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Biochemistry

ts A1S9 locus in mouse L cells may encode a novobiocin binding protein that is required for DNA topoisomerase II activity

(DNA replication eukaryotes/purification of novobiocin-sensitive enzyme of DNA structure modification)

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ABSTRACT **Nuclear novobiocin binding proteins (NBPs) from a set of mouse L cells have been extensively purified by affinity chromatography on novobiocin-Sepharose columns. The NBPs, specifically eluted with 100 μg of novobiocin per ml, exhibited equivalent DNA topoisomerase activities (measured as ATP-dependent relaxation or catenation of φX174 replicative-form I DNA substrate) when extracted from equal numbers of wild-type (WT-4) mouse L cells growing logarithmically at 34°C or at 38.5°C, from *ts* A1S9 cells similarly cultivated at the low, permissive temperature or from revertant ts^+ AR cells in exponential growth at either temperature. The NBPs isolated from similar numbers of *ts* A1S9 cells grown to midlogarithmic phase and then incubated for 24 hr at 38.5°C (the nonpermissive temperature) showed no topoisomerase II activity. Preliminary $NaDodSO_4$/polyacrylamide gel electrophoretic analysis of enzymatically active material revealed that the NBPs of WT-4 and ts^+ AR cells grown at 34°C comprised three major polypeptides of 76,000, 74,000, and 30,000 daltons and a number of larger molecular mass components present in trace amounts. The NBP of *ts* A1S9 cells grown at the permissive temperature was similar, except that the 30,000-kilodalton polypeptide was not detected. Such enzymatically active NBPs from WT-4 and ts^+ AR cells were unaffected by 100 μg of novobiocin per ml, whereas the analogous preparation from *ts* A1S9 cells was totally inhibited. On the basis of these and other considerations, it is postulated that the *ts* A1S9 locus of mouse L cells encodes a temperature-sensitive polypeptide that is required for normal DNA topoisomerase II activity.**

The *ts* A1S9 L cell (1) is mutant in a gene required for nuclear DNA replication (2, 3) and for normal progression through the S phase of the cell cycle (4). Temperature-inactivated *ts* A1S9 cells synthesize "Okazaki fragments" and convert them to progeny single-strand DNA of $\geq 5 \times 10^6$ daltons (2). In addition they support full replication of monomeric and multimeric (up to 34×10^7 daltons) polyoma DNA (5, 6). Therefore, it was concluded that the enzyme proteins of polydeoxyribonucleotide synthesis must be present and functional in temperature-inhibited *ts* A1S9 cells. Supporting evidence came from studies with isolated nuclei (ref. 7; unpublished data), which revealed that the chromatin-bound DNA is rendered inactive as an *in situ* template for replication, as a result of heat denaturation of the *ts* A1S9 gene product.

These findings, and others which indicated that expression of the *ts* A1S9 defect results in modification of chromatin structure (8) and supercoiling of the nuclear DNA (9), suggested that the *ts* A1S9 mutation may affect a protein which determines DNA conformation—i.e., a topoisomerase.

Two quite distinct DNA topoisomerase enzyme activities have been described in mammalian cells (10, 11): a DNA topoisomerase I and a DNA topoisomerase II. These can be distinguished by their properties and by the mechanism by which they modify the topology of DNA. The DNA topoisomerase I more closely resembles the nicking-closing, ω protein activity already well characterized biochemically and genetically in bacteria (12, 13). Whereas the topoisomerase I enzyme has long been recognized to function in eukaryotes (14), evidence for the topoisomerase II eukaryotic analogue for the DNA gyrase of prokaryotes was reported later (15–17) and, indeed, after the studies described herein were initiated.

No evidence for abnormal topoisomerase I activity had been seen in earlier studies of temperature-inactivated *ts* A1S9 cells (cf. refs. 4 and 6; unpublished data). Therefore, attention was focused on DNA topoisomerase II. The first hint that this enzyme might be affected came with the demonstration that *ts* A1S9 cells are hypersensitive to novobiocin (9) when compared with wild-type (WT-4) cells and the revertant ts^+ AR cells (18). Novobiocin, long known to block the function of the *gyr* B subunit of the *Escherichia coli* DNA gyrase (19), has recently been reported to inhibit DNA topoisomerase II activity of HeLa (15) and *Drosophila melanogaster* cells (16).

