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A recombinant topoisomerase I used for autoantibody detection in sera from patients with systemic sclerosis

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SUMMARY

We report the expression of a cDNA clone encoding 695 carboxyl-terminal amino acids of human DNA topoisomerase I (topoI) in *Escherichia coli*. More than 96% of the anti-HeLa topoI-positive sera from patients with a connective tissue disease displayed also an immunoreactivity with this recombinant protein (the HTopoA protein). Sera from patients with a definite diagnosis systemic sclerosis and reacting with HeLa topoI, all reacted with the HTopoA protein as well. Sera from patients with systemic sclerosis that did not contain anti-topoI antibodies (about 30% of the systemic sclerosis sera), as concluded from HeLa immunoblot, displayed also no immunoreactivity with our recombinant antigen. By expressing different fragments of HTopoA, we were able to assign at least three different autoimmune epitope regions on the HTopoA protein and we show that over a period of 5 years the amount of anti-topoI antibodies against these regions may fluctutate.

Keywords topoisomerase I systemic sclerosis fusion proteins epitope regions

INTRODUCTION

In the eukaryotic cell, the topological states of DNA are modulated by two enzymes known as type I and type II DNA topoisomerase. The type I enzyme (topoisomerase I; topoI) interconverts different topological forms of DNA by creating a transient single-stranded nick in the DNA backbone, passing the unbroken strand of the DNA through the nick, and resealing the original scission (Wang, 1985; Osheroff, 1989). Although topoI is not necessary for the viability of eukaryotic cells (Thrash *et al.*, 1985; Uemura & Yanagida, 1984), it does appear to play important roles in chromatin organization (Uemura & Yanagida, 1984), mitosis (Maul *et al.*, 1986), DNA replication (Snapka, 1986; Yang *et al.*, 1987), recombination (Bullock, Champoux & Botchan, 1985; McCoubrey & Champoux, 1986) and transcription (Brill *et al.*, 1987; Garg, DiAngelo & Jacob, 1987).

Autoantibodies directed against DNA topoI in sera of patients with systemic sclerosis have been described by several investigators (Douvas, Achten & Tan 1979; van Venrooij *et al.*, 1985; Jarzabek-Chorzelska *et al.*, 1986; Shero *et al.*, 1986; Guldner *et al.*, 1986; Shero *et al.*, 1987; Kumar *et al.*, 1988). These autoantibodies are usually detected by the immunodiffusion or by the more sensitive immunoblotting technique (Kumar *et al.*, 1988). Thymus extracts (Kumar *et al.*, 1988) and HeLa cell extracts (Maul *et al.*, 1986) are the most widely used antigen sources in these tests. In the present study we report the expression of a cDNA clone encoding 695 carboxyl-terminal amino acids (=91%) of human topoI. The DNA sequence of this clone is identical with nucleotides 418–3409 of the earlier-described topoI clone T1B (D'Arpa *et al.*, 1988). Our recombinant topoI protein (HTopoA protein) displayed an immunoreactivity with 82 of 85 anti-topoI-positive sera (=96%) indicating the usefulness of the HTopoA protein in routine detection of anti-topoI antibodies in sera from patients with connective tissue diseases.

Furthermore, we were able to assign at least three different autoimmune epitope regions on the HTopoA protein and show that over a period of 5 years the amount of anti-topoI antibodies against these regions may fluctuate.

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MATERIALS AND METHODS

Sera

Most patient sera were obtained from the Department of Rheumatology of the University Hospital St. Radboud at Nijmegen, The Netherlands. Some additional sera were received from hospitals in Enschede, Deventer and Groningen, the Netherlands.

Cells
Culturing of HeLa S3 suspension cells as well as preparation of HeLa total nuclear protein fraction has been described (Habets *et al.*, 1983).

Bacteria and growth media
Escherichia coli strains Y1089 and RR1 were purchased from Promega Biotec (Madison, WI). The strains HMS174 and BL21(DE3) were provided by Dr F. W. Studier (Brookhaven National Laboratory, NY) and grown as described (Studier & Moffatt, 1986; Rosenberg *et al.*, 1987). When growing phage- or plasmid-containing cells, ampicillin (Sigma) was added to the medium at a final concentration of 100 μg/ml.

