0026-895X/95/050986-11$3.00/0
Copyright © by The American Society for Pharmacology and Experimental Therapeutics
All rights of reproduction in any form reserved.
MOLECULAR PHARMACOLOGY, 47:986–996 (1995).

MOL PHARM

Investigation of the Mechanism of Higher Order Chromatin Fragmentation Observed in Drug-Induced Apoptosis

HELEN M. BEERE,[1] CHRISTINE M. CHRESTA,[1] ALI ALEJO-HERBERG, ANDRZEJ SKLADANOWSKI, CAROLINE DIVE, ANNETTE KRAGH LARSEN, and JOHN A. HICKMAN

Cancer Research Campaign Molecular and Cellular Pharmacology Group, School of Biological Sciences, University of Manchester, Manchester M13 9PT, UK (H.M.B., C.M.C., A.A.-H., C.D., J.A.H.), and Institut Gustave Roussy, CNRS URA 147, Villejuif, France (A.S., A.K.L.)

Received December 19, 1994; Accepted February 14, 1995

SUMMARY

Apoptosis is characterized by the nonrandom cleavage of DNA. After continuous treatment of MOLT-4 human T lymphoblastoid cells with the topoisomerase II inhibitor etoposide (50 μM) and the nongenotoxic agent *N*-methylformamide (300 mM), apoptosis was confirmed by electron microscopy. Analysis of DNA integrity by conventional gel electrophoresis failed to detect internucleosomal DNA cleavage. Resolution of DNA by field inversion gel electrophoresis showed fragments of 50 kilobases (kb). Etoposide induced the transient appearance of an additional DNA band of >600 kb, which was temporally coincident with DNA-protein complex formation and was rapidly reversible upon drug removal. This DNA band was not observed after *N*-methylformamide treatment. *In situ* DNA end-labeling showed the incorporation of biotinylated dUTP into 50-kb DNA fragments but not etoposide-induced DNA fragments of >600 kb. DNA end-labeling with terminal deoxynucleotidyltransferase was therefore not dependent upon internucleosomal DNA cleavage, and fragments of approximately 50 kb were characterized by free 3′-OH termini that were not occluded by topoisomerase II protein. Although we considered that topoisomerase II potentially played an active role in the fragmentation of higher order chromatin during apoptosis, the results showed that DNA cleavage by topoisomerase II induced reversible, protein-associated fragments of >600 kb and not irreversible cleavage to 50-kb fragments. The reversible cleavage of DNA to fragments of >600 kb appears to be a signal for the engagement of apoptosis and is not an initial step in the sequential unwinding of chromatin.

The realization that apoptotic cell death occurs by a conserved and genetically programmed mechanism has generated a hope that selective intervention in a process that can be modulated may influence the course of the major degenerative diseases and cancer. However, remarkably little is known about the biochemical events that irreversibly commit cells to death by apoptosis. To date, the principal biochemical feature that has been characterized is the nonrandom cleavage of DNA, a process that was thought to be associated with one of the most highly conserved morphological features of apoptosis, the condensation of chromatin (1). It was reported that DNA cleavage in cells undergoing apoptosis was mediated by the activity of an endonuclease, yielding internucleosomal integer DNA fragments of approximately 180–200 base pairs that are visualized as the characteristic "laddering" pattern when separated by conventional gel electrophoresis (2). Recently, however, FIGE analysis of the fragmentation pattern of DNA from different cell types has shown that other changes in the integrity of chromatin may be observed before, concomitantly with,[2] or even in the absence of internucleosomal DNA cleavage (3–6). The formation of discrete DNA fragments of approximately 300 kb and/or 30–50 kb has suggested that cleavage of "looped domains" of DNA, possibly at attachment points on the nuclear matrix, may occur independently of internucleosomal cleavage (4, 5). The suggestion that internucleosomal DNA cleavage and higher order DNA fragmentation are independent events is supported by recent studies examining the ionic dependence of apoptosis in murine thymocytes and isolated rat liver nuclei (7, 8).

A number of reports suggest that the appearance of an apoptotic morphology, characterized by chromatin condensa-

This research was supported by grants from the Cancer Research Campaign to J.A.H. (SP1518) and C.M.C. (SP2234) and by a travel grant from Zeneca Pharmaceuticals to H.M.B. A.S. is a fellow of l'Association pour la Recherche sur le Cancer.

[1] H.M.B. and C.M.C. contributed equally to this work.

[2] H. M. Beere, C. M. Chresta, and J. A. Hickman. Investigation of a role for topoisomerase II in the cleavage of DNA during apoptosis of human promyelocytic HL-60 leukemia cells. Submitted for publication.

ABBREVIATIONS: FIGE, field inversion gel electrophoresis; b-dUTP, biotin-16-dUTP; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ICE, interleukin-converting enzyme; kb, kilobase(s); NMF, *N*-methylformamide; PK, proteinase K; SDS, sodium dodecyl sulfate; TdT, terminal deoxynucleotidyltransferase; PBS, phosphate-buffered saline; mAMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide.

MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

tion, is not necessarily accompanied by internucleosomal DNA cleavage and may instead reflect higher order DNA degradation to 50-kb fragments. These reports include studies on DU-145 prostatic carcinoma cells (5), oligodendrocytes (9), neuroblastoma cells (10), hepatocytes (11), and MOLT-4 human T lymphoblastoid cells (12, 13). The biochemical nature of these fragments and the mechanism of their release are of interest, not least with respect to the possibility that they might be formed by the enzymatic activity of topoisomerase II. Topoisomerase II is considered to play a pivotal role in the attachment of looped domains of DNA to the nuclear matrix via interaction with specialized A/T-rich regions of DNA, termed matrix attachment regions (14). This raised the possibility that DNA cleavage to approximately 50-kb fragments might be the result of double-strand cuts mediated by the activity of topoisomerase II. If this was the case, it might provide essential clues as to the how the commitment to apoptosis occurs. We chose to investigate whether morphological apoptosis in MOLT-4 cells was accompanied by the appearance of high molecular weight DNA fragments and whether this might be indicative of the cleavage of DNA loop domains by the enzymatic activity of topoisomerase II.

TdT reactions are now commonly used for the detection of apoptotic cells using appropriately labeled deoxynucleotides, such as b-dUTP (15). We postulated that this reaction could be used to distinguish topoisomerase II-mediated loop release from that produced by endonuclease activity. Topoisomerase II covalently binds to the 5′-phosphate group of DNA and conceals the 3′-OH ends. The TdT reaction relies on the availability of free 3′-OH DNA ends, and therefore concealment of DNA breaks by associated protein would prevent the incorporation of deoxynucleotides. Consequently, DNA fragment ends generated by, and presumably associated with, topoisomerase II would not be labeled. The question also arises as to whether the TdT reaction is able to label high molecular weight DNA fragments, including those of approximately 50 kb in size, or whether it is appropriate only for cells in which blunt-ended DNA fragments are generated by the endonuclease to produce internucleosomal cleavage (16), as suggested previously (15). This is apposite to those cell types that undergo morphological apoptosis without concomitant internucleosomal DNA cleavage and those in which detection of apoptosis using the TdT method of detection has produced anomalous results, for example, breast epithelia (17).

We have used the MOLT-4 human lymphoblastoid cell line, which was previously shown to undergo apoptosis with a typical morphology but without internucleosomal DNA cleavage (12, 13). Our study shows that MOLT-4 cells stimulated to undergo apoptosis after both genotoxic and nongenotoxic damage exhibit 50-kb DNA fragments in the absence of internucleosomal cleavage. The nongenotoxic NMF was used because of our concern that agents that directly or indirectly damage DNA are widely used to evaluate changes in DNA integrity during apoptosis and that the damage directly imposed by these agents may be superimposed upon that which represents the apoptotic process itself. Data presented here support this concern and allow discrimination between changes in DNA integrity that are agent specific, and that may act as a "trigger" for apoptosis, and those that represent the conserved features of apoptosis.

Experimental Procedures

Materials

All materials were obtained from Sigma Chemical Co. (Poole, Dorset, UK) unless otherwise stated. Anti-topoisomerase α and β monoclonal antibodies were very generous gifts from Drs. F. Drake (SmithKline Beecham, King of Prussia, PA) and I. Hickson (Imperial Cancer Research Fund, Oxford, UK), respectively.

Cell Culture

MOLT-4 human T lymphoblastoid and human HL-60 promyelocytic leukemia cells were both maintained in RPMI 1640 medium (Imperial Laboratories, Andover, Hampshire, UK) supplemented with 2 mM L-glutamine and 10% fetal calf serum (Advanced Protein Products), at 37° in an atmosphere of 95% air/5% CO_2 with 100% humidity. Cells were routinely maintained in logarithmic phase growth and had an approximate doubling time of 24 hr. Stock cultures of cells were maintained for approximately 20 passages before reestablishment of stocks from frozen banked cultures. NMF or etoposide was added to cells in the logarithmic phase of growth.

