PHOSPHORYLATION OF DNA TOPOISOMERASE I:
ROLE IN MAMMALIAN CELL SIGNAL TRANSDUCTION

by

Scott Samuels

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF MOLECULAR AND CELLULAR BIOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1991
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Phosphorylation of DNA topoisomerase I: Role in mammalian cell signal transduction

Samuels, Scott, Ph.D.
The University of Arizona, 1991
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As members of the Final Examination Committee, we certify that we have read
the dissertation prepared by D. Scott Samuels
entitled Phosphorylation of DNA Topoisomerase I:
Role in Mammalian Cell Signal Transduction

and recommend that it be accepted as fulfilling the dissertation requirement
for the Degree of Doctor of Philosophy.

Nobuyoshi Shimizu 2/8/91
Date

Thomas Lindell 2/8/91
Date

H. V. Aposhian 2/8/91
Date

Jennifer Hall 2/8/91
Date

Final approval and acceptance of this dissertation is contingent upon the
candidate's submission of the final copy of the dissertation to the Graduate
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I dedicate this dissertation in honor of Min Sobel and in memory of Sarah Samuels, Sid Samuels and Abe Sobel.

Life may be sweeter for this....
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The tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) induces an altered program of gene expression, followed by DNA synthesis and cell division, in quiescent murine fibroblasts. The phosphorylation of DNA topoisomerase I, a nuclear enzyme involved in transcription and replication, was characterized in quiescent 3T3-L1 cells treated with TPA. An anti-DNA topoisomerase I antibody from sera of patients with the autoimmune disease diffuse scleroderma was used to isolate phosphorylated DNA topoisomerase I.

Phosphorylation was stimulated slightly within 10 min and 6-fold by 2 h of TPA treatment; TPA at 100 ng/ml maximally enhanced phosphorylation. DNA topoisomerase I was modified primarily on serine, with a minor phosphothreonine component. The half-life of incorporated phosphate on DNA topoisomerase I was approximately 40 min in both TPA-treated and control cells, suggesting that stimulation of a kinase was the mechanism for increased phosphorylation. In addition, the phosphorylation of DNA topoisomerase I was enhanced in rat H-35 hepatoma cells treated with insulin as well as Swiss/3T3 fibroblasts treated with epidermal growth factor.

DNA topoisomerase I phosphorylation in vitro by protein kinase C, the cellular receptor for TPA, was also characterized. DNA topoisomerase I from HeLa cells was purified to apparent homogeneity and was phosphorylated in a Ca\(^2\)\(^+\) and phospholipid-dependent fashion by type III protein kinase C, purified 860-fold from mouse brain; the reaction was stimulated by TPA. Approximately 1.3 moles of phosphate were incorporated per mole of substrate, predominately on a tryptic peptide that comigrated with the major in vivo phosphopeptide, although other sites were phosphorylated to a lesser extent. Serine was the primary amino acid modified, however phosphothreonine was also detected. The incorporation of phosphate into DNA topoisomerase I was linear in the range of time and protein kinase C examined. The apparent \(K_m\) and \(V_{max}\) for the reaction were 0.4 \(\mu\)M and 0.7 \(\mu\)mol phosphate per min per mg, respectively. Thus, phosphorylation, possibly mediated by
protein kinase C, was postulated to be a physiologically significant means of regulating DNA topoisomerase I during mammalian cell proliferation.
INTRODUCTION

Phosphorylation is an important and ubiquitous mechanism for transducing intracellular signals; this posttranslational modification often modulates the activity of the substrate. We have studied the phosphorylation state of DNA topoisomerase I, a nuclear enzyme involved in a myriad of DNA-related processes, during the induction of mammalian cell growth by a phorbol ester tumor promoter, in order to elucidate the early events involved in signal transduction. Our results demonstrate that phosphorylation of DNA topoisomerase I, which is known to activate the enzyme, is rapidly enhanced after the stimulation of cell proliferation and that protein kinase C, a central component of signal transduction networks, can directly phosphorylate DNA topoisomerase I \textit{in vitro}. These results suggest that phosphorylation may be the mechanism for activating DNA topoisomerase I \textit{in vivo} and that protein kinase C may function, possibly directly, in the phosphorylation of DNA topoisomerase I during the induction of mammalian cell growth.

Topoisomerases have been implicated in nearly every aspect of the macromolecular metabolism of DNA: replication, transcription, recombination, repair, nucleosome assembly and chromosome segregation (Liu, 1983; Vosberg, 1985; Wang, 1985; Wang, 1987; Liu, 1990; Wang \textit{et al.}, 1990). These nuclear enzymes regulate the conformation of DNA by transiently nicking one or both strands of the double helix (Liu, 1983; Vosberg, 1985; Wang, 1985). This function is necessary to resolve the topological problems that develop during transcription and replication (Wang, 1987; Liu, 1990). The type I topoisomerase breaks and rejoins one strand of the double helix, thus catalyzing conformational transformations including the conversion of supercoiled DNA to its relaxed form (Wang, 1971; Champoux and Dulbecco, 1972; Keller, 1975; Liu and Miller, 1981; Liu, 1983; Vosberg, 1985; Wang, 1985). Although the physiological role of DNA topoisomerase I has not been fully clarified, the
enzyme is thought to modulate local supercoiling during RNA transcription (Liu and Wang, 1987; Zhang et al., 1988) and DNA replication (Champoux and Dulbecco, 1972; Zeng et al., 1985; Yang et al., 1987).

DNA topoisomerase I is associated with transcriptionally active nucleosomes as isolated by chromatography on a high-mobility-group protein column (Weisbrod, 1982). DNA topoisomerase I is also associated with actively transcribed Drosophila heat shock genes as detected by immunocytochemistry (Fleischmann et al., 1984), photo-crosslinking (Gilmour et al., 1986) and camptothecin-induced cleavage (Gilmour and Elgin, 1987). Camptothecin, an inhibitor of DNA topoisomerase I that stabilizes the covalent protein-DNA reaction intermediate (Liu, 1990), has also been used to demonstrate the presence of DNA topoisomerase I during transcription of the mammalian genes for tyrosine aminotransferase (Stewart and Schütz, 1987), ribosomal RNA (Zhang et al., 1988) and c-fos (Stewart et al., 1990). Other studies have localized DNA topoisomerase I to the ribosomal RNA genes of Tetrahymena (Bonven et al., 1985), chicken (Muller et al., 1985; Muscarella et al., 1987), Xenopus (Culotta and Sollner-Webb, 1988), Dictyostelium (Ness et al., 1988) and rat (Rose et al., 1988). Ribosomal RNA synthesis is blocked by camptothecin in vitro (Garg et al., 1987) as well as anti-DNA topoisomerase I antibodies in vivo (Egyházi and Durban, 1987) and in vitro (Rose et al., 1988).

In addition to the apparent role DNA topoisomerase I has in the transcription of eukaryotic genes, it also can promote replication of adenovirus DNA (Nagata et al., 1983) and simian virus 40 DNA (Yang et al., 1987) in cell free systems. Camptothecin induces cleavage of simian virus 40 DNA at replication forks (Snapka, 1986; Avemann et al., 1988) and DNA synthesis is arrested during the early phase of elongation in a temperature-sensitive DNA topoisomerase I mutant of BALB/3T3 cells (Zeng et al., 1985). The activities of topoisomerases increase when cells are stimulated to proliferate or as they traverse the cell cycle (Poccia et al., 1978; Miskimins et al., 1983; Duguet et al., 1983; Taudou et al., 1984; Tricoli et al., 1985; Kafiani et al., 1986; Sullivan et al., 1986; Zwelling et al., 1987;
Nelson et al., 1987; Roca and Mezquita, 1989). Therefore DNA topoisomerase I is presumably regulated during mammalian cell proliferation, a cascade of events that requires coordinated expression of certain genes and replication of the genome.

The phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) is a potent tumor promoter that induces numerous metabolic and biochemical changes in cultured fibroblasts, including gene expression, DNA synthesis and cell division (Dicker and Rozengurt, 1978; Shimizu et al., 1986; Rozengurt, 1986; Angel et al., 1987; Herschman, 1989). Protein kinase C, the Ca\(^{2+}\)/phospholipid-dependent serine/threonine protein kinase, is directly activated by TPA (Castagna et al., 1982). In fact, protein kinase C is the major receptor for TPA (Niedel et al., 1983; Leach et al., 1983; Kikkawa et al., 1983) and is thought to mediate the cellular response to phorbol ester tumor promoters (Nishizuka, 1984a; Ashendel, 1985). Diacylglycerol, a product of phosphatidylinositol turnover generated after the binding of certain growth factors, also activates protein kinase C (Nishizuka, 1984b; Berridge, 1984). The exact nature of the signal transduction to the nucleus is not known. Treatment of cells with TPA results in the translocation of some protein kinase C to the nucleus (Halsey et al., 1987; Thomas et al., 1988; Shimizu and Shimizu, 1989; Leach et al., 1989) and the phosphorylation of histones H1, H2B and H4 (Patskan and Baxter, 1985; Butler et al., 1986), nuclear matrix proteins NP80 and NP33 (Macfarlane, 1986), DNA topoisomerase II (Rottmann et al., 1987), lamin B (Hornbeck et al., 1988; Fields et al., 1988) and the thyroid hormone receptor (Goldberg et al., 1988). In addition, many nuclear proteins are phosphorylated \textit{in vitro} by protein kinase C, including histone H1 (Iwasa et al., 1980; Schatzman et al., 1983; Sahyoun et al. 1983), high-mobility-group proteins 1, 2, 14 and 17 (Ramachandran et al., 1984; Kimura et al., 1985), the transcription factor CREB (Yamamoto et al., 1988), lamin B (Hornbeck et al., 1988) and nuclear protein P1 (Walaas et al., 1989) as well as the enzymes DNA topoisomerase II (Sahyoun et al., 1986; Rottmann et al., 1987), DNA methyltransferase (DePaoli-Roach et al., 1986), DNA polymerase \(\alpha\) (Krauss et al., 1987), RNA polymerase II (Chuang et al., 1987) and DNA topoisomerase I (Samuels et al., 1988; Samuels et al., 1989;
Protein kinase C has an apparently central function in mammalian cell signal transduction; its activation constitutes an important step in the induction of cell proliferation, secretion and exocytosis, ion and sugar transport, smooth muscle contraction and relaxation, and other cellular responses (Nishizuka, 1984a; Kuo et al., 1984; Bell, 1986; Nishizuka, 1986). Protein kinase C consists of a family of proteins with at least seven distinct members, encoded by at least six discrete genes, that have different patterns of tissue expression and slightly different enzymatic properties (Nishizuka, 1988). The activities of protein kinase C \( \gamma \) (type I), \( \beta \), and \( \beta_\alpha \) (type II) and \( \alpha \) (type III) are dependent on \( \text{Ca}^{2+} \). Type I protein kinase C is expressed in the brain and spinal cord only while type II protein kinase C is found in many tissues and cell types. The type III subspecies is expressed universally and has been detected in the nucleus as well as the cytoplasm and plasma membrane of murine fibroblasts (Shimizu and Shimizu, 1989; Leach et al., 1989). Its activation in quiescent cells by diacylglycerol or TPA results in the phosphorylation of several substrates on serine and threonine residues, followed by a program of gene expression, DNA synthesis, mitosis and cell division.

The activities of eukaryotic topoisomerases are regulated by transcriptional and posttranslational mechanisms. The steady-state amount of DNA topoisomerase I mRNA is increased after treatment of quiescent fibroblasts with TPA (Hwong et al., 1989) or fresh serum and is due to an elevated rate of transcription (Romig and Richter, 1990). Noncovalent interactions include the stimulation of DNA topoisomerase I activity in vitro by histone H1 (Rowe et al., 1981; Ross et al., 1983; Javaherian and Liu, 1983), high-mobility-group proteins 1, 2 and 17 (Javaherian and Liu, 1983) and adenovirus E1A protein (Chow and Pearson, 1985) as well as its inhibition by ATP (Rowe et al., 1981; Castora and Kelly, 1986) and single-stranded DNA (Tricoli and Kowalski, 1983). Poly(ADP-ribosylation) (Ferro et al., 1983; Jongstra-Bilen et al., 1983; Ferro and Olivera, 1984; Kasid et al., 1989) and phosphorylation on tyrosine residues (Tse-Dinh et al., 1984) also inhibit DNA topoisomerase I.

We hypothesized that DNA topoisomerase I would be phosphorylated during the induction of mammalian cell growth because DNA topoisomerase I has been implicated in transcription and replication, nuclear processes that are required during cell proliferation, and because DNA topoisomerase I is activated, as previously demonstrated *in vitro*, by phosphorylation. This dissertation describes the modulation of the phosphorylation state of DNA topoisomerase I *in vivo* (Samuels and Shimizu, manuscript in preparation) as well as the characterization of DNA topoisomerase I phosphorylation catalyzed *in vitro* by protein kinase C (Samuels and Shimizu, manuscript submitted). The data suggest that phosphorylation is a physiological means of activating DNA topoisomerase I during signal transduction for proliferation in cultured mammalian cells.
CHAPTER 2

EXPERIMENTAL PROCEDURES

Materials

Calf thymus DNA topoisomerase I was from BRL (Gaithersburg, MD). Epidermal growth factor (EGF) was from Collaborative Research (Bedford, MA), 12-O-tetradecanoylphorbol-13-acetate (TPA, phorbol-12-myristate-13-acetate) was from Sigma (St. Louis, MO), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) was from Sigma or Seikagaku (Tokyo, Japan), n-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide (H-8) was from Seikagaku and camptothecin was from Yakuruto (Kunitachi, Japan). Agarose (low EEO) was from Fischer (Fairlawn, NJ), sucrose (enzyme grade) was from BRL, and Triton X-100 and sodium dodecyl sulfate (SDS) were from Bio-Rad (Richmond, CA). NaCl, KCl, MgCl₂, CaCl₂, K₂HPO₄, KH₂PO₄, NaH₂PO₄, NaOH, HCl, acetic acid, formic acid, pyridine, n-butanol and glycerol were from either Mallinckrodt (Paris, KY), MCB (Cincinnati, OH), EM (Cherry Hill, NJ) or Fischer. All other chemicals were from Sigma unless otherwise indicated. Aqueous solutions were made with double-distilled deionized water (ddH₂O). The following stock solutions were diluted immediately before use: leupeptin at 0.5 mg/ml, pepstatin A at 0.7 mg/ml in ethanol, aprotinin at 2 mg/ml, dithiothreitol (DTT) at 1 M or 50 mM and dithioerythritol (DTE) at 1 M were stored at -20°C, Na₂VO₄ (Aldrich, Milwaukee, WI) at 0.5 M and 2-mercaptoethanol (2-ME) were stored at 4°C, and phenylmethylsulfonyl fluoride (PMSF) at 0.1 M in isopropanol and NaF at 1 M were stored at 22°C. Radi isotopes ([γ-³²P]ATP, [³²P]orthophosphate, [³H]thymidine) were from ICN (Costa Mesa, CA). Phosphocellulose (P81) paper was from Whatman (Clifton, NJ). SDS-polyacrylamide gel molecular weight markers from Sigma included: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa). Supercoiled plasmid DNA was isolated by cesium chloride/ethidium bromide density gradient
centrifugation; ΔBpMTVdhfrHiHi3#1 and pUC13 were prepared by Telsa Mittelmeier and Dr. Yoshiko Shimizu, respectively. The pH of all Tris buffers was measured at 25°C.

Chromatography

Fast performance liquid chromatography (FPLC) system was from Pharmacia (Piscataway, NJ). Hydroxyapatite (Bio-Gel HTP) and Bio-Rex 70 (weak cationic exchanger) were from Bio-Rad, phosphocellulose (P11) and DEAE-cellulose (DE52) were from Whatman, Sephacryl S-200 (SF), DNA-cellulose (single-stranded) and phenyl-Sepharose were from Sigma, Mono Q (FPLC anion exchanger) was from Pharmacia and high performance hydroxyapatite (KB type S) was from Koken (Tokyo, Japan).