Screening of a λgt11 expression library with antibody probes
A systemic sclerosis serum (diluted 1/500) with a high titre of anti-topoI antibodies was used to screen a human placental cDNA library (Clontech) constructed with the λgt11 vector as described previously (Habets *et al.*, 1987). To detect specifically bound antibody, ^{125}I-labelled sheep anti-human immunoglobulin F(ab)$_2$ fragment (Amersham) was used.

Expression of topoI as fusion proteins
The clone λHTopoB (see Results) was transferred to the lysogenic host *E. coli* Y1089. The lysogen was induced by temperature shift and addition of 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) allowing maximal fusion protein synthesis (Sillekens *et al.*, 1987).

The cDNA HTopoA (see Results) was inserted into the *Bam*HI site of the pET-3c expression vector (Studier & Moffatt, 1986; Rosenberg *et al.*, 1987) to yield pEHTopoA. *E. coli* HMS174 was used as host strain for initial cloning of the target DNA into the pET vector and for maintaining the plasmids. *E. coli* BL21(DE3) was used as host for expression of pEHTopoA. BL21(DE3) contains a single copy of the gene for T7 RNA polymerase in the chromosome under control of the inducible *lac*UV5 promotor. Transcription of pET-3c is controlled by the strong φ10 promotor for T7 RNA polymerase. Addition of 0·4 mM IPTG to a growing culture of BL21(DE3)/pEHTopoA induces T7 RNA polymerase, which in turn transcribes HTopoA in the pET-3c plasmid.

In this study we also used a topoI cDNA clone which was isolated from a HeLa cell DNA library (Oddou *et al.*, 1988). The isolated clone, referred to as the A-sequence, contains an insert of 2177 bp, corresponding to nucleotides 1241–3425 of the sequence published by D'Arpa *et al.* (1988). The cDNA of this clone contains an open reading frame encoding amino acids 344–589 of topoI (see Results) and was cloned into the pEV-vrf1 expression vector (Crowl *et al.*, 1985). Plasmids carrying the A-sequence were introduced into *E. coli* RR1(pRK248*c*Its) expressing a temperature-sensitive λcI repressor. Upon heat induction from 30°C to 42°C for 3 h, large amounts of a 33-kD polypeptide (the A-fragment) were synthesized. In addition to the 246 amino acids homologous to topoI, the polypeptide contains four amino-terminal and 21 carboxyl-terminal amino acids not related to the topoI sequence.

Gel electrophoresis, protein blotting and detection of antigens
SDS–PAGE and transfer of proteins from 13% polyacrylamide gels onto nitrocellulose sheets was performed as described by Habets *et al.* (1983). For the detection of antigen, the protein blots were treated and processed as described (Verheijen *et al.*, 1986).

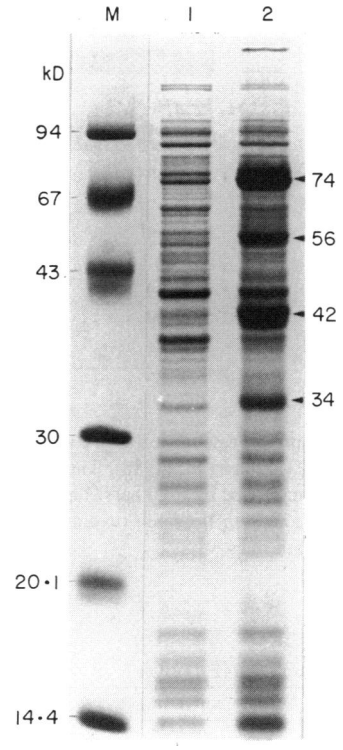

Fig. 1. Expression of pEHTopoA in BL21(DE3). Coomassie brilliant blue stained 13% SDS/polyacrylamide gel of a crude BL21(DE3) lysate containing pET-3c as a control (lane 1) or pEHTopoA (lane 2). In both cases the bacteria were harvested after induction for 3 hours at 37°C with 0·4 mM IPTG. M, mol. wt markers (in kD).

DNA sequence analysis
cDNA fragments were digested with a variety of restriction enzymes. DNA fragments were ligated into the polylinker region of M13 mp18 (Messing, 1983). Sequence analysis of the DNA fragments was performed by the dideoxy chain termination method (Sanger, Nicklen & Coulson, 1977).

RESULTS

Isolation and expression of cDNA clones
Serum from a patient with systemic sclerosis was used to screen a λgt11 cDNA expression library of human placenta for clones encoding topoI using standard methods previously described by Habets *et al.* (1987). One putative topoI clone with an insert of 1·2 kbp, referred to as λHTopoB, was identified.

