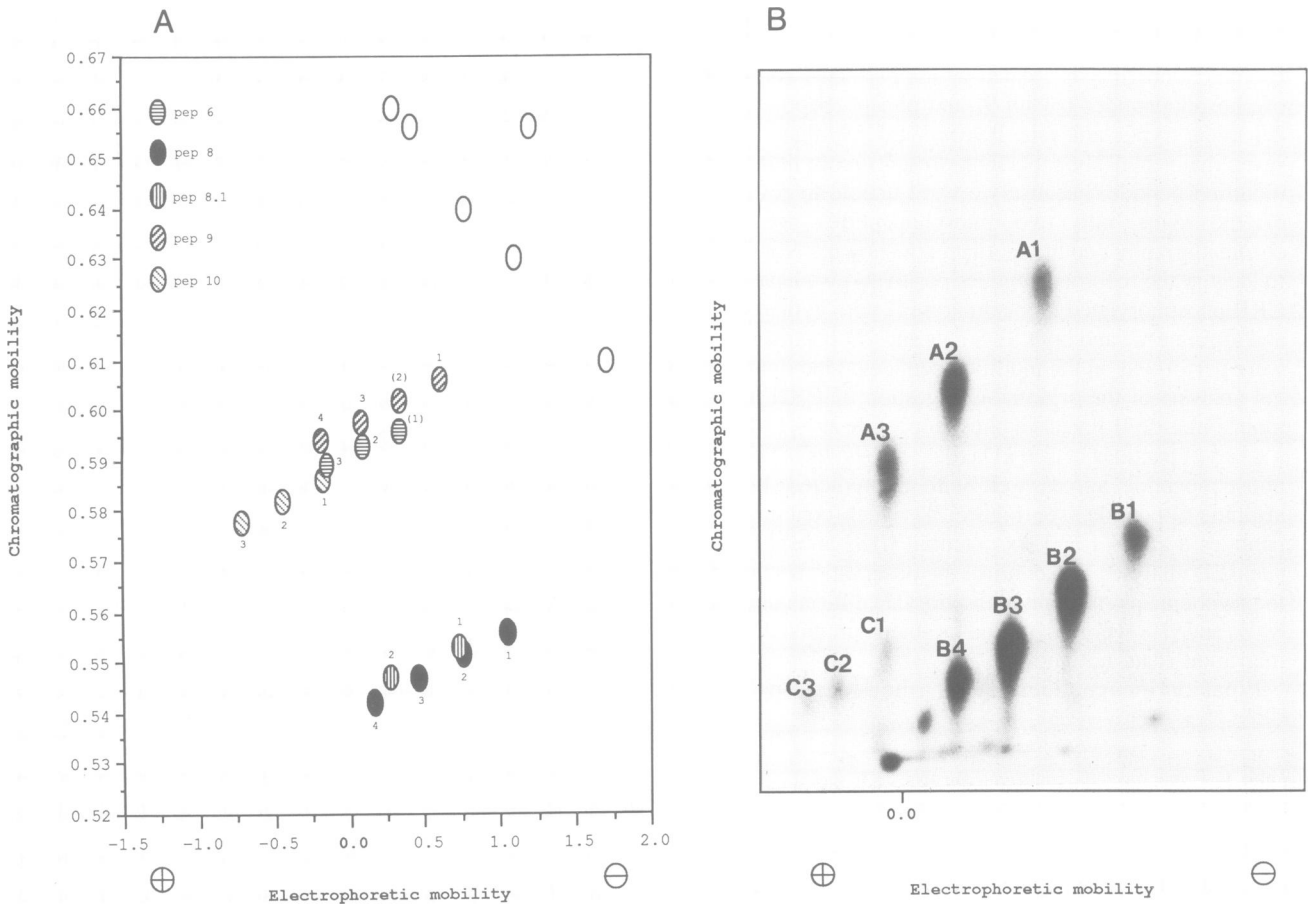

Fig. 8. Predicted and actual 2D phosphopeptide maps after tryptic digestion of yeast topoisomerase II. (**A**) The predicted tryptic fragments of yeast topoisomerase II with CKII acceptor sites (see list in Table I) are plotted here based on their predicted migration in two-dimensional chromatography (Boyle *et al.*, 1991). The empty circles in the upper right hand corner represent peptides 1, 2, 3, 4, 5 and 7, all of which have no corresponding spot in the actual 2D phosphopeptide map. Peptides 6, 8, 8.1, 9 and 10 each contain more than one potential acceptor site, and thus the migration is calculated for the peptide with one, two, three and in some cases four modified residues. The predicted migration of the corresponding peptides are encoded as shown in the upper lefthand corner; the small number adjacent to the spot indicates the number of phosphorylated residues. The small numbers in parentheses indicate spots that appear to be absent from the actual 2D map, shown in panel B. (**B**) Yeast topoisomerase II was labelled with yeast CKII as described in Materials and methods. Elution from the gel and tryptic digestion were performed as in Figure 4B. The origin is indicated by the point labelled 0.0. Identification of spots is given by the letter and number indicated.

1988) and epidermal growth factor (Ackerman *et al.*, 1990), appear to stimulate CKII activity. In yeast the disruption of both of the CKII α subunits is lethal and a synchronous culture of the *cka*1 *cka*2 ts mutant used in this study arrests at the non-permissive temperature either as large budded cells or as unbudded cells, depending on the point of the switch to non-permissive temperature (C.V.C.Glover, unpublished observations). The large budded cell phenotype is consistent with a G_2-M arrest point, which may in part be due to the requirement that CKII activate topoisomerase II, which in turn is required for the proper traversal of mitosis.

Other cellular targets of CKII are also nuclear proteins intimately associated with cell division processes. A number of nuclear-localized oncogene-encoded proteins, including c-myb, c-myc, c-fos, E1A, the HPV E7 protein and SV40 large T antigen have been reported to contain CKII recognition sites (Meisner and Czech, 1991). Most of these have been demonstrated to be substrates *in vitro*; for c-myb (Lüscher *et al.