Antibodies

Normal human IgG was from Cappel (Durham, NC), scleroderma sera L702 was a generous gift from Dr. William C. Earnshaw (Johns Hopkins University School of Medicine, Baltimore, MD) and scleroderma autoantibodies FO, MI and KK were kindly provided by Drs. Takeshi Tojo and Mitsuo Homma (Keio University School of Medicine, Tokyo, Japan). FO, MI and KK were purified from the sera of scleroderma patients by ammonium sulfate precipitation and DEAE-cellulose column chromatography. Protein A-Sepharose CL-4B (Pharmacia) was incubated with the antibodies in 10 mM sodium phosphate (pH 7.5) and 150 mM NaCl (PBS) for ~16 h at 4°C and washed twice with PBS before use.

Cell Culture

Human fibroblasts (HFO; American Tissue Type Culture Collection, Rockville, MD) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS; Gibco), 0.3 mg/ml glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco). Swiss/3T3 (American Tissue Type Culture Collection), 3T3-L1 (American Tissue Type Culture Collection) and VT-1 (Shimizu et al.,
1986) mouse fibroblasts were grown in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were passed at 1:10 or 1:20 when 90% to 95% confluent. Cultures at 80% to 90% confluence (5.5 to 6.5 x 10⁴ 3T3-L1 cells/cm²) in 100 mm dishes were incubated in 10 ml DMEM containing 5% FBS for four to six days at 37°C in 5% CO₂ and 100% humidity. EGF was added to a final concentration of 15 ng/ml. TPA was added to a final concentration of 100 ng/ml from a 0.1 mg/ml stock in dimethylsulfoxide (DMSO), unless otherwise indicated. HeLa cells (American Tissue Type Culture Collection) were grown in RPMI 1640 (Gibco) supplemented with 5% heat-inactivated calf serum (Gibco), 0.3 mg/ml glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Metabolic Labeling

Confluent 100 mm dishes of murine fibroblasts were incubated at 37°C in 5 ml phosphate-free DMEM (Hazelton Biologies, Lenexa, KS) containing 0.1 mCi/ml [³²P]orthophosphate (carrier-free) for 2.5 to 4 h at 37°C. EGF (100 ng/ml), TPA (100 ng/ml) or DMSO (0.1%) were added and cultures were incubated for an additional 1 hr, unless otherwise indicated.

Cell Fractionation

For some experiments, confluent cultures (either labeled or unlabeled) treated with EGF, TPA or DMSO were washed twice on ice with 5 ml cold PBS containing 0.1 mM PMSF, detached with a Teflon-covered razor blade into 2.5 ml cold PBS containing 10 mM NaF, 0.5 mM Na₃VO₄ and 0.5 mM PMSF and rinsed with an additional 2.5 ml. The cell pellets were frozen at -70°C for one to five days. The pellets were thawed at 37°C for 15 s and resuspended in 1 ml cold 0.25 M sucrose, 1 mM EDTA, 10 mM acetic acid, 10 mM triethanolamine (pH 7.4), 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin A, 2 μg/ml aprotinin, 1 mM Na₃VO₄, 40 mM NaF and 1 mM PMSF (SEAT buffer) by pipeting 40 times through a P1000 tip (1.3 mm internal diameter; Rainin, Woburn, MA) as described by Miskimins and Shimizu.
(1982). The lysate was pelleted at either 180 x g (1000 rpm in an IEC HN-SII with a 958 rotor) for 10 min (4°C) or 14,000 x g (Eppendorf 5414) for 10 s (22°C). The cytoplasmic fraction was the supernatant and the nuclear fraction was either resuspended in 1 ml 10 mM Tris-HCl (pH 7.5) and 5 mM MgCl₂ (TM buffer) or extracted as described below.

**Preparation of Nuclear Extract**

For most experiments, labeled cells were washed twice on ice with 5 ml cold PBS (10 mM sodium phosphate (pH 7.5) and 150 mM NaCl) containing 0.1 mM PMSF and detached by scraping with a Teflon-covered razor blade into 2.5 ml PBS containing 10 mM NaF, 0.5 mM Na₃VO₄ and 0.5 mM PMSF twice. The cells were collected at 180 x g (1000 rpm in an IEC HN-SII with a 958 rotor) for 5 min (4°C). The cell pellets were frozen in liquid N₂ and stored at -70°C for one to five days. Cells were thawed at 37°C for 15 s, resuspended in 200 µl 20 mM sodium phosphate (pH 7.5), 0.25 M sucrose, 0.5% Triton X-100, 2 mM DTT, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin A, 2 µg/ml aprotinin, 1 mM Na₃VO₄, 40 mM NaF and 1 mM PMSF (N buffer) by pipeting ten times through a P1000 tip (1.3 mm internal diameter; Rainin) and incubated on ice for 30 min in a siliconized 1.5 ml tube (RPI, Mt. Prospect, IL). Nuclei were collected at 14,000 x g (Eppendorf 5414) for 10 s (22°C), washed with 200 µl N buffer by pipeting five times through a P200 tip (0.6 mm internal diameter; Rainin) and resuspended with 40 µl N buffer (final volume=65 µl). Nuclei were mixed with 10 µl 50 mM EDTA (pH 8.0), incubated 15 min on ice and lysed by adding 25 µl 0.2 M HEPES (pH 7.5) and 4 M NaCl (NE buffer). After 15 min on ice, DNA was precipitated by adding 40 µl 20% (w/v) polyethylene glycol (M, 8000) and removed at 14,000 x g for 30 min at 4°C. Note that nuclear lysis at a higher NaCl concentration may result in increased efficiency of DNA topoisomerase I extraction; Dr. Yoshiko Shimizu uses 2.5 M NaCl (see below) which also extracts all of the histones but 1.25 M NaCl is adequate: lyse nuclei by adding 35 µl NE buffer and precipitate DNA by adding 45 µl 20% (w/v) polyethylene glycol (M, 8000).
Immunoprecipitation

The nuclear supernatant (or protein kinase C reaction) was diluted with 500 μl of 20 mM sodium phosphate (pH 8.0), 0.5% Triton X-100, 1 mM EDTA, 2 mM DTT, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin A, 2 μg/ml aprotinin, 1 mM Na₃VO₄, 40 mM NaF and 1 mM PMSF (ipD buffer) in a siliconized 1.5 ml tube (final concentration=0.15 M NaCl), added to 5 μg antibody bound to 10 μl of Protein A-Sepharose CL-4B (Pharmacia) and incubated with rotation for 1 h at 22°C and for 16 to 24 h at 4°C. The immunoprecipitate was pelleted at 14,000 x g (Eppendorf 5414) for 1 s (22°C) and washed on ice, by a modification of Fambrough and Bayne (1983), six times with 250 μl PBS containing 0.5% Triton X-100, 1 mM EDTA and 0.1 mM PMSF (W buffer), then once with 250 μl 10 mM sodium phosphate (pH 7.5), 0.3 M NaCl, 0.1% Triton X-100, 0.1% SDS and 0.1 mM PMSF, followed by once with 250 μl 10 mM sodium phosphate (pH 7.5), 1 M NaCl, 0.5% Triton X-100, 1 mM EDTA and 0.1 mM PMSF and finally twice with 250 μl W buffer. The washed immunoprecipitate was resuspended in 25 μl 74 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 26 μg/ml bromophenol blue, heated at 100°C for 5 min, pelleted and washed with 25 μl 37 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 13 μg/ml bromophenol blue. The supernatants were pooled and electrophoresed on 7% SDS-polyacrylamide slab gels with 1.5 cm 3.5% SDS-polyacrylamide stacking gels (Hoefer, San Francisco, CA) at 4°C as described by Laemmli (1970). Dried gels were exposed to Konica type A film with Cronex intensifying screens (DuPont, Wilmington, DE) and quantified with a Betascope 603 blot analyzer (Betagen, Waltham, MA). Note that if 1.25 M NaCl is used for nuclear lysis, immunoprecipitation can be performed in a final concentration of ~0.2 M NaCl by maintaining 500 μl ipD buffer for dilution of the nuclear supernatant.

Preparation of Nuclear Extract from H-35 Hepatoma Cells

H-35 cells were cultured and extracted by Dr. Yoshiko Shimizu as follows: 10⁶ cells
were plated on a 100 mm dish in DMEM containing 5% FBS and 5% heat-inactivated calf serum. When the cells were approximately 50% confluent, the media was changed to serum-free DMEM. After two or more days, the media was again changed with serum-free DMEM. After one more day, the cells were washed twice with phosphate-free DMEM and incubated for 2 h in 5 ml phosphate-free DMEM containing 0.1 mCi/ml $^{32}$Porthophosphate. Insulin (Eli Lilly) was added at 600 ng/ml and incubation was continued for 1, 10, 30 or 60 min. The cultures were washed three times with Dulbecco's phosphate-buffered saline (without divalent cations) and frozen at -70°C for 1 day. The following steps were performed at 0 to 4°C. The frozen cells were lysed by the addition of 0.5 ml 0.1 M Tris-HCl (pH 7.5), 2 mM Na$_2$VO$_4$, 1 mg/ml aprotinin, 0.5% Triton X-100 and 1 mM PMSF, scraped off the dish and collected at 14,000 x g for 30 min. The pellet was resuspended in 1 ml 0.25 M sucrose, 1 mM EDTA, 10 mM acetic acid, 10 mM triethanolamine (pH 7.4) and 1 mM PMSF and collected at 14,000 x g for 2 min. The pellet was resuspended in 200 μl 2.5 M NaCl, 0.125 M sucrose, 0.5 mM EDTA, 5 mM acetic acid, 5 mM triethanolamine (pH 7.4), and 1 mM PMSF, incubated for 60 min, mixed with 50% (w/v) polyethylene glycol (M, 8000) to a final concentration of 6%, incubated for 30 min, and centrifuged at 14,000 x g for 30 min. The supernatant was frozen at -70°C. The H-35 cell nuclear supernatant was diluted 1:9 in ipD buffer and immunoprecipitated with scleroderma anti-DNA topoisomerase I antibody.

**DNA Topoisomerase I Assay**

Relaxation of supercoiled plasmid DNA by DNA topoisomerase I was measured by a modification of Liu and Miller (1981), Liu (1983) and Halligan et al. (1985). Reactions contained 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM MgCl$_2$, 0.5 mM DTT, 0.5 mM EDTA, 30 μg/ml bovine serum albumin and 20 μg/ml supercoiled plasmid DNA (pUC13, ΔBpMTVdhfrHlHl3#1 or pERAK) in a final volume of 20 μl. Reactions were initiated by addition of 2 or 5 μl column fraction, cell lysate, nuclear extract or purified protein diluted in the reaction mixture without DNA and incubated at 30°C for 30 min unless otherwise
indicated. Reactions were terminated on ice by addition of 5 µl 5% (w/v) n-lauroylsarcosine, 50 mM EDTA, 20% Ficoll (Type 400), 0.25 mg/ml bromophenol blue and 0.25 mg/ml xylene cyanole, heated to 60°C for 2 min and cooled briefly on ice. Samples (15 µl with 16 wells or 10 µl with 20 wells) were loaded onto a 0.7% (for ΔBpMTVdhfrHiHi3#1 and pERAK) or 1% (for pUC13) agarose gel and electrophoresed for 12 to 16 h at 20 V (2.2 V/cm) in 40 mM Tris-acetate (pH ~7.9) and 1 mM EDTA at room temperature. The gels were stained in 0.5 µg/ml ethidium bromide for 30 to 60 min, destained in distilled deionized water for 30 to 60 min, visualized on a long wave UV transilluminator (Ultraviolet Products, San Gabriel, CA) and photographed through Wratten filters No. 25 and 12 (Kodak, Rochester, NY) as well as 5 mm of UV opaque plexiglass (Fotodyne, New Berlin, WI) with a MP-3 Land Camera (Polaroid, Cambridge, MA) using type 55 or type 57 film (Polaroid) at 14.7 for 2.5 to 5 min (type 55) or 0.5 to 2 s (type 57). The negative (type 55 film) was rinsed in distilled deionized water before and after treatment with hypoclearing agent (Kodak) for 1 min.

Quantification was performed by scanning negatives with a densitometer (E-C, St. Petersburg, FL) and employing a standard curve (optical density vs. amount of DNA) to calculate percent supercoiled DNA converted to relaxed DNA. One unit was defined as the amount of DNA topoisomerase I required to relax 50% (0.2 µg) of the supercoiled plasmid DNA in the standard reaction.

Protein Kinase C Assay

Phosphorylation of a lysine-rich histone fraction by protein kinase C was measured as described by Shimizu and Shimizu (1989) using phosphocellulose paper as described by Witt and Roskoski (1975). Reactions contained 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.4 mg/ml histone (calf thymus type III-S), 9 µM [γ-³²P]ATP (1 µCi) and either 0.1 mM CaCl₂, 25 µg/ml phosphatidylserine (PS) and 100 ng/ml TPA or 2 mM EGTA in a final volume of 100 µl. Reactions were initiated by adding 1 to 3.5 µl column fraction and incubated at 30°C for 15 min. Reactions were terminated by placing in an ice-water bath and applying 75 µl to
P81 phosphocellulose papers (2.5 x 2.5 cm). The papers were deposited in 10 ml water per paper, washed with six changes of water and dried at 80°C for 30 min. The incorporation of [32P]phosphate was measured by scintillation counting. Protein kinase C activity was determined by subtraction of kinase activity measured in the presence of EGTA from kinase activity measured in the presence of CaCl₂, PS and TPA. One unit was defined as the amount of protein kinase C required to transfer 1 nmol of 32P, from [γ-32P]ATP to histone in 1 min.

Purification of Human DNA Topoisomerase I

DNA topoisomerase I was purified to apparent homogeneity by a modification of Liu and Miller (1981). HeLa cells were grown in suspension (2-10 x 10⁵ cells/ml) in T150 flasks; approximately 30 g of cells were harvested on 11 occasions (values given are for total amounts). Cells were collected at 160 x g (1000 rpm in a Sorvall RC-5B with a GSA rotor) for 12 min at 22°C. All subsequent procedures were performed at 0 to 4°C. The cells were resuspended in 1000 ml PBS (10 mM sodium phosphate (pH 7.5) and 150 mM NaCl), collected at 180 x g (1000 rpm in an IEC HN-SII with a 958 rotor) for 10 min, washed with 500 ml PBS containing 0.1 mM PMSF, collected at 180 x g for 10 min, frozen in liquid N₂ and stored at -70°C for less than 1 year. Frozen cell pellets were resuspended in 150 ml 5 mM potassium phosphate (pH 7.5), 2 mM MgCl₂, 1 mM PMSF, 1 mM 2-ME, 0.5 mM DTT, 0.1 mM EDTA, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin and 0.7 μg/ml pepstatin A and incubated for 30 min. The cells were then lysed in four aliquot batches of ~45 ml by 20 strokes (1 stroke=up and down) in a 50 ml Dounce homogenizer (pestle A). The nuclei were collected at 900 x g (2200 rpm in an IEC HN-SII with 958 rotor) for 10 min, washed with 150 ml 5 mM potassium phosphate (pH 7.5), 1 mM PMSF, 1 mM 2-ME, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin and 0.7 μg/ml pepstatin A (NW buffer), resuspended in 150 ml NW buffer and incubated for 1 h after the addition of 0.5 M EDTA (pH 8.0) to a final concentration of 4 mM (1.2 ml). The nuclei were lysed by slowly adding, with stirring, 150 ml 2 M NaCl, 100 mM
Tris-HCl (pH 7.5), 10 mM 2-ME and 1 mM PMSF. DNA was then precipitated by slowly adding, with stirring, 150 ml 18% (w/v) polyethylene glycol (M, 8000), 1 M NaCl and 50 mM Tris-HCl (pH 7.5) and pelleted at 15,000 x g (9500 rpm in a Sorvall RC-5B with a GSA rotor) for 25 min. The supernatants were pooled and loaded onto a hydroxyapatite column (1.5 x 26 cm) equilibrated with 1 M NaCl, 50 mM Tris-HCl (pH 7.5), 6% polyethylene glycol (M, 8000), 10 mM 2-ME and 1 mM PMSF at 15 cm/hr (0.45 ml/min). The column was washed with 150 ml 0.2 M potassium phosphate (pH 7.0) in 10% glycerol, 10 mM 2-ME and 1 mM PMSF (HA buffer) and eluted with 200 ml of a 0.2-0.7 M potassium phosphate (pH 7.0) linear gradient in HA buffer. Fractions (4 ml) containing DNA topoisomerase I activity (between 0.5 M and 0.6 M) were pooled, diluted with an equal volume (40 ml) of 5 mM potassium phosphate (pH 7) in HA buffer containing 0.1 mM EDTA (P buffer) and loaded onto a phosphocellulose column (1 x 1.2 cm) equilibrated with 0.2 M potassium phosphate (pH 7.0) in P buffer at 9.5 cm/hr (0.12 ml/min). The column was washed with 5 ml 0.2 M potassium phosphate (pH 7.0) in P buffer and eluted with 15 ml of a 0.2-0.7 M potassium phosphate (pH 7.0) linear gradient in P buffer. Fractions (0.3 ml) containing DNA topoisomerase I activity (between 0.45 M and 0.7 M) were pooled, concentrated from 8 ml to 1.5 ml with a minicon-B15 (Amicon), loaded onto a Sephacryl S-200 column (1.5 x 50 cm) equilibrated with 0.1 M KCl in 40 mM Tris-HCl (pH 7.5), 10% glycerol, 10 mM 2-ME, 1 mM PMSF and 0.1 mM EDTA (S buffer) and eluted at 15 cm/hr (0.45 ml/min). Fractions (2 ml) containing DNA topoisomerase I activity were pooled and loaded onto a single-stranded DNA-cellulose column (1 x 0.3 cm) equilibrated with 0.1 M KCl in S buffer at 3.6 cm/hr (0.05 ml/min). The column was washed with 4 ml each of 0.1 M, 0.2 M, 0.3 M, 0.4 M and 1 M KCl in S buffer. Fractions (0.25 ml) containing DNA topoisomerase I activity (0.3 M KCl wash) were pooled, dialyzed against two changes (2 h each) of 100 ml 70 mM potassium phosphate (pH 7.0), 50% glycerol, 0.5 mM DTT, 0.1 mM EDTA (T buffer) and stored at -20°C. Note that DNA topoisomerase I usually elutes between 0.5 M and 0.6 M from the phosphocellulose column, concentration with a minicon-B15 decreases yield and a 2 x 50 cm
gel filtration column would more adequately accommodate the sample volume. However, this purification scheme should be replaced by the one described below, due to ease and yield.