MATERIALS AND METHODS

Cells and Their Cultivation. WT-4 mouse L cells, *ts* A1S9 cells, and ts^+ AR cells were maintained as described (2) by growth in suspension culture at 34°C, the permissive temperature for *ts* A1S9 cells. The nonpermissive temperature was 38.5°C. Where indicated, L-[4,5-^{3}H]leucine (1 μCi/ml, 40–60 Ci/mmol; 1 Ci = 3.7×10^{10} Bq; Amersham) was included in the growth medium for three or four generations, to generally label cell protein.

Preparation of Nuclear Protein. Cells cultured at 34°C or 38.5°C for 24 hr, as indicated in the figure legends, were collected by centrifugation at 800 × *g* for 15 min at 4°C. All subsequent steps were carried out at 4°C in the presence of 2 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$). The pelleted cells were washed twice in phosphate-buffered saline (17) with $PhMeSO_2F$, resuspended in 20 ml of buffer A (50 mM sodium phosphate/10 mM EDTA/3 mM dithiothreitol/2 mM $PhMeSO_2F$, pH 7.8) to permit hypotonic swelling, and then lysed by repeated passage through a 22-gauge needle until, as judged by phase-contrast microscopy, 95% of the cells were broken. Glycerol [to 10% (wt/vol)] was added to the lysate and the nuclei were collected by centrifugation at 800 × *g* for 15 min.

The nuclear pellet was washed twice and resuspended in 5 ml of buffer B [buffer A with 20% (wt/vol) glycerol]. This was slowly agitated while the nuclei were gently lysed by the dropwise addition of an equal volume of buffer C (buffer B with 4.0 M NaCl). The nuclear lysate was stirred for a further 30 min and then centrifuged at 30,000 rpm in a SW 50.1 rotor (Beckman)

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Abbreviations: NBP, novobiocin binding protein; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; RF, replicative form.

for 12 hr. The supernate, with the desired nuclear proteins, was mixed with 2.0 g of hydroxylapatite (BDH) and equilibrated in buffer D (equal parts of buffer B and buffer C) to remove residual nucleic acids. The hydroxylapatite was allowed to settle and the supernate was recovered by decantation. The hydroxylapatite–nucleic acid complex was washed with 2 ml of buffer D and the DNA-free, protein-containing supernates were combined and dialyzed for 6 hr against each of two changes (2 liters) of buffer E [50 mM Tris·HCl/20 mM $MgCl_2$/150 mM KCl/3 mM dithiothreitol/2 mM $PhMeSO_2F$/10% (wt/vol) glycerol, pH 7.0]. The dialyzed samples constituted the nuclear protein extracts.

Affinity Chromatography. *Preparation of novobiocin-coupled Sepharose.* Novobiocin was coupled to activated CH-Sepharose 4B in 0.1 M $NaHCO_3$ according to the procedure suggested by the manufacturer (Pharmacia). Washed novobiocin-Sepharose was resuspended in buffer E and distributed into 0.7 × 15 cm columns. The efficacy of the novobiocin-Sepharose so prepared was kindly verified for us by M. Gellert (National Institutes of Health), who found that the columns could bind the B subunit of *E. coli* DNA gyrase (10) and that this protein was specifically eluted with novobiocin. Novobiocin-Sepharose columns have also recently been used to purify the DNA gyrase of *E. coli* in a single step (20).

Fractionation of nuclear protein extracts. Nuclear proteins prepared from the various cells were applied to novobiocin-Sepharose affinity columns at 4°C. The unadsorbed material was washed through with 100 ml of buffer E. To elute the NBPs, the columns were developed with 100 μg of novobiocin per ml in buffer E. The latter was permitted to enter the column, the flow was stopped, and the antibiotic-containing buffer was allowed to equilibrate with the column overnight. Flow was reinitiated and the proteins specifically eluted with novobiocin [novobiocin binding proteins (NBPs)] were collected and concentrated by ultrafiltration through Amicon filters to ≯300 μl and dialyzed against three changes of buffer E before being frozen at −70°C pending further use. After elution of the NBPs additional material was removed from the columns with 1% $NaDodSO_4$ in buffer E at room temperature.