*, 1990), c-myc (Lüscher *et al.*, 1989) and HPV E7 (Barbosa *et al.*, 1990), the *in vitro* phosphorylated sites have been shown to be phosphoacceptor sites in intact cells. Two other targets implicated in growth control and known to be physiological substrates are the anti-oncogene p53 (Meek *et al.*, 1990) and the serum response factor, which binds to the serum response element upstream of c-*fos* to activate c-*fos* expression (Manak *et al.*, 1991). Taken

Table II. Phosphoamino acid analysis of tryptic phosphopeptides containing CKII sites

Peptide	%PSer	%PThr
A1	73	27
A2	65	35
A3	49	51
B1	<1	>99
B2	45	55
B3	71	29
B4	63	27

Results are shown from phosphoamino acid analysis of the major phosphopeptides after CKII labelling and tryptic digestion of yeast topoisomerase II. The hydrolysed phosphoamino acids were separated by TLC; commercial standards were used to identify phosphothreonine and phosphoserine. The relative intensities were quantified by densitometric scanning using a Herschman Elscript 400. A1–3 and B1–4 correspond to the phosphopeptides indicated in the tryptic map shown in Figure 8B.

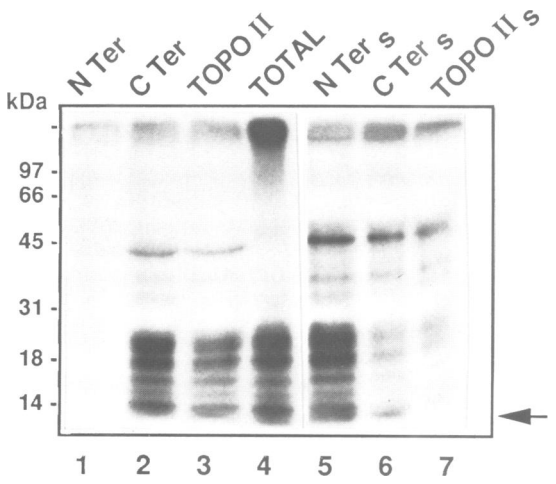

Fig. 9. An antiserum against the extreme C-terminus of topoisomerase II precipitates the major CKII modified fragments after hydroxylamine cleavage. Yeast topoisomerase II was labelled with yeast CKII, eluted from the gel and cleaved by hydroxylamine as described in Materials and methods. Immunoprecipitation was performed using sera raised against the C-terminus (C Ter), the N-terminus (N Ter) or intact topoisomerase II (TOPO II). The domain specific sera have been shown to react with the region used as antigen and precipitate the appropriate Coomassie Blue stainable bands (data not shown). Shown is the autoradiogram of a 12% SDS gel which separates the topoisomerase II peptides immunoprecipitated with the indicated sera. Lane 1, precipitation with anti-N-Ter; lane 2, precipitation with anti-C-Ter; lane 3, precipitation with antiTopoII; lane 4, total labelled and cleaved topoisomerase II. Lanes 5–7 are the supernatants of lanes 1–3. The molecular weight markers are indicated to the left of the gel and an arrow indicates the potential 6.7 kDa hydroxylamine cleavage fragment that is not precipitated by the anti-C-Ter serum, probably containing tryptic peptide 6.