A number of longer cDNAs were obtained by rescreening the cDNA library with λHTopoB as hybridization probe. One of these clones, referred to as λHTopoA, contained an insert of 3·0 kbp. The isolated insert of λHTopoA was recloned into the *Bam*HI-site of the pET-3c plasmid vector (see Materials and Methods) to yield pEHTopoA.

Lysogens containing phage λHTopoB produced a β-galactosidase fusion protein (HTopoB protein) with an apparent mol. wt of about 125 kD.

Induced BL21(DE3) harboring pEHTopoA produced a polypeptide (HTopoA protein) with an apparent mol. wt of 74

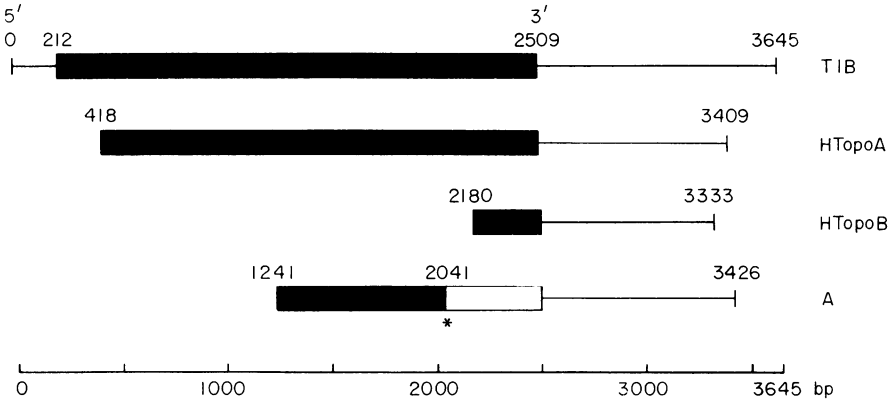

Fig. 2. Schematic representation of the cDNA clones HTopoA and HTopoB and comparison with the full-length cDNA clone T1B published by D'Arpa *et al.* (1988). The relative position of the cDNA A-sequence is indicated as well. Regions coding for topoI sequences are indicated by solid bars. The open box in the A-sequence represents the out of frame part of the coding sequence of topoI. The premature stop in the open reading frame in the A-sequence is indicated by an asterisk. The 5′ and 3′ noncoding regions are indicated by thin lines.

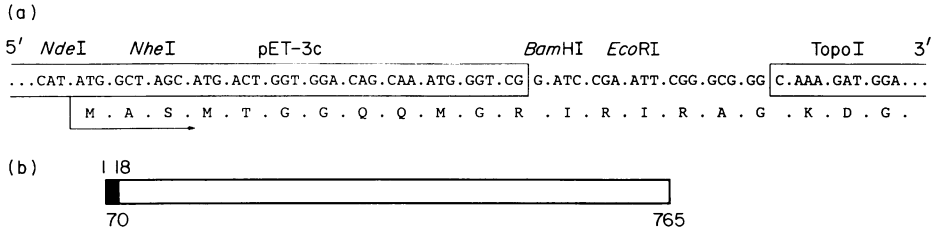

Fig. 3. (a) Nucleotide and amino acid sequences at the protein start point of cloned HTopoA in the pET-3c translation vector (pEHTopoA). The boxed nucleotide sequences at the 5′- and 3′-sites indicate the sequences belonging to the pET-3c vector and the topoI cDNA clone region, respectively. The first 11 amino acids of the fusion protein are derived from the gene 10 protein. The intervening sequence originated from linkers. Locations of the *Nde*I, *Nhe*I, *Bam*HI and *Eco*RI sites are shown. (b) Schematic representation of the HTopoA protein. The fusion part consists of 18 amino acids followed by amino acids 70–765 of the native topoI protein.

kD (Fig. 1). Next to a large amount of this protein the induced pEHTopoA lysates contained three additional protein products of 56, 42, and 34 kD, respectively, that could not be detected in lysates of the induced wild type. We assume that these proteins are proteolytic degradation fragments of the 74-kD HTopoA protein.