Enzyme Assay. DNA topoisomerase II activity (10, 15, 16) was detected by monitoring the ATP-dependent relaxation or catenation (or both) of supercoiled φX174 replicative form (RF) I DNA (Bethesda Research Laboratories). Relaxation of 1.5 μg of DNA was carried out in 20 μl of an assay mixture containing 50 mM Tris·HCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 1.0 mM ATP, 25 μg of bovine serum albumin per ml, 1.0 mM $PhMeSO_2F$, and 10% (wt/vol) glycerol at pH 7.8. Catenation was assessed in the same reaction mixture, supplemented with 5 mM spermidine and 2 μg of histone 1 (generously provided by P. Lewis, Dept. of Biochemistry, University of Toronto) per ml. The NBPs (5 μl) extracted from cells incubated for 24 hr at 34°C or 38.5°C were added to the reaction mixtures, which were then incubated for 30 min at 34°C or 38.5°C, respectively. The reactions were terminated by addition of 5 μl of 5% (wt/vol) $NaDodSO_4$/30 mM EDTA/250 μg of bromophenol blue per ml/30% (wt/vol) glycerol in 50 mM Tris·HCl (pH 7.8). Analysis of DNA interconversions was performed by electrophoresis.

Electrophoresis. *DNA.* The DNA forms present in each topoisomerase reaction mixture were analyzed by electrophoresis in 1% agarose gels in TEA buffer (10 mM Tris·HCl/10 mM EDTA/20 mM sodium acetate, pH 8.3) for 3 hr at 6 V/cm (21). Relaxed and supercoiled forms were detected by comigration with their known counterparts in the commercial preparation of φX174 RF I DNA. Catenanes were recognized as a slow migrating species of DNA (15, 22), which was unaffected by treatment with proteinase K or $NaDodSO_4$ prior to electrophoresis. Gels were stained with 10 μg of ethidium bromide per ml in TEA buffer and were destained briefly in TEA buffer to reveal the DNA, which was photographed under ultraviolet illumination.

Polypeptides. $NaDodSO_4$/polyacrylamide gel electrophoresis of the polypeptides in the NBP fractions [and of molecular mass marker proteins (Bethesda Research Laboratories) phosphorylase B, bovine serum albumin, ovalbumin, α-chymotrypsinogen, and β-lactoglobulin of 92,000, 68,000, 43,000, 27,500, and 18,000 daltons, respectively] was performed according to the procedure of Laemmli (23). Gels were fixed and stained with 0.25% (wt/vol) Coomassie blue in 7% (vol/vol) acetic acid and 10% (vol/vol) ethanol. When radiolabeled material was to be analyzed, the gels were cut into 1-mm slices; each slice was digested overnight with 0.5 ml of H_2O_2 before radioactivity was determined by liquid scintillation counting.

RESULTS

Novobiocin-Sepharose Chromatography of Mouse L-Cell Nuclear Proteins. Nuclear proteins were prepared from separate 3-liter lots of WT-4, *ts* A1S9, and ts^+ AR cells (at 3×10^5 cells per ml) grown at 34°C in medium containing [^{3}H]leucine to facilitate monitoring the recovery of protein in the course of subsequent cell fractionation and isolation of NBPs. Table 1 indicates that about the same proportion of cellular protein was ultimately adsorbed to novobiocin-Sepharose in each case: i.e., 3.08%, 3.11%, and 3.09% for WT-4, *ts* A1S9, and ts^+ AR cells, respectively. Specific elution of the affinity columns with novobiocin released 0.46% of the WT-4 protein and 0.48% of the ts^+ AR protein, as judged by the recovery of ^{3}H-labeled material. Only 0.27% of *ts* A1S9 cell protein was so recovered, suggesting that the NBPs present in the nuclear protein fraction of *ts* A1S9 cells differ from those in WT-4 and ts^+ AR cells.