together these data suggest that signal transduction pathways initiated by growth factors may activate CKII to phosphorylate nuclear proteins involved in cell division processes. Thus CKII may play a complementary role to that of p34^{cdc2} kinase in the control of cell proliferation.

CKII modifies the C-terminal domain of topoisomerase II

Two-dimensional phosphopeptide mapping has allowed us positively to identify most of the CKII phosphorylation sites and to localize them to the extreme C-terminal 350 aa of DNA topoisomerase II. The highly conserved regions of the enzyme comprise nearly 1000 aa at the N-terminus, and these sequences show high homology with the two subunits of bacterial gyrase (Figure 7; Wyckoff *et al.*, 1989). None of the potential acceptor sites in the N-terminal two-thirds of the protein are modified by CKII. These observations suggest that the C-terminal domain with its multiple phosphoacceptor sites defines a regulatory domain that has evolved in eukaryotic DNA topoisomerase II to permit a degree of regulation that is absent and presumably unnecessary in prokaryotic gyrase.

The amino acids identified as phosphoacceptors are indicated by double asterisks in Table I and in Figure 7. Single asterisks represent sites that are probably modified, but about which there remains some ambiguity. Despite the general divergence in the C-terminus among eukaryotic topoisomerase II coding regions, it is noteworthy that in the vicinity of each C-terminal CKII site, similar sites are conserved in the *Schizosaccharomyces pombe*, *Drosophila* and human enzymes (Figure 7). This would suggest that the regulation of topoisomerase II by CKII is characteristic of a large variety of eukaryotic organisms.

Truncation of the *S.cerevisiae TOP*2 gene at aa 979, which removes all of the major CKII phosphoacceptor sites, renders the enzyme unable to complement a *S.cerevisiae top*2 ts mutant, even though the truncated form of the enzyme contains all regions of homology to gyrase A and B subunits including the active site tyrosine residue (M.E.Cardenas, unpublished results). This is consistent with the results of Shiozaki and Yanagida (1991), who found that a truncation of the *S.pombe* topoisomerase II at aa 922 was inviable in the absence of the genomic *top*2$^+$ gene. On the other hand, truncation of the *S.pombe* topoisomerase II at aa 1198 allowed complementation of the *top*2 gene disruption (Shiozaki and Yanagida, 1991). This truncation leaves intact potential CKII target sites in the region corresponding to the *S.cerevisiae* peptide 6 (see Figure 7). It is not known, however, whether these acceptor sites are modified in fission yeast.

Phosphorylation by CKII varies through the cell cycle

Two-dimensional phosphopeptide analysis has shown that the phosphorylation patterns of topoisomerase II in G_1 phase and at mitosis are related, but not identical. Among major phosphate accepting peptides *in vivo*, all but two comigrate with those phosphorylated by purified CKII *in vitro*. These two exceptions (indicated by arrowheads in Figure 3) are major phosphoacceptor sites in G_1, yet are also modified weakly in metaphase. Although in any cell cycle arrest study one risks provoking non-physiological effects by arresting the cell cycle, there are several reasons why we believe that these results are physiologically significant. First of all, in a non-synchronized culture we also see that the 2D tryptic phosphopeptide map corresponds well to the CKII pattern (M.E.Cardenas, unpublished). Secondly, the increase in mitotic labelling was previously observed using elutriated chicken lymphoblastoid cells (Heck *et al.*, 1989), suggesting that the mitotic increase is not an artefact of the nocodazole treatment. Finally, a CKII-like pattern of phosphopeptides was obtained from a [^{32}P]phosphate-labelled culture of *cdc*28-1, a strain that blocks in G_1 upon temperature shift, suggesting that the observed modifications are G_1 specific and not due to α-factor arrest.

