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A topoisomerase II-like protein is part of an inducible DNA-binding protein complex that binds 5′ of an immunoglobulin promoter

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ABSTRACT

We previously identified a B cell-specific protein-DNA complex 5′ of an immunoglobulin μ heavy chain promoter. The sequences to which this protein complex bound were required for induction of immunoglobulin mRNA levels with interleukin-5 + antigen. Further studies identified a second sequence 5′ of these regulatory sequences that bound to both the nuclear matrix and to a similar interleukin-5 + antigen inducible protein complex. Therefore, we sought to identify the putative regulatory proteins that comprised this DNA-binding complex. In this study, we have used anti-topoisomerase II antibodies to demonstrate that one of the proteins found in the interleukin-5 + antigen inducible complexes is serologically related to topoisomerase II. To our knowledge, this is the first report where a topoisomerase II related protein participates in an inducible mobility shifted protein-DNA complex. These data suggest a model in which the enhanced immunoglobulin gene transcription observed after treatment with interleukin-5 + antigen might be explained by the induction of a protein complex that acts to relieve torsional stress along the gene.

INTRODUCTION

The nuclear matrix has been defined as the nuclear subfraction that forms a proteinaceous scaffold for chromosomes after histone depletion (1). Matrix associated regions (MARs) have been functionally defined as the A+T rich DNA sequences that are preferentially retained by the chromosomal scaffold, and that bind to nuclear matrix proteins. These sequences are usually 200–300 base pairs (bp) long, typically contain topoisomerase II cleavage sites, and occur on the average of every 30 kilobases in eukaryotic DNA (1–4). MARs have also been shown to occur near enhancer and promoter regions (5–8), and may even play an important role in transcriptional activation (9).

Topoisomerase II is one of the major protein components of the nuclear matrix (10), and is thought to play an important role in replication, recombination, and transcriptional processes (11). It acts to relieve torsional stress in DNA molecules by breaking both strands and relaxing supercoils in an ATP-dependent process. Two distinct isoforms of topoisomerase II (α and β) have been identified in humans and in mice (12,13). The relative amounts of each isoform present in cells appear to be differentially regulated (14,15), and their intracellular localization may differ (16). It is not known whether these two forms are preferentially used in different functions.

Our previous studies showed that interleukin-5 + antigen (IL-5 + Ag) increased μ immunoglobulin heavy chain mRNA 3- to 6-fold in an antigen-specific cell line (17). The increase in mRNA correlated with an increased abundance of a DNA-binding protein complex that bound to sequences required for induction of μ mRNA levels (18). The sequences to which the induced protein complex bound occurred 200 bp 5′ of the transcription start site and included a 45 bp A+T rich protein binding site (18). Deletion analyses showed that these sequences were necessary for induction of μ mRNA in response to IL-5 + Ag. A second protein binding site also bound to an IL-5 + Ag inducible protein complex and occurred approximately 500 bp 5′ of the transcription start site. This region was >74% A+T rich, could function as a nuclear MAR (5), and UV cross-linking revealed that it bound to some of the same proteins bound by the more 3′ transcription-associated region (18).

The present study was undertaken to begin to identify the protein components of the IL-5 + Ag inducible DNA-binding protein complexes that bind 5′ of the μ promoter. Because both protein-binding sequences were A+T rich and apparently bound some of the same proteins (18), we asked whether any previously identified nuclear matrix proteins might be components of our inducible mobility shifted complexes. Topoisomerase II seemed a likely candidate due to its known association with MARs and its possible role in transcription. Furthermore, one of the proteins that bound to these two DNA fragments by UV cross-linking exhibited an apparent molecular weight of nearly 200 kD,

consistent with the published size of murine topoisomerase II (14,19). The current data show that a topoisomerase II-like protein is a component of both of the B cell-specific, IL-5 + Ag inducible protein complexes that bind 5′ of the basal V_H S107 immunoglobulin promoter.