Detection of DNA Fragmentation

Conventional gel electrophoresis. After drug treatment, 10^6 cells were resuspended in 20 μl of lysis buffer (10 mM EDTA, 50 mM Tris·HCl, pH 8, 0.5% sodium lauryl sarcosinate, 0.5 mg/ml PK) and incubated for 1 hr at 50°. RNase A was added (10 μl, at 0.5 mg/ml) and the incubation was continued for an additional 1 hr at 50°. After addition of 10 μl of 10 mM EDTA, pH 8, containing 1% low-melting point agarose, cell samples were loaded into dry wells of a 2% agarose gel stained with ethidium bromide (0.2 μg/ml). Samples were then resolved by electrophoresis (40 V for 3–4 hr) using a 0.09 M Tris phosphate/0.002 M EDTA buffer system, and DNA was visualized using a UV transilluminator.

FIGE. Preparation of agarose plugs. Agarose plugs were prepared according to a previously published method (18). Briefly, 4×10^6 cells were resuspended in 60 μl of nuclear buffer (10 mM Tris·HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA) and 70 μl of 1.5% low-melting point agarose prepared in nuclear buffer containing 0.4 mg/ml PK. Agarose plugs were then incubated overnight at 37° in sarcosyl lysis buffer (10 mM Tris·HCl, pH 9.5, 10 mM NaCl, 25 mM EDTA, 1% sodium sarcosinate) containing 200 μg/ml PK, washed three times (1 hr each time) in 10 mM Tris·HCl, 1 mM EDTA, and stored in 0.04 M EDTA at 4° before analysis.

Gel electrophoresis. Samples, including a commercially prepared DNA standard (50–1000 kb), were analyzed on a 1.5% agarose gel using a horizontal electrophoresis system (model H4; BRL, Gaithersburg, MD) in conjunction with a Pulsewave 760 electrophoretic field switcher, programming block, and power supply (model 200/2.0; Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK). The pulsewave switcher was programmed to provide 0.5-sec forward and 10-sec reverse pulses for the first 19 hr (3:1 ratio), after which the forward pulse was increased to 10 sec and the reverse pulse to 60 sec for an additional 19 hr (3:1 ratio). A constant voltage of 150 V was maintained throughout the total run time and a buffer system of 10 mM Tris acetate, 1 mM EDTA, pH 8.6, was used. The buffer was recirculated continuously and the temperature was maintained at 4° throughout. After staining with ethidium bromide (0.5 μg/ml), the gel was incubated with RNase A (20 μg/ml) in buffer for a minimum of 3 hr at 37° and photographed.

Immunoblotting

After drug treatment, cells were washed once in PBS containing protease inhibitors (1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 50 μg/ml leupeptin, and 1% aprotinin) and were resuspended at approximately 1×10^7 cells/ml. Samples were then sonicated on ice to lyse the cells and disrupt the nuclear matrices. DNase was added to each sample to a concen-

Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

tration of 340 units/ml, in the presence of 10 mM $MgCl_2$, and samples were incubated on ice for 1 hr, to prevent the retardation of topoisomerase II by etoposide-induced DNA-protein complexes. The protein content of each whole-cell extract was then estimated using the Bio-Rad protein assay reagent, and 75 μg of protein/lane were analyzed by SDS-polyacrylamide gel electrophoresis (7% acrylamide resolving gel/4% acrylamide stacking gel). After the electrophoretic transfer of proteins to nitrocellulose Immobilon-P polyvinylidene difluoride membranes (50 mM CAPS, pH 11), gels were stained with Coomassie blue to visualize remaining protein and to ensure that the gels had been equally loaded. Topoisomerase IIα was detected using affinity-purified antibody raised against a dodecapeptide specific to human topoisomerase IIα (170 kDa) (19), provided by Dr. F. Drake, and topoisomerase IIβ (180 kDa) was detected with the mouse monoclonal antibody 8D7 (20), supplied by Dr. I. Hickson. After an additional 45-min incubation with goat anti-mouse IgG-horseradish peroxidase conjugate (1/1000 dilution) and repeated washings with 0.3% and 0.1% Tween 20 in Tris-buffered saline, the antibody-specific proteins were visualized using the enhanced chemiluminescence detection system (Amersham International), according to the manufacturer's instructions.

Determination of Topoisomerase II Activity

Nuclear extracts. After drug treatment, approximately 4×10^7 exponentially growing MOLT-4 cells were washed once with 10 ml of buffer A (5 mM KH_2PO_4, pH 6.4, 150 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μM pepstatin, 10 μM leupeptin), resuspended in 2.5 ml of buffer A containing 0.3% Triton X-100, and then rotated gently for 10 min. The nuclear suspension was centrifuged (1000 $\times$ *g* at 4° for 10 min) and the pellet was washed with 5 ml of buffer A, resuspended in 0.4 ml of nuclear extraction buffer (buffer A containing 0.35 M NaCl), and incubated on ice for 30 min, with gentle stirring. Nuclear extracts were then centrifuged for 15 min (12,000 $\times$ *g* at 4°) and the protein concentration of each sample was determined using the Bio-Rad acid protein assay (Bio-Rad Laboratories, Munich, Germany). Nuclear extracts were frozen immediately and stored at −80°, and topoisomerase II activity was measured within 4 days.

Decatenation assay. Nuclear extract, of the appropriate protein concentration, was added to a reaction mixture containing 50 mM Tris·HCl, pH 8, 10 mM $MgCl_2$, 100 mM KCl, 5 mM EDTA, 5 mM dithiothreitol, 1 mM ATP, and 200 ng of kinetoplast DNA, and the reaction was allowed to proceed at 37° for 15 min. Reactions were arrested by the addition of loading buffer (1% SDS, 0.5% bromphenol blue, 30% glycerol), after which the samples were electrophoresed in 1.2% agarose gels at 1 V/cm for 16 hr in Tris/borate/EDTA buffer, pH 8. Liberated minicircles were quantified by densitometric analysis of photographic negatives of the ethidium bromide-stained agarose gels, using a Joyce-Loebl Chromoscan 3 densitometer.

Measurement of DNA-Protein Complexes

MOLT-4 cells were seeded at approximately 3×10^6/ml and incubated with 0.2 μCi/ml [^{14}C]leucine and 0.6 μCi/ml [^{3}H]thymidine for approximately 18 hr at 37°. After prelabeling, cells were exposed to 300 mM NMF or 50 μM etoposide for the appropriate incubation time. Cells were then harvested by centrifugation and the reaction was arrested by the addition of 0.5 ml of warm stop buffer (2.5% SDS, 10 mM EDTA, 0.8 mg/ml salmon sperm DNA). Cell lysates were passed 10 times through a 22-gauge syringe needle, incubated for 15 min at 65°, and after the addition of 112 μl of 1 M KCl, were incubated for 10 min on ice. Samples were centrifuged (12,000 $\times$ *g*) for 5 min at 4°, 1 ml of wash solution (10 mM Tris·HCl, pH 8, 100 mM KCl, 1 mM EDTA, 0.1 mg/ml salmon sperm DNA) was added, and the pellets were heated at 65° for 15 min and centrifuged at room temperature for 10 sec. This washing procedure was repeated three times. After the final wash, the pellet was dissolved in 0.5 ml of water by heating to 65°. The samples were then transferred to scintillation vials containing 5 ml of INSTA-GEL scintillation cocktail and the amount of radioactivity was determined. Data were expressed as the ratio of [^{3}H]thymidine radioactivity (dpm) to [^{14}C]leucine radioactivity.

Transmission Electron Microscopy

After drug treatment, MOLT-4 cells were collected by centrifugation and prepared for transmission electron microscopy according to a previously published method (21). Briefly, cells were washed twice with 0.1 M sodium cacodylate buffer, fixed for 1 hr at room temperature with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, washed an additional three times with 0.1 M sodium cacodylate buffer, and then incubated for 1 hr with 1% osmium tetroxide. Cells were then maintained overnight at 4° in the presence of 0.5% magnesium uranyl acetate and the following day were dehydrated through an alcohol gradient. Cells were resuspended in Spurr's resin and maintained overnight at room temperature. After three resin changes, cells were resuspended in fresh resin, transferred to Been-type embedding capsules, and allowed to polymerize overnight at 60°. Sections (60 nm) were cut and examined using an AE1 801/1 Phillips 201 transmission electron microscope.

TdT Assay

Cells were prepared according to a previously published method (15). After drug treatment, MOLT-4 cells were collected by centrifugation and fixed in 1% formaldehyde in PBS for 15 min on ice. After centrifugation, the pellet was resuspended in 200 μl of PBS, and 1 ml of 70% ice-cold ethanol was added immediately. Samples were then stored at 4° until analysis (1–4 days). After rehydration in PBS for 15 min, the cells were resuspended in 50 μl of cacodylate buffer containing 5 units of TdT (Boehringer-Mannheim, Indianapolis, Indiana), 2.5 mM $CoCl_2$, 0.2 M potassium cacodylate, 25 mM Tris·HCl, pH 6.6, 0.25 mg/ml bovine serum albumin, and 0.5 nmol of b-dUTP (Boehringer-Mannheim) and were incubated at 37° for 30 min. Cells were then washed in PBS, resuspended in 100 ml of staining buffer (2.5 ng/ml fluoresceinated avidin, 4× saline sodium citrate buffer, (1× = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) 0.1% Triton X-100, 5%, w/v, nonfat dry milk), and incubated for 30 min at room temperature in the dark. The cells were then rinsed in PBS containing 0.1% Triton X-100 and were resuspended in 0.5 ml of PBS containing 67 μl of propidium iodide (3.2 mM), for 5 min before analysis. Briefly, incorporated b-dUTP was visualized with fluoresceinated avidin, and red propidium-DNA fluorescence was measured with a FACS Vantage fluorescence activated cell sorter (Becton Dickinson, San Jose, CA). The data from 10^4 cells were collected, stored, and analyzed using LYSYS II software. Cell cycle distribution was analyzed using Cellfit software (15, 22).