Topoisomerase II Activity of WT-4, *ts* A1S9, and ts^+ AR NBPs. Topoisomerase II activity of NBPs prepared from the three cell types was assayed as described under *Materials and Methods.* As indicated in Fig. 1*A* the NBPs of WT-4 cells grown at 34°C catalyzed the relaxation of φX174 RF I DNA to the form II in the presence (Fig. 1*A*, lane b) but not in the absence (Fig. 1*A*, lane a) of ATP. As seen in Fig. 1*A*, lanes e and f, the catenation reaction was also dependent upon ATP. NBP prepared from WT-4 cells incubated at 38.5°C for 24 hr similarly displayed topoisomerase II activity in relaxation (Fig. 1*A*, lane c) and catenation (Fig. 1*A*, lane g) assays.

The NBPs obtained from *ts* A1S9 cells grown at 34°C also exhibited relaxation and catenation activities in the presence of ATP (Fig. 1*B*). However, if these cells were incubated at 38.5°C for 24 hr prior to preparation of these proteins, the NBPs showed little or no topoisomerase II activity (Fig. 1*B*, lanes c and g). The results of assays of topoisomerase II activity of the ts^+ AR cell NBP fraction are shown in Fig. 1*C*. Like the NBPs of WT-4 cells, those of the revertant cell displayed ATP-dependent relaxation (Fig. 1*C*, lanes b and c) and catenation (Fig. 1*C*, lanes f and g) of the substrate φX174 RF I DNA, regardless of whether the cells from which they were isolated had been incubated at 34°C or 38.5°C.

Two general comments may be made on the data shown in Fig. 1. The first is that all of the NBPs are devoid of detectable topoisomerase I activity. This is indicated by the fact that there is no accumulation of relaxed RF II DNA in the absence of ATP (Fig. 1, lanes a and f). The absence of ATP-independent nicking-closing activity is not surprising, in view of the finding that topoisomerase I activity is adsorbed to hydroxylapatite under the conditions used herein (24).

Table 1. Distribution of cellular proteins generally labeled with [^{3}H]leucine during isolation of nuclear NBPs

Cells examined	[^{3}H]Leucine, dpm per cell fraction						
				Novobiocin-Sepharose			
	Whole cells	Nuclei	Nuclear protein extract	Adsorbed to column	Novobiocin eluate	$NaDodSO_4$ eluate	Residue on column
WT-4	5.4×10^7 (100)	3.08×10^7 (57)	2.86×10^7 (53)	1.68×10^6 (3.08)	2.48×10^5 (0.46)	1.40×10^6 (2.6)	1.06×10^4 (0.02)
ts A1S9	5.3×10^7 (100)	2.65×10^7 (50)	2.59×10^7 (47)	1.6×10^6 (3.11)	1.43×10^5 (0.27)	1.5×10^6 (2.9)	0.98×10^4 (0.02)
ts^+ AR	5.4×10^7 (100)	2.87×10^7 (53)	2.79×10^7 (51)	1.68×10^6 (3.09)	2.50×10^5 (0.48)	1.41×10^6 (2.7)	1.05×10^4 (0.02)

Numbers in parentheses represent percent of total protein. Each entry represents the average of duplicate determinations in at least three separate experiments. In all cases, the standard error was <10%.

The second point of interest is the finding that the NBP isolated from equivalent numbers of WT-4 and ts^+ AR cells yields similar DNA topoisomerase II activity in both the relaxation and catenation assays. The same relative activity was observed with extracts from *ts* A1S9 cells grown at 34°C.

We showed earlier that *ts* A1S9 cells plated at 34°C are very sensitive to novobiocin as compared with WT-4 and ts^+ AR cells (9). Therefore, it was of interest to determine the effect of novobiocin on the topoisomerase II activity of the NBPs extracted from *ts* A1S9, WT-4, and ts^+ AR cells grown at 34°C. Fig. 2 gives the results of one such experiment. The NBP fraction of WT-4 (Fig. 2, lanes b and c) and ts^+ AR cells (Fig. 2, lanes f and g) were active in the production of catenanes in the absence and presence of 100 μg of novobiocin per ml. No topoisomerase II activity was detected in *ts* A1S9 cell NBPs treated with this same concentration of novobiocin (Fig. 2, lane e). Essentially similar results (data not shown) were obtained with NBPs incubated with 50 μg of novobiocin per ml.