MATERIALS AND METHODS

Cell lines and antibodies

The B cell transfectant, BCg3R-1d, has been described in detail elsewhere (17). All cell lines were maintained in RPMI 1640 with 7.5% fetal bovine serum and were induced as previously described (17). Topoisomerase II antibodies were prepared by immunizing rabbits with Keyhole limpet hemocyanin-conjugated synthetic peptides from the predicted sequences of topoisomerase II α and β (13). Antibody FHD29 was directed against a highly conserved sequence common to both subtypes (SNYDDDEKKVTGGRN; corresponding to amino acids 148−162 of topoisomerase II α). Antibody FHD22 was directed at a unique sequence from topoisomerase II α (DNMGRAGEMELKPFN; amino acids 195−209), and antibody 37 was directed against the corresponding region of topoisomerase II β (NNMMKTSEAKIKHFD). Antibodies FHD20 and 21 were from two rabbits immunized with the same topoisomerase II β peptide (EIFVVDRNTVETT; amino acids 928−942), and have been previously described (13). Antibody FHD9 was generated by immunization of a rabbit with topoisomerase II α purified from P388 murine leukemia cells (12). Where indicated, antibodies were affinity purified on the immobilized peptides as previously described (13). Rabbit anti-serum against 5′-nucleotidase, affinity purified rabbit anti-mouse IgG, and affinity purified rabbit anti-p18 were generously provided by Drs L.Thompson and P.Kincade (Oklahoma Medical Research Foundation, OK).

Nuclear extracts and proteins

Nuclear extracts were prepared by hypotonic lysis as previously described (20). Protein concentrations were measured using the Bradford assay according to the manufacturer's suggestions (Bio-Rad Laboratories, Richmond, CA). Proteins were stored at −70°C in 20 mM HEPES, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 500 μM PMSF, and 1 μg/ml leupeptin. Purified topoisomerase II of both α and β isoforms was prepared exactly as described (14).

Mobility shift assays and cleavage reactions

Mobility shift assays were performed by incubating 5 μg of total nuclear protein with γ-^{32}P-end-labeled DNA at 37°C for 15 minutes as described (21). In some instances, antibodies were also added to the reaction mixture and were allowed to preincubate for 5 minutes at 37°C. Samples were electrophoresed in 4% polyacrylamide gels, dried, and autoradiographed. The DNA fragments used were a 150 bp *BamHI-FokI* fragment (bf150) spanning the sequence from −574 to −425 relative to the S107 variable region transcription start site, a 125 bp fragment spanning bases −251 to −124 (TX125) relative to the transcription start site (18), and a 160 bp *Nco I-MboII* fragment containing the octamer binding site (21).

Western blotting

Purified topoisomerase II α and β isoforms were subjected to 7% SDS/PAGE and were transferred to Immobilon membranes as previously described (12). Immunoblotting was performed with the designated anti-sera and goat anti-rabbit IgG alkaline phosphatase as described (12).

RESULTS

IL-5 and antigen induce B cell-specific protein-DNA complexes

We previously identified two sequences 5′ of the S107 V_H region promoter that bound to protein complexes by mobility shift assay and that were induced in response to IL-5 and antigen and to some degree by lipopolysaccharide (LPS) (18). Earlier studies implied that some of the proteins that bound to these sequences were similar. In this study we wished to determine whether topoisomerase II was a component of these protein complexes. Previously, we demonstrated that the protein complexes we observed after induction with IL-5 + antigen were not found in the T cell line AKR117 or in fibroblasts. Because the purified topoisomerase II used in these studies was produced in the monocyte line P388D1, we extended our earlier studies to determine whether the inducible complexes might be found in P388D1 or other murine non-B cell lines. Figure 1 shows the inducible gel-retarded complexes bound to both the TX125 fragment (Figure 1a) whose presence correlates with increased mRNA levels after induction, and the bf150 fragment (Figure 1b) that acts as a MAR (5). An asterisk identifies the inducible B cell-specific protein complexes bound to each fragment. Several other DNA-protein species can be detected in the B cell extracts and in extracts from other cell types, but only the complex that migrated with the slowest mobility in the B cell extracts was inducible (18).