**Purification of Murine DNA Topoisomerase I**

DNA topoisomerase I was purified to near homogeneity by a modification of A. Bodley and L. F. Liu (personal communication). Livers were excised from BALB/cByJ mice (Jackson Laboratories, Bar Harbor, ME), rinsed in saline and frozen at -70°C (by Dr. Yoshiko Shimizu). All procedures were performed at 0 to 4°C. Frozen livers (~150 g) were added to 600 ml 25 mM HEPES (pH 7.5), 0.25 M sucrose, 5 mM KCl, 1 mM MgCl₂, 1 mM DTE, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, 0.7 μg/ml pepstatin A and 1 mM PMSF (H buffer) and homogenized in a Waring blender at full speed (15 pulses of 1 s each) until ~80% of the cells were lysed as monitored by phase contrast microscopy. The homogenate was filtered successively through 2, 4 and 8 layers of cheesecloth and mixed with 10% Triton X-100 to a final concentration of 0.5% (35 ml). Nuclei were collected at 8000 x g (7000 rpm in a Sorvall RC-5B with a GSA rotor) for 10 min, washed with 600 ml 25 mM HEPES (pH 7.5), 0.25 M sucrose, 0.01% Triton X-100, 1 mM DTE, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, 0.7 μg/ml pepstatin A and 1 mM PMSF (E buffer), resuspended with 240 ml E buffer and extracted for 1 h after adding 5 M NaCl to a final concentration of 0.35 M (25 ml). Residual nuclei were pelleted at 10,000 x g (8000 rpm in a Sorvall RC-5B with a GSA rotor) for 30 min. The supernatant was mixed with 100% glycerol to a final concentration of 10% (36 ml) as well as 0.5 M EDTA (pH 8.0) to a final concentration of 1 mM (0.7 ml) and then loaded onto a Bio-Rex 70 column (5 x 10 cm) equilibrated with 0.4 M NaCl in 25 mM HEPES (pH 7.5), 10% glycerol, 0.01% Triton X-100, 1 mM EDTA, 1 mM DTE and 0.5 mM PMSF (B buffer) at 8.6 cm/hr (2.8 ml/min). The column was washed with 400 ml 0.4 M NaCl in B buffer and 200 ml of a 0.4-0.5 M NaCl linear gradient in B buffer and then eluted with 1000 ml 0.6 M NaCl in B buffer. The 0.6 M NaCl eluate was loaded onto a hydroxyapatite column (2.5 x 5.5 cm) equilibrated with 0.2 M potassium phosphate (pH 7.0) in 10% glycerol, 2 mM DTT and 0.5
mM PMSF (C buffer) at 23 cm/hr (1.9 ml/min). The column was washed with 60 ml 0.2 M potassium phosphate (pH 7.0) in C buffer and eluted with 300 ml of a 0.2-0.7 M potassium phosphate (pH 7.0) linear gradient in C buffer. Fractions (5 ml) containing DNA topoisomerase I activity (between 0.45 M and 0.55 M) were pooled, diluted with an equal volume (70 ml) of 1.5 M potassium phosphate (pH 7.0) in C buffer and loaded onto a phenyl-Sepharose column (2.5 x 6.2 cm) equilibrated with 1.0 M potassium phosphate (pH 7.0) in C buffer at 22 cm/hr (1.8 ml/min). The column was washed with 30 ml 1.0 M potassium phosphate (pH 7.0) in C buffer and eluted with 150 ml of a 1.0-0.7 M potassium phosphate (pH 7.0) linear gradient in C buffer followed by 150 ml of a 0.7-0.001 M potassium phosphate (pH 7.0) linear gradient in C buffer. Fractions (4.85 ml) containing DNA topoisomerase I activity (between 0.35 M and 0.25 M) were pooled, diluted with 0.5 volume (25 ml) 0.01 M potassium phosphate (pH 7.0) in C buffer and loaded onto a Bio-Rex 70 column (1 x 3 cm) equilibrated with 0.2 M potassium phosphate (pH 7.0) in C buffer and equipped with a flow adaptor at 20 cm/hr (0.26 ml/min) from bottom to top. The column was washed with 15 ml 0.2 M potassium phosphate (pH 7.0) in C buffer from bottom to top and eluted with 10 ml 0.7 M potassium phosphate (pH 7.0) in C buffer from top to bottom. Fractions (0.25 ml) containing DNA topoisomerase I activity were pooled, dialyzed against two changes (2 h each) of 100 ml 30 mM potassium phosphate (pH 7.0), 50% glycerol, 2 mM DTT, 0.5 mM EDTA (mT buffer) and stored at -20°C. Note that this purification scheme can be used to purify DNA topoisomerase I from other sources including HeLa cells, in which case tissue homogenization in a Waring blender and filtration through cheesecloth would be replaced with disruption in a Dounce homogenizer. Also, proteolytic degradation of DNA topoisomerase I might be avoided by washing livers (or HeLa cells) twice in PBS containing 1 mM PMSF, freezing the sample rapidly in liquid N₂, and avoiding long-term storage (Liu and Miller, 1981; Vosberg, 1985). The purification can be scaled down 50% using ~50 g cells or ~75 g tissue for convenience and still obtain a large amount of DNA topoisomerase I (A. Bodley, personal communication). The nuclear extract (from tissue) should be filtered over siliconized glass
wool before loading onto the Bio-Rex 70 column in order to avoid clogging the column with particulates. The first Bio-Rex 70 column should be washed with 400 ml 0.4 M NaCl in B buffer and eluted with 2000 ml of a 0.4-1.2 M NaCl linear gradient in B buffer with DNA topoisomerase I eluting between 0.8 M and 1.1 M (A. Bodley and L. F. Liu, personal communication) to decrease eluted sample volume and also to allow for copurification of DNA topoisomerase II. Alternatively, eluted sample volume might be decreased by washing with 600 ml each of 0.4 M, 0.5 M, 0.6 M and 0.7 M NaCl in B buffer with DNA topoisomerase I eluting in the 0.6 M wash. The phenyl-Sepharose column should be eluted with 300 ml of a 1.0-0.001 M potassium phosphate (pH 7.0) linear gradient in C buffer because DNA topoisomerase I elutes at a low salt concentration. DTT can be substituted for DTE in H, E and B buffers.

**Purification of Protein Kinase C**

Protein kinase C was purified by a modification of Jeng et al. (1986) and Kikkawa et al. (1987). Brains were excised from BALB/cByJ mice (Jackson Laboratories), rinsed in saline and frozen at -70°C for 1.5 years (by Dr. Yoshiko Shimizu). Frozen brains from 14 mice (~6 g) were thawed in 12 ml 20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin and 0.7 μg/ml pepstatin A and disrupted by 5 strokes in a 30 ml Potter-Elvehjem homogenizer (Wheaton, Millville, NJ) at a speed of 3.8/10 on ice. The homogenate was clarified at 100,000 x g (38,000 rpm in Beckman L8-80 with a Ti80 rotor) for 60 min at 4°C. The supernatant was diluted with two volumes (25 ml) of 20 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 1 mM DTT and 0.05% Triton X-100 (D buffer) and loaded onto a DE52 column (1.5 x 9 cm) equilibrated with D buffer at 14 cm/hr (0.38 ml/min) at 4°C. The column was washed with 100 ml D buffer and developed with 60 ml of a 0-0.4 M NaCl linear gradient in D buffer. The fractions (1 ml) containing protein kinase C activity (between 0.05 M and 0.22 M) were pooled, dialyzed against 500 ml D buffer for 4 h at 4°C, clarified at 900
x g (2200 rpm in an IEC HN-SII with 958 rotor) for 10 min (4°C) and loaded onto a Mono Q HR5/5 column (0.5 x 5 cm) equilibrated with D buffer at 0.5 ml/min using a FPLC system at 22°C. The column was washed with 35 ml D buffer and developed with 20 ml of a 0-0.3 M NaCl linear gradient in D buffer. The fractions (1 ml) containing protein kinase C activity (between 0.20 M and 0.26 M) were pooled, diluted with two volumes (6 ml) of 0.02 M potassium phosphate (pH 7.5) in 0.5 mM EGTA, 0.5 mM EDTA, 1 mM DTT, 0.05% Triton X-100 and 10% glycerol (K buffer), clarified at 900 x g (2200 rpm in an IEC HN-SII with 958 rotor) for 10 min (4°C) and loaded onto a high performance hydroxyapatite column (0.78 x 10 cm) equilibrated with 0.02 M potassium phosphate (pH 7.5) in K buffer (PKC buffer) at 0.5 ml/min using a FPLC system at 22°C. The column was washed with 15 ml PKC buffer and developed with 50 ml of a 0.02-0.3 M potassium phosphate (pH 7.5) linear gradient in K buffer. Fractions (1 ml) containing the three protein kinase C peaks, corresponding to the types I (between 0.10 M and 0.11 M), II (between 0.12 M and 0.14 M) and III (between 0.18 M and 0.20 M) enzymes, were pooled separately, desalted and concentrated with a minicon-B15 (Amicon) and stored in PKC buffer at -70°C. Note that increasing the size of the DE52 column to 1.5 x 15 cm, washing it with 250 ml D buffer and eluting it with 100 ml of a 0-0.3 M NaCl linear gradient in D buffer will probably decrease background and increase resolution.

A phenyl-Sepharose column (0.7 x 6.5 cm) can be employed between the DE52 and Mono Q columns instead of dialysis in order to increase purity (add ~0.45 volumes 3 M NaCl in D buffer to 1 M final concentration, load in 1.0 M NaCl in D buffer, wash with 25 ml 1.0 M NaCl in D buffer and elute with 30 ml of a 1.0-0 M NaCl linear gradient in D buffer followed by 25 ml D buffer). To further enhance purity, fractions from the Mono Q column should contain 0.5 ml and the high performance hydroxyapatite column should be washed with 25 ml PKC buffer and eluted with 50 ml of a 0.02-0.25 M potassium phosphate (pH 7.5) linear gradient in K buffer collecting 0.25 ml per fraction. D buffer before and during phenyl-Sepharose chromatography should probably not contain Triton X-100, which alters the behavior of proteins on the column. Sequential phenyl-Sepharose chromatography in the
absence and presence of Triton X-100 may augment separation.

**DNA Topoisomerase I Phosphorylation Reaction**

Reactions contained 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂ and either 0.15 mM CaCl₂, 25 µg/ml PS and 100 ng/ml TPA or 2 mM EGTA in 30 µl. Reactions were in the presence of 7.5 ng type III protein kinase C (320 units/mg) and 420 ng human DNA topoisomerase I unless otherwise indicated. In addition, the enzyme buffers contributed 9 mM potassium phosphate (pH 7.1), 6% glycerol, 150 µM DTT, 60 µM EDTA, 50 µM EGTA and 0.05 µl/ml Triton X-100. The reaction was initiated by adding 1 or 10 µM [γ-³²P]ATP (2-20 µCi) and incubated at 30°C for the indicated time (0.5-30 min). The reaction was terminated by placing in an ice water bath and adding 1.5 µl 0.1 M EGTA (pH 7.5).

Alternatively, the reaction was stopped on ice and 67 µl 3 mM EGTA (pH 7.5) and 5 mM EDTA (pH 8.0) was added followed by 3 µl 3 mg/ml bovine serum albumin and 10 µl 100% trichloroacetic acid; the precipitate was incubated on ice for 30 min, collected at 14,000 x g for 15 min, washed three times with 0.25 ml cold acetone and resuspended in 30 µl water. The samples were then mixed with 30 µl 74 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 26 µg/ml bromophenol blue, heated in a boiling water bath for 5 min and electrophoresed on 3.5% SDS-polyacrylamide stacking gels (1.5 cm) and 7% SDS-polyacrylamide resolving gels at 4°C as described by Laemmli (1970). Dried gels were subject to autoradiography with Cronex intensifying screens (DuPont) and quantified with a Betascope 603 blot analyzer (Betagen). The protein kinase C inhibitor H-7 was added prior to incubation from a 10 mM aqueous stock solution; TPA at 10 µg/ml and PS at 2.5 mg/ml were in DMSO.