Results

Expression of morphological features associated with apoptosis of MOLT-4 cells. We first examined the morphology of MOLT-4 cells by transmission electron microscopy (Fig. 1) after treatment with 300 mM NMF, a nongenotoxic toxin,[2] and 50 μM etoposide, a topoisomerase II poison that induces protein-associated, double-strand DNA breaks (23–25). Untreated cells (Fig. 1A) contained large nuclei surrounded by a thin margin of cytoplasm. In contrast, after incubation with NMF (Fig. 1B) or etoposide (Fig. 1C), cells were observed to have shrunken nuclei, nucleolar disintegration, and extensive margination and condensation of the heterochromatin toward one pole of the cell. The integrity of the plasma membrane was apparently maintained and the cells themselves did not appear shrunken. This was confirmed by flow cytometric analysis, which showed a mainte-

Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

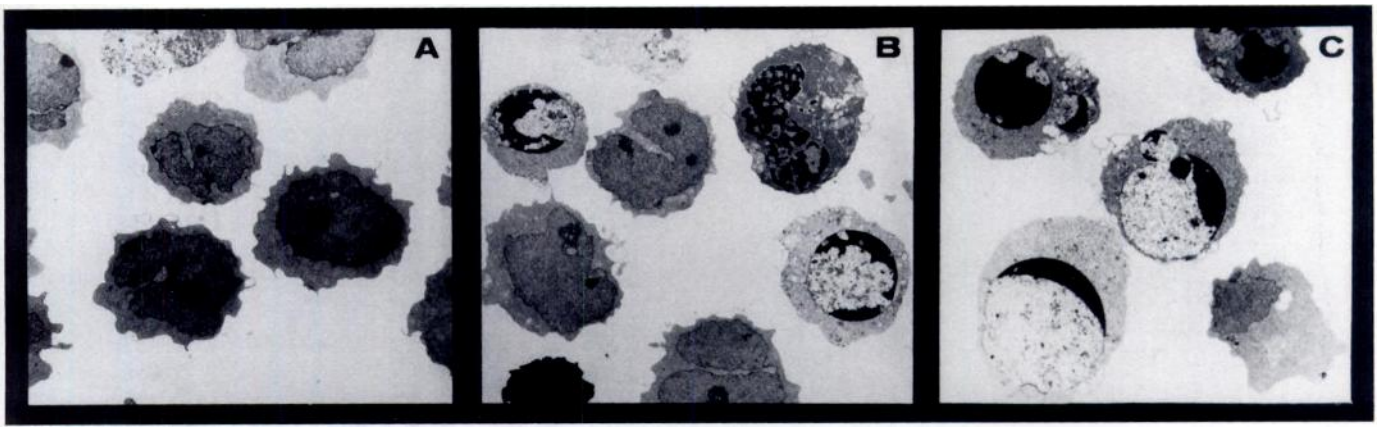

Fig. 1. MOLT-4 cell morphology. MOLT-4 cells were incubated in the absence of drug (A) or in the presence of 300 mM NMF (B) or 50 μM etoposide (C) for 8 hr. Cells were prepared as described in Experimental Procedures, and ultrathin sections were examined for changes in morphology. After exposure to NMF and etoposide, several of the morphological features associated with apoptosis, including extensive margination and fragmentation of chromatin, were evident. Magnification, 7500×.

nance of the forward-scatter signal, an indicator of cell size (data not shown).

Induction by NMF and etoposide of the formation of approximately 50-kb DNA fragments. Although it was previously shown that apoptotic MOLT-4 cells do not undergo internucleosomal DNA cleavage (12, 13), the pattern of higher order chromatin fragmentation in this cell type had not been characterized. We investigated DNA integrity in MOLT-4 cells after incubation with NMF (300 mM) or etoposide (50 μM), using FIGE (Fig. 2A). Exposure of cells to NMF (Fig. 2A, *lanes 4-6*) or etoposide (Fig. 2A, *lanes 7-9*) induced the formation of discrete DNA fragments, of approximately 50 kb in both cases. Evidence of DNA fragmentation was observed after a 4-hr exposure to 300 mM NMF (Fig. 2A, *lane 5*) and was significantly increased by 8 hr (Fig. 2A, *lane 6*). Treatment of cells with etoposide also induced 50-kb DNA fragmentation, which was easily detectable within 2 hr (Fig. 2A, *lane 7*). Exposure of cells to etoposide also induced the transient appearance of a larger, unresolved, DNA band of >600 kb. This band was clearly visible within 2 hr of drug exposure but, in contrast to the formation of 50-kb DNA fragments, was seen to progressively decrease with time (Fig. 2A, *lanes 7-9*). This decrease correlated with a progressive increase in the formation of 50-kb DNA fragments. The >600-kb band was not detected in cells treated with NMF. The integrity of DNA isolated from untreated cells was clearly maintained (Fig. 2A, *lanes 1-3*). We were unable to detect internucleosomal DNA fragmentation in MOLT-4 cells (Fig. 2B) after treatment with either NMF (Fig. 2B, *lanes 6-8*) or etoposide (Fig. 2B, *lanes 9-11*). In contrast, the human myelocytic leukemia cell line HL-60 clearly showed internucleosomal DNA cleavage after an 8-hr incubation with 300 mM NMF (Fig. 2B, *lane 2*).

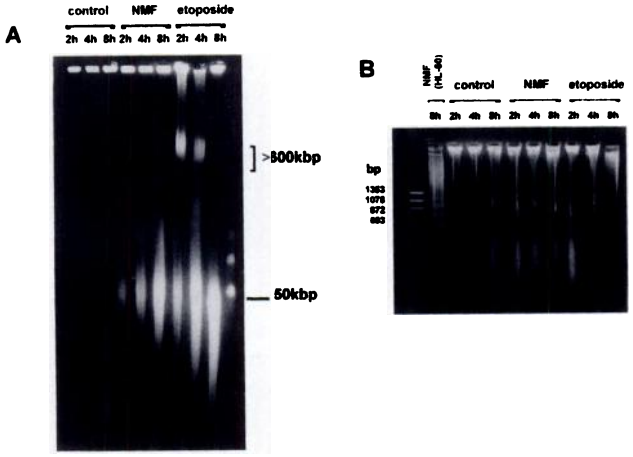

Fig. 2. NMF- and etoposide-induced DNA fragmentation. A, FIGE of DNA isolated from untreated MOLT-4 cells (*lanes 1-3*) or cells exposed to 300 mM NMF (*lanes 4-6*) or 50 μM etoposide (*lanes 7-9*). *Lane 10*, DNA markers. DNA fragments of approximately 50 kb were produced in response to both agents. An additional, unresolved, DNA band of >600 kb appeared transiently in cells incubated with etoposide (*lanes 7-9*). B, Conventional gel electrophoresis, which failed to demonstrate internucleosomal DNA cleavage in MOLT-4 cells in response to either NMF (*lanes 6-8*) or etoposide (*lanes 9-11*). Exposure of HL-60 cells to 300 mM NMF for 8 hr clearly showed cleavage of DNA into 200-base pair integers, to produce laddering (*lane 2*). Molecular weight markers (*lane 1*) and untreated cells (*lanes 3–5*).

Characterization of DNA damage associated with etoposide- and NMF-induced apoptosis. FIGE showed that exposure of MOLT-4 cells to etoposide, but not NMF, induced the formation of an unresolved band containing DNA fragments of >600 kb, in addition to those of 50 kb (Fig. 2A). We wished to determine whether these DNA fragments were protein associated, because etoposide induces covalent binding between topoisomearase II and DNA (23, 25). The frequency of topoisomerase II-DNA complexes can be determined using an SDS/KCl assay, which specifically precipitates protein-bound but not free DNA. Exposure of MOLT-4 cells to 300 mM NMF for up to 8 hr failed to induce the formation of DNA-protein complexes (Fig. 3A), suggesting that the 50-kb fragments were not protein associated. In contrast, exposure of cells to 50 μM etoposide for 30 min induced a rapid 6-fold increase in the number of DNA-protein complexes (Fig. 3A). After 8 hr the frequency of DNA-protein complexes decreased to approximately twice the basal level. The rapid rise and subsequent decline in protein-DNA complex formation after exposure to etoposide (Fig. 3A) was paralleled temporally by the transient appearance of the >600-kb band observed with FIGE (Fig. 2A) and proteolytic degradation of topoisomerase II (see below). However, analysis of DNA isolated from cells exposed to 300 mM NMF showed no evidence of the >600-kb DNA band (Fig. 2A), and