Neither the T cell line EL-4, or the monocyte line P388D1 contained protein complexes with the same mobility as the inducible B cell complex. However, they both contained nuclear

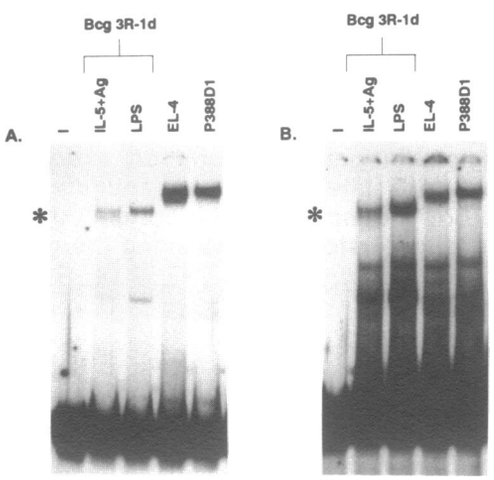

Figure 1. Nuclear extracts from induced B cells contain B cell-specific protein complexes. **(A)** The TX125 fragment containing DNA from -251 to −125 5′ of the S107 V_H region promoter was analyzed by mobility shift assay with induced extracts from the BCg3R-1d transfected cell line, or with extracts from the T cell line (EL-4) and monocyte cell line (P388D1). Lane 1 contains labeled fragment without extract. An asterisk indicates the inducible protein complex. **(B)** Mobility shift assays were performed with the bf150 fragment containing DNA from −575 to −425 5′ of the S107 V_H region gene. The extracts used were exactly as in (A). An asterisk indicates the inducible complex.

proteins that bound to both bf150 and TX125 and migrated even more slowly (Figure 1). To date, we have not identified any cell types other than B cells that contain protein complexes with mobilities similar to the inducible B cell complex. Stromal cells, fibroblasts, heart, and several other T cell and monocyte lines did not contain this complex, while at least five other B cell lines did contain proteins of this mobility (data not shown).

Reactivity with anti-topoisomerase II antibodies

To determine whether topoisomerase II might be a component of the induced mobility shifted complexes, we asked whether anti-topoisomerase II antibodies could inhibit binding of the induced proteins to DNA. Figure 2 shows the topoisomerase II isoform specificity of four of the antibodies. Affinity purified antibody 29 reacted with both isoforms while antibodies 20 and 21 (not shown) reacted only with the β isoform. Anti-sera 9 reacted primarily with the α isoform, and affinity purified antibody 22 was specific for the α isoform. Antibody 37 did not react by Western blotting. Western blotting of both induced and uninduced extracts from BCg3R-1d cells using antibodies specific for either the α or β isoform indicated that immunoreactive species representative of both isoforms were present at approximately the same levels both before and after induction with IL-5 + Ag (data not shown).

Antibody FHD29 was used first in these studies because it was raised against a highly conserved peptide common to both the α and β isoforms of topoisomerase II and reacted equally well with both forms. Figure 3a, lane 2, shows the IL-5 + Ag inducible protein complex (asterisk), the constitutive mobility shifted complex, and an intermediate complex with a mobility between the inducible and constitutive forms, bound to the bf150 fragment. Previous experiments indicated that this intermediate complex is probably a degradation product of the inducible complex (18). While the addition of high concentrations of FHD29 to extracts prior to analysis by mobility shift assay actually abrogated binding of the inducible proteins and the intermediate complex to the DNA, (Figure 3a, lane 3), lower concentrations of antibody (lanes 4–7) produced a super-shifted band consistent with antibody binding directly to the DNA binding proteins. None of the unrelated affinity purified polyclonal rabbit antibodies shown in lanes 8–13 exhibited any inhibition of binding in this assay.

Similarly, addition of anti-topoisomerase II antibodies to the TX125 transcription-associated fragment and induced extracts (Figure 3b, lane 4) produced super-shifted complexes suggesting that a topoisomerase II-like protein was a component of this complex as well. Preimmune serum (lane 3) did not effect binding of this mobility shifted complex or the complex observed with bf150 (data not shown). Thus, a protein serologically related to topoisomerase II is a component of the IL-5 + Ag inducible B cell-specific complex that binds to the bf150 and TX125 fragments.