**Two-Dimensional Phosphoamino Acid Analysis**

Phosphoamino acids were identified as described by Hunter and Sefton (1980). Phosphorylated DNA topoisomerase I from ³²P-labeled 3T3-L1 cells treated with 100 ng/ml
TPA for 1 or 2 h (four 150 mm dishes) or from the protein kinase C reaction were eluted from a SDS-polyacrylamide gel. The gel slices were homogenized with a teflon micro-probe (RPI) in 250 μl 50 mM NH₄HCO₃ and 0.1% SDS with 20 μg bovine serum albumin, heated in a boiling water bath for 10 min after the addition of 2-ME to 5% (12.5 μl), incubated with rotation at 22°C for 6 to 12 hr, pelleted at 14,000 x g (Eppendorf 5414) for 15 min, resuspended in 250 μl 50 mM NH₄HCO₃, 0.1% SDS and 5% 2-ME, incubated with rotation at 22°C for 6 to 12 hr and pelleted at 14,000 x g for 15 min. The supernatants were pooled, filtered through a Millex GV 0.22 μm filter (Millipore, Bedford, MA) and precipitated after adding 20 μg bovine serum albumin by the addition of 100% trichloroacetic acid to 20% (125 μl). After 30 min on ice, the precipitate was collected at 14,000 x g for 15 min (4°C), washed three times with 500 μl cold acetone and dried at 22°C in a fume hood. The pellet was resuspended in 100 μl 6 N HCl and heated at 110°C under N₂ for 2 h. The hydrolysate was diluted with 200 μl ddH₂O, dried with heat under reduced pressure in a Speed Vac (Savant, Farmingdale, NY), resuspended in 50 μl ddH₂O, mixed with 2.4 μg each of phosphotyrosine, phosphoserine and phosphothreonine and dried with heat under reduced pressure to 2 to 5 μl. The amino acids were analyzed on 160 μm-thin cellulose plates (Kodak) by electrophoresis in 7.8% acetic acid and 2.2% formic acid (pH 1.9) at 400 V for 1 h in the first dimension (10 cm) and 5% acetic acid and 0.5% pyridine (pH 3.5) at 400 V for 2.5 h in the second dimension (20 cm). Thin-layer electrophoresis was performed in a 2117 Multiphor (LKB) maintained at ~16°C by circulating water from a Precision water bath (Chicago, IL) set at 30°C through a KR-60 cooler (Polyscience, Evanston, IL) with a Lauda T-1 circulator (W. Germany) set at 30°C. The phosphoamino acid markers were visualized by spraying the plates with 0.3% ninhydrin in n-butanol and the incorporated [³²P]phosphate was detected by autoradiography as well as quantified with a Betascope blot analyzer. The plates were dried at 50 to 60°C for 10 to 20 min subsequent to separation in each dimension and staining.
Two-Dimensional Tryptic Phosphopeptide Mapping

Phosphopeptides from complete digests with trypsin were characterized as described by Hunter and Setton (1980) and Iwashita and Fox (1984). Phosphorylated DNA topoisomerase I was excised from a SDS-polyacrylamide gel as described above, resuspended in 75 μl cold performic acid for 2 h on ice (generated immediately before use by incubating 180 μl 99% formic acid (Sigma) with 20 μl 30% hydrogen peroxide for 1 h at 22°C), diluted with 1 ml ddH₂O and dried with heat under reduced pressure in a Speed Vac. The pellet was resuspended in 500 μl 50 mM NH₄HCO₃ and digested with 30 μg TPCK-treated trypsin for ~18 h at 37°C followed by an additional 20 μg TPCK-treated trypsin for ~6 h at 37°C. The digest was diluted with 0.5 to 1 ml ddH₂O, dried with heat under reduced pressure, resuspended in 300 μl 1% ammonium carbonate, dried with heat under reduced pressure, resuspended in 50 μl 1% ammonium carbonate and dried with heat under reduced pressure to 2 to 5 μl. [Alternatively, as described by Durban et al. (1985), gel slices were incubated in 10% methanol for 4 h at 22°C, dried with heat under reduced pressure, resuspended in 500 μl 50 mM NH₄HCO₃ and digested as described]. The phosphopeptides were resolved on 160 μm-thin cellulose plates (Kodak) by electrophoresis in 1% ammonium carbonate (pH 8.9) at 800 V for 25 min in the first dimension (20 cm) and ascending chromatography in 37.5% n-butanol, 25% pyridine and 7.5% acetic acid for 4 h in the second dimension (10 cm). Thin-layer electrophoresis was performed as described above; the plates were dried at 50 to 60°C for 10 to 20 min after separation in each dimension and the phosphopeptides were visualized by autoradiography.

Comparative Tryptic Phosphopeptide Mapping

Tryptic phosphopeptides were generated and analyzed in the first dimension as described above except thin-layer electrophoresis in 1% ammonium carbonate at 800 V was performed at ~16°C for 30 min instead of 25 min. Equal amounts of radioactivity were spotted in each lane; serial dilutions were in 1% ammonium carbonate.
**Photo-crosslinking**

Quiescent 3T3-L1 cells in 100 mm dishes were ³²P-labeled, treated with TPA or DMSO for 1 h, washed twice and covered with 10 ml cold Dulbecco's phosphate-buffered saline (Gibco). The cultures (covers removed) were then irradiated for 10 min on ice 10 cm under an inverted UV transilluminator (Ultraviolet Products) with filter removed (1.2 J/m²/s) as described by Gilmour et al. (1986). Control dishes were exposed for 10 min on ice 2.5 m under fluorescent lights. Cells were collected and DNA topoisomerase I was immunoprecipitated from nuclear supernatants as described above.

**DNA Synthesis**

Incorporation of [³H]thymidine was measured as described by Shimizu and Shimizu (1980, 1989). Quiescent 3T3-L1 cells in 12-well culture plates were treated with 0.1% DMSO or various concentrations of camptothecin dissolved in DMSO for 5 min at 37°C followed by the addition of 0.1% DMSO or 100 ng/ml TPA and incubation for 20 h. The cultures were washed twice with 1 ml DMEM and incubated in 0.5 ml DMEM containing 1 μCi/ml [³H]thymidine (80 Ci/mmol) for 1 h at 37°C. The labeled cells were washed three times on ice with 1 ml cold Dulbecco's phosphate-buffered saline (Gibco), incubated for ~16 h at 4°C after the addition of 1 ml 10% trichloroacetic acid, washed twice with cold 5% trichloroacetic acid and solubilized by incubation for ~16 h at 37°C after the addition of 0.5 ml 1 N NaOH. The solubilized cells were transferred to scintillation vials, neutralized with 0.1 ml 6 N HCl and mixed vigorously with 3 ml 3a70B (RPI). Incorporation of [³H]thymidine was measured by scintillation counting.

**Protein Concentration**

Protein concentrations were determined as described by Bradford (1976). Proteins in 800 μl ddH₂O were mixed with 200 μl Bradford dye reagent concentrate (Bio-Rad). The
optical density of the solution was measured at 595 nm. Bovine serum albumin diluted in the same buffer as the assayed protein was employed to generate a standard curve (optical density vs. amount of protein). Non-linear curve fitting and regression were performed with LOWRY (McPherson, 1985). Alternatively, proteins in 50 μl were incubated with 1 ml BCA* working reagent (50 parts reagent A and 1 part reagent B) at 60°C for 30 min (Pierce, Rockford, IL). The optical density of the solution was measured at 562 nm after cooling to 22°C and protein concentration was determined as described.

Detection of Proteins in SDS-Polyacrylamide Gels

Proteins in SDS-polyacrylamide gels were visualized by silver staining as described by Merril et al. (1981). Glass trays used for staining and glass plates were washed in warm detergent (1% 7X or Contrad), rinsed extensively in ddH₂O and wiped with 100% ethanol. Approximately 100 ml of solution and gentle agitation on a Genesis rocker platform (Javelina, Tucson, AZ) were employed for each procedure; gloves were utilized. After electrophoresis, gels were fixed in 50% methanol and 12% acetic acid for at least 1 h, rinsed twice with ddH₂O briefly, rinsed three times in 10% ethanol and 5% acetic acid for 10 min each, rinsed twice with ddH₂O briefly, incubated in 3.4 mM potassium dichromate and 3.2 mM nitric acid for 5 min, rinsed four times with ddH₂O for 0.5 min each and incubated in 12 mM silver nitrate for 30 min. Gels were then rinsed twice with ddH₂O briefly, transferred to a clean glass tray, rinsed with 0.28 M sodium carbonate and 0.05% formaldehyde (not older than 1 month) briefly and developed in 0.28 M sodium carbonate and 0.05% formaldehyde until the intensity of staining was appropriate. Staining was terminated with 1% acetic acid and gels were photographed on a light box (Graphic Technology, Newburgh, NY) with bottom illumination using type 55 film (Polaroid) at f4.7 for 1/8 to 1 s. The negative was processed with hypoclearing agent (Kodak) as described above.
CHAPTER 3

ANTI-DNA TOPOISOMERASE I ANTIBODIES FROM SCLERODERMA SERA

Anti-DNA topoisomerase I antibodies were extensively employed to analyze the phosphorylation state of DNA topoisomerase I. These antibodies were obtained from the sera of human patients with the autoimmune disease scleroderma. We and several other laboratories have demonstrated that the scleroderma autoantibodies recognize DNA topoisomerase I by a variety of criteria. The clinical significance of anti-DNA topoisomerase I antibodies in the sera of scleroderma patients is not yet understood. In this section, we show that these antibodies neutralize the DNA relaxation activity of DNA topoisomerase I as well as immunoprecipitate phosphorylated DNA topoisomerase I.

History of Scleroderma Autoantibodies and Scl-70

Scleroderma (progressive systemic sclerosis) is one of several autoimmune diseases characterized by the presence of autoantibodies to nuclear antigens (Tan, 1982; Samuels, 1987). The major autoantigen recognized by sera from several scleroderma patients was initially purified 250-fold as a 70 kDa basic non-histone chromosomal protein, termed Scl-70, that copurified with histone H1; in addition, the autoantibodies were found to be monospecific for Scl-70 (Douvas et al., 1979). Antibodies to Scl-70 were recognized by immunodiffusion in the sera from 23% of patients with the diffuse form of scleroderma and from 13% of patients with the CREST variant but not in the sera of any patients with the autoimmune diseases systemic lupus erythematosus or rheumatoid arthritis (Tan et al., 1980). Scl-70 was determined to be a degradation product of a larger autoantigen, Scl-86; antibodies to Scl-86 were detected by immunoblotting in the sera from 59% of patients with diffuse scleroderma (van Venrooij et al., 1985).

Scl-70 was demonstrated by several independent criteria to be DNA topoisomerase I
(Shero et al., 1986; Guldner et al., 1986; Maul et al., 1986; Samuels et al., 1986; D'Arpa et al., 1988). The actual molecular mass of Scl-70 was determined to be 100 kDa (Shero et al., 1986; Guldner et al., 1986; Maul et al., 1986). The presence of both ~70 kDa and 100 kDa forms (Shero et al., 1986), the distribution of nuclear staining (Guldner et al., 1986) as well as the molecular mass of 100 kDa and isoelectric point of 8.4 (Maul et al., 1986) suggested that Scl-70 was DNA topoisomerase I. In addition, DNA topoisomerase I is a basic nonhistone chromosomal protein that often copurifies with histone H1 (L. F. Liu, personal communication). The identification was corroborated by: binding of Scl-70 sera to purified DNA topoisomerase I in a solid-phase radioimmunoassay (Shero et al., 1986), inhibition of DNA relaxation activity from chromosomal extracts, purified DNA topoisomerase I and cell lysates by anti-Scl-70 (Shero et al., 1986; Maul et al., 1986; Samuels et al., 1986), binding of anti-DNA topoisomerase I and anti-Scl-70 to the same set of peptides produced by partial digestion of nuclear extracts with α-chymotrypsin (Shero et al., 1986), immunoblotting of purified DNA topoisomerase I with anti-Scl-70 (Guldner et al., 1986), immunoprecipitation of purified DNA topoisomerase I with anti-Scl-70 (Guldner et al., 1986; Samuels et al., 1989) and cDNA cloning of the gene encoding DNA topoisomerase I from a λgt11 expression library screened with anti-Scl-70 (D'Arpa et al., 1988). Recognition of various DNA topoisomerase I fragments, expressed from the cloned gene, has demonstrated that the scleroderma autoantibodies are polyclonal and multi-focal (D'Arpa et al., 1990).

**Scleroderma Autoantibodies Inhibit DNA Topoisomerase I Activity**

DNA topoisomerase I activity is measured as the ability to relax supercoiled (form I) plasmid DNA. The relaxation reaction results in the formation of a series of topoisomers, DNA molecules with the same nucleotide sequence but differing in topological conformation. The relaxed molecules migrate slower in agarose gels due to their decreased compactedness (Keller, 1975). Completely relaxed covalently closed plasmids comigrate under these electrophoretic conditions with nicked (form II) plasmids.
A supercoiled plasmid (a 7 kb derivative of pMTVdhfr) was relaxed by 180 ng of purified calf thymus DNA topoisomerase I in a 40 μl reaction (Figure 3.1, lane 3). Preincubation of DNA topoisomerase I for 30 min at 4°C in 20 μl with normal human IgG had no effect on its activity (Figure 3.1, lane 4); however, scleroderma IgG (anti-Scl-70) at high concentrations occasionally caused a tailing of nicked DNA (Figure 3.1, lanes 2 and 5). In a titration of purified scleroderma IgG from FO sera with purified DNA topoisomerase I (Figure 3.1, lanes 5 to 10), inhibition first appeared at 1 μg IgG and DNA relaxation activity was completely neutralized by 10 μg IgG. IgG from MI sera had a similar response while a third sera, KK, inhibited DNA topoisomerase I at only 0.2 μg IgG. KK sera may have a higher titer of anti-DNA topoisomerase I autoantibodies or the antibodies may have a higher affinity for the enzyme.

Cultured human and murine fibroblasts were collected and fractionated by pipeting in SEAT buffer and centrifugation. The post-nuclear (cytoplasmic) fraction in SEAT buffer from EGF-treated cells and the nuclear fraction in TM buffer contained DNA topoisomerase I activity as assayed by the relaxation of supercoiled plasmid DNA. DNA topoisomerase I activity in the cytoplasm of human HFO cells treated for 8 h with 15 ng/ml EGF or in the nucleus of cells treated for 12.5 h was effectively inhibited by scleroderma IgG from FO sera. FO antibodies also inhibited the relaxation activity from the cytoplasm of Swiss/3T3 mouse cells treated with 15 ng/ml EGF for 6 h and from the nucleus of untreated cells.

Scleroderma Autoantibodies Recognize DNA Topoisomerase I Phosphorylated in Vitro by Protein Kinase C

DNA topoisomerase I was purified to near homogeneity from HeLa cells by hydroxyapatite, P11 phosphocellulose and single-stranded DNA-cellulose column chromatography (Samuels et al., 1989). The 100 kDa DNA topoisomerase I was incubated with protein kinase C, partially purified from mouse brain using DE52-cellulose and Mono Q column chromatography (by Dr. Yoshiko Shimizu), [γ-32P]ATP and the protein kinase C
Figure 3.1. Inhibition of purified calf thymus DNA topoisomerase I by scleroderma autoantibodies. Reactions contained in 40 μl either no DNA topoisomerase I (lanes 1 and 2) or 180 ng of purified calf thymus DNA topoisomerase I (Topo I; lanes 3-10). Lanes 1 and 3 contained no IgG; lane 4, 20 μg of control IgG from normal human sera (Normal Ab); lanes 2 and 5, 20 μg of FO IgG; lane 6, 10 μg FO IgG; lane 7, 2 μg FO IgG; lane 8, 1 μg FO IgG; lane 9, 0.2 μg FO IgG; and lane 10, 0.1 μg FO IgG (Sci Ab). The DNA topoisomerase I reactions were analyzed by 0.7% agarose gel electrophoresis and visualized by staining with ethidium bromide. The positions of nicked and completely relaxed (upper arrow) as well as supercoiled (lower arrow) plasmid DNA are indicated.
cofactors Ca\(^{2+}\), PS and TPA. The phosphorylation reaction was diluted and incubated with a mixture of 2 \(\mu\)g each FO, MI and KK IgG bound to 20 \(\mu\)l Protein A-Sepharose CL-4B. The flow-through was precipitated with trichloroacetic acid and contained about 10 phosphorylated proteins including one with a molecular mass of 77 kDa, presumably the autophosphorylated protein kinase C (Figure 3.2, lane 2). The high background may be due to absorption of \(^{32}\)P by PS. The immunoprecipitate contained a single phosphorylated protein with a molecular mass of 100 kDa, identical to genuine DNA topoisomerase I (Figure 3.2, lane 1). The 100 kDa protein was phosphorylated in the reaction only if DNA topoisomerase I, protein kinase C and the protein kinase C cofactors were present (Samuels et al., 1989; chapter 6).

Scleroderma antibodies recognize a 100 kDa phosphoprotein in nuclear extracts from murine fibroblasts (chapters 4 and 5) and rat hepatoma cells (chapter 5).
Figure 3.2. Immunoprecipitation of *in vitro* phosphorylated DNA topoisomerase I with *scleroderma* autoantibodies. The protein kinase C phosphorylation reaction contained 270 ng of human DNA topoisomerase I and 180 ng murine protein kinase C. After incubation at 30°C for 15 min, the reaction was immunoprecipitated with immobilized scleroderma IgG. The immunoprecipitate (IP; lane 1) and the flow-through (FT; lane 2) were analyzed by 7% SDS-polyacrylamide gel electrophoresis and autoradiography.
CHAPTER 4

CHARACTERIZATION OF DNA TOPOISOMERASE I PHOSPHORYLATION IN MURINE FIBROBLASTS TREATED WITH 12-O-TETRADECANOYLPHORBOL-13-ACETATE

We hypothesized that the phosphorylation state of DNA topoisomerase I would be modulated in murine fibroblasts treated with the phorbol ester tumor promoter TPA because DNA topoisomerase I has been implicated in transcription and replication, which occur following the induction of cell growth, and because phosphorylation enhances the activity of DNA topoisomerase I in vitro. Addition of TPA to quiescent 3T3-L1 cells rapidly induces phosphorylation of several proteins (most prominently an 80 kDa protein) and expression of early genes (initially the c-fos proto-oncogene) followed by stimulation of DNA synthesis and mitosis. We found that treatment of quiescent 3T3-L1 and variant VT-1 cells with TPA rapidly stimulates the phosphorylation of DNA topoisomerase I and postulated that this posttranslational modification might be the mechanism for regulating the enzyme during the early events in TPA-induced cell proliferation.