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

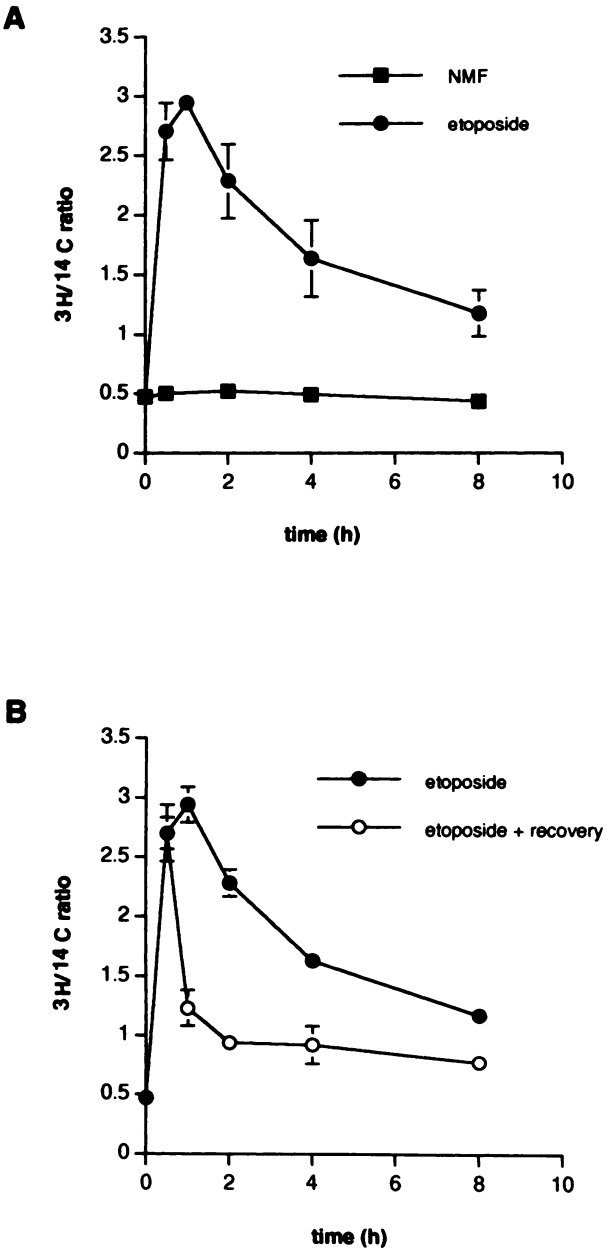

Fig. 3. Protein-associated DNA breaks induced by NMF and etoposide. MOLT-4 cells were prelabeled with [^{3}H]thymidine and [^{14}C]leucine before exposure to NMF or etoposide. The number of protein-associated DNA breaks was quantitated by scintillation counting after salt precipitation (see Experimental Procedures). A, Cells were continuously exposed to NMF (300 mM) or etoposide (50 μM) and samples were removed after 30 min, 1, 2, 4, and 8 hr of treatment. B, To determine the reversibility of etoposide-induced DNA breaks, cells were exposed to etoposide for 30 min before drug removal and continued incubation in drug-free medium. Samples were removed at 1, 2, 4, and 8 hr after drug removal. The mean ± standard error values, expressed as ^{3}H/^{14}C ratios, of three independent experiments are shown.

protein-DNA complexes were not detected (Fig. 3A). Transient exposure of cells to 50 μM etoposide for 30 min, followed by drug removal and continued incubation in drug-free medium, showed rapid reversibility of the protein-associated DNA breaks (Fig. 3B). FIGE analysis of DNA also showed the reversibility of the >600-kb DNA band (see below). The observations shown in Fig. 3 are consistent with the formation of etoposide-mediated DNA strand breaks, induced by the stabilization of topoisomerase II-DNA cleavable complexes.

Detection of higher order DNA fragmentation by end labeling with TdT. The morphological appearance of apoptosis in MOLT-4 cells was coincident with the release of 50-kb DNA fragments (Figs. 1 and 2A) and occurred in the absence of internucleosomal DNA degradation (Fig. 2B). Earlier studies, using rat thymocytes and chicken erythroblastoid cells, suggested to us a potential role for topoisomerase II in the release of 50–300-kb loops of chromatin (18, 26). During topoisomerase II-mediated cleavable complex formation (see above), the enzyme molecule forms a covalent link with the 5′-phosphate of DNA and is noncovalently bound to the 3′-OH end of DNA. In an attempt to determine the nature of the termini of DNA fragments detected during apoptosis (Fig. 2A) and the potential role of topoisomerase II in their release, we used the TdT assay. Fig. 4A shows representative flow cytometric data from three independent experiments, expressed as a two-dimensional frequency contour plot showing green (TdT-incorporated b-dUTP) versus red (DNA) fluorescence, collected from analysis of untreated MOLT-4 cells or after an 8-hr exposure to etoposide or NMF. Treatment of cells with either etoposide or NMF induced the appearance of a subpopulation of cells exhibiting increased green fluorescence, compared with control (Fig. 4A, *subpopulation above the dashed line*). The DNA content of this subpopulation, measured by the binding of propidium to DNA, was essentially unchanged. The kinetics of b-dUTP incorporation are shown in Fig. 4B. Etoposide-induced DNA damage, which was detected within 2 hr of drug treatment (Figs. 2A and 3A), was not labeled by this assay, presumably because the 3′-OH termini of the DNA breaks were concealed by topoisomerase II. Significant b-dUTP incorporation was observed only after a 4-hr continuous exposure to etoposide and was seen to increase significantly by 8 hr. Similarly, NMF-induced DNA fragmentation was labeled by TdT, although the percentage of b-dUTP incorporation was significantly less than that observed with etoposide. Comparison of the kinetics of the appearance of TdT-positive cells (Fig. 4B) and the release of 50-kb DNA fragments (Fig. 2A) suggests that these fragments are the substrates for TdT.

Investigation of the reversibility of DNA fragmentation. DNA damage induced by topoisomerase II inhibitors is characterized by its reversibility after reincubation in drug-free medium (27). If DNA breaks observed with FIGE during apoptosis are the product of functional topoisomerase II molecules, then it might be argued that removal of toxin would allow their resealing. In an attempt to distinguish between DNA breaks associated with apoptosis and those resulting directly from etoposide-mediated topoisomerase II poisoning, we investigated the reversibility of DNA fragment formation upon drug removal (Fig. 5). The reversibility of DNA damage was studied using two topoisomerase II inhibitors, etoposide and its more potent congener teniposide, which had already been shown to induce the release of 200–300-kb DNA fragments (18, 26).

MOLT-4 cells were treated with 50 μM etoposide either continuously or for 60 min, followed by reincubation in drug-free medium (Fig. 5A). Continuous incubation with 50 μM etoposide (Fig. 5A, *lanes 12-14*) induced 50-kb DNA fragment formation after 2–4 hr, as well as an unresolved DNA band of >600 kb. This high molecular weight band showed a time-