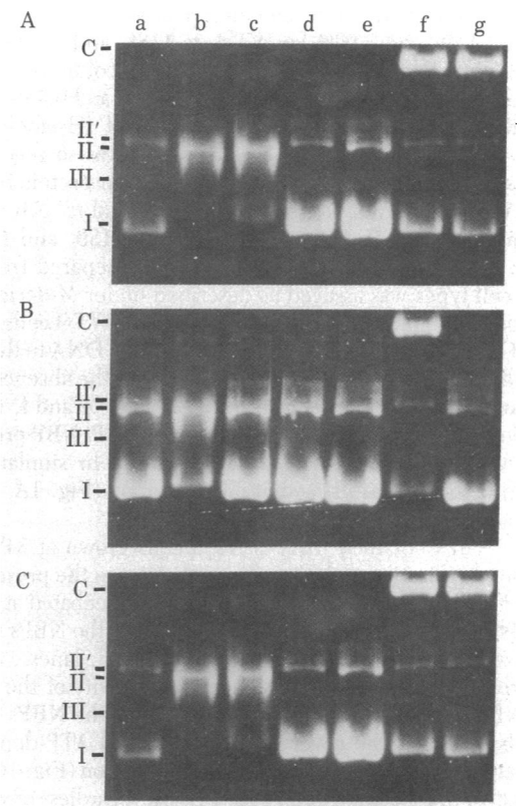

FIG. 1. Topoisomerase activity of the NBP fraction of mouse L cells. NBPs of WT-4 (*A*), *ts* A1S9 (*B*), and ts^+ AR (*C*) were assayed for topoisomerase activity. In *A–C*, lane d carries a sample of φX174 RF DNA, of which ≈90% is RF I and the rest is RF II. Lanes a–c present the results of relaxation assays, and lanes e–g, the results of catenation assays. The reactions in lanes a and e were carried out in the absence of ATP to test for DNA topoisomerase I activity. All other reaction mixtures contained ATP to permit measurement of DNA topoisomerase II activity. NBPs used in assays shown in lanes a, b, e, and f were extracted from cells incubated at 34°C, whereas lanes c and g used NBPs prepared from cells incubated at 38.5°C for 24 hr.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis of Mouse L Cell NBPs. We have carried out a preliminary analysis of the polypeptides of the NBPs of WT-4, *ts* A1S9, and ts^+ AR cells grown at 34°C in the presence of [^{3}H]leucine to generally label cellular proteins. For this purpose the ^{3}H-labeled NBPs were prepared, lyophilized, resuspended in $NaDodSO_4$ sample buffer, and electrophoresed on 8% acrylamide gels. Fig. 3 presents profiles of recovery of ^{3}H-labeled protein. The NBPs of WT-4 cells (Fig. 3 *Top*) and of ts^+ AR cells (Fig. 3 *Bottom*) comprised three main polypeptides with approximate molecular masses of 76,000, 74,000, and 30,000 daltons, as judged by comparison with the migration of molecular mass standards; these polypeptides accounted for 48%, 26%, and 14%, respectively, of the total radioactivity recovered from the gel.

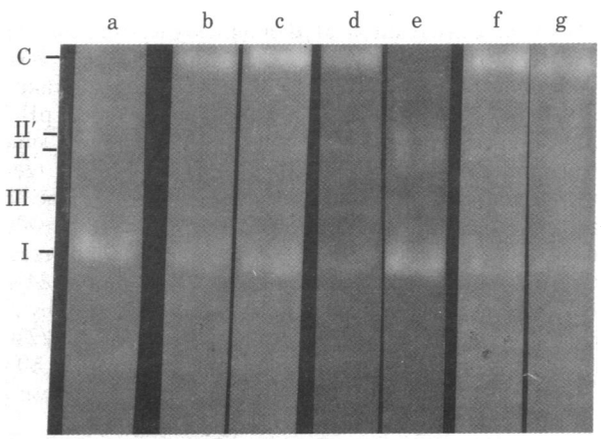

FIG. 2. Novobiocin sensitivity of topoisomerase II activity of the NBPs of WT-4, *ts* A1S9, and ts^+ AR cells. Topoisomerase II activity of the NBPs of the various cells incubated at 34°C was assayed in the presence and absence of novobiocin (100 μg/ml) by monitoring catenation of φX174 RF DNA. Lanes: a, φX174 RF DNA standard alone; b, WT-4 cell NBP added; c, WT-4 cell NBP with novobiocin; d, *ts* A1S9 cell NBP added; e, *ts* A1S9 cell NBP with novobiocin; f, ts^+ AR cell NBP added; and g, ts^+ AR cell NBP with novobiocin.