Scleroderma Autoantibodies Recognize Phosphorylated DNA Topoisomerase I from TPA-Treated 3T3-L1 Cells

Patients with the diffuse form of the autoimmune disease scleroderma have high sera titers of antibodies that recognize DNA topoisomerase I (chapter 3). These autoantibodies were utilized in this study to isolate DNA topoisomerase I from cultured murine 3T3-L1 fibroblasts. Quiescent cultures that had been incubated in 5% fetal bovine serum for 3 days after reaching confluence were equilibrated with [32P]orthophosphate and then treated with TPA. The nuclear lysates from these cells were incubated with immobilized normal human antibodies or autoantibodies from four scleroderma patients, FO, KK, MI and L702 (Figure 4.1). DNA topoisomerase I has a molecular mass of 100 kDa and was immunoprecipitated
Figure 4.1. Phosphorylated DNA topoisomerase I is immunoprecipitated from TPA-treated 3T3-L1 cells with scleroderma autoantibodies. Quiescent 3T3-L1 fibroblasts were equilibrated with [\(^{32}\)P]orthophosphate and incubated with 100 ng/ml TPA for 1 h at 37°C. DNA topoisomerase I was immunoprecipitated from nuclear extracts with 5 \(\mu\)g purified IgG from non-scleroderma humans (hIgG; lane 1), 5 \(\mu\)g purified IgG from scleroderma patients FO (lane 2), KK (lane 3) and MI (lane 4), or 1 \(\mu\)l antiserum from scleroderma patient L702 (lane 5) and was subject to 7% SDS-polyacrylamide gel electrophoresis and autoradiography.
by the autoantibodies as the major phosphoprotein. KK antibodies immunoprecipitated the most amount of phosphorylated DNA topoisomerase I (Figure 4.1, lane 3) and MI antibodies immunoprecipitated the least amount (Figure 4.1, lane 4). This suggests that KK antibodies have the strongest affinity for phosphorylated DNA topoisomerase I, in agreement with their relative ability to inhibit DNA relaxation activity (chapter 3), because the amount of antibody is in 1000-fold molar excess. The normal human immunoglobulin did not strongly bind to any phosphorylated protein (Figure 4.1, lane 1). Thus, DNA topoisomerase I from TPA-treated murine fibroblasts is a phosphoprotein that can be recognized by scleroderma anti-DNA topoisomerase I autoantibodies.

**DNA Topoisomerase I Phosphorylation Is Rapidly Enhanced in TPA-Treated 3T3-L1 Cells**

DNA topoisomerase I from quiescent fibroblasts was phosphorylated at a basal level after a 4 h pre-incubation with [³²P]orthophosphate (Figure 4.2A, lanes 1 and 2). The amount of [³²P]orthophosphate incorporated into DNA topoisomerase I reached equilibrium within 3.5 h of labelling in quiescent cells (Figure 4.3). Treatment of cells with 100 ng/ml TPA for as little as 10 min stimulated the incorporation of phosphate into DNA topoisomerase I (Figure 4.2A and B). The degree of phosphorylation increased linearly during 2 h following TPA treatment; DNA topoisomerase I phosphorylation was stimulated 2-fold at 20 min after the addition of TPA and 6-fold by 2 h (Figure 4.2B). The effect of 0.1% DMSO (the diluent for TPA) alone was not appreciable (less than 1.2-fold stimulation at 1 h).

A 67 kDa phosphoprotein was immunoprecipitated in two experiments and demonstrated phosphorylation kinetics similar to authentic DNA topoisomerase I. Although protease inhibitors were employed, this protein could be the abundant proteolytic product of the 100 kDa DNA topoisomerase I (Liu and Miller, 1981). This observation suggests that the site of phosphorylation may be on the carboxyl terminal 67 kDa, consistent with *in vitro* experiments demonstrating protein kinase C-mediated phosphorylation of a 67 kDa DNA topoisomerase I (Pommier *et al.*, 1990; chapter 6) but potentially discrepant with comparative
Figure 4.2A. Time course of DNA topoisomerase I phosphorylation in TPA-treated 3T3-L1 cells. Quiescent 3T3-L1 fibroblasts were labeled with [\(^{32}\)P]orthophosphate for 4 h and treated at 37°C with 0.1% DMSO for 40 min (lane 1), no treatment (lane 2), or 100 ng/ml TPA for 10 min (lane 3), 20 min (lane 4), 30 min (lane 5) and 60 min (lane 6). DNA topoisomerase I was immunoprecipitated from nuclear extracts, resolved in a 7% SDS-polyacrylamide gel and visualized by autoradiography.
Figure 4.2B. Time course of DNA topoisomerase I phosphorylation in TPA-treated 3T3-L1 cells. The amount of $^{32}$P incorporated into the 100 kDa DNA topoisomerase I was directly counted from dried gels with a Betascope analyzer. Data plotted is the mean of five independent experiments relative to no treatment (mean=1800 counts) for each experiment. Two of the experiments included a 15 min point, three experiments used 10 min, 20 min and 120 min points and all five experiments contained 30 min and 60 min points. Error bars represent standard error of the mean.
Figure 4.3. Time course of phosphate Incorporation into DNA topoisomerase I in 3T3-L1 cells. Quiescent 3T3-L1 fibroblasts were incubated for 1.5 h, 3.5 h or 5.5 h in phosphate-free DMEM containing 0.1 mCi/ml [32P]orthophosphate. DNA topoisomerase I was immunoprecipitated and electrophoresed on a 7% SDS-polyacrylamide gel. The dried gel was quantified directly with a Betascope analyzer.
tryptic phosphopeptide mapping (chapter 7). If the 67 kDa phosphoprotein is the 67 kDa fragment of DNA topoisomerase I, it may have been phosphorylated in the cell as the intact protein and degraded during storage or isolation; however, the presence of the 67 kDa species may have physiological significance.

The level of DNA topoisomerase I phosphorylation parallels the induction of early genes in response to TPA treatment (Greenberg and Ziff, 1984; I. Stuiver, Y. Shimizu and N. Shimizu, manuscript in preparation). Although the exact relationship between DNA topoisomerase I phosphorylation and gene expression has not been established, the kinetic correlation suggests a role for phosphorylated DNA topoisomerase I in the transcription of early genes. The enhancement of DNA topoisomerase I phosphorylation predates the expression of the DNA topoisomerase I gene (Hwong et al., 1989), suggesting that phosphorylation and not de novo synthesis of the enzyme provides the required topoisomerase activity.

**DNA Topoisomerase I Phosphorylation in 3T3-L1 Cells Is Dependent on TPA Concentration**

TPA at 1 ng/ml for 1 h slightly stimulated DNA topoisomerase I phosphorylation (relative to DMSO), while 100 ng/ml TPA was saturating (Figure 4.4). The dose-response profile is analogous to the stimulation of DNA synthesis by TPA in 3T3-L1 cells (Shimizu and Shimizu, 1989). Therefore, phosphorylation of DNA topoisomerase I appears to be an element of the cellular response to TPA and could be involved in the signal transduction leading to cell proliferation. However, the connection between TPA-induced DNA topoisomerase I phosphorylation and DNA synthesis has not been rigorously verified.

**DNA Topoisomerase I Is Phosphorylated Primarily on Serine in TPA-Treated 3T3-L1 Cells**

Acid hydrolysis and two-dimensional thin-layer electrophoresis of DNA topoisomerase I from 3T3-L1 cells treated with 100 ng/ml TPA for 1 h indicated that predominantly serine was phosphorylated (Figure 4.5). However, 10% of the radioactivity incorporated into
Figure 4.4. Effect of TPA concentration on DNA topoisomerase I phosphorylation in quiescent 3T3-L1 cells. Quiescent 3T3-L1 cells labeled with $[^{32}P]$orthophosphate were treated for 1 h at 37°C with 0.1% DMSO or 1 ng/ml, 10 ng/ml, 100 ng/ml and 1000 ng/ml TPA in 0.1% DMSO. DNA topoisomerase I was isolated by immunoprecipitation and subject to electrophoresis. The dried gel was quantified with a Betascope analyzer and the amount of $^{32}$P incorporated into 100 kDa DNA topoisomerase I was plotted relative to the DMSO treatment (800 counts); the graph shows the results of a typical experiment.
Figure 4.5. Phosphoamino acid analysis of DNA topoisomerase I phosphorylated in TPA-treated 3T3-L1 cells. DNA topoisomerase I immunoprecipitated from $^{32}$P-labeled 3T3-L1 cells treated with 100 ng/ml TPA for 1 h was isolated from a SDS-polyacrylamide gel and hydrolyzed with acid. The hydrolysate was resolved by two-dimensional thin-layer electrophoresis and visualized by autoradiography. The positions of the phosphoamino acid markers (P-Ser, P-Thr and P-Tyr), detected by ninhydrin staining, and inorganic phosphate ($^{32}$P) are indicated; other spots are probably incompletely hydrolyzed phosphopeptides.
phosphoamino acids was on threonine while phosphotyrosine was absent (quantified directly from the thin-layer plate with a Betascope analyzer). This result is compatible with *in vitro* data that DNA topoisomerase I was phosphorylated primarily on serine but also on threonine by protein kinase C (chapter 6). The spot that migrates slowest at pH 1.9 and with phosphotyrosine at pH 3.5 was also observed after two-dimensional phosphoamino acid analysis of DNA topoisomerase I phosphorylated by protein kinase C *in vitro* (chapter 6). This spot apparently does not represent a phosphorylated basic amino acid due to its stability in acidic conditions and possibly represents an incompletely hydrolyzed phosphopeptide.

*Phosphate Incorporated into DNA Topoisomerase I Has a Short Half-life*

The turnover of phosphate incorporated into DNA topoisomerase I was assayed to determine if TPA treatment induced *de novo* phosphorylation (by stimulating a kinase) or prevented dephosphorylation (by inhibiting a phosphatase). We would expect that the phosphorylation state of DNA topoisomerase I would be stable during a post-TPA chase if inhibition of a phosphatase was the mechanism for enhancing phosphorylation. Cells labeled with $[^{32}\text{P}]$orthophosphate were washed with unlabeled media and treated with DMSO or TPA. The level of phosphate incorporated into DNA topoisomerase I decreased to about 30% after 2 h of either treatment (Figure 4.6). The half-life of phosphate on DNA topoisomerase I was about 40 min and was not reduced by treatment of cells with TPA (Figure 4.6). Therefore the increased amount of phosphorylated DNA topoisomerase I was most likely due to stimulation of phosphorylation mediated by a kinase. The phosphorylated DNA topoisomerase I remaining after 2 h was presumably generated from labeled phosphate that persisted in the intracellular ATP pool, but it could represent a population of molecules resistant to dephosphorylation. The short half-life of incorporated phosphate is consistent with the rapid equilibration of incorporated phosphate on DNA topoisomerase I in quiescent cells (Figure 4.3).
Figure 4.6. Turnover of phosphate incorporated into DNA topoisomerase I from TPA-treated 3T3-L1 cells. Quiescent 3T3-L1 fibroblasts equilibrated with $[^{32}P]$orthophosphate were washed twice with unlabeled oMEM and incubated in unlabeled oMEM containing either 0.1% DMSO (hollow circle) or 100 ng/ml TPA (filled circle). DNA topoisomerase I was immunoprecipitated from cells after 30 min, 60 min or 120 min at 37°C and subject to electrophoresis on 7% SDS-polyacrylamide gels. The amount of $^{32}P$ incorporated into the 100 kDa DNA topoisomerase I was quantified from dried gels with a Betascope analyzer. Data plotted is the mean of three independent experiments relative to no treatment (mean=1700 counts) for each experiment. Error bars represent standard error of the mean.
Phosphorylated DNA Topoisomerase I Is Depleted From Nuclear Extracts by Photocrosslinking

Photo-crosslinking of labeled cells after TPA treatment was attempted to test if phosphorylated DNA topoisomerase I was bound to DNA and thus involved in transcription or other DNA-related processes. Irradiation with UV light generates covalent linkages between DNA and proteins associated with DNA (Gilmour et al., 1986). DNA and DNA-protein adducts were precipitated from nuclear extracts with polyethylene glycol and the supernatants were immunoprecipitated. The phosphorylated DNA topoisomerase I in the post-DNA fraction from DMSO-treated cells was not affected by photo-crosslinking (Figure 4.7). However, irradiation of TPA-treated cells with UV light reduced the level of unbound phosphorylated DNA topoisomerase to that of the control (Figure 4.7), indicating that the phosphorylated DNA topoisomerase I was tightly associated with DNA. Inefficiency of crosslinking may have prevented, to some extent, the precipitation and depletion of DNA topoisomerase I from the post-DNA supernatants. However some phosphorylated DNA topoisomerase I, or nearly all in the DMSO-treated cells, was probably not active on DNA and thus was not crosslinked and precipitated. We were unable to liberate labeled DNA topoisomerase I from the precipitated DNA fraction.

DNA Topoisomerase I Phosphorylation Is Rapidly Enhanced in TPA-Treated VT-1 Cells, a Variant of 3T3-L1 Cells That Have a Limited Response to TPA

The role of DNA topoisomerase I phosphorylation in signal transduction was further investigated utilizing VT-1 cells, a genetic variant of 3T3-L1 cells. VT-1 fibroblasts were isolated by their lack of mitogenic response to phorbol ester tumor promoters (Shimizu et al., 1986). Treatment of VT-1 with TPA induces early effects including c-fos and c-myc gene expression (I. Stuiver, Y. Shimizu and N. Shimizu, manuscript in preparation) and protein kinase C translocation to the nucleus but not phosphorylation of the 80 kDa protein and DNA synthesis (Shimizu and Shimizu, 1989). TPA stimulated the phosphorylation of DNA
Figure 4.7. Depletion of phosphorylated DNA topoisomerase I from nuclear extracts of TPA-treated 3T3-L1 cells by photo-crosslinking. Quiescent 3T3-L1 cells labeled with \(^{32}\)P-orthophosphate were treated for 1 h at 37°C with either 0.1% DMSO or 100 ng/ml TPA and were either irradiated with UV light or not irradiated. DNA topoisomerase I was immunoprecipitated from the nuclear supernatants and subjected to electrophoresis. The dried gel was quantified with a Betascope analyzer and the relative amount of \(^{32}\)P incorporated into the 100 kDa DNA topoisomerase I was plotted.
topoisomerase I in VT-1 cells with kinetics similar to that in parental 3T3-L1 cells (Figure 4.8). Therefore DNA topoisomerase I phosphorylation correlates with early gene expression and may be necessary but is not sufficient for stimulation of DNA synthesis. The defect in VT-1 cells is either downstream of DNA topoisomerase I phosphorylation or is on a different branch of the signal transduction network.

