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Human Autoantibody to Topoisomerase II

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The rheumatic diseases are characterized by the production of autoantibodies that are usually directed againts components of the cell nucleus. In this communication, we describe autoantibodies that recognize DNA topoisomerase II (anti-topoII) present in the serum of a patient with systemic lupus erythematosus. Several lines of evidence indicate that this antibody recognizes topoisomerase II. First, it binds to the native enzyme in soluble extracts prepared from isolated chromosomes and effectively depletes such extracts of active enzyme. Second, the serum binds to topoisomerase II in immunoblots of mitotic chromosomes and chromosome scaffolds. Finally, the antiserum binds strongly to a fusion protein encoded by a cloned cDNA and expressed in *Escherichia coli* that (based on immunological evidence) represents the carboxy-terminal portion of chicken topoisomerase II. Autoantibodies such as the one described here may provide useful reagents for the study of human topoisomerase II.

The importance of autoantibodies to cell and molecular biologists has been considerable (for reviews see Refs. [1–3]). The pioneering characterization of the RNP and Sm autoantibodies from sera of patients with systemic lupus erythematosus led to the discovery of an abundant new class of small nuclear RNPs [4]. Subsequent studies indicated that these may be essential for mRNA splicing [5]. More recently, autoantibodies from patients with scleroderma have led to the first identification [6, 7] and subsequent cloning [8] of polypeptide components of the centromere. Other autoantigens that have been identified, characterized, and cloned at the cDNA level using autoimmune patient sera, include La [9], PCNA/cyclin [10–12], ADP-ribose polymerase [13, 14], and topoisomerase I [15–19].

In this communication we report the identification of a new autoantibody specificity directed against DNA topoisomerase II. Topoisomerase II was first identified as a soluble enzyme in nuclear extracts (reviewed in [20]). This enzyme had a unique catalytic activity. By creating transient protein-linked double strand breaks in duplex DNA, it could pass DNA strands through one another, thus altering the topological state of the DNA [20]. More recent cell fractionation and

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immunocytochemical evidence indicates that topoisomerase II is an abundant (perhaps the most abundant) component of the insoluble residual fractions derived from the eukaryotic cell nucleus (the nuclear matrix [21]) and mitotic chromosomes (the chromosome scaffold [22, 23]). Thus this enzyme may have structural as well as catalytic functions. Interest in the enzyme has recently blossomed following the further discoveries that it is a highly specific marker for proliferating cells [24–26] and may be the primary target of the potent amsacrine and etoposide anti-tumor drugs [27, 28].

The cell biology of topoisomerase II has been little studied, largely due to a lack of suitable antibodies and the limited interspecies cross-reactivity of those that are available [22, 23, 29, 30]. Clearly a more general source of antibody is needed. Autoantibodies of the sort that we describe below may fill this need, since they exhibit little species specificity. The serum we describe reacts equally well with both the chicken and human enzymes.

EXPERIMENTAL PROCEDURES

Sera. The patient is a 36-year-old black female who has had systemic lupus erythematosus (SLE) since age 24 with photosensitive rash, arthritis, Raynaud's phenomenon with digital ulcers, digital vasculitis, seizures, proteinuria, positive antinuclear antibodies, hypocomplementemia (low C3, C4, and CH50), anemia, anti-dsDNA antibodies, and hyper-γ-globulinemia. The VDRL was negative as were tests for anti-Ro, anti-La, anti-Sm, anti-RNP, rheumatoid factor, anti-centromere antibodies, and anti-Scl-70 (topoisomerase I). An identical response against topoisomerase II was seen in six sequential serum samples obtained between 8/84 and 5/87.

Control sera from 67 patients with SLE were chosen at random from those stored in the frozen serum bank at the University of Connecticut. Details of the characteristics of these SLE patients are available on request.