Identification of the cDNA clones

DNA sequencing of λHTopoA and λHTopoB established that we had isolated clones encoding part of topoI. The DNA sequences of HTopoA and HTopoB were completely identical with the corresponding parts in the full-length clone T1B published by D'Arpa *et al.* (1988). The relative position of the HTopoI sequences to T1B are shown in Fig. 2. The clone HTopoB codes for 109 amino acids located at the carboxy-terminal end of topoI. HTopoA contains in its 5′-section an open reading frame of 2091 bp flanked by 900 non-coding nucleotides at the 3′-end. The HTopoA protein contains amino acids 70–765 of the topoI protein fused to only 18 amino acids encoded by the vector and linker sequences (Fig. 3). The calculated mol. wt of this protein is 84·2 kD, comprised of a 1·9 kD peptide encoded by the vector linked to 82·3 kD of the topoI protein.

Reactivity of autoimmune sera with the HTopoA protein

Using the immunoblotting technique with a HeLa S3 total nuclear protein extract as antigen source (Habets *et al.*, 1983), sera from patients with several connective tissue diseases were screened for the presence of anti-topoI antibodies (de Rooij *et al.*, 1988). Fig. 4a shows an example of such an analysis. In this way we selected 85 anti-topoI sera which were subsequently probed on immunoblots containing the HTopoA protein. A typical example of the staining pattern of these sera on such immunoblots is shown in Fig. 4b, lane 2. A positive immuno-reaction with the 74-kD protein was obtained with 82 of the 85 sera (=96%). All these sera not only recognized the 74 kD protein but also the putative degradation products of 56, 42, and 34 kD (see Fig. 1, lane 2). By comparison, only 85% of the sera that were positive on the immunoblot reacted positively in the Scl-70 immunodiffusion test (Kurata & Tan, 1976). Also the three sera that were anti-topoI-positive on a HeLa immunoblot but did not react with the recombinant antigen were negative in this immunodiffusion test.

Reactivity of systemic sclerosis sera with the HTopoA protein

From 40 patients in the group of 85 studied we could obtain a definite diagnosis systemic sclerosis according to the ARA

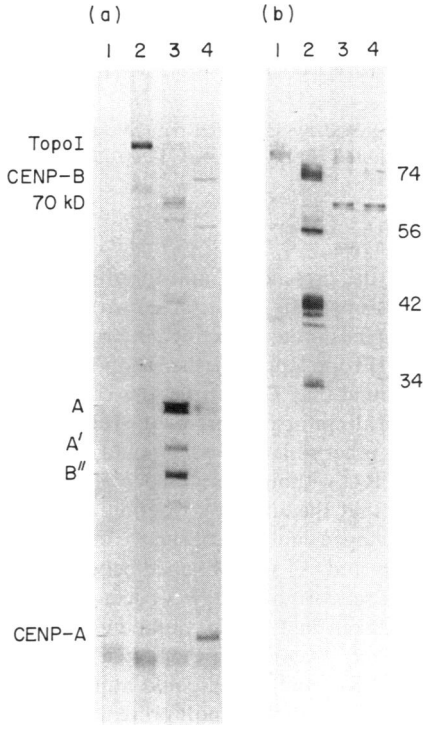

Fig. 4. (a) Characterization of human autoimmune sera. Western blots containing HeLa total nuclear proteins were probed with a normal human serum (lane 1), systemic sclerosis serum Z28 showing the topoI band at around 100 kD (lane 2), anti-U1, U2 RNP serum V26 showing the RNP proteins 70K, A, A′ and B″ (lane 3), or CREST serum B39 decorating the CENP-A band (19 kD) and the CENP-B band (80 kD) (lane 4).
(b) Analysis of human autoimmune sera on immunoblots containing total proteins of induced BL21(DE3)/pEHTopoA. Lane 1, normal human serum, lane 2, systemic sclerosis serum Z28; lane 3, anti-U1, U2 RNP serum V26; lane 4, CREST serum B39. The antibody–antigen complexes were detected with horseradish peroxidase-conjugated second antibodies.

criteria (Masi *et al.*, 1980). All these sera showed a strong immunoreactivity with the HTopoA protein. An analogous control experiment was performed with 23 sera from patients with a definite diagnosis of systemic sclerosis that were negative for anti-topoI antibodies on a HeLa immunoblot. None of these 23 sera did react with HTopoA protein or its degradation products. These results indicate that both types of immunoblots display a comparable sensitivity for detecting anti-topoI autoantibodies in patient sera.