The most highly labelled phosphopeptides in metaphase coincide with CKII target sites, but are not identical to the most highly labelled CKII-modified phosphopeptides in G_1. For instance, the modification of peptide 9 on Ser1354, 1357, 1364 and Thr1366 is highly enriched in mitosis and is thus probably metaphase-specific. The modification of peptide 8 presents a more complicated cell cycle pattern: in α-factor arrest the modified peptides have three or four modified residues, instead of one or two as seen in metaphase, despite the fact that the overall level of topoisomerase II phosphorylation is lower in G_1. This suggests that fewer topoisomerase molecules are modified in G_1, but those that are have more residues modified in this particular region (aa 1259, 1273, 1270 and 1267, forming spots B3 and B4). This could be explained by a slight change in conformation or in availability of the sites for modification in metaphase. In any case, since quantification of $^{32}PO_4$ incorporation shows that there is a 6- to 10-fold increase in radioactivity

incorporated in topoisomerase II in metaphase, and since only a few additional target sites (e.g. on peptides 9 and/or 6) are modified, there must also be an increase in the number of modified topoisomerase II molecules. This may reflect a preference for phosphorylation of newly synthesized topoisomerase II, which in yeast shows an S-phase specific peak in transcription. Since no other metaphase-specific modifications were observed, our results suggest that the mitotic increase in phosphate incorporation is due primarily to a quantitative increase (number of molecules modified) and secondly to a qualitative variation (shift of sites modified), of CKII phosphorylation.

The metaphase-specific increase in CKII phosphorylation of topoisomerase II may either reflect an enhanced kinase activity or a G_2-M-specific inactivation of a phosphatase. There is no clear documentation of cell cycle variation in either CKII or phosphatase activities in the literature to date. The former possibility is supported, however, by the recent report that $p34^{cdc2}$ kinase phosphorylates the β subunit of CKII and stimulates the kinase activity *in vitro* (Mulner-Lorillon *et al.*, 1990).

A role for other kinases?

From the *in vivo* analysis we identify at least two significant phosphoacceptor sites that do not correspond to CKII sites observed *in vitro*. These probably represent acceptor sites for another kinase. *In vitro* topoisomerase II is a substrate for a variety of kinases, and in all cases the modification has a stimulatory effect on the decatenation activity.

Two explanations may account for the fact that these additional sites show no significant phosphorylation in the *cka*1 *cka*2 ts mutant at the non-permissive temperature. First, CKII may be required to modify and activate a second kinase. Alternatively, the CKII modification may be required to render topoisomerase II a proper substrate for the second kinase. Glycogen synthase (GS) provides a precedent for this model: phosphorylation by CKII does not directly activate this enzyme, but is a prerequisite for modification of GS by the glycogen synthase kinase III, which triggers the critical regulatory event (Picton *et al.*, 1982). One intriguing possibility is that CKII phosphorylation of topoisomerase II is a necessary prerequisite for modification by a cell cycle specific kinase, such as the $p34^{cdc2}$ kinase. We have shown that yeast topoisomerase II as isolated is a good substrate for $p34^{cdc2}$ kinase (Figure 4B). The potential modification by $p34^{cdc2}$ is likely to be specific for the G_2-M transition and might alter topoisomerase II to promote chromatin condensation into metaphase chromosomes. Previous studies show that the topoisomerase II recovered in the mitotic chromosomal scaffold of HeLa cells is highly phosphorylated, although it is not known on which residues these modifications occur (S.M.Gasser and U.Laemmli, unpublished).

In view of the evidence implicating topoisomerase II in metaphase chromosome organization, it is tempting to speculate that the hyperphosphorylation of the C-terminal portion by CKII might aid in the cell cycle regulated condensation of chromatin. Several cold-sensitive mutants in yeast topoisomerase II map to this C-terminal region, although none of those sequenced to date affects a phosphoacceptor site (Thomas *et al.*, 1991). The development of an *in vitro* assay for chromosome condensation now allows us to test whether the modifications of the C-terminus influence the efficiency with which topoisomerase II catalyzes the condensation of chromosomes *in vitro* (Adachi *et al.*, 1991).