Immunoprecipitation of topoisomerase II from soluble extracts. Soluble extracts containing topoisomerase II activity were prepared from crude preparations of mitotic chromosomes as previously described [22, 31]. Briefly, mitotic HeLa cells (blocked overnight with 0.1 μg/ml colcemid) were centrifuged (600×*g* for 3 min) and resuspended in RSB (10 m*M* Tris : HCl, pH 7.4, 10 m*M* NaCl, 5 m*M* $MgCl_2$) to allow hypotonic swelling. After 5 min at room temperature, swollen cells were centrifuged as before and then lysed by resuspension and dounce homogenization in buffer A (15 m*M* Tris : HCl, pH 7.4, 80 m*M* KCl, 2 m*M* K-EDTA, 0.3 m*M* spermine, 0.75 m*M* spermidine, 0.1% digitonin, plus Trasylol and PMSF). This and all subsequent operations were performed at 4°C. Chromosomes were sedimented at 200*g* for 30 min and resuspended in 1 ml RSB. The bulk of chromosomal proteins were then solubilized by addition of NaCl to 1 *M* and β-mercaptoethanol to 10 m*M*. (This treatment solubilizes the chromosome scaffold [36].) Upon addition of PEG 8000 to 6% the DNA formed a precipitate that was removed by centrifugation at 12,000*g* for 30 min, leaving the final soluble extract, which contained both active topoisomerases I and II.

Immunoprecipitation was performed as described [25, 32]. Serum was added at a concentration of 20 μl for the chromosomes from 10^8 cells and incubated with mixing for 2 h at 4°C. Immune complexes were subsequently removed by adsorption to fixed *Staphylococcus aureus* [33].

Topoisomerase assays. Immunoadsorbed soluble extracts prepared as described above were assayed for topoisomerase activity as follows. To assay topoisomerase II, three serial dilutions of extract were tested for their ability to decatenate 500 ng of trypanosome mitochondrial kinetoplast DNA (gift of Kathy Ryan and Paul Englund [32]) at 37°C for 1 h in reaction volumes totaling 40 μl in the presence of topoisomerase II reaction buffer (50 m*M* Tris : HCl, pH 7.4, 60 m*M* KCl, 0.5 m*M* ATP, 9.5 m*M* $MgCl_2$, 0.5 m*M* Na-EDTA, 0.5 m*M* dithiothreitol, 0.005% Triton X-100). The extracts were also tested for topoisomerase I activity (the ability to relax supercoiled plasmid DNA in the presence of EDTA) in a buffer containing 50 m*M* Tris : HCl, pH 7.4, 60 m*M* KCl, 0.5 m*M* Na-EDTA, 0.5 m*M* dithiothreitol, and 0.005% Triton X-100 [34]. The reactions were stopped by adding DNA sample buffer (40% sucrose in 40 m*M* Tris : HCl, 16 m*M* Na-acetate, pH 8.1, 1.7 m*M* Na-EDTA, 0.25% bromphenol blue), and heating to 65°C before loading onto a 1% agarose gel.

Immunoblotting. Electrophoresis of pellets and supernatants from immunoprecipitations in 10% polyacrylamide gels containing SDS and subsequent immunoblotting procedures were performed as described previously [22, 35].

RESULTS AND DISCUSSION

Identification of an autoantiserum that binds to a 170-kDa chromosomal antigen. In an ongoing effort to identify the major autoantigens of mitotic chromosomes, we have used immunoblotting to screen large numbers of autoimmune sera for binding to chromosomal components. In these experiments, one serum, BS, showed strong binding to a protein of M_r ~170 kDa (Fig. 1*B*). This protein was present both in isolated mitotic chromosomes and in the non-histone chromosomal scaffold fraction obtained from them by nuclease digestion and subsequent high salt extraction (Fig. 1*B*) [22, 36].

Two observations suggested that the 170-kDa autoantigen might be DNA topoisomerase II. First, the autoantigen is present in both chromosomes and chromosome scaffolds (Fig. 1*B*). (Topoisomerase II is a major component of the latter [22, 30].) Second, the autoantiserum recognized both the 170-kDa species and a ladder of smaller polypeptides (Fig. 1*B*). One of the hallmarks of many antibodies to topoisomerase II is that they recognize both the 170-kDa antigen and a characteristic ladder of proteolytic fragments derived from it [22].

Antibodies that recognize topoisomerase II from a variety of species are not widely available to the large number of investigators who study this important enzyme. Autoantibodies, if available, could potentially fill this need. We therefore decided to determine whether the 170-kDa antigen recognized by serum BS is indeed topoisomerase II.