Table 1. Immunoreactivity of anti-topoI-positive sera with the HTopoA protein, the HTopoB protein or the A-fragment

Immunoreactivity of anti-topoI sera with:			
HTopoA protein	HTopoB protein	A-fragment	*n* (sera)
+	+	+	38
+	−	+	7
+	−	−	8
+	+	−	0

Serum F14 is placed in the group of sera that gave a positive immunoreaction with all three proteins.

Autoimmune epitope distribution

To obtain more information about the autoimmune epitope distribution on topoI, immunoblots containing either the HTopoA protein, the HTopoB protein or the A-fragment were probed with anti-topoI positive sera. All proteins were expressed as described in Materials and Methods. As shown in Table 1 three reaction patterns were found, indicating that the various sera recognized different epitopes. Seventy-two percent of the sera were found to be immunoreactive with all three proteins, 15% reacted with both the HTopoA protein and the A-fragment but contained no detectable level of antibody directed against the protein encoded by HTopoB, whereas another 13% of the sera reacted only with the HTopoA protein. This finding indicates that topoI contains at least three different autoimmune epitope regions, which are distributed over the entire protein (Fig. 5). One epitope region (ER) has to be situated on the A-fragment (ER-2), formed by amino acids 344–589, whereas a second region has to be located on the HTopoB protein which contains amino acids 657–765 (ER-3). As the HTopoA protein bears also one or more epitopes which are not present on the HTopoB protein or the A-fragment, amino acids 70–344 and/or amino acids 589–657 are necessary to form a third ER (ER-1). In none of the sera used in this study could antibodies be detected merely directed against ER-3 without the simultaneous presence of autoantibodies directed against ER-1 and/or ER-2.

We also performed a preliminary study on the epitope distribution of anti-topoI positive sera from systemic sclerosis patients which had been followed longitudinally for about 4 to 8 years. In most of these sera the antibody pattern appeared not to have changed in that period. In some patients, however, the titre of antibody against the various epitopes was found to have been changed significantly during the course of the disease. This was observed, for example, in the serum samples of patient F14 (Fig. 6) who came to the hospital in 1984 as a severe case of Raynaud's disease. The serum of 1984 of this patient showed a very weak immunoreaction with both the HTopoA protein and the A-fragment. From 1985 up to 1988 this reaction became stronger and clearly positive. In contrast, a significant level of antibody directed against the HTopoB protein (ER-3) was not seen until 1987. A three-fold increase of the HTopoB protein concentration on the immunoblots to enhance the sensitivity of the reaction gave identical results.

DISCUSSION

Using autoantibodies from a patient with systemic sclerosis we isolated a human cDNA clone (HTopoB) encoding DNA topoI from a λgt11 expression library. Screening of the same library with this clone revealed additional recombinants with a longer cDNA insert, the longest being HTopoA. The cDNA HTopoA covered 2091 nucleotides (695 amino acids) of the coding sequence of topoI which consists of 2297 nucleotides (765 amino acids) (D'Arpa *et al.*, 1988). This cDNA was expressed in the pET-3c expression vector giving rise to a protein (HTopoA protein) of 84·2 kD. On SDS–PAGE the HTopoA protein migrated as a polypeptide of approximately 74 kD. A similar

Fig. 5. Map of the different epitope regions (ER) on the HTopoA protein as recognized by sera from patients with anti-topoI autoantibodies. The numbering of amino acids (70–765) is derived from the full-length clone T1B (D'Arpa *et al.*, 1988). □, ER-1; ■, ER-3; ▩, ER-2.

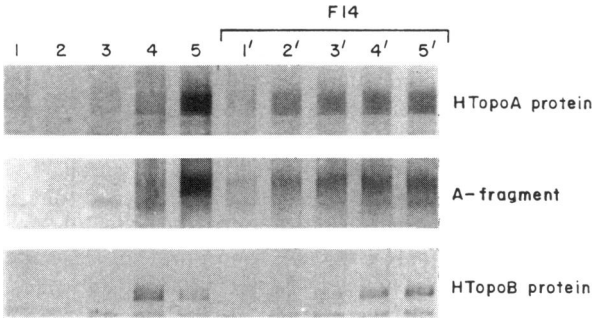

Fig. 6. Characterization of several samples of the SSc serum of patient F14 on immunoblots containing the HTopoA protein (74 kD), the HTopoB protein (125 kD) or the A-fragment (33 kD). Samples were collected in 1984 (lanes 1′), 1985 (lanes 2′), 1986 (lanes 3′), 1987 (lanes 4′) and 1988 (lanes 5′). Control sera used were: a normal human serum (lanes 1), anti-Sm serum C45 (lanes 2), CREST serum B39 (lanes 3) and SSc sera Z28 (lanes 4) and R25 (lanes 5). The antibody-antigen complexes were detected with horseradish peroxidase-conjugated second antibodies.