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

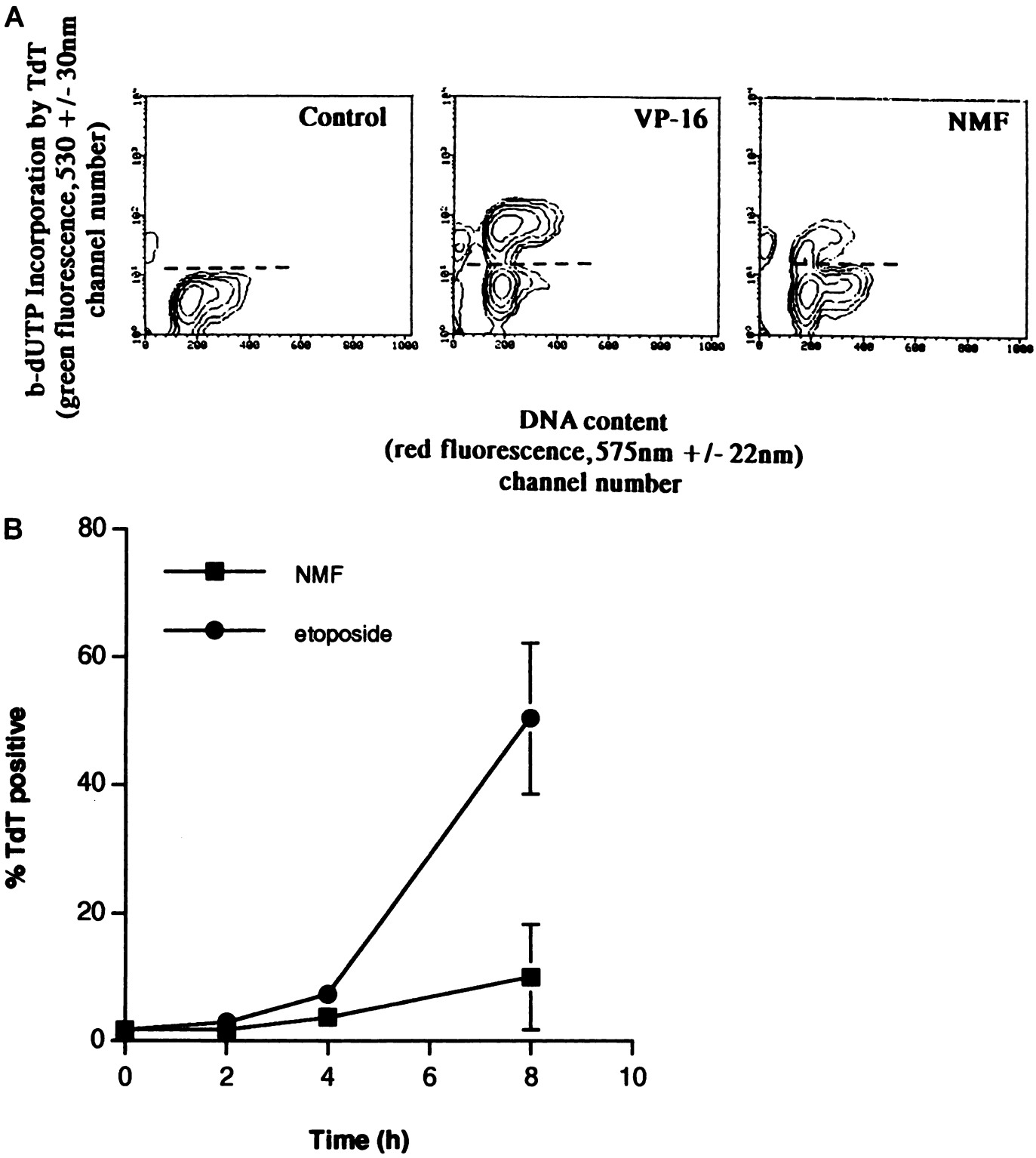

Fig. 4. A, Incorporation of b-dUTP, by exogenous TdT, into the DNA of MOLT-4 cells treated for 8 hr with either etoposide (*VP-16*) (50 μM) or NMF (300 mM). For each time point 10^4 cells were analyzed by multiparameter flow cytometry. *Cells with green fluorescence above the dashed line*, cells considered positive for the TdT assay. DNA content was assessed simultaneously by staining with propidium. The result is a representative example of three independent experiments. B, Frequency of apoptotic cells, determined by TdT labeling of formaldehyde-fixed cells. Cells were treated for the indicated times with either etoposide (50 μM) or NMF (300 mM) and the percentage of TdT-positive cells was determined as described for A. The means ± standard errors of three independent experiments are shown.

dependent decrease in intensity concomitant with the progression to 50-kb DNA fragments (Fig. 5A, *lanes 11-14*) (also see Fig. 2A). After transient exposure to etoposide, drug-specific DNA damage was also detected as an unresolved band of >600 kb (Fig. 5A, *lane 4*). Removal of etoposide after 60 min and continued incubation in drug-free medium (Fig.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

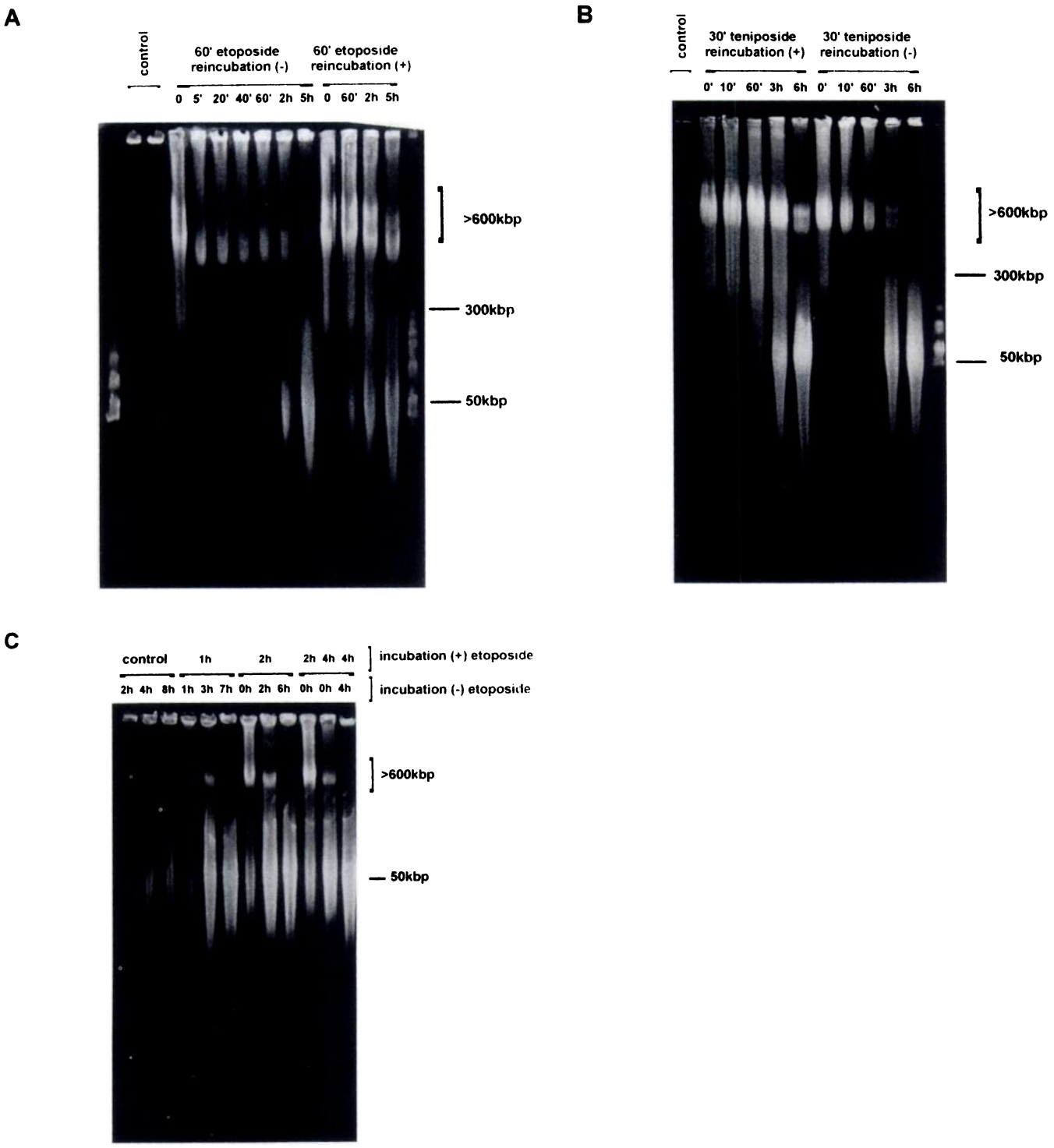

Fig. 5. Reversibility of DNA fragmentation induced by etoposide (50 μM) and teniposide (20 μM). Cells were transiently exposed to etoposide or teniposide before drug removal and continued incubation in drug-free medium. DNA fragments of approximately 300 kb and >600 kb were readily reversible after the removal of both etoposide (A, *lanes 5-10*) (–) and teniposide (B, *lanes 8-11*) (–). However, 50-kb fragmentation still proceeded with kinetics identical to those observed in cells continuously exposed to etoposide (A, *lanes 11-14*) (+) or teniposide (B, *lanes 2-6*) (+). Incubation of cells with etoposide for 1, 2, or 4 hr before continued incubation in the absence of drug showed that the formation of 50-kb DNA breaks was irreversible (C).

5A, *lanes 8-10*) showed a significant and rapid (within 5 min) reduction in the amount of this higher order DNA damage (>600 kb) (Fig. 5A, *lane 5*). This reversal was maintained thereafter (Fig. 5A, *lanes 5-10*). However, it did not prevent the subsequent formation of a discrete 50-kb DNA fragmentation pattern (Fig. 5A, *lanes 7-10*). Comparison of the kinetics of 50-kb DNA fragmentation observed in cells exposed transiently to etoposide (Fig. 5A, *lanes 5-10*) and those exposed continuously to drug (Fig. 5A, *lanes 11-14*) showed no detectable difference. The reversal of fragmentation to >600

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

kb is consistent with data showing the rapid resealing of etoposide-induced, protein-associated DNA breaks (Fig. 3B).

Continuous exposure of MOLT-4 cells to 20 μM teniposide (Fig. 5B, *lanes 3-7*) also induced the appearance of an unresolved DNA band of >600 kb in size, in addition to 300-kb DNA fragments (Fig. 5B, *lane 3*). Removal of teniposide after a 30-min exposure (Fig. 5B, *lane 7*) and continued incubation in teniposide-free medium showed the reversal of both 300- and >600-kb DNA fragment formation within 10 min (Fig. 5B, *lane 8*). DNA fragments of 50 kb were not detected until after 3 hr and were found both in cells continuously exposed to teniposide (Fig. 5B, *lanes 5* and *6*) and in those transiently exposed to teniposide (Fig. 5B, *lanes 10* and *11*). The detection of 50-kb DNA fragments was coincident with the morphological appearance of apoptosis (data not shown).