Ability of serum BS to bind functional topoisomerase II in soluble extracts. Use of an indirect immunoadsorption protocol demonstrated that serum BS recognizes topoisomerase II in soluble extracts prepared from isolated mitotic chromosomes. Such extracts contain topoisomerase II activity, which we measured as the ability of the extract to liberate monomer circles from mitochondrial kinetoplast networks (Fig. 2). When serum BS was added to such an extract and immune complexes were subsequently removed by adsorption to fixed *S. aureus* [33] a significant decrease in the level of active topoisomerase II was observed. Six independent serum samples obtained from patient BS were all equally effective. Two of these are compared in Figs. 2*a* and *b*. A guinea pig antibody to topoisomerase II (obtained as described below) also removed the bulk of the topoisomerase II activity from the extract (Fig. 2*h*).

In control experiments, sera from several other individuals had no effect on topoisomerase II activity in the extracts. These controls included an autoantiserum that recognizes DNA topoisomerase I (which did remove a substantial percentage of the topoisomerase I activity—Fig. 2*f*, lower panel) as well as two sera from individuals with anti-centromere antibodies (Figs. 2*c* and *d*). Finally, two sera from normal individuals (Figs. 2*e* and *g*) also had no effect on the activity of either topoisomerase in these extracts.

The pellets and supernatants from the immunoprecipitations shown in Fig. 2

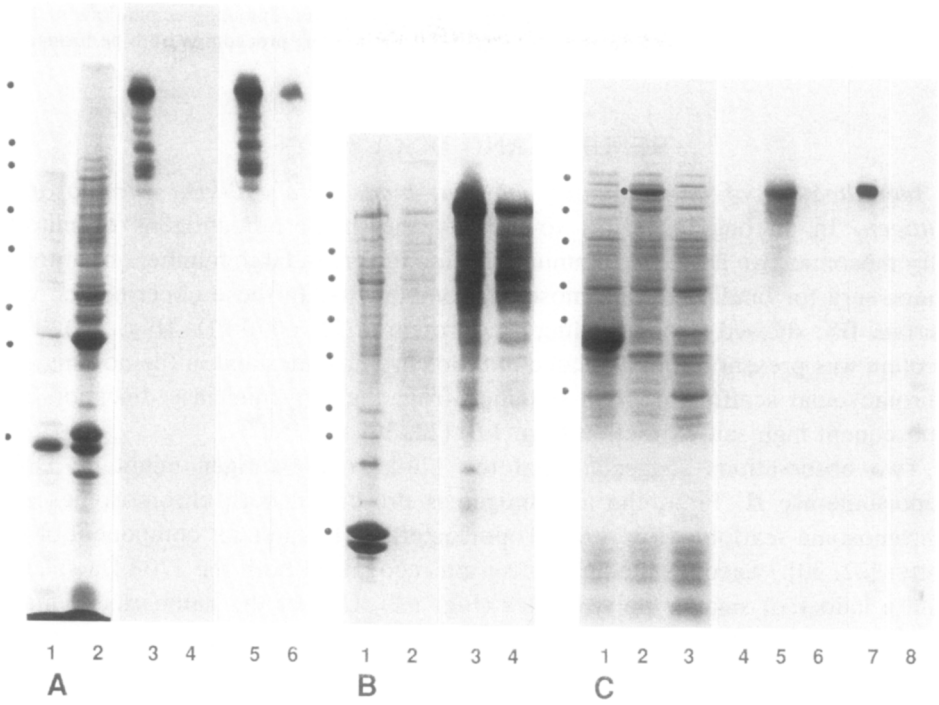

Fig. 1. Binding of autoimmune and experimental anti-topoII to mitotic chromosomes, chromosome scaffolds, and bacterial fusion proteins. (*A*) Binding of experimental antibodies recognizing topoisomerase II to chicken (lanes 1, 3, 5) and human (lanes 2, 4, 6) mitotic chromosomes. Lanes 1 and 2, Coomassie blue staining. Lanes 3 and 4, Immunoblot with serum 2b2 (dilution 1 : 1000). Note the failure of this antibody (which was used to clone chicken topoisomerase II) to recognize the human antigen. Lanes 5 and 6, Immunoblot with serum 24b1 (dilution 1 : 1000). This serum (raised against the cloned fusion protein) recognizes both the chicken and human antigens. (*B*) Binding of autoimmune serum BS to mitotic chromosomes (lanes 1 and 3) and chromosome scaffolds (lanes 2 and 4). Lanes 1 and 2, Coomassie blue staining. Lanes 3 and 4, Immunoblot with serum BS (dilution 1 : 1000). (*C*) Binding of experimental and autoimmune sera to cloned topoisomerase II. Lanes 1 and 4, induced bacteria harboring the pATH vector without cloned insert. The trpE protein is the heavy band in lane 1. Lanes 2, 5, 7, 8, induced bacteria harboring a pATH vector into which is cloned 1.9 kb of the chicken topoisomerase II cDNA. The fusion protein is indicated by a dot to the left of lane 2. Lanes 3 and 6, bacteria harboring the recombinant plasmid prepared under conditions where expression of the trp operon was not induced. Lanes 1–3 are stained with Coomassie blue. Lanes 4–8 are immunoblots with serum 24b1 diluted 1 : 1000 (lanes 4–6); serum BS diluted 1 : 1000 (lane 7); control normal serum WE diluted 1 : 1000 (lane 8). The markers shown at the left of *A* and *B* are (from top to bottom) 200 kDa (myosin), 116 kDa (β-galactosidase), 95 kDa (phosphorylase *b*), 68 kDa (bovine serum albumin), 60 kDa (catalase), 43 kDa (actin), 40 kDa (aldolase), and 29 kDa (carbonic anhydrase). The upper marker shown to the left of (*C*) is 95 kDa.

were subjected to SDS–PAGE, transferred to nitrocellulose [35], and probed with the following sera (Fig. 3): BS (panel *A*); human anti-topoisomerase I (serum SC, panel *B*); and guinea pig anti-topoisomerase II (serum 24b1, panel *C*). The original serum used in the precipitation is indicated above each blot. As predicted from the data of Fig. 2, only sera BS and 24b1 precipitated topoisomerase II (compare panels *A* and *C*). In all other precipitations the antigen remained in the supernatant fraction (compare blots *1* and *6* with blots *2–5*). In contrast, autoantiserum