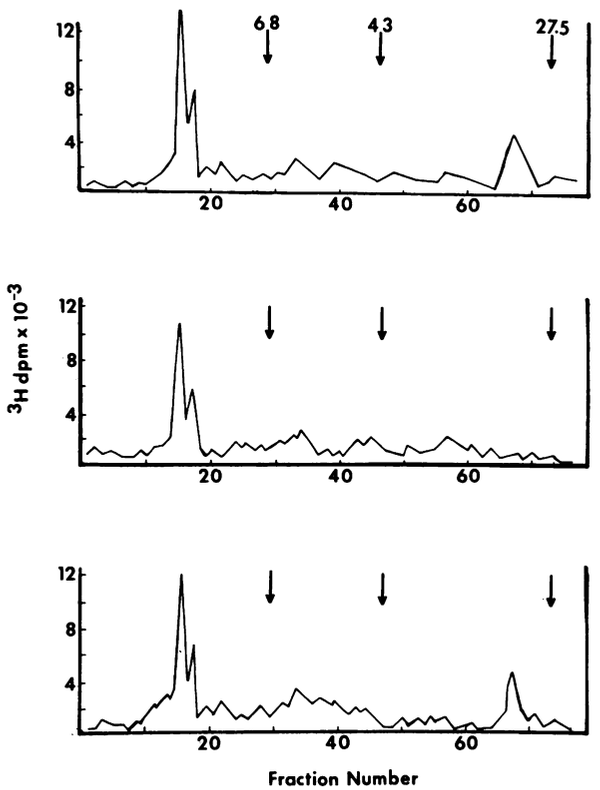

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis of mouse L-cell NBP fractions. Mouse L-cell NBPs from cells growing at 34°C were electrophoresed along with molecular mass markers (whose size in kilodaltons is noted) on 8% polyacrylamide gels in the presence of $NaDodSO_4$ and dithiothreitol. The gels were fixed and sliced and the radioactivity due to [^{3}H]leucine used to generally label cellular proteins was determined for each 1-mm slice. The results obtained for WT-4, *ts* A1S9, and *ts*$^+$ AR cell NBPs are shown in *Top*, *Middle*, and *Bottom*, respectively.

The *ts* A1S9 cell NBP fraction (Fig. 3 *Middle*) yielded only two main polypeptide species that comigrated with the 76,000- and 74,000-dalton NBPs of WT-4 cells. The ratio of the amount of [^{3}H]leucine in the 76,000-dalton/74,000-dalton polypeptides was 1.79, as compared with 1.84 for the analogous WT-4 NBPs. The labeled proteins that remained adsorbed to the columns after novobiocin treatment were eluted with $NaDodSO_4$. Such eluates, whether derived from WT-4, *ts* A1S9, or *ts*$^+$ AR cells, presented a complex series of polypeptides in $NaDodSO_4$/polyacrylamide gel electrophoresis (data not shown), in which no obvious difference among cell types was immediately apparent.

DISCUSSION

In this paper we report the isolation and preliminary characterization of NBPs from a pool of proteins extracted with 2 M NaCl from the nuclei of mouse L cells. Three cell types were examined: wild-type, WT-4 cells, the *dna*^{ts}/*S*^{ts} *ts* A1S9 mutant; and its *ts*$^+$ AR revertant.