Filipski *et al.* (18) previously showed the early induction (30 min) and reversibility of 50-kb DNA fragmentation induced after the treatment of rat thymocytes with the topoisomerase inhibitor mAMSA. We were unable to demonstrate the early induction of 50-kb DNA fragmentation in MOLT-4 cells after mAMSA treatment, although higher order DNA damage of >600 kb and 300 kb was evident and was shown to be reversible (data not shown). We examined the possibility that the DNA cleavage pattern to 50-kb fragments produced in cells exhibiting morphological features of apoptosis might be reversible (Fig. 5C). MOLT-4 cells were treated with 50 μM etoposide for 1, 2, or 4 hr. The drug was then removed and DNA samples were prepared after an appropriate drug-free recovery period (Fig. 5C). Exposure of cells to etoposide for 1 hr (Fig. 5C, *lane 4*) did not immediately induce 50-kb fragments. However, after either a 2-hr (Fig. 5C, *lane 7*) or 4-hr (Fig. 5C, *lane 10*) incubation with etoposide, 50-kb DNA fragmentation was evident. Removal of etoposide after exposure times of 1 hr, 2 hr, and 4 hr all showed progressive 50-kb DNA fragmentation (Fig. 5C, *lanes 4-12*), with kinetics identical to those seen in cells continuously exposed to etoposide (Figs. 2A and 5A), demonstrating that these fragments are irreversible (compare with those at >600 kb in Fig. 5C, *lanes 4* and *10*).

Coincidence of the formation of 50-kb DNA fragments with the degradation of topoisomerase II proteins. Evidence suggests that topoisomerase II may have a structural role in the attachment of chromatin loops to the nuclear matrix (14). We therefore determined whether there was a temporal relationship between the release of 50-kb DNA loops and the level and activity of topoisomerase II proteins in the MOLT-4 cell line, after exposure to NMF or etoposide. It was previously reported that internucleosomal cleavage of DNA in HL-60 myeloid cells was associated with degradation of topoisomerase II (28). Representative Western blot analyses of the levels of topoisomerase IIα and -β proteins in whole-cell extracts after 2-, 4-, and 8-hr exposures to either NMF or etoposide are shown in Fig. 6. The level of topoisomerase IIα and -β in untreated cells remained essentially unchanged throughout the experiment (Fig. 6, *lanes 1-3*). Exposure of cells to 300 mM NMF induced a significant decrease in the level of topoisomerase IIα by 8 hr (Fig. 6, *lane 9*). Evidence of a slight decrease was observed by 4 hr (Fig. 6, *lane 8*). Etoposide also induced a time-dependent decrease in the level of topoisomerase IIα protein (Fig. 6, *lanes 4-6*), which, like the appearance of DNA fragmentation (Fig. 2A), preceded that observed with NMF. Etoposide induced a less rapid degradation of topoisomerase IIβ protein (Fig. 6, *lower*), which may reflect isoform specificity of the drug-enzyme interaction, as already reported for teniposide (29). NMF induced a decrease in the level of both topoisomerase IIα and -β proteins. To establish whether the decrease in the level of topoisomerase II protein (Fig. 6) was coincident with a loss of its enzymatic activity, we determined the ability of nuclear extracts, isolated from MOLT-4 cells treated with NMF, to decatenate kinetoplast DNA (Fig. 7). A representative gel is shown in Fig. 7A. After densitometric analysis, data are expressed as the protein concentration required to induce 50% decatenation of the substrate DNA (Fig. 7B). NMF induced a time-dependent decrease in the ability of nuclear extracts to decatenate kinetoplast DNA, showing a temporal correlation with the degradation of topoisomerase II protein after NMF treatment (Fig. 6).

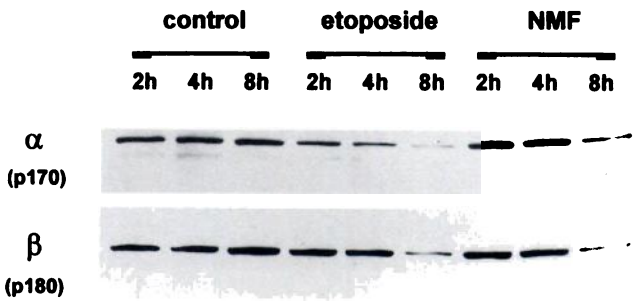

Fig. 6. Representative Western blot analysis of the levels of topoisomerase IIα (*upper*) and -β (*lower*) proteins after exposure of MOLT-4 cells to 50 μM etoposide (*lanes 4-6*) or 300 mM NMF (*lanes 7-9*). NMF and etoposide were shown to induce a time-dependent decrease in the levels of both topoisomerase II isoforms.

Discussion

MOLT-4 lymphoblastoid cells provide an excellent model for the detailed study of the mechanism of higher order DNA fragmentation observed during apoptosis. We and others have shown previously that the formation of large DNA fragments may precede internucleosomal DNA cleavage and may represent the initial stages of apoptosis (3, 5). Using MOLT-4 cells, a high percentage of apoptosis was induced in a relatively short time in the absence of significant spontaneous apoptosis and, unlike in other cell types such as murine thymocytes or myeloid cells (30), there was no subsequent or concomitant appearance of internucleosomal DNA fragmentation (Fig. 2), which can complicate data interpretation. Treatment of MOLT-4 cells with genotoxic etoposide and treatment with nongenotoxic NMF both induced the appearance of 50-kb DNA fragments (Fig. 2A), supporting the view that cleavage of DNA, to release what may be looped domains, is a conserved process in response to agents with disparate modes of action.

The formation of 300- and 50-kb DNA fragments in rat thymocytes treated with calcium ionophore and the topoisomerase II inhibitor mAMSA and in isolated liver nuclei under conditions of autodigestion suggested to Filipski *et al.* (18) that there was a gradual unfolding and cleavage of chromatin from higher order forms. In this study we showed that etoposide- and teniposide-induced apoptosis in MOLT-4 cells was associated with the appearance of additional high

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

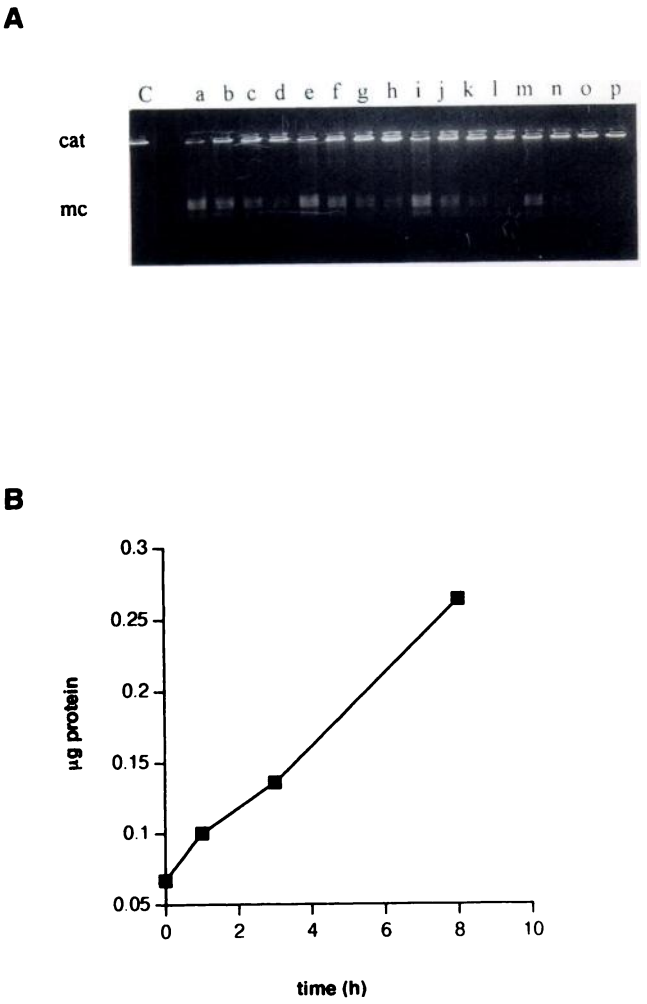

Fig. 7. A, Decatenation of kinetoplast DNA from *Crithidia fasciculata* in the presence of nuclear extracts from MOLT-4 cells treated with 300 mM NMF for 0 (*lanes a–d*), 1 (*lanes e–h*), 3 (*lanes i–l*), or 8 hr (*lanes m–p*). Kinetoplast DNA (*lane C*) was incubated with 0.16, 0.08, 0.053, or 0.04 µg of protein from nuclear extracts, corresponding to 1/25, 1/50, 1/75, and 1/100 dilutions. *cat*, kinetoplast DNA; *mc*, minicircles. B, Graphical representation of data, showing the amount of protein (µg) required to produce 50% decatenation of the substrate DNA.

molecular weight DNA fragments (Figs. 2A and 5), which were visualized as an intense but unresolved band of DNA fragments of >600 kb and a less discrete band of approximately 300 kb. The progressive decrease in the intensity of the >600-kb fragments appeared to occur concomitantly with the appearance of the 50-kb cleavage products (Fig. 2A) and a gradual loss of protein-associated DNA fragments (>600 kb) (Fig. 3), suggesting sequential degradation. However, concomitant degradation of topoisomerase II protein (Fig. 6) complicates the interpretation of any relationship between the >600-kb fragments and those at 50 kb, because it is conceivable that loss of topoisomerase II-associated strand breaks to form naked fragments may promote conformational changes promoting cleavage to 50 kb. In addition, analysis of DNA integrity after treatment of MOLT-4 cells with the non-DNA-damaging toxin NMF failed to show the formation of >600-kb DNA fragments (Fig. 2A). Only the 50-kb band was observed (Fig. 2A), suggesting that the DNA fragments of >600 kb were not a conserved feature of DNA breakdown during apoptosis.