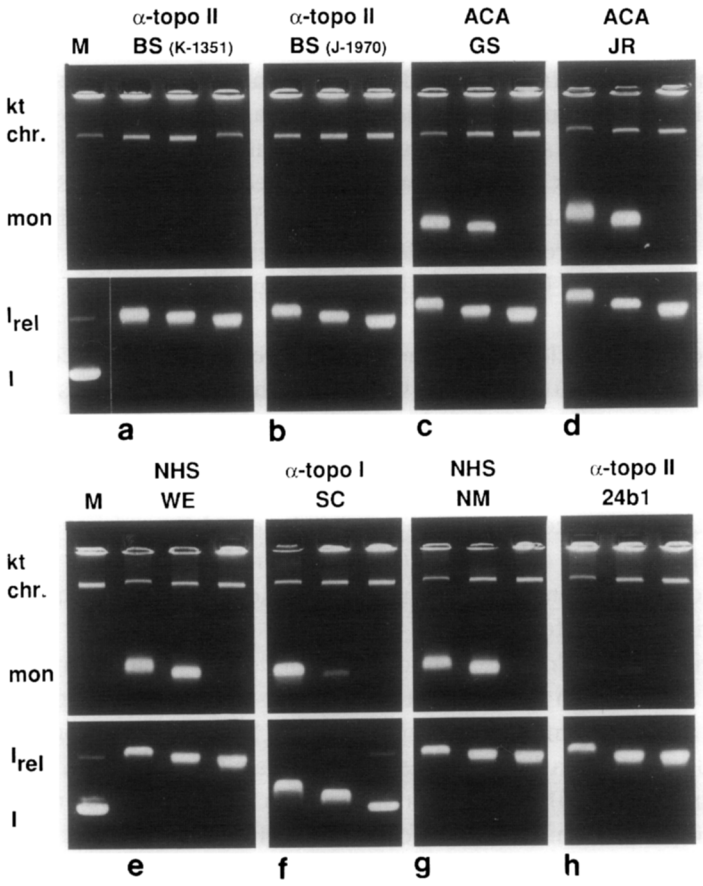

Fig. 2. Autoimmune anti-topoII precipitates functional topoisomerase II from chromosomal extracts. Soluble enzyme extracts were incubated with the indicated antibodies, and immune complexes were subsequently removed by absorption with fixed *S. aureus* [33]. The extracts were then tested for their ability to decatenate kinetoplast DNA ([31] topoisomerase II activity, upper panels) or to relax supercoiled plasmid DNA in the presence of EDTA ([34] topoisomerase I activity, lower panels). The antibodies used for the absorptions were (*a* and *b*) two independent sera from patient BS; (*c* and *d*) sera from two patients with anti-centromere antibodies; (*e* and *g*) sera from two individuals lacking rheumatic disease; (*f*) serum from an individual with antibodies to topoisomerase I (ScI-70); and (*h*) serum from an experimental animal injected with cloned chicken topoisomerase II fusion protein. Each panel shows a series of three serial dilutions of the adsorbed enzyme extract. (Dilutions were 1:12, 1:24, and 1:120.) Lane *M* in (*a*) and (*e*) shows the input DNA. The abbreviations for the DNA forms shown (at left) are kt, kinetoplast DNA network (remains trapped in well); chr., contaminating chromosomal DNA running at the exclusion limit of the gel; mon, released monomer DNA; $_{rel}$, relaxed covalently closed circular plasmid DNA; and I, supercoiled plasmid DNA.

SC (anti-topoisomerase I, panel *B*) caused significant precipitation of topoisomerase I (compare blot 5 with blots *1–4* and 6).

The experiments presented in Figs. 2 and 3 thus indicate that serum BS recognizes human DNA topoisomerase II both in solution and in immunoblots.

cDNA cloning of chicken topoisomerase II. We have previously described the preparation of an antibody, 2b, against gel purified topoisomerase II from chicken

chromosome scaffolds [22]. Antibody 2b is highly species specific. It strongly recognizes the chicken enzyme, but not its human counterpart (Fig. 1*A*, lanes *3* and *4*). In experiments performed with the intention of more readily gaining access to larger amounts of topoisomerase II antigen, antibody 2b was used to screen a chicken cDNA expression library constructed in λgt11 [37, 38]. In the initial experiment, 4×10^5 phage were screened, yielding 13 strong positives. Twelve of these were subsequently plaque purified. The recombinant phage were grown by standard procedures [39], and the inserted chicken sequences were excised with *Eco*R1. The largest insert, designated pTOPOII$_1$ was 1900 bp in length.