Materials and methods

Strains and plasmids

The strains used are GA-24 (MAT**a**, *ura*3, *his*, *bar*1, *suc*2-9, *pep*4-3, *GAL*, provided by H.Riezman) and YDH8 [MAT**a**, *cka*1::*HIS*3, *cka*2::*TRP*1, *ura*3, pDH8 (*CEN6/ARS*H4 *cka*2-8^{ts}) (D.E.Hanna and C.V.C.Glover, unpublished)]. The plasmid YEpTOP2-PGAL1 was generously given by J.Wang (Giaever *et al.*, 1988). The *pep*4-3 mutation was used because topoisomerase II is particularly susceptible to proteolysis after cell lysis. We have observed no secondary effects of the *pep*4-3 mutation in labelling studies.

Topoisomerase II antibody production

For an antibody against the entire enzyme, topoisomerase II was purified to apparent homogeneity from yeast carrying the overexpression plasmid YEpTOP2-PGAL1. Two hundred micrograms of purified protein was separated from potential contaminants in a preparative 8% polyacrylamide gel and stained with Coomassie blue in the absence of acetic acid. The only major band (migrating at 170 kDa) was excised and electroeluted from the gel in SDS–PAGE running buffer (Laemmli, 1970) at 70 V for 3 h. The eluted protein was emulsified with complete Freund's adjuvant containing 100 mM KCl. The injection and bleeding schedule was as described (Daum *et al.*, 1982). For immunofluorescence, topoisomerase II specific antibodies were affinity purified as described (Gasser *et al.*, 1986), while for immunoprecipitation the total antiserum was used.

The production of antibodies against specific domains of topoisomerase II required the expression of *trpE* fusion proteins in which either an *Eco*RI–*Sau*IIIA fragment of the *TOP*2 gene encoding aa 156–488, or a *Hin*dIII–*Bam*HI fragment extending from aa 1195 to the end of the gene, were inserted in frame downstream of the bacterial *trpE* gene (Spindler *et al.*, 1984). The fusion proteins were purified as inclusion bodies, excised from SDS gels and injected as described above. The sera were used directly for immunoprecipitation as described above.

Metabolic labelling

For ^{32}P-labelling, cells carrying the topoisomerase II expression plasmid YEpTOP2-PGAL1 were grown in synthetic media lacking uracil, supplemented with 3% glycerol and 2% lactic acid. From this medium phosphate had been precipitated as indicated by Rubin (1975). Cultures in exponential growth were diluted 5-fold into the same medium. When the cultures reached a density of 10^7 cells/ml they were harvested by centrifugation and 3×10^8 cells were resuspended in 10 ml of the conditioned medium containing 2% galactose. Following 1 h of galactose induction, 5 mCi of [^{32}P]orthophosphate (Amersham) were added and growth was continued for 1 h. Cells were collected by centrifugation and lysed for immunoprecipitation as described below. For cell cycle arrest in G_1 or at mitosis, α-factor (1 μg/ml, kindly provided by Howard Riezman) or nocodazole (15 μg/ml and 1% dimethyl sulphoxide) respectively, were added to the cultures 1.5 h prior to galactose induction, and were present during the labelling period. Following incubation with the inhibitors, the percentage of unbudded (G_1-arrested) or large budded (metaphase-arrested) cells was $\geq$85%. If this was not the case, the cultures were not used for the labelling study.

For [^{35}S]sulphate-labelling, the same culture manipulations were performed except that cells were grown in selective synthetic media lacking uracil and low in sulphate (Douglas *et al.*, 1979) supplemented with 0.3% glucose, 3% glycerol, 2% lactic acid. 30 min prior to galactose addition, cells were labelled with 1 mCi [^{35}S]sulphate (Amersham).

For temperature shift experiments in the *cka*1 *cka*2 ts mutant strain YDH8, cultures were divided in two. One half was incubated at 37°C for 1 h prior to galactose induction, and the other at 25°C. Topoisomerase II was induced for 1 h and then half of each culture was labelled with [^{32}P]phosphate as described above. The 3 h period required for the arrest and labelling is less than one generation time for *cka*1 *cka*2 ts mutant at 25°C in the media used, so the cell numbers remain roughly the same. The non-labelled cultures were processed and used for immunoprecipitation in parallel to the ^{32}P-labelled fractions, in order to quantify topoisomerase II levels under the conditions used. We show in Figures 1 and 6 that our standardized immunoprecipitation procedure precipitates equal amounts of topoisomerase II, which we must assume is representative of the topoisomerase II population in the cell. Thus small variations in the numbers of cells used for each sample are of no consequence.