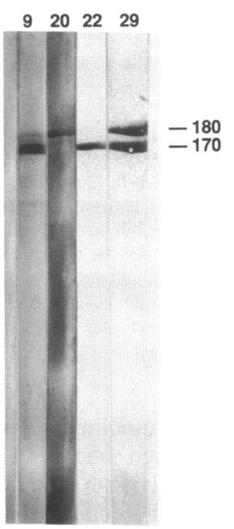

Figure 2. Reactivity of anti-topoisomerase II antisera with α and β isoforms. Western blot analyses of purified topoisomerase α (170 kDa) and β (180 kDa) isoforms were performed with antisera generated against purified α isoform (lane 9), peptides specific for the 180 and 170 isoforms (lanes 20 and 22, respectively), and a peptide common to both α and β (lane 29).

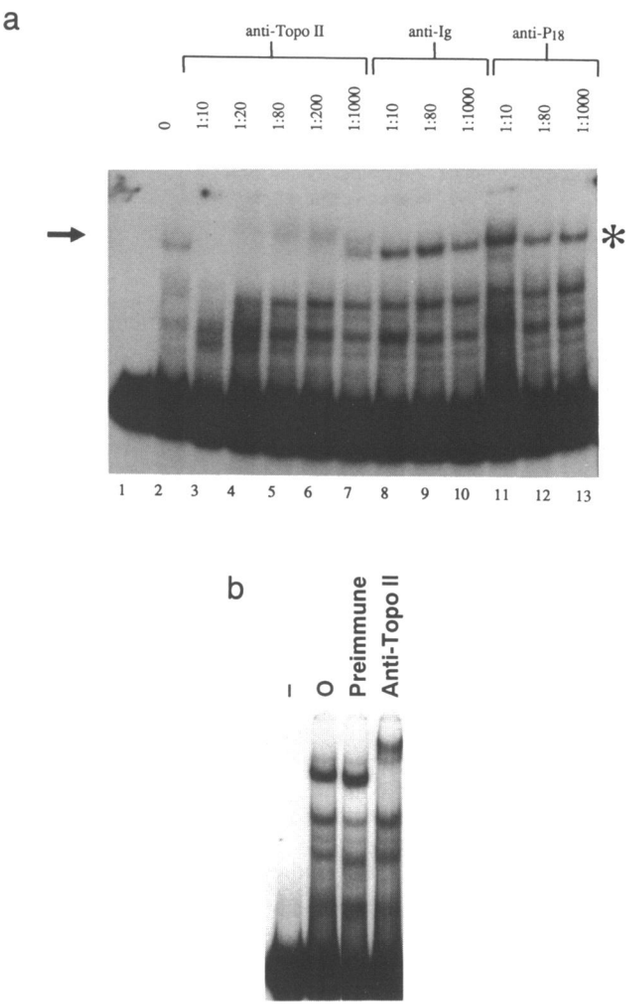

Figure 3. Anti-topoisomerase II antibodies inhibit binding of IL-5 + Ag inducible complexes. **(a)** Mobility shift assays were performed using bf150 and IL-5 + antigen induced extracts. The asterisk marks the inducible mobility shifted band. Lane 1 contains only fragment DNA, lane two contains no antibody, lanes 3–7 contain dilutions of the anti-topoisomerase II antibody FHD29, and lanes 8–13 contain dilutions of two unrelated antibodies. The arrow shows the super-shifted bands in lanes 5 and 6. **(b)** Mobility shift assays were performed as in (a) using the TX125 fragment and IL-5 + antigen induced extracts. A super-shifted band representing affinity purified antibody FHD29 bound to the inducible complex is visible in lane 4. Preimmune serum did not affect binding of this complex.