This insert was subcloned into the appropriate pATH vector (constructed by T. J. Koerner, Duke University), in which the chicken sequences are transcribed and translated to produce a hybrid protein fused to 32 kDa of the bacterial trpE protein [8]. When bacteria containing this plasmid (which we have designated pTOPOII$_1$) were exposed to indole acrylic acid (to induce expression of the trp operon), they produced a major new polypeptide species that migrated in SDS–PAGE with a M_r of 74.5 kDa (Fig. 1*C*, lane *2*). This fusion protein contains 42.5 kDa (or ~386 amino acid residues) of chicken polypeptide fused to 32 kDa of the bacterial trpE protein.

The region containing the fusion polypeptide was excised from a polyacrylamide gel and used to immunize a guinea pig. This animal responded strongly, producing an antiserum that we designate 24b1. Serum 24b1 recognizes a 170-kDa polypeptide present in both chicken and human chromosomes in immunoblots (Fig. 1*A*, lanes *5* and *6*). In addition, it is able to bind the native enzyme in soluble extracts prepared from human chromosomes (Fig. 2*h*).

The properties of the two sera 2b2 and 24b1 suggest strongly that at least two independent epitopes are shared by *bona fide* chromosomal topoisomerase II and the fusion polypeptide encoded by pTOPOII$_1$. One epitope(s), recognized by antibody 2b2, is not conserved between species. This epitope(s) is present on chicken, but not human, chromosomal topoisomerase II. In contrast, the other epitope(s), recognized by antibody 24b1, is apparently conserved between chicken and human type II topoisomerases. Assuming a minimal epitope size of 6 amino acid residues and that all amino acids occur with an equal probability, the odds that two unrelated proteins of length 386 amino acid residues will share two epitopes are roughly 2×10^{-12}. Thus, the existence of two independent epitopes shared by both the cloned fusion protein and *bona fide* chromosomal topoisomerase II strongly suggests that pTOPOII$_1$ does in fact encode a portion of topoisomerase II.