Indirect immunofluorescence

Immunofluorescence was performed, as described in Klein *et al.* (1992), on cells grown and induced identically to the labelled cultures except that no radioactivity was added to the culture. Photography was done using a Bio-Rad confocal image processing system.

Immunoprecipitation

Radiolabelled cultures were centrifuged and the cell pellets were washed once with cold water. Cell pellets were resuspended in the same volume (usually 150–200 μl) of ice-cold lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA) containing protease inhibitors (1 mM phenyl methyl sulphonylfluoride, 5 μg/ml pepstatin, 100 U/ml aprotinin) and phosphatase inhibitors (30 mM sodium pyrophosphate, 5 mM β-glycerolphosphate). Cells were disrupted by vigorous vortexing with 0.5 mm acid washed glass beads (Sigma) and lysed by addition of 1% SDS and incubation 3 min at 95°C. Cell lysates were diluted 10-fold with lysis buffer plus detergents (1% Nonidet-P40, 0.5% deoxycholate, sodium salt) and clarified by centrifugation for 30 min in an Eppendorf microfuge. For immunoprecipitation, the cell lysates were incubated for 1 h at 4°C with preimmune antibodies attached to protein A–Sepharose (PAS). After a brief spin to eliminate the beads, anti-topoisomerase II antiserum was added and incubation continued for another hour. Immunocomplexes were collected by addition of PAS and subsequent centrifugation, and were digested with 2 μg/ml RNase A in lysis buffer for 30 min at room temperature. The agarose beads were washed four times in lysis buffer plus detergents, twice in lysis buffer plus detergents supplemented with 2 M urea and 1% β-mercaptoethanol, and once in 10 mM Tris–HCl, pH 7. The immunocomplexes were eluted from the PAS beads by incubation for 5 min at 95°C in 4-fold concentrated PAGE sample buffer and subjected to SDS–PAGE. Dried gels were exposed to Kodak XAR-5 films.

Phosphorylation reactions

In vitro phosphorylation reactions were carried out with the glycerol gradient-purified topoisomerase II, which is devoid of copurifying kinase activity (see Cardenas *et al.*, submitted). CKII purified from chicken embryonic nuclei (Nakagawa *et al.*, 1989) was kindly provided by Dr J.Nakagawa. Protein kinase C purified from bovine brain (Marais and Parker, 1989) was a gift of Dr P.Parker. Purified cdc2 kinase, consisting of $p34^{cdc2}$–cyclin B in a 1:1 complex that was isolated from starfish by affinity chromatography on p13-coated beads (Labbé *et al.*, 1989) was provided by Dr M.Dorée.

The kinase assay buffers were as follows. For CKII: 50 mM Tris–HCl pH 7.4, 150 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 10 μM ATP; for protein kinase C: 20 mM HEPES–NaOH pH 7.4, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 μg/ml decaproin (Sigma), 5 μM ATP; for cdc2 kinase: 50 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$, 6 mM EGTA, 10 μM ATP. The reactions were incubated for 15 min at 30°C in a total volume of 20 μl of kinase buffer containing 5 μCi of [γ-^{32}P]ATP (Amersham), stopped by addition of SDS-sample buffer, and the products were separated by SDS–PAGE (Laemmli, 1970).

Two-dimensional tryptic phosphopeptide and phosphoamino acid analysis

These analyses were carried out as described by Beemon and Hunter (1978) and Cooper *et al.* (1983) with minor modifications (Peter *et al.*, 1990). Hydroxylamine cleavage was performed as follows. After labelling 10 μg of purified yeast topoisomerase II by reaction with CKII for 45 min at 30°C in a total volume of 20 μl of kinase buffer containing 5 μCi of [γ^{32}P]ATP (Amersham), the sample was diluted by addition of 0.5 ml 6 M guanidine–HCl and 2 M hydroxylamine–HCl, adjusted to pH 9.0 with saturated LiOH. The reaction was incubated for 4 h at 45°C, and then 50 μl of 100% formic acid were added. The labelled topoisomerase II fragments were recovered from a NAP-5 column (Pharmacia) and the protein-containing fractions were lyophilized. The lyophilized material was either dissolved in SDS loading buffer for gel electrophoresis or in lysis buffer with detergents (see above), containing protease and phosphatase inhibitors for immunoprecipitation.

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