aberrant behaviour of a topoI recombinant protein on an SDS polyacrylamide gel has been described by D'Arpa *et al.* (1988). Next to the 74-kD protein, lysates of bacteria expressing HTopoA contained several smaller prominent polypeptides (Fig. 1). Their presence can be explained either by assuming partial proteolytic cleavage of the 74-kD protein or by the use of alternative ATG initiation codons.

The HTopoA protein was recognized by 96% of our anti-topoI-positive sera. Three sera, scored as weakly positive with topoI on a HeLa immunoblot, did not react with the HTopoA protein. This discrepancy can be explained in several ways. One explanation is that these sera had been scored as false-positive for topoI because of an immunoreaction with another protein migrating in the same mol. wt region as HeLa topoI. As these sera were also negative in the Scl-70 immunodiffusion test, this possibility seems the most likely one. Another possible explanation is that the sera do react with topoI but with an epitope present on amino acids 1–70, i.e. that part of topoI that is missing in the HTopoA protein. Nevertheless, the finding that the HTopoA protein is recognized by at least 96% of our anti-topoI sera makes this recombinant protein a good substitute for native eukaryotic topoI in the screening for anti-topoI antibodies of sera from patients with connective tissue diseases. Since large amounts of purified HTopoA protein can be obtained in a relative simple and easy way this antigen might be very useful in future diagnostic tests. Furthermore, our results confirm an earlier report that about 30% of systemic sclerosis patients do not seem to contain detectable levels of anti-topoI antibodies (Jarzabek-Chorzelska *et al.*, 1986). Even the use of a very sensitive ELISA test with the recombinant topoI as antigen failed to show the presence of anti-topoI antibodies in these systemic sclerosis patients (data not shown).

In analysing the immunoreaction patterns of anti-topoI sera with either the HTopoA protein, the HTopoB protein or the A-fragment we found that 72% of the sera contained antibodies directed against all epitope regions on the HTopoA protein. The fact that in the other sera antibodies could be detected against ER-1 and/or ER-2 without the presence of antibodies against ER-3 suggests that the antibody reaction against the various ERs is not developed simultaneously.

A follow-up study of sera obtained between 1984 and 1988 from a systemic sclerosis patient (F14) demonstrated that in the serum sample taken in 1984 no antibodies could be detected against ER-3. Only 3 years after the first detection of autoantibodies against ER-1 and ER-2, could autoantibodies against ER-3 be dectected. As the HTopoB protein is a β-galactosidase fusion protein we considered the possibility that these results were due to a development of antibodies against β-galactosidase. However, all serum samples were negative for the presence of such antibodies (data not shown). One of the possible explanations for our finding is that the autoimmune epitope on ER-3 was developed in a later phase of the disease as compared with the other autoimmune epitopes on topoI. However, this conclusion should be interpreted with great care, as in these experiments one is comparing the immunoreactivity of different types of antibodies on different antigens.

Recently, Eng, Pandit & Sternglanz (1989) and Lynn *et al.* (1989) have mapped the tyrosine residue in yeast topoI that is responsible for the formation of the covalent enzyme–DNA intermediate. On basis of alignment of topoI sequences of human and yeast, these investigators proposed Tyr-723 in human topoI to be the equivalent active site tyrosine. This means that only autoantibodies against the HTopoB protein (ER-3) may interact with or near the active centre and possibly inhibit topoI activity. Further studies are now in progress to map the epitope regions in more detail in order to select those autoimmune sera that recognize the active site of topoI. Such knowledge may contribute to a better insight into the progression of systemic sclerosis.

NOTE IN PROOF

Shortly after this paper was accepted for publication, another description B cell epitope on DNA topoisomerase I appeared (Maul, G.G., Jimenez, S.A., Riggs, E. & Ziemnicka-Kotula, D. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 8492). This autoepitope is contained in the region defined by us as ER-3 (see Fig. 5).

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