Evidence from molecular analysis of the cDNA is also consistent with the identification of pTOPOII$_1$ as a clone for chicken DNA topoisomerase II. In RNA (Northern) blotting experiments, pTOPOII$_1$ was found to hybridize to a single mRNA of 6.2 kb (Ratrie, Heck, and Earnshaw, unpublished data). This is large enough to encode the 175-kDa topoisomerase II polypeptide with 1400 bases of 5′ and 3′ untranslated sequences left over. In a parallel experiment, no cross-hybridization to human mRNA was detected. Finally, preliminary DNA se-

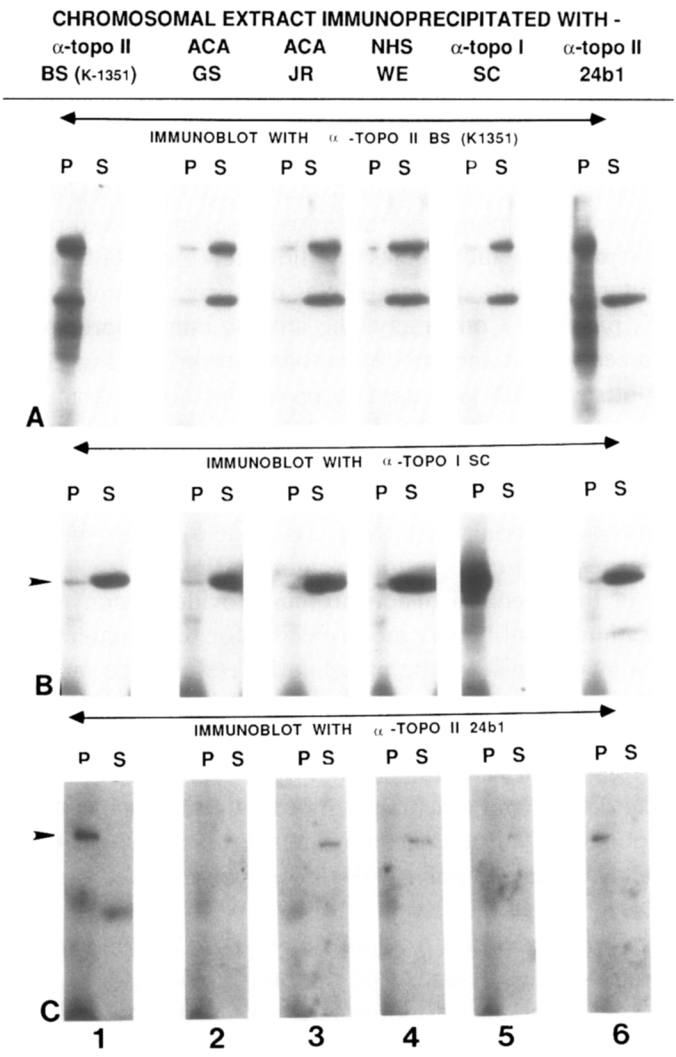

Fig. 3. Autoimmune anti-topoisomerase II immunoprecipitates topoisomerase II polypeptide from chromosomal extracts. Aliquots of the soluble extracts assayed for enzyme activity in Fig. 2 were analyzed by SDS–PAGE together with aliquots of the precipitated material. The samples were electrophoresed in three parallel 10% SDS–polyacrylamide gels [22], transferred to nitrocellulose filters [35], and probed with (*A*) autoimmune anti-topoII; (*B*) autoimmune anti-topoisomerase I; and (*C*) experimental antibody raised against cloned chicken topoisomerase II. The immunoprecipitations were carried out with the following sera: (panel 1) autoimmune anti-topoisomerase II serum BS; (panels 2 and 3) autoimmune anti-centromere antibodies GS and JR; (panel 4) normal human serum; (panel 5) autoimmune anti-topoisomerase I serum SC; (panel 6) experimental anti-topoisomerase II. Because of the large excess of other extract proteins not precipitated by the antibodies, the loading of lanes *P* (pellet of the immunoprecipitation) and *S* (supernatant of the immunoprecipitation) was different. Lanes *P* contain the antigen from 10^6 cells, while lanes *S* contain antigen from 10^4 cells.

quence analysis of the 5′ terminus of the cDNA yielded a sequence with significant similarity to the published sequence of topoisomerase II from *Saccharomyces cerevisiae* [40].