**Camptothecin, a Specific Inhibitor of DNA Topoisomerase I, Suppresses TPA-Stimulated DNA Synthesis in 3T3-L1 Cells**

The effect of the DNA topoisomerase I inhibitor camptothecin on the stimulation of DNA synthesis by TPA was examined to probe the requirement for DNA topoisomerase I in signal transduction. Quiescent 3T3-L1 fibroblasts were treated with various concentrations of camptothecin followed by either 0.1% DMSO or 100 ng/ml TPA for 20 h. DNA synthesis was assayed by incorporation of \([^3H]thymidine\) during a 1 h incubation. Thymidine incorporation in the absence of camptothecin was more than 10-fold greater in cells treated with TPA than in cells treated with DMSO; camptothecin at 25 \(\mu M\) suppressed TPA-stimulated DNA synthesis (Figure 4.9). This suggests that DNA topoisomerase I was involved in the mitogenic response of the cell to TPA and is consistent with other reports that camptothecin inhibits replication in cultured mammalian cells (Liu, 1990). However, 1 \(\mu M\) camptothecin stimulated the incorporation of thymidine in quiescent cells (Figure 4.9). The effects of 100 ng/ml TPA and 1 \(\mu M\) camptothecin on thymidine incorporation were additive; camptothecin at 0.2 to 5 \(\mu M\) apparently enhanced the stimulation of DNA synthesis in TPA-treated cells (Figure 4.9). The increase in thymidine incorporation by camptothecin treatment, particularly in quiescent (DMSO-treated) cells, was unexpected but may reflect repair of DNA damaged by camptothecin-induced DNA topoisomerase I-DNA cleavable complexes or transcriptional inhibition of a cell cycle suppressor.
Figure 4.8. Time course of DNA topoisomerase I phosphorylation in TPA-treated variant VT-1 cells. Quiescent $^{32}$P-labeled VT-1 fibroblasts were treated with 0.1% DMSO for 45 min (lane 1) or with 100 ng/ml TPA for 20 min (lane 2) and 60 min (lane 3). DNA topoisomerase I was immunoprecipitated, resolved in a 7% SDS-polyacrylamide gel and visualized by autoradiography.
Figure 4.9. Effect of camptothecin on TPA-stimulated DNA synthesis in 3T3-L1 cells. Quiescent 3T3-L1 fibroblasts were treated with 0, 0.04 mM, 0.2 mM, 1 mM, 5 mM and 25 mM camptothecin in 0.1% DMSO followed by either 0.1% DMSO (hollow circle) or 100 ng/ml TPA (filled circle) and incubated for 20 h. Incorporation of $^3$H-thymidine was measured for 1 h after washing twice with DMEM. Data plotted is the mean of four trials; error bars represent standard error of the mean.
CHAPTER 5

PHOSPHORYLATION OF DNA TOPOISOMERASE I IN CULTURED CELLS TREATED WITH GROWTH FACTORS

Cultured cells treated with polypeptide growth factors were examined to assess whether DNA topoisomerase I is phosphorylated during the stimulation of cell proliferation by other treatments besides TPA. Epidermal growth factor (EGF) effectively induces DNA synthesis and cell division in quiescent Swiss/3T3 mouse fibroblasts. Physiological concentrations of insulin stimulate proliferation of H-35 rat hepatoma cells. The mechanism by which these growth factors transduce signals to the nucleus has not been completely elucidated. However, the transmembrane receptors for each contain an intracellular tyrosine kinase which is activated by extracellular binding of the ligand. Phospholipase C, and phosphatidylinositol 3 kinase are substrates for activation by tyrosine phosphorylation and catalyze the turnover of inositol phospholipids, generating the protein kinase C activators \( \text{Ca}^{2+} \) (via inositol triphosphate) and diacylglycerol. The experiments in this section demonstrate that EGF and insulin can also enhance the phosphorylation of DNA topoisomerase I, suggesting that DNA topoisomerase I phosphorylation may be a common response to the induction of mammalian cell growth. Results from non-responsive variants indicate that although DNA topoisomerase I phosphorylation may be necessary, it is not sufficient for the stimulation of DNA synthesis. In addition, the only cell line examined that was lacking an enhancement in DNA topoisomerase I phosphorylation was also the only one deficient for the induction of c-fos transcription.

*DNA Topoisomerase I Phosphorylation Is Rapidly Enhanced in Insulin-Treated H-35 Cells*

H-35 hepatoma cell nuclear extracts were prepared by Dr. Yoshiko Shimizu. Scleroderma autoantibodies recognized the phosphorylated 100 kDa DNA topoisomerase I in
rat hepatoma cell nuclear extracts. DNA topoisomerase I was phosphorylated in quiescent H-35 cells and treatment with insulin immediately enhanced the amount of incorporated phosphate (Figure 5.1). The level of DNA topoisomerase I phosphorylation doubled after 1 h of insulin treatment (Figure 5.2).

**DNA Topoisomerase I Phosphorylation in H-35 Cell Variants With an Altered Response to Insulin**

Several genetic variants have been previously isolated by their resistance to an insulin-diphtheria A fragment conjugate (Shimizu and Shimizu, 1986). Insulin does not stimulate DNA synthesis, but does enhance autophosphorylation of the insulin receptor, in the variants DTal-b and 14-1; in addition, insulin induces tyrosine aminotransferase production in H-35 cells and 14-1 variants but not in DTal-b variants (Shimizu and Shimizu, 1986). Insulin also induces expression of the early gene c-fos in H-35 and DTal-b but not in 14-1; however transcription of the early gene c-myc is induced in all three cell lines (Y. Shimizu, D. S. Samuels and N. Shimizu, manuscript in preparation).

The basal level of DNA topoisomerase I phosphorylation in quiescent DTal-b and 14-1 pre-incubated for 2 h with $[^{32}P]$orthophosphate was approximately half the level in H-35 cells (Figure 5.2). Treatment with insulin stimulated the phosphorylation of DNA topoisomerase I in DTal-b but not in 14-1 (Figure 5.2). The H-35 variant 14-1 is the only cell line tested that is deficient in DNA topoisomerase I phosphorylation, suggesting a correlation between induction of DNA topoisomerase I phosphorylation and c-fos expression.

**DNA Topoisomerase I Phosphorylation is Enhanced in Swiss/3T3 Cells Treated with Epidermal Growth Factor**

Scleroderma autoantibodies immunoprecipitated the 100 kDa phosphorylated DNA topoisomerase I from Swiss/3T3 mouse fibroblasts. EGF at 100 ng/ml for 1 h stimulated the phosphorylation of DNA topoisomerase I 2.5-fold (Figure 5.3).
Figure 5.1. Time course of DNA topoisomerase I phosphorylation in insulin-treated H-35 cells. Quiescent $^{32}$P-labeled H-35 rat hepatoma cells were incubated at 37°C without insulin for 30 min (lane 1) or treated at 37°C with 600 ng/ml insulin for 1 min (lane 2), 10 min (lane 3), 30 min (lane 4) and 60 min (lane 5). DNA topoisomerase I was immunoprecipitated from 2.5 M NaCl nuclear extracts, resolved in a 7% SDS-polyacrylamide gel and visualized by autoradiography.
Figure 5.2. Time course of DNA topoisomerase I phosphorylation in H-35 cells and variants treated with Insulin. Quiescent $^{32}$P-labeled H-35 (filled circle), 14-1 (hollow circle) and DTal-B (hollow square) rat hepatoma cells were incubated at 37°C without insulin or treated at 37°C with 600 ng/ml insulin for 1 min, 10 min, 30 min and 60 min. DNA topoisomerase I was immunoprecipitated from 2.5 M NaCl nuclear extracts, subjected to electrophoresis in a 7% SDS-polyacrylamide gel and quantified directly with a Betascope analyzer.
Figure 5.3. Phosphorylation of DNA topoisomerase I in EGF-treated Swiss/3T3 cells.

Quiescent $^{32}$P-labeled Swiss/3T3 mouse fibroblasts were not treated (lane 1) or treated with 100 ng/ml EGF (lane 2) at 37°C for 1 h. The cells were pretreated with 50 μM H-7 for 1 h. DNA topoisomerase I was isolated by immunoprecipitation from nuclear extracts and visualized by autoradiography following 7% SDS-polyacrylamide gel electrophoresis.
CHARACTERIZATION OF DNA TOPOISOMERASE I PHOSPHORYLATION BY PROTEIN KINASE C IN VITRO

We hypothesized that protein kinase C might directly phosphorylate DNA topoisomerase I because protein kinase C is the major cellular receptor for TPA, is translocated to the nucleus of murine fibroblasts following TPA treatment and is known to phosphorylate several nuclear components in vitro. Protein kinase C and DNA topoisomerase I were purified and the transfer of phosphate to DNA topoisomerase I catalyzed in vitro by protein kinase C was biochemically characterized. The reaction was dependent on the known cofactors for protein kinase C. Phosphorylation was primarily on serine, which was also the residue phosphorylated in vivo in TPA-treated murine fibroblasts. The $K_m$ and $V_{max}$ indicated that the reaction was physiologically feasible, suggesting that protein kinase C may directly phosphorylate DNA topoisomerase I in TPA-treated mammalian cells.

Protein Kinase C Phosphorylates Human DNA Topoisomerase I in a Ca$^{2+}$ and Phospholipid-Dependent Fashion

DNA topoisomerase I was purified from HeLa cells using hydroxyapatite, P11 phosphocellulose, Sephacryl S-200 and single-stranded DNA-cellulose column chromatography (see chapter 2). SDS-polyacrylamide gel electrophoresis and silver staining indicated that the purified 100 kDa protein was essentially homogeneous (Figure 6.1). Human DNA topoisomerase I, a nuclear enzyme, was examined as a substrate for phosphorylation by incubation with type III protein kinase C, protein kinase C cofactors and [γ-32P]ATP followed by 7% SDS-polyacrylamide gel electrophoresis and autoradiography. The murine type III protein kinase C was purified 860-fold by DE52-cellulose, Mono Q and high
Figure 6.1. SDS-polyacrylamide gel electrophoresis of human DNA topoisomerase I.

One µg of DNA topoisomerase I purified from HeLa cells was applied to a 3.5% polyacrylamide stacking gel and electrophoresed through a 10% polyacrylamide resolving gel. Protein was detected by silver staining.
performance hydroxyapatite column chromatography based on its activation by Ca\(^{2+}\), phospholipid and TPA (chapter 2).

The phosphorylation of DNA topoisomerase I by type III protein kinase C was dependent on Ca\(^{2+}\) and PS in the absence of TPA (Figure 6.2, lane 8). The addition of TPA stimulated the extent of phosphorylation (Figure 6.2, lane 12) and diminished the requirement for Ca\(^{2+}\) (Figure 6.2, lanes 7 and 11); however PS was absolutely required for the reaction (Figure 6.2, lane 10). This cofactor requirement profile establishes DNA topoisomerase I as a class C (Ca\(^{2+}\) and phospholipid-dependent) protein kinase C substrate (Bazzi and Nelsestuen, 1987). Ca\(^{2+}\), PS and TPA stimulated the phosphorylation of a 77 kDa protein, putatively protein kinase C. The autophosphorylation was slightly enhanced in the presence of DNA topoisomerase I (detected upon overexposure of the autoradiogram). This may reflect the ability of substrates, particularly basic proteins, to alter the cofactor dependency of protein kinase C (Bazzi and Nelsestuen, 1987). The effect of DNA topoisomerase I on protein kinase C activity in vitro may be worth further pursuit. No other phosphoproteins were observed. Phosphorylated DNA topoisomerase I has been immunoprecipitated from the protein kinase C reaction with anti-DNA topoisomerase I autoantibodies from scleroderma patients (Samuels et al., 1989; chapter 3).

**Protein Kinase C Phosphorylates Murine DNA Topoisomerase I**

DNA topoisomerase I was purified to near homogeneity from mouse liver by Bio-Rex 70, hydroxyapatite (Figure 6.3A) and phenyl-Sepharose (Figure 6.3B) column chromatography (chapter 2). SDS-polyacrylamide gel electrophoresis and silver staining revealed a 67 kDa protein (Figure 6.4). A 67 kDa species is an abundant proteolytic fragment of DNA topoisomerase I (Liu and Miller, 1981). The murine DNA topoisomerase I was most likely degraded from 100 kDa to 67 kDa during either storage of the raw material or isolation of the enzyme because other eukaryotic type I DNA topoisomerases are approximately 100 kDa as well as sensitive to proteolysis (Liu, 1983; Vosberg, 1985; Wang, 1985), and the liver is
Figure 6.2. Effect of cofactors on the phosphorylation of DNA topoisomerase I by type III protein kinase C. Phosphorylation reactions were incubated at 30°C for 2 min in 30 μl with 420 ng human DNA topoisomerase I (Topo I; lanes 1, 2 and 5-12), 7.5 ng type III protein kinase C (PKC; lanes 3-12), 0.15 mM CaCl₂ (lanes 2, 4, 6, 8, 10 and 12), 2 mM EGTA (lanes 1, 3, 5, 7, 9 and 11), 25 μg/ml PS (lanes 2, 4, 7, 8, 11 and 12), 100 ng/ml TPA (lanes 2, 4 and 9-12) and 10 μM [γ-³²P]ATP. Reactions were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography.
Figure 6.3A. Purification of murine DNA topoisomerase I. The 0.6 M NaCl wash from the Bio-Rex 70 column containing DNA topoisomerase I activity was loaded onto a hydroxyapatite column and the column was eluted with a linear gradient of potassium phosphate (pH 7.0) (dotted line). DNA topoisomerase I activity was assayed by relaxation of supercoiled DNA using serial dilutions (filled circle); activities greater than 120 units were out of range and were plotted as 120 units (1 unit equals the amount of DNA topoisomerase I that relaxes 50% of the supercoiled DNA in the standard reaction). Protein concentration was assessed by optical density at 280 nm (solid line).
Figure 6.3B. Purification of murine DNA topoisomerase I. Fractions 29-42 from the hydroxyapatite column were loaded onto a phenyl-Sepharose column and the column was eluted with a gradient of potassium phosphate (pH 7.0) (dotted line). DNA topoisomerase I activity (filled circle) and protein (solid line) was measured as in Figure 6.3A. No DNA topoisomerase I activity was detected in fractions 1-30. Fractions 44-53 were pooled and concentrated on a second Bio-Rex 70 column.
Figure 6.4. SDS-polyacrylamide gel electrophoresis of murine DNA topoisomerase I.

One µg of DNA topoisomerase I purified from mouse liver was applied to a 3.5% polyacrylamide stacking gel and electrophoresed through a 10% polyacrylamide resolving gel. Protein was detected by silver staining.
particularly rich in proteases. No differences in the enzymatic properties of the 67 kDa and 100 kDa species are detected in vitro (Liu and Miller, 1981), but the 33 kDa fragment is now thought to constitute a regulatory domain (A. Bodley and L.F. Liu, personal communication). However, a 67 kDa DNA topoisomerase I was previously purified from mouse L1210 cells (Ross et al., 1983), has previously been shown to be the target of activation by protein kinase C (Pommier et al., 1990), and appears to be phosphorylated occasionally in TPA-treated 3T3-L1 cells (chapter 4). Type III protein kinase C phosphorylated murine DNA topoisomerase I in the presence of Ca$^{2+}$, PS and TPA (Figure 6.5).

**Human DNA Topoisomerase I IsPhosphorylated Primarily on Serine by Type III Protein Kinase C**

Partial acid hydrolysis of labeled human DNA topoisomerase I followed by two-dimensional thin-layer electrophoresis at pH 1.9 and 3.5 demonstrated that phosphorylation occurred primarily on serine (Figure 6.6), but also a minor component on threonine (about 25% as quantified directly from the thin-layer plate with a Betascope analyzer). The conspicuous spot migrating slowest at pH 1.9 and between phosphotyrosine and phosphothreonine at pH 3.5 was also observed after two-dimensional phosphoamino acid analysis of DNA topoisomerase I phosphorylated in vivo (chapter 4). This spot is probably an incompletely hydrolyzed phosphopeptide, as its stability under acidic conditions precludes the possibility that it is a phosphorylated basic amino acid.

**Kinetics of Human DNA Topoisomerase I Phosphorylation by Type III Protein Kinase C**

Approximately 1.3 moles of phosphate were transferred per mole of human DNA topoisomerase I during extended incubation (30 min) with 1500-fold molar excess ATP. The phosphorylation reaction was linear with respect to protein kinase C concentration up to 2.5 ng/µl (Figure 6.7A) and time from 0.5 min to 16 min (Figure 6.7B), and appeared to demonstrate Michaelis-Menten kinetics when human DNA topoisomerase I concentration was
Figure 6.5. Phosphorylation of murine DNA topoisomerase I by type III protein kinase C. The 67 kDa carboxyl terminus of DNA topoisomerase I, purified from mouse liver, was incubated at 30°C for 5 min with 7.5 ng protein kinase C, 1 µM [γ-<sup>32</sup>P]ATP and 2 mM EGTA (lane 1) or 0.15 mM CaCl<sub>2</sub>, 25 µg/ml PS and 100 ng/ml TPA (lane 2) in 30 µl. Reactions were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography.
Figure 6.6. Phosphoamino acid analysis of DNA topoisomerase I phosphorylated by type III protein kinase C. Phosphorylated human DNA topoisomerase I was isolated from a SDS-polyacrylamide gel and acid hydrolyzed. The amino acids were resolved by two-dimensional thin-layer electrophoresis and visualized by autoradiography. The positions of the phosphoamino acid markers (P-Ser, P-Thr and P-Tyr), detected by ninhydrin staining, and inorganic phosphate (\(^{32}\text{P}\)) are indicated; other spots are probably incompletely hydrolyzed phosphopeptides.
Figure 6.7A. Evaluation of the phosphorylation of DNA topoisomerase I by type III protein kinase C. Phosphate incorporated into human DNA topoisomerase I, quantified directly from dried SDS-polyacrylamide gels with a Betascope analyzer, as a function of protein kinase C concentration. The reactions contained 420 ng DNA topoisomerase I, 1 μM [γ-32P]ATP, Ca²⁺, PS and TPA in 30 μl, and were incubated at 30°C for 2 min.
Figure 6.7B. Evaluation of the phosphorylation of DNA topoisomerase I by type III protein kinase C. Phosphate incorporated into human DNA topoisomerase I, quantified directly from dried SDS-polyacrylamide gels with a Betascope analyzer, as a function of time. The reactions contained 420 ng DNA topoisomerase I, 7.5 ng type III protein kinase C, 1 μM [γ-32P]ATP, Ca²⁺, PS and TPA in 30 μl, and were incubated at 30°C.
varied from 90 to 370 nM (Figure 6.7C). The apparent $K_m$ was 0.36 $\mu$M and the apparent $V_{\text{max}}$ was 0.74 $\mu$mol P$_i$/min/mg protein kinase C determined from linear regression of the Lineweaver-Burk plot (Figure 6.7D). Although a short reaction time (5 min) was employed, these results should be interpreted with caution because limited amounts of DNA topoisomerase I restricted the maximum substrate concentration to only slightly greater than the estimated $K_m$.