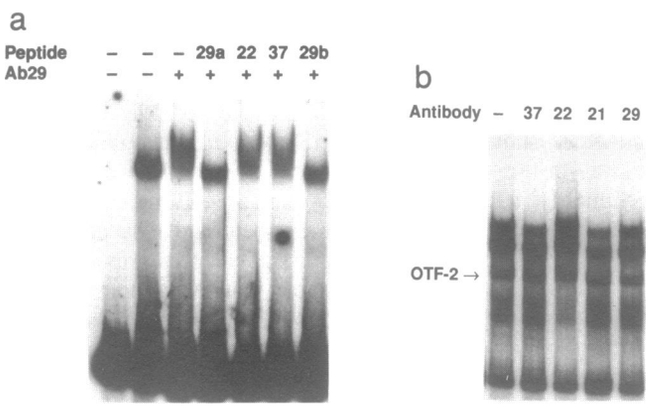

Figure 4. Anti-topoisomerase antibodies interact specifically with proteins in the IL-5 + Ag inducible complex. **(a)** Mobility shift assays were performed with TX125 and IL-5 + Ag induced extract in the presence of antibody FHD29 and 10 ng of peptides 29, 37, and 22. Addition of peptides 37 and 22 did not effect the supershifted complexes, while both 10 ng (29a) and 1 ng (29b) of peptide 29b abolished the supershifted species. **(b)** Mobility shift assays were performed using an octamer-containing DNA fragment and IL-5 + antigen induced extracts. The previously identified B cell DNA-binding protein OTF-2 is labeled. The first lane contains extract alone. The remaining lanes contain extract and anti-topoisomerase II antibodies. Antibodies 37 and 29 were used at a final dilution of 1:200, antibody 22 was used at 1:20 and antibody 21 was used at 1:1000.

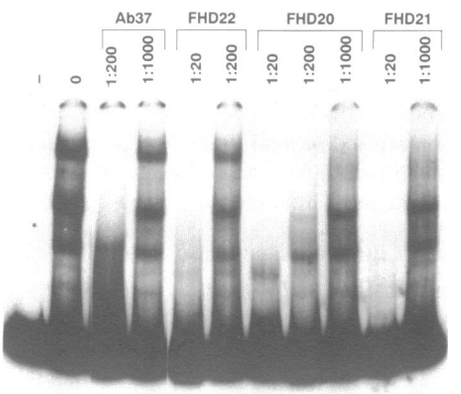

Figure 5. The mobility shifted complex reacts with isoform-specific antibodies. Mobility shift assays were performed using bf150 and Il-5 + antigen induced extracts and four antibodies shown to be isoform specific by Western analyses. Lane one contains fragment without extract. Lane 2 contains IL-5 + antigen induced extract without antibody. Ab 37, FHD20, and FHD21 were unpurified antisera raised against the β isoform. FHD22 was highly concentrated affinity purified, α isoform-specific antibody, raised against the corresponding peptide used to prepare the β isoform-specific Ab 37.

Specificity of antibody interaction

Addition of the purified peptide 29, used for generating antibody FHD29, to samples containing the supershifted complexes shown in Figure 3b completely eliminated the ability of the antibodies to bind to the protein complex (Figure 4a). Peptides used to prepare antibodies FHD22 and 37 did not inhibit binding of antibody FHD29 to the inducible complex.

Furthermore the anti-topoisomerase II antibodies used to produce the super-shifted complexes did not react with all protein-DNA complexes in a non-specific manner. Figure 4b (lane 5) shows that mobility shifted complexes containing octamer proteins were not affected by the concentrations of FHD29 that produced the super-shifted bands of the IL-5 + Ag inducible complexes in Figure 2. Likewise, none of the isoform specific Abs inhibited binding of octamer proteins (lanes 2–4) at concentrations that effected binding of the IL-5 + Ag inducible complex in Figure 5. We have used octamer-containing DNA fragments as negative controls for binding of our inducible protein complexes in several other instances (18).

Effect of isoform-specific antibodies

To confirm the presence of topoisomerase II in the complex and to examine which isoform of topoisomerase might be involved, we tested the effects of several other antibodies on the mobility shifted complex (Figure 5). Antisera (Ab 37), directed against a peptide of the β isoform inhibited binding of the inducible complex at a 1:200 dilution. Affinity purified antibodies produced against the homologous peptide sequence of the α isoform (FHD22) were only effective in inhibiting binding at much higher concentrations. A second antisera recognizing primarily the α isoform (FHD9) had no effect upon the inducible concentration even at a 1:10 final dilution (data not shown). However, two different polyclonal sera (FHD20 and FHD21) directed against one peptide from the β isoform inhibited binding of the induced complex to the bf150 fragment even at a 1:1000 dilution