It is therefore highly likely that pTOPOII$_1$ is derived from the *bona fide* cDNA encoding chicken topoisomerase II.

Binding of autoimmune anti-topoII to cloned chicken topoisomerase II. The data of Figs. 3 *a* and *c* demonstrate that autoantiserum BS and rabbit serum 24b1 recognize the same antigenic species in whole cell extracts. That is, the antigen immunoprecipitated by autoantiserum BS is recognized in immunoblots by serum 24b1 (Fig. 3 *C*, panel *1*). Conversely, the antigen immunoprecipitated by serum 24b1 is recognized in immunoblots by autoantiserum BS (Fig. 3 *A*, panel *6*).

When autoantiserum BS was used to probe bacterial extracts expressing the cloned chicken topoisomerase II fusion protein a single species was recognized (Fig. 1 *C*, lane *7*). This corresponded to the antigen recognized by experimental serum 24b1 (Fig. 1 *C*, lane *5*). Normal human serum did not recognize any polypeptide in this bacterial lysate (Fig. 1 *C*, lane *8*). Thus the human autoantiserum recognizes cloned chicken topoisomerase II.

Availability of the bacterial fusion protein provides a convenient method for affinity purification of antibodies monospecific for topoisomerase II from serum BS (data not shown). This will be particularly useful since, as shown in Fig. 3, serum BS also recognizes an autoantigen of ~116 kDa. (This is seen most clearly in the last lane of panel *A*. Serum BS recognizes this species in the supernatant of the immunoprecipitation with antiserum 24B1, which is specific for topoisomerase II.) This autoantigen is presumably not related to topoisomerase II, as it is neither a component of mitotic chromosomes nor is it recognized by serum 24b1 (Fig. 3 *A*, panel *6*). It may be poly(ADP-ribose) polymerase, a known autoantigen [13] of 116 kDa.

Prevalence of autoantibodies to topoisomerase II. We have screened sera from 67 other individuals with a diagnosis of systemic lupus erythematosus, looking for additional sera that recognize topoisomerase II. When these sera were used to screen immunoblots of total human nuclear proteins, 15 appeared to recognize high-molecular-weight polypeptides that were in size similar to topoisomerase II. To determine whether, in fact, these 15 sera did recognize topoisomerase II, they were screened for their ability to recognize the proteins immunoprecipitated from cultured HeLa cells by serum BS. None of the 15 sera showed significant binding to the 170-kDa polypeptide immunoprecipitated by serum BS (data not shown). In a control experiment, serum 24b1 bound strongly to the immunoprecipitated 170-kDa antigen, confirming the result presented in Fig. 3 (data not shown).

It thus appears that none of the 67 randomly chosen sera from patients with systemic lupus erythematosus recognizes topoisomerase II. Therefore autoantibodies to topoisomerase II may be rare in these patients ($\leq$1.5%).

Conclusions. Because the DNA topoisomerases are well known at the biochemical level, we suggest that the terminology used to describe the autoantibodies that recognize them be revised to reflect this. Thus, we will refer to anti-Scl-70 [15] autoantibodies (which recognize DNA topoisomerase I [16–18]) as anti-

topoI. Similarly, sera such as BS, which recognize DNA topoisomerase II, will be referred to as anti-topoII.

It is likely that the utility of the anti-topoII autoantibodies will be primarily in their role as specific reagents with which to study this important nuclear structural enzyme in a number of species. Such reagents may permit further analysis of the correlation between growth rate, topoisomerase II content, as sensitivity to amsacrine and etoposide drugs in human tumors. The correlation between cellular content of topoisomerase II and proliferative state is by now well known [24–26], and a correlation between topoisomerase II content and growth rate has been reported for the rat Dunning tumor model [40]. Such studies may eventually lead to the development of strategies for deciding when particular drugs are likely to be the reagents of choice for treatment in the clinic.

In addition, the use of topoisomerase II as a specific immunocytochemical marker for proliferating cells [24–26] may permit determination of when cells embarked on pathways of terminal differentiation complete their final division. Such studies are now limited to the detection of cells in S phase (by [^{3}H]-thymidine autoradiography or by immunostaining with autoantibodies for PCNA/cyclin [10, 11]). The wide species cross-reactivity of autoimmune anti-topoII, may render the autoantibodies suitable for these experiments.

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