The transient nature of the etoposide- or teniposide-induced DNA cleavage products of >600 kb suggested that these breaks either were resealed or were degraded further to lower molecular weight fragments (Fig. 2A). DNA damage produced by the topoisomerase II inhibitors has been shown to be rapidly reversed after drug removal (27). Our own data measuring DNA protein complex formation confirm this (Fig. 3B). To address this further, we investigated the patterns of DNA cleavage induced by transient exposure to etoposide (Fig. 5). We observed that a 60-min exposure to etoposide induced the immediate appearance of >600-kb (but not 50-kb) fragments. After the removal of etoposide, we observed a rapid (within 5 min) and significant decrease in the unresolved >600-kb band, which could not be accounted for by the concomitant increase in the formation of 50-kb fragments (Fig. 5A). We therefore consider that the decrease in high molecular weight fragments (>600 kb), after drug removal, was not due to sequential degradation as chromatin was unfolded but was, instead, due to the resealing of topoisomerase II-induced DNA cleavage products. However, although resealed, these initial breaks appear to act as triggers for subsequent apoptotic cleavage of DNA to yield 50-kb fragments. The onset and progression of 50-kb fragmentation was temporally indistinguishable in cells transiently exposed and those continuously exposed to etoposide (Fig. 5A) or teniposide (Fig. 5B). Removal of etoposide and continued incubation in drug-free medium after the formation of 50-kb DNA fragments (2–4 hr) demonstrated that these breaks were irreversible (Fig. 5C). This is in contrast to one previous study showing reversal of the 50-kb DNA fragmentation induced by a 30-min exposure of thymocytes to mAMSA (18), but we were unable to demonstrate reversal of 50-kb fragmentation or to detect 50-kb DNA fragments in MOLT-4 cells transiently exposed to mAMSA (data not shown).

It is possible that the products generated by the trapping of topoisomerase II cleavable complexes may be "sensed" by the cell as damage and this initiates apoptosis. In contrast, NMF produces damage at some locus other than DNA, probably the cell membrane (31), initiating cleavage only to 50-kb fragments. The finding that etoposide initiates the cleavage of DNA to fragments of >600 kb before the appearance of 50-kb apoptotic fragments (Fig. 2A) is perhaps surprising, in view of observations that topoisomerase II molecules have been shown to be associated with A/T-rich binding sites at the bases of DNA looped domains (32, 33). These domains have been estimated to be approximately 50–80 kb in size (26). Why do etoposide and teniposide not trap cleavable complexes formed at the bases of these looped domains, so as to produce rapidly detected and reversible cleavage products of 50–80 kb upon FIGE? And why are the cleavable complexes that are trapped observable at >600 kb? Similar high molecular weight fragments of approximately 700 kb were reported in rat thymocytes treated with etoposide, although dexamethasone-treated cells showed only 50-kb fragments (34), supporting our view that these >600-kb fragments are the result of topoisomerase II inhibitor-specific cleavage. It is possible that there are discrete pools of topoisomerase II associated with discrete domains of DNA and that those at the bases of loops are less accessible to inhibitors such as etoposide.

Alternatively, topoisomerase II may be limited at the bases of the loops, in comparison with other sites. Studies have

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

shown that topoisomerase II is able to form complexes with other proteins, including scaffold protein 2 and casein kinase II (35, 36), and it is conceivable that such associations may modify the accessibility of topoisomerase II to agents such as etoposide and teniposide. However, the formation of discrete and specific DNA fragments in response to topoisomerase II inhibitors clearly indicates that topoisomerase IIα and/or -β must be present and, more importantly, accessible at well defined and nonrandom positions along the chromatin, which may be representative of higher order chromatin structure.

Because apoptosis can occur without internucleosomal DNA cleavage, a critical question arises as to the mechanism of DNA fragmentation. How it is initiated and controlled? It is tantalizing to speculate that topoisomerase II may play a role in this process, because it is localized at the bases of A/T-rich looped domains (32) and actively cleaves DNA to produce double-strand breaks. However, data described here, and reported elsewhere for the HL-60 cell line,[2] do not support an active role for topoisomerase II in apoptotic DNA cleavage. In the HL-60 cell line we showed that the topoisomerase II poison ICRF-193 effectively inhibited etoposide-mediated topoisomerase II cleavable complex formation and subsequent DNA fragmentation associated with apoptosis but had no effect on NMF-induced apoptosis, characterized by the formation of 50-kb DNA fragments or the condensation of chromatin.[2] Here we have shown that the 50-kb breaks have free 3′-OH ends, because they are able to undergo TdT reactions to incorporate labeled nucleotides (Fig. 4). If the DNA were cleaved by topoisomerase II to reveal topoisomerase II-associated 3′-OH ends, this labeling would not occur. However, we cannot completely exclude the possibility that further degradation of DNA, evident as fragments of <50 kb (Fig. 2A), may contribute in part to the observed levels of end labeling (Fig. 4A).

NMF treatment of MOLT-4 cells showed that the formation of 50-kb fragments (Fig. 2A) occurred in the absence of protein-associated DNA complexes (Fig. 3) and permitted incorporation of b-dUTP. This latter result is particularly important because TdT labeling reactions of this type are increasingly being used to detect apoptotic cells. We were concerned that condensed chromatin may have been associated with protein-concealed breaks and that the TdT reaction might detect only the products of internucleosomal cleavage. This is clearly not the case in MOLT-4 cells.

Other studies have suggested a role for topoisomerase II in the anchorage of chromatin loop domains to the nuclear matrix (14, 37). It may be envisioned that specific and rapid proteolysis of an integral structural protein may promote the uncoiling or release of chromatin loops from their attachment points on the nuclear matrix, which may in turn facilitate the access of endonucleases. Topoisomerase II, when complexed with DNA, undergoes rapid proteolysis in HL-60 cells after treatment with etoposide (38). This may account for the more rapid degradation of topoisomerase II protein and earlier onset of DNA fragmentation observed in cells exposed to etoposide, compared with those incubated with NMF (Fig. 6). Proteolysis of nuclear matrix-associated proteins, including topoisomerase II, may be integral to the progression of apoptosis (39, 40). To date, two specific proteases have been characterized that appear to have a critical role in the apoptotic process, i.e., ICE, which has been shown to induce apoptosis in fibroblasts (41), and, more recently, a protease resembling ICE (42), which specifically cleaves and inactivates polyADP-(ribose) polymerase, an enzyme involved in DNA repair. It is likely that ICE and the protease resembling ICE may be only representative of one or more families of specific proteases that are able to mediate the proteolytic cleavage of an array of proteins integral to the maintenance of cellular function and chromatin structure.

In conclusion, we have shown that the morphological features of apoptosis in MOLT-4 cells are associated with cleavage of DNA to 50-kb fragments, in the absence of internucleosomal DNA cleavage. Comparison of the kinetics of TdT labeling and the irreversible cleavage of DNA to fragments of approximately 50 kb suggests that these fragments, the termini of which are not concealed by protein, are the substrates for b-dUTP incorporation. The degradation of topoisomerase II protein concomitantly with the cleavage of DNA to 50-kb fragments, during both etoposide- and NMF-induced apoptosis, may lead to exposure of naked DNA and may promote subsequent DNA cleavage. It is therefore possible that the degradation of nuclear matrix-associated proteins, including topoisomerase II, may be an important step in the commitment of cells to apoptosis. Reversible formation of protein-associated DNA fragments of >600 kb is not a feature of the conserved process of apoptosis. Instead, these appear to provide a trigger for the initiation of apoptotic DNA cleavage, as is typical for the topoisomerase II inhibitors. Elucidation of the enzymatic mechanism of DNA cleavage to 50-kb fragments is the subject of our ongoing studies.

Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

aspet

References

1. Arends, M. J., R. G. Morris, and A. H. Wyllie. Apoptosis: the role of the endonuclease. *Am. J. Pathol.* **136**:593–608 (1990).
2. Compton, M. M. A biochemical hallmark of apoptosis: internucleosomal degradation of the genome. *Cancer Metastasis Rev.* **11**:105–119 (1992).
3. Brown, D. G., X. M. Sun, and G. M. Cohen. Dexamethasone-induced apoptosis involves cleavage of DNA to large fragments prior to internucleosomal fragmentation. *J. Biol. Chem.* **28**:3037–3039 (1993).
4. Cohen, G. M., X. M. Sun, R. T. Snowden, D. Dinsdale, and D. N. Skilleter. Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. *Biochem. J.* **286**:331–334 (1992).
5. Oberhammer, F., J. W. Wilson, C. Dive, I. D. Morris, J. A. Hickman, A. E. Wakeling, R. P. Walker, and M. Sikorska. Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50kbp fragments prior to or in the absence of internucleosomal fragmentation. *EMBO J.* **12**:3679–3684 (1993).
6. Sun, X. M., R. T. Snowden, D. Dinsdale, M. G. Ormerod, and G. M. Cohen. Changes in nuclear chromatin precede internucleosomal DNA cleavage in the induction of apoptosis by etoposide. *Biochem. Pharmacol.* **2**:187–195 (1993).
7. Sun, X. M., and G. M. Cohen. Mg^{2+}-dependent cleavage of DNA into kilobase pair fragments is responsible for the initial degradation of DNA in apoptosis. *J. Biol. Chem.* **269**:14857–14860 (1994).
8. Zhivotovsky, B., D. Wade, A. Gahm, S. Orrenius, and P. Nicotera. Formation of 50 kbp chromatin fragments in isolated liver nuclei is mediated by protease and endonuclease activation. *FEBS Lett.* **351**:150–154 (1994).
9. Barres, B. A., I. K. Hart, H. S. Coles, J. F. Burne, J. T. Voyvodic, W. D. Richardson, and M. C. Raff. Cell death and control of cell survival in the oligodendrocyte lineage. *Cell* **70**:31–46 (1992).
10. Castle, V. P., K. P. Heidelberger, J. Bromberg, X. Ou, M. Dole, and G. Nunez. Expression of the apoptosis-suppressing protein *bcl*-2, in neuroblastoma is associated with unfavorable histology and N-*myc* amplification. *Am. J. Pathol.* **143**:1543–1549 (1993).
11. Oberhammer, F., G. Fritsch, M. Schmied, M. Pavelka, D. Printz, T. Purchio, H. Lassmann, and R. Schulte-Herman. Condensation of the chromatin at the membrane of an apoptotic nucleus is not associated with activation of an endonuclease. *J. Cell Sci.* **104**:317–326 (1993).
12. Catchpoole, D. R., and B. W. Stewart. Etoposide-induced cytotoxicity in two human T-cell leukemic lines: delayed loss of membrane permeability rather than DNA fragmentation as an indicator of programmed cell death. *Cancer Res.* **53**:4287–4296 (1993).
13. Falcieri, E., A. M. Martelli, R. Bareggi. A. Cataldi, and L. Cocco. The protein kinase inhibitor staurosporine induces morphological changes typical of apoptosis in MOLT-4 cells without concomitant DNA fragmentation. *Biochem. Biophys. Res. Commun.* **193**:19–25 (1993).