The NBP fractions derived from all three types of cells grown to midlogarithmic phase at the permissive temperature of 34°C yielded equivalent levels of ATP-dependent topoisomerase II activity. Such activity was also detected in the NBPs of WT-4 and *ts*$^+$ AR cells incubated for an additional 24 hr at 38.5°C. In contrast, the NBPs of temperature-inactivated *ts* A1S9 cells (similarly incubated at 38.5°C) were incapable of effecting ATP-dependent relaxation or catenation of superhelical ϕX174 RF I DNA. Entirely similar results were obtained in analyses of DNA topoisomerase II activity in proteins extracted with 1 M NaCl from nuclei isolated by classical techniques of cell fractionation, whether the DNA topoisomerase II activity was measured in terms of ATP-dependent relaxation of a double-stranded, covalently closed plasmid DNA (even in the presence of ATP-independent topoisomerase I activity) or in terms of catenation (unpublished data). These findings are compatible with the hypothesis that the *ts* A1S9 mutation encodes a temperature-sensitive topoisomerase II, a subunit thereof, or an auxillary polypeptide that is required for topoisomerase II function.

The novobiocin sensitivity of the topoisomerase II activity in the NBP prepared from Wt-4, *ts* A1S9, and *ts*$^+$ AR cells growing at 34°C is very similar to that exhibited by the intact cells plated at 34°C in the presence of this drug (9). This observation is in accord with the finding (2, 4) that the *ts* A1S9 mutation is partially expressed at the permissive temperature. Indeed, the functional biochemical defect (2) is seen in *ts* A1S9 cells grown at 34°C as the transient accumulation of a pool of newly made, low molecular weight, single-strand DNA, which is slowly converted to mature chromosomal DNA (refs. 2 and 4; unpublished data).

One of the most interesting observations derives from our preliminary characterization of the NBPs from cells grown at 34°C. Thus, analysis by $NaDodSO_4$/polyacrylamide gel electrophoresis revealed the presence of three major polypeptides of 76,000, 74,000, and 30,000 daltons in the NBP extracts from Wt-4 cells and the *ts*$^+$ AR revertant. Several larger molecular weight components were also detected, but in trace amounts. The NBPs from *ts* A1S9 cells appear to lack the 30,000-dalton component. It is conceivable that this material is absent from the NBP fraction of *ts* A1S9 cells because it has an altered affinity for DNA, such that it is not extracted from nuclei with 2 M NaCl. More acceptable is the hypothesis that the 30,000-dalton polypeptide of *ts* A1S9 cells has been so modified, even at 34°C, that its affinity for novobiocin is enhanced. This would result in tighter binding to the novobiocin-Sepharose column, precluding elution with the drug under the conditions used. This model accommodates the observed novobiocin sensitivity of intact cells plated at 34°C and the noted drug sensitivity of the DNA topoisomerase II activity of NBP derived from *ts* A1S9 cells grown at the permissive temperature. It is also possible that the modified polypeptide is to be found among the many proteins eluted from novobiocin-Sepharose with $NaDodSO_4$ or amid the few that remain very tightly bound to this material. It may be present as part of a larger polypeptide of the NBP fraction *per se* in the form of a large molecular weight precursor whose processing is modified in *ts* A1S9 cells.

Other topoisomerase II enzymes have been isolated by using protocols that do not depend upon novobiocin affinity chromatography. The most extensively studied is that of *E. coli*, which contains two protein subunits (10). The topoisomerase of bacteriophage T4 is reported to be a complex of three polypeptides (25). The HeLa cell enzyme is thought to function as a dimer of 172,000-dalton subunits (15). Little is yet known about the physical properties of topoisomerase II enzymes detected in *D. melanogaster* (16, 21), *Xenopus laevis* (22), and some cultured mammalian cells (ref. 21; unpublished data). However, all are sensitive to novobiocin. Clearly, additional experiments are necessary to clarify the relationship of these several topoisomerase II enzymes with that detected herein, in mouse L cells.

The present studies provide compelling biochemical and genetic evidence for involvement of the mouse L-cell DNA topoisomerase II in an essential function of chromatin replication. It has been suggested that this enzyme participates in initiation of DNA synthesis (10, 26), segregation of daughter molecules (11, 22), unwinding of parental DNA (15, 27), and

compaction of chromatin for chromosome condensation (16). We anticipate that further studies with *ts* A1S9 cells will help to determine whether any or all of these functions are directly or indirectly served by the biochemical action of a eukaryotic topoisomerase II.

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