H-7, an Inhibitor of Protein Kinase C, Suppresses DNA Topoisomerase I Phosphorylation in Vitro

The protein kinase C inhibitor H-7 suppressed the phosphorylation of human DNA topoisomerase I by type III protein kinase C (Figure 6.8). The amount of phosphate incorporated was decreased to 73% and 29% of the control by 10 $\mu$M and 50 $\mu$M H-7, respectively, as quantified directly with a Betascope analyzer. H-7 has a $K_i$ of 6 $\mu$M for protein kinase C (Hidaka et al., 1984).

DNA Topoisomerase I Is Phosphorylated by Three Types of Protein Kinase C

Type I and II protein kinase C were purified 220-fold and 440-fold as the first and second peaks on high performance hydroxyapatite column chromatography (Figure 6.9A). All three protein kinase C subtypes were able to phosphorylate human DNA topoisomerase I in a Ca$^{2+}$, PS and TPA-dependent fashion (Figure 6.9B). The $K_m$ was similar for all three reactions (0.4 $\mu$M), but the $V_{\text{max}}$ was about 1.3 $\mu$mol/min/mg for type I protein kinase C and 0.4 $\mu$mol/min/mg for type II protein kinase C.
Figure 6.7C. Evaluation of the phosphorylation of DNA topoisomerase I by type III protein kinase C. Phosphate incorporated into human DNA topoisomerase I, quantified directly from dried SDS-polyacrylamide gels with a Betascope analyzer, as a function of DNA topoisomerase I concentration. The reactions contained 7.5 ng type III protein kinase C, 1 µM [γ-32P]ATP, Ca²⁺, PS and TPA in 30 µl, and were incubated at 30°C for 5 min. The line represents a third order regression of the data.
Figure 6.7D. Evaluation of the phosphorylation of DNA topoisomerase I by type III protein kinase C. Phosphate incorporated into human DNA topoisomerase I, quantified directly from dried SDS-polyacrylamide gels with a Betascope analyzer, as a function of DNA topoisomerase I concentration. The reciprocal of the data from Figure 6.7C were plotted and subjected to linear regression.
Figure 6.8. Inhibition of DNA topoisomerase I phosphorylation by the protein kinase C inhibitor H-7. Phosphorylation reactions contained 0, 10 μM or 50 μM H-7 and were incubated at 30°C for 5 min. The amount of phosphate incorporated into human DNA topoisomerase I was quantified directly from a dried 7% SDS-polyacrylamide gel with a Betascope analyzer.
Figure 6.9A. Phosphorylation of DNA topoisomerase I by three subspecies of protein kinase C. The fractions from the Mono Q column containing protein kinase C activity were loaded onto a high performance hydroxyapatite column and the column was eluted with a linear gradient of potassium phosphate (pH 7.5) (dotted line). Kinase activity was assayed in the presence of 0.1 mM CaCl₂, 25 μg/ml PS and 100 ng/ml TPA (filled circle) or 2 mM EGTA (hollow circle). Fractions 11-12, 14-16 and 23-26 were pooled as types I, II and III protein kinase C, respectively.
Figure 6.9B. Phosphorylation of DNA topoisomerase I by three subspecies of protein kinase C. Phosphorylation reactions were incubated at 30°C for 2 min with 270 ng human DNA topoisomerase I, 0.0024 units type I (lanes 1 and 2), type II (lanes 3 and 2), type III (lanes 5 and 6) protein kinase C, 2 mM EGTA (lanes 1, 3, and 5), 0.15 mM CaCl₂, 25 μg/ml PS and 100 ng/ml TPA (lanes 2, 4 and 6), and 10 μM [γ-³²P]ATP in 30 μl. Reactions were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.
CHAPTER 7

SITE OF PHOSPHORYLATION ON DNA TOPOISOMERASE I

The site of phosphorylation on DNA topoisomerase I was mapped by treatment with the protease trypsin to compare *in vivo* and *in vitro* phosphorylation. Trypsin cleaves at the carboxyl side of lysine and arginine residues and complete digestion of DNA topoisomerase I (23% lysine and arginine) generates 177 peptides. The major phosphorylation site in TPA-treated murine fibroblasts and *in vitro* by protein kinase C appeared to be the same, suggesting that protein kinase C may directly phosphorylate DNA topoisomerase I in TPA-treated murine fibroblasts.

*The Pattern of Tryptic Phosphopeptides Generated from DNA Topoisomerase I Phosphorylated in Vitro and in Vivo Is Similar*

Digestion of DNA topoisomerase I, isolated from TPA-treated 3T3-L1 cells, to completion with trypsin generated a major phosphopeptide, a secondary phosphopeptide and minor phosphopeptides as resolved by high-voltage thin-layer electrophoresis (Figure 7.1, lane 3). The tryptic phosphopeptide map of the 100 kDa human DNA topoisomerase I phosphorylated *in vitro* by protein kinase C revealed a single major, three secondary as well as minor phosphopeptides (Figure 7.1, lane 1). The major phosphopeptides *in vitro* and *in vivo* had analogous mobilities during thin-layer electrophoresis (Figure 7.1, lane 2). In addition, the secondary *in vivo* phosphopeptide comigrated with one of the secondary *in vitro* phosphopeptides. Therefore the major site of phosphorylation on DNA topoisomerase I seems to be the same in TPA-treated fibroblasts and *in vitro* by protein kinase C.

The tryptic phosphopeptide map of the 67 kDa murine DNA topoisomerase I phosphorylated by protein kinase C *in vitro* was distinct from the other patterns, although one or two minor phosphopeptides comigrated with minor *in vivo* phosphopeptides.
Figure 7.1. Comparative tryptic phosphopeptide mapping of DNA topoisomerase I phosphorylated *in vivo* and *in vitro*. Human DNA topoisomerase I phosphorylated by type III protein kinase C (lanes 1 and 2) and DNA topoisomerase I immunoprecipitated from \(^{32}P\)-labeled 3T3-L1 cells treated with 100 ng/ml TPA for 2 h (lanes 2 and 3) were digested with trypsin. The phosphopeptides were resolved by thin-layer electrophoresis and visualized by autoradiography. The major (→) and shared secondary (*) phosphopeptides are indicated.
The Sites Phosphorylated by Protein Kinase C on the 100 kDa Human and 67 kDa Murine DNA Topoisomerase I Appear To Be Distinct

The 100 kDa human DNA topoisomerase I and the 67 kDa murine DNA topoisomerase I produce different phosphopeptide patterns when phosphorylated in vitro by protein kinase C, digested to completion with TPCK-trypsin and separated by thin-layer electrophoresis (Figure 7.2, lanes 1 and 3). The sequence of murine DNA topoisomerase I is not yet available, but the divergence between the two phosphopeptide maps can probably be attributed to the disparity in the molecular masses rather than the phylogenic origin of the two mammalian proteins because the in vivo phosphorylated DNA topoisomerase is murine and shares more phosphopeptides in common with the in vitro phosphorylated 100 kDa human enzyme than with the in vitro phosphorylated 67 kDa murine enzyme. The 67 kDa DNA topoisomerase I produced one major, at least two secondary and numerous minor phosphopeptides upon digestion with trypsin (Figure 7.2, lane 3). Two secondary phosphopeptides from the 100 kDa and 67 kDa proteins comigrate, but the major phosphopeptides from each protein have different mobilities in thin-layer electrophoresis (Figure 7.2, lane 2).

Several mechanisms could account for the variation in tryptic phosphopeptide patterns between the 100 kDa and 67 kDa DNA topoisomerase I phosphorylated by protein kinase C: the major phosphorylation site might be on the 33 kDa amino terminus and protein kinase C would primarily phosphorylate another site in its absence; the major phosphorylation site might be on the 67 kDa carboxyl terminus but its recognition would require tertiary structure that is lost upon cleavage; or the major phosphorylation site might be on the same peptide as the cleavage site which creates the 67 kDa fragment and cleavage would affect the mobility of the peptide. The first two explanations appear unlikely because the major site on the 67 kDa protein is not phosphorylated on the 100 kDa protein in vitro or in vivo, although other phosphorylation sites on the 67 kDa protein are phosphorylated on the 100 kDa protein; this implies that protein kinase C would primarily phosphorylate a de novo site rather than a
Figure 7.2. Comparative tryptic peptide mapping of the 100 kDa and 67 kDa DNA topoisomerase I phosphorylated in vitro by type III protein kinase C. Human 100 kDa DNA topoisomerase I (lanes 1 and 2) and murine 67 kDa DNA topoisomerase I (lanes 2 and 3) phosphorylated by type III protein kinase C were digested with trypsin. The phosphopeptides were resolved by thin-layer electrophoresis and visualized by autoradiography.
secondary site upon either loss of or inability to recognize the authentic site. However the last explanation also seems dubious because the chemical milieu at the site would be drastically altered by cleavage, implying that recognition is based entirely on downstream sequences and is unaffected by putative structural changes; in addition, although the cleavage site (amino terminus of the 67 kDa protein) is not known, the only candidate phosphorylation site would be serine 250 which is on a tryptic peptide of only 4 amino acids. Phosphatase-mediated loss of activity from a 67 kDa mammalian DNA topoisomerase I combined with protein kinase C-mediated reactivation (Pommier et al., 1990) argues for the second or third mechanism: DNA topoisomerase I may be phosphorylated on its 67 kDa, carboxyl terminus in the intact protein in vivo and by protein kinase C in vitro, but another major site is phosphorylated by protein kinase C in vitro when DNA topoisomerase I lacks its 33 kDa amino terminus. Even if protein kinase C does not directly phosphorylate DNA topoisomerase I in the cell, the similarity between the migration of the in vivo and in vitro tryptic phosphopeptides suggests that phosphorylation of the 100 kDa human DNA topoisomerase I in vitro by protein kinase C may be a satisfactory model for further probing the regulation of DNA topoisomerase I by phosphorylation.
EFFECT OF PROTEIN KINASE C MODULATION IN VIVO ON DNA TOPOISOMERASE I PHOSPHORYLATION

Protein kinase C activity in vivo was inhibited in order to determine if protein kinase C has a physiological role in DNA topoisomerase I phosphorylation. Two techniques available for suppressing protein kinase C activity are down-regulation of the enzyme by long-term treatment with TPA and direct inhibition using the isoquinolinesulfonamides H-7 and H-8; the caveat is that these methods have not been established for the modulation of protein kinase C in the nucleus, where DNA topoisomerase I phosphorylation occurs in vivo. Overnight TPA pretreatment slightly enhanced DNA topoisomerase I phosphorylation in response to a second TPA treatment. H-8, but not H-7, suppressed TPA-induced DNA topoisomerase I phosphorylation. These results suggest that either protein kinase C does not function in the induction of DNA topoisomerase I phosphorylation in vivo or that a nuclear form of protein kinase C is not susceptible to inhibition by these treatments.

Down-Regulation of Protein Kinase C by Long-Term TPA-Treatment Does Not Diminish DNA Topoisomerase I Phosphorylation

Overnight treatment of cultured cells with the phorbol ester tumor promoter TPA results in the depletion of protein kinase C, the major cellular receptor for TPA. The level of protein kinase C in the plasma membrane and cytoplasm of 3T3-L1 cells is decreased to less than 10% by treatment with TPA for 30 h (Yoshiko Shimizu, personal communication). Quiescent 3T3-L1 fibroblasts were treated with either 100 ng/ml TPA or 0.1% DMSO in 10 ml DMEM containing 5% FBS. After 30 h, cultures were washed three times with 5 ml DMEM containing 0.1% BSA, labeled in phosphate-free DMEM containing 0.1 mCi/ml $[^{32}\text{P}]$orthophosphate and treated with either 100 ng/ml TPA or 0.1% DMSO. Overnight TPA
treatment had no effect on the basal level of DNA topoisomerase I phosphorylation (Figure 8.1, lanes 1 and 3); however, the 30 h TPA pretreatment slightly enhanced TPA-induced phosphorylation (Figure 8.1, lanes 2 and 4). This observation suggests that either protein kinase C is not involved in DNA topoisomerase I phosphorylation or that the nuclear protein kinase C is not down-regulated by overnight TPA treatment. The first explanation seems incongruous because substantial evidence indicates that protein kinase C mediates the biochemical effects of TPA, although TPA may have other targets. Thus, even if protein kinase C did not directly phosphorylate DNA topoisomerase I in the nucleus, it would be expected to have a fundamental role in the TPA-induced signal transduction that results in the enhancement of DNA topoisomerase I phosphorylation. Therefore, we favor the explanation that nuclear protein kinase C is not down-regulated and may be able to function in the induction of DNA topoisomerase I phosphorylation after long-term TPA treatment.

The Protein Kinase Inhibitor H-8, But Not H-7, Suppresses DNA Topoisomerase I Phosphorylation in TPA-Treated 3T3-L1 Cells

H-7 and H-8 are both isoquinolinesulfonamides that inhibit cyclic nucleotide-dependent protein kinases and protein kinase C (Hidaka et al., 1984). H-7 has been used as an inhibitor of protein kinase C, for which it has a $K_i$ of 6.0 $\mu$M, but it also inhibits cAMP-dependent protein kinase with a $K_i$ of 3.0 $\mu$M and cGMP-dependent protein kinase with a $K_i$ of 5.8 $\mu$M. H-8 is used in tandem with H-7 to determine which kinase is responsible for an effect suppressed by the inhibitor; however, neither kinase inhibitor is very specific. H-8 inhibits protein kinase C with a $K_i$ of 15 $\mu$M, cAMP-dependent protein kinase with a $K_i$ of 1.2 $\mu$M and cGMP-dependent protein kinase with a $K_i$ of 0.5 $\mu$M. Neither H-7 nor H-8 effectively inhibit casein kinase II ($K_i$ of 780 and 950 $\mu$M, respectively). H-7 inhibits DNA topoisomerase I phosphorylation in vitro by type III protein kinase C (chapter 6); but there is no evidence that these compounds can enter the nucleus of intact cells.

Quiescent 3T3-L1 fibroblasts were $^{32}$P-labeled, pretreated for 1 h with 50 $\mu$M H-7 or
Figure 8.1. DNA topoisomerase I phosphorylation in 3T3-L1 cells after long-term TPA treatment. Quiescent 3T3-L1 fibroblasts were treated for 30 h with 100 ng/ml TPA (lanes 1 and 2) or 0.1% DMSO (lanes 3 and 4), washed, labeled, and treated with 100 ng/ml TPA (lanes 2 and 4) or 0.1% DMSO (lanes 1 and 3) for 1 h. DNA topoisomerase I was immunoprecipitated from nuclear extracts, resolved by 7% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.
50 μM H-8 followed by treatment with 100 ng/ml TPA or 0.1% DMSO for 1 h. H-7 had no effect on the relative stimulation of DNA topoisomerase I phosphorylation by treatment with TPA (Figure 8.2, lanes 3 and 4), but it slightly enhanced the basal level of phosphorylation (Figure 8.2, lanes 1 and 3). In contrast, H-8 significantly suppressed the relative stimulation of phosphate incorporation into DNA topoisomerase I by TPA treatment (Figure 8.2, lanes 5 and 6), although it also slightly enhanced the basal phosphorylation level (Figure 8.2, lanes 1 and 5). A 5 min pretreatment with 50 μM H-7 or 1 h pretreatment with 10 μM H-7 and H-8 yielded analogous results.

Several mechanisms could account for these results: protein kinase C does not have a physiological role in DNA topoisomerase I phosphorylation; nuclear protein kinase C is resistant to H-7 inhibition (as well as TPA-induced down-regulation) due to either its subcellular localization or a posttranslational modification; and H-8, but not H-7, penetrates the nucleus in whole cells. As mentioned above, an abundance of data implicating protein kinase C as the mediator of TPA-induced cellular phenomena argues against the first explanation, although this interpretation should not be completely discounted on the basis of precedence. The last two explanations are feasible but lack supporting experimentation and cannot currently be distinguished. The inhibition of DNA topoisomerase I phosphorylation by H-8 argues against the sole involvement of casein kinase II; however, whether casein kinase II directly phosphorylates DNA topoisomerase I following an H-8 sensitive step has not been addressed. The H-8 sensitivity may reflect a requirement for cyclic nucleotide-dependent protein kinases in the stimulation of DNA topoisomerase I phosphorylation. However, 10 μM H-8 suppressed DNA topoisomerase I phosphorylation and the similar $K_i$ for H-7 and H-8 suggests that some inhibition would occur at 50 μM H-7, unless H-7 was not transported into the nucleus as freely as H-8. We cannot dismiss the possibilities that a currently unknown kinase which is much more sensitive to H-8 than H-7 phosphorylates DNA topoisomerase I in the nucleus of TPA-treated fibroblasts or that a nuclear protein kinase C which is resistant to H-7 functions in the phosphorylation of DNA topoisomerase I in TPA-treated fibroblasts.
Figure 8.2. Effect of H-7 and H-8 on DNA topoisomerase I phosphorylation in TPA-treated 3T-L1 cells. Quiescent $^{32}$P-labeled cells were treated with 50 μM H-7 (lanes 3 and 4) or 50 μM H-8 for 1 h (lanes 5 and 6) followed by treatment with 100 ng/ml TPA (lanes 2, 4 and 6) or 0.1% DMSO (lanes 1, 3 and 5) for 1 h. Immunoprecipitated DNA topoisomerase I was analyzed by 7% SDS-polyacrylamide gel electrophoresis and autoradiography.
CONCLUSIONS

Treatment of quiescent 3T3-L1 fibroblasts with the phorbol ester tumor promoter TPA stimulates gene expression and DNA synthesis. This is the first demonstration that the phosphorylation state of DNA topoisomerase I is modulated in vivo; it is significant because the activity of DNA topoisomerase I has been previously shown to be regulated by phosphorylation in vitro. Phosphorylation of DNA topoisomerase I is enhanced shortly after the addition of TPA and increases to about 6-fold over control levels after 2 h (chapter 4; Samuels and Shimizu, manuscript in preparation). The response exhibits the same dose dependency as the induction of DNA synthesis (Shimizu and Shimizu, 1989), reaching a maximum at 100 ng/ml TPA. The major site of phosphorylation is a serine residue although other sites, including at least one threonine residue, are also phosphorylated. TPA-stimulated DNA synthesis is blocked by the DNA topoisomerase I-specific inhibitor camptothecin, suggesting that DNA topoisomerase I functions in some facet of the signal transduction. These results and the data presented from DNA-crosslinking and the genetic variants suggest that DNA topoisomerase I is active in early gene expression (particularly c-fos) and that phosphorylation is probably the molecular mechanism for DNA topoisomerase I activation in vivo. In addition, the enhancement of DNA topoisomerase I seems to be a common response to the induction of mammalian cell growth; our experiments with murine fibroblasts and rat hepatoma cells suggest that although the increase in phosphorylation of DNA topoisomerase may be necessary, it is not sufficient for the stimulation of DNA synthesis.

Identification of the 100 kDa phosphoprotein as DNA topoisomerase I was based on its recognition by four anti-DNA topoisomerase I antibodies from Japanese and American scleroderma patients. The immunoreactivity of the scleroderma autoantibodies has been previously demonstrated by several criteria utilizing solid phase radioimmunoassay, peptide
mapping, immunoblotting, DNA relaxation activity assay and immunocytochemistry (Shero et al., 1986; Guldner et al., 1986; Maul et al., 1986; Samuels et al., 1986; chapter 3). These antibodies were employed to clone the gene for DNA topoisomerase I from an expression library (D'Arpa et al., 1988) and to immunoprecipitate phosphorylated DNA topoisomerase I from an in vitro protein kinase C reaction (Samuels et al., 1989; chapter 3).

Protein kinase C mediates many of the biological effects of TPA and has a significant role in mammalian cell signal transduction (Nishizuka, 1984a; Ashendel, 1985; Nishizuka, 1986). DNA topoisomerase I activity is enhanced by protein kinase C-catalyzed phosphorylation in vitro (Samuels et al., 1989; Pommier et al., 1990). The phosphorylation reaction in vitro is dependent on Ca²⁺ and PS, stimulated by TPA and inhibited by H-7, corroborating the involvement of protein kinase C (chapter 6; Samuels and Shimizu, manuscript submitted). The major site of phosphorylation is a serine residue; albeit other sites, including threonine residues, are phosphorylated, presumably some as in vitro artifacts. Both human and murine DNA topoisomerase I can serve as phosphate acceptors and types I, II and III protein kinase C can catalyze the reaction.

The apparent $K_m$ of 0.4 µM for the phosphorylation reaction denotes a high affinity of protein kinase C for DNA topoisomerase I ($k_f << k_b$). The reaction kinetics are similar to that of other in vitro topoisomerase phosphorylation reactions. Protein kinase C phosphorylates DNA topoisomerase II with a $K_m$ of 0.1 µM (Sahyoun et al., 1986) and casein kinase II phosphorylates DNA topoisomerase I with a $K_m$ of 0.3 µM and a $V_{max}$ of 0.7 µmol/min/mg (Mills et al., 1982) as well as DNA topoisomerase II with a $K_m$ of 0.4 µM and a $V_{max}$ of 3.3 µmol/min/mg (Ackerman et al., 1985). These values indicate that phosphorylation of topoisomerases by protein kinase C and casein kinase II is physiologically feasible.

Serine phosphorylation stimulates the activities of topoisomerases (Durban et al., 1983; Kaiserman et al., 1988; Samuels et al., 1989; Pommier et al., 1990; Coderoni et al., 1990a,b; Ackerman et al., 1985; Sahyoun et al., 1986; Rottmann et al., 1987; Saijo et al., 1990) and this posttranslational modification plausibly regulates the conformational state of...
DNA in the cell, especially during transcription and replication. DNA topoisomerase I is phosphorylated in vivo (Durban et al., 1981, 1983, 1985; chapters 4 and 5) and dephosphorylation inhibits or abolishes its activity (Durban et al., 1983; Kaiserman et al., 1988; Pommier et al., 1990; Coderoni et al., 1990b). Phosphorylation of native DNA topoisomerase I from HeLa cells by protein kinase C (Samuels et al., 1989) and from Novikoff cells by casein kinase II (Durban et al., 1983) increased DNA relaxation activity about 3-fold suggesting that the phosphorylation state is not saturated in vivo.

Several other nuclear proteins are also substrates for protein kinase C including DNA topoisomerase II (Sahyoun et al., 1986; Rottmann et al., 1987), DNA methyltransferase (DePaoli-Roach et al., 1986), DNA polymerase α (Krauss et al., 1987), RNA polymerase II (Chuang et al., 1987) and transcription factor CREB (Yamamoto et al., 1988). The activities of these nuclear components are enhanced by protein kinase C-mediated phosphorylation in vitro.

DNA topoisomerase I may serve as a target of several signal transduction pathways, as suggested for the nuclear factors AP-2 (Imagawa et al., 1987), thyroid hormone receptor (Goldberg et al., 1988) and CREB (Gonzalez et al., 1989), and thus could be a substrate for protein kinase C-mediated activation by phosphorylation in vivo. Casein kinase II probably is the principal kinase phosphorylating DNA topoisomerase I in rapidly growing tumorigenic cells (Durban et al., 1985) while protein kinase C may function to phosphorylate DNA topoisomerase I during TPA-induced cell proliferation (chapter 4). Alternatively, phosphorylation of DNA topoisomerase I by the two kinases could act at different times in the cell cycle, possibly affecting the expression of particular genes. Phosphoamino acid and phosphopeptide analyses indicate that the major site of phosphorylation in TPA-treated fibroblasts is most likely the same as the major site phosphorylated in vitro by protein kinase C (chapter 7). DNA topoisomerase I phosphorylated in vitro and in vivo also shared a secondary phosphopeptide in common. A small amount of phosphothreonine was detected both in vitro and in vivo (chapters 4 and 6). In addition, protein kinase C copurifies with
DNA topoisomerase I in Chinese hamster DC3F/9-OHE cells (Pommier et al., 1990).

Several studies have revealed the presence of protein kinase C in the nucleus. Protein kinase C was localized to the nuclei of rat neurons utilizing light and electron microscopic immunocytochemistry (Girard et al., 1985; Wood et al., 1986; Kose et al., 1988; Huang et al., 1988); staining was adjacent to the inner nuclear envelope (Wood et al., 1986) and type I and II protein kinase C subtypes were specifically identified (Huang et al., 1988). In rat liver nuclei, the presence of protein kinase C was demonstrated by immunoblotting (Capitani et al., 1987), protein kinase C was activated by prolactin (Buckley et al., 1988) and the nuclear protein kinase C was purified 50-fold (Masmoudi et al., 1989). Nuclei from HL-60 human promyelocytic leukemia cells bind phorbol esters (Kraft et al., 1987) and TPA induced the translocation of protein kinase C to the nuclear envelope (Kiss et al., 1988). Lamin B, a component of the nuclear envelope, was phosphorylated and nuclear translocation of protein kinase C was observed in HL-60 cells treated with bryostatin 1 (Fields et al., 1988); nuclear matrix proteins were phosphorylated after incubation of HL-60 cells with TPA (Macfarlane, 1986). One of the nuclear proteins phosphorylated in HL-60 cells has a molecular mass of about 100 kDa (Kiss et al., 1988; Fields et al., 1988), similar to DNA topoisomerase I. Signals transduced by cAMP in B lymphocytes stimulate nuclear translocation of protein kinase C (Cambier et al., 1987). Lamin B (Hornebeck et al., 1988) and histones H2B and H4 (Patskan and Baxter, 1985) were phosphorylated in TPA-treated lymphocytes. Histone phosphorylation, particularly H2B and H1\(^{\circ}\), was also reported in H-35 rat hepatoma cells activated by TPA (Butler et al., 1986). Translocation of protein kinase C to the nucleus of murine 3T3 fibroblasts occurred subsequent to TPA treatment (Halsey et al., 1987; Thomas et al., 1988; Leach et al., 1989; Shimizu and Shimizu, 1989). The type III subspecies was localized to the nuclear envelope, although in an altered conformation which masked an epitope on the regulatory domain (Leach et al., 1989). This altered conformation may explain the putative resistance of TPA-induced DNA topoisomerase I phosphorylation to H-7 inhibition and TPA-mediated down-regulation (chapter 8). TPA also provokes nuclear
translocation of protein kinase C in VT-1 cells (Shimizu and Shimizu, 1989).

Therefore ample evidence indicates that a form of protein kinase C is localized to the nucleus where it would be able to directly phosphorylate DNA topoisomerase I. Durban et al. (1985) have suggested, based on HPLC of tryptic phosphopeptides, that casein kinase II is responsible for DNA topoisomerase I phosphorylation \textit{in vivo}. Casein kinase II can phosphorylate DNA topoisomerase I from Novikoff hepatoma cells (Durban et al., 1983, 1985; Mills et al., 1982), \textit{Xenopus} oocytes (Kaiserman et al., 1988) and calf thymus (Coderoni et al., 1990a,b) \textit{in vitro}. Casein kinase II has also been found in the nucleus and phosphorylates several nuclear proteins (Hathaway and Traugh, 1982). In fact, treatment of BRK cells with TPA activates casein kinase II (Carroll et al., 1988) intimating that casein kinase II may mediate DNA topoisomerase I phosphorylation in TPA-treated 3T3-L1 cells. However, as mentioned above, we have hypothesized that DNA topoisomerase I may be phosphorylated \textit{in vivo} by both kinases, possibly on the same site, either in response to different signals or at different times in the cell cycle. Although unequivocal proof of which, if either, kinase physiologically catalyzes the phosphate transfer reaction and presumably modulates DNA topoisomerase I \textit{in vivo} will require further studies (by someone else).

TPA treatment of HL-60 cells (Gorsky et al., 1989) and 3T3-L1 cells (Y. Shimizu, D. S. Samuels and N. Shimizu, unpublished data) increases DNA topoisomerase I activity, consistent with activation of the enzyme by phosphorylation (Durban et al., 1983; Kaiserman et al., 1988, Samuels et al., 1989; Pommier et al., 1990; Coderoni et al., 1990b). Treatment of H-35 cells with insulin and Swiss/3T3 cells with EGF also stimulates gene expression, DNA synthesis and phosphorylation of DNA topoisomerase I (chapter 5). The kinetics of DNA topoisomerase I phosphorylation in stimulated 3T3-L1 and H-35 cells are similar. The induction of DNA topoisomerase I phosphorylation is genetically correlated with \textit{c-fos} gene expression: the only cell line examined that was deficient in the enhancement of DNA topoisomerase I phosphorylation following treatment with a growth factor or tumor promoter, the H-35 variant 14-1, was also the only cell line deficient in the induction of \textit{c-fos}.
transcription. The transcription of the c-fos gene has been kinetically correlated with the activity of DNA topoisomerase I in vivo on the c-fos gene (Stewart et al., 1990). Thus, we propose that phosphorylated DNA topoisomerase I is probably required for transcription of the early gene c-fos, which is required for signal transduction and cell proliferation. The early stimulation of phosphorylation coincides with the induction of c-fos expression (Greenberg and Ziff, 1984; I. Stuiver, Y. Shimizu and N. Shimizu, manuscript in preparation), but then the level of incorporated phosphate continues to rise, possibly reflecting transcription of other early genes (Lau and Nathans, 1985; I. Stuiver, Y. Shimizu and N. Shimizu, manuscript in preparation). The gene for DNA topoisomerase I is one of the early genes whose expression is induced in cultured fibroblasts after 2 to 3 h of TPA treatment (Hwong et al., 1989), suggesting a further requirement for DNA topoisomerase I in transcription and replication.

DNA topoisomerase I is most likely involved in the transcription of the early genes and might also be essential in later events such as DNA synthesis. DNA topoisomerase I protein has a half life of about one cell cycle (Heck et al., 1988) so its activity in vivo is presumably modulated by its phosphorylation state. The rapid equilibration of $^{32}$P orthophosphate on DNA topoisomerase I in quiescent cells (~2 h) and the rapid turnover of incorporated phosphate from DNA topoisomerase I ($t_{1/2}$=40 min) indicates that the phosphorylation state is tightly regulated by the action of one or more kinases and phosphatases (chapter 4).

Despite a dearth of explicit evidence, the stimulation of DNA topoisomerase I phosphorylation in TPA-treated quiescent fibroblasts implies that protein kinase C has a role, in addition to the regulation of a myriad of cellular phenomena, as the activator of nuclear processes associated with the induction of gene expression in cell proliferation (Figure 9.1).
Figure 9.1. Nuclear translocation of protein kinase C and regulation of nuclear components by phosphorylation.
APPENDIX A

ABBREVIATIONS

2-ME, 2-mercaptoethanol
DMEM, Dulbecco's modified Eagle's medium
DMSO, dimethylsulfoxide
DTE, dithioerythritol
DTT, dithiothreitol
EGF, epidermal growth factor
EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid
FBS, fetal bovine serum
FPLC, fast performance liquid chromatography
H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine
H-8, n-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide
PMSF, phenylmethylsulfonyl fluoride
PS, phosphatidylserine
SDS, sodium dodecyl sulfate
TPA, 12-O-tetradecanoylphorbol-13-acetate
TPCK, tosylphenylalanyl chloromethyl ketone
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