14. Adachi, Y., E. Kas, and U. K. Laemmli. Preferential, cooperative binding of DNA topoisomerase II to scaffold-associated regions. *EMBO J.* **8:**3997–4006 (1989).
15. Gorczyca, W., J. Gong, B. Ardelt, F. Traganos, and Z. Darzynkiewicz. The cell cycle related differences in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. *Cancer Res.* **53:**3186–3192 (1993).
16. Peitsch, M. C., C. Muller, and J. Tschopp. DNA fragmentation during apoptosis is caused by frequent single strand cuts. *Nucleic Acids Res.* **21:**4206–4209 (1993).
17. Gavrieli, Y., Y. Sherman, and S. A. Ben-Sassons. Identification of programmed cell death *in situ* via specific labelling of nuclear DNA fragmentation. *J. Cell Biol.* **119:**493–501 (1992).
18. Filipski, J., J. Leblanc, T. Youdale, M. Sikorska, and P. R. Walker. Periodicity of DNA folding in higher order chromatin structures. *EMBO J.* **9:**1319–1327 (1990).
19. Chung, T. D., F. H. Drake, K. B. Tan, S. R. Per, S. T. Crooke, and C. K. Mirabelli. Characterization and immunological identification of cDNA clones encoding two human DNA topoisomerase II isozymes. *Proc. Natl. Acad. Sci. USA* **86:**9431–9435 (1989).
20. Jenkins, J. R., P. Ayton, T. Jones, S. L. Davies, D. L. Simmons, A. L. Harris, D. Sheer, and I. D. Hickson. Isolation of cDNA clones encoding the *beta*-isozyme of human DNA topo II and localisation of the gene to chromosome 3p24. *Nucleic Acids Res.* **20:**5587–5592 (1992).
21. Liptrot, C., and K. Gull. in *Cell and Tissue Culture: Laboratory Procedures* (A. Doyle, J. B. Griffith, and D. G. Newall, eds.). John Wiley and Sons, New York, 2.1–2.14 (1993).
22. Chapman, R. C., C. M. Chresta, A. Alejo-Herberg, H. M. Beere, S. Heer, A. D. Whetton, J. A. Hickman, and C. Dive. Flow cytometric analysis of apoptosis in drug resistant and drug sensitive leukaemic cells; further characterisation of the *in situ* terminal deoxynucleotidyl transferase (TdT) assay. *Cytometry*, in press.
23. Chen, G. L., L. Yang, T. C. Rowe, B. D. Halligan, K. M. Tewey, and L. F. Liu. Nonintercalative antitumor drugs interfere with the breakage-reunion of mammalian topoisomerase II. *J. Biol. Chem.* **259:**13560–13566 (1984).
24. Liu, L. F. DNA topoisomerase poisons as antitumor drugs. *Annu. Rev. Biochem.* **58:**351–375 (1989).
25. Ross, W., T. Rowe, B. Glisson, J. Yalowich, and L. Liu. Role of topoisomerase II in mediating epipodophyllotoxin-induced DNA cleavage. *Cancer Res.* **44:**5857–5860 (1984).
26. Razin, S. V., P. Petrov, and R. Hancock. Precise localization of the α-globin gene cluster within one of the 20- to 300-kilobase DNA fragments released by cleavage of chicken chromosomal DNA at topoisomerase II sites *in vivo*: evidence that the fragments are DNA loops or domains. *Proc. Natl. Acad. Sci. USA* **88:**8515–8519 (1991).
27. Pommier, Y., D. Kerrigan, R. Schwartz, and L. A. Zwelling. The formation and resealing of intercalator-induced DNA strand breaks in isolated L1210 cell nuclei. *Biochem. Biophys. Res. Commun.* **107:**576–583 (1982).
28. Kaufmann, S. H. Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin and other cytotoxic anticancer drugs: a cautionary note. *Cancer Res.* **49:**5870–5878 (1989).
29. Drake, F. H., G. A. Hofmann, H. F. Bartus, M. R. Mattern, S. T. Crooke, and C. K. Mirabelli. Biochemical and pharmacological properties of p170 and p180 forms of topoisomerase II. *Biochemistry* **28:**8154–8160 (1989).
30. Bicknell, G. R., R. T. Snowden, and G. M. Cohen. Formation of high molecular mass DNA fragments is a marker of apoptosis in the human leukaemic cell line, U937. *J. Cell Sci.* **107:**2483–2489 (1994).
31. Dibner, M. D., K. A. Ireland, L. A. Koerner, and D. C. Dexter. Polar solvent-induced changes in membrane lipid lateral diffusion in human colon cancer cells. *Cancer Res.* **45:**4998–5003 (1985).
32. Earnshaw, W. C., and M. S. Heck. Localization of topoisomerase II in mitotic chromosomes. *J. Cell Biol.* **100:**1716–1725 (1985).
33. Gasser, S. M., and U. K. Laemmli. The organization of the higher-order chromatin loop: specific DNA attachment sites on nuclear scaffold. *EMBO J.* **5:**511–518 (1986).
34. Walker, P. R., C. Smith, T. Youdale, J. Leblanc, J. F. Whitfield, and M. Sikorska. Topoisomerase II-reactive chemotherapeutic drugs induced apoptosis in thymocytes. *Cancer Res.* **51:**1078–1085 (1991).
35. Bojanowski, K., O. Filhol, C. Cochet, E. M. Chambaz, and A. K. Larsen. DNA topoisomerase II and casein kinase II associate in a molecular complex that is catalytically active. *J. Biol. Chem.* **268:**22920–22926 (1993).
36. Ma, X., N. Saitoh, and P. J. Curtis. Purification and characterization of a nuclear DNA-binding factor complex containing topoisomerase II and chromosome scaffold protein 2. *J. Biol. Chem.* **268:**6182–6188 (1993).
37. Earnshaw, W. C., B. Hallighan, C. A. Cooke, M. S. Heck, and F. Liu. Topoisomerase II is a structural component of mitotoic chromosome scaffolds. *J. Cell Biol.* **100:**1706–1715 (1985).
38. Boege, F., F. Giesler, and E. Kjeldsen. Treatment of HL-60 cells with amsacrine or VP16 induces rapid proteolysis-associated elimination of DNA-complexed topoisomerase II from the nuclei. *Proc. Am. Assoc. Cancer Res.* **35:**362 (1994).
39. Bruno, S., P. Lassota, W. Giaretti, and Z. Darzynkiewicz. Apoptosis of rat thymocytes triggered by prednisolone, camptothecin or teniposide is selective to G_0 cells and is prevented by inhibitors of proteases. *Oncol. Res.* **4:**29–35 (1992).
40. Weaver, V. M., B. Lach, P. R. Walker, and M. Sikorska. Role of proteolysis in apoptosis: involvement of serine proteases in internucleosomal DNA fragmentation in immature thymocytes. *Biochem. Cell. Biol.* **71:**488–500 (1993).
41. Miura, M. H., R. Zhu, R. Rotella, E. A. Hartweig, and J. Yuan. Induction of apoptosis in fibroblasts by IL-1β-converting enzyme, a mammalian homolog of the *C. elegans* cell death gene *ced-3*. *Cell* **75:**653–660 (1993).
42. Lazebnik, Y. A., S. H. Kaufmann, S. Desnoyers, G. G. Poirier, and W. C. Earnshaw. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature (Lond.)* **371:**346–347 (1994).

Send reprint requests to: John Hickman, Cancer Research Campaign Molecular and Cellular Pharmacology Group, School of Biological Sciences, G38 Stopford Building, University of Manchester, Manchester M13 9PT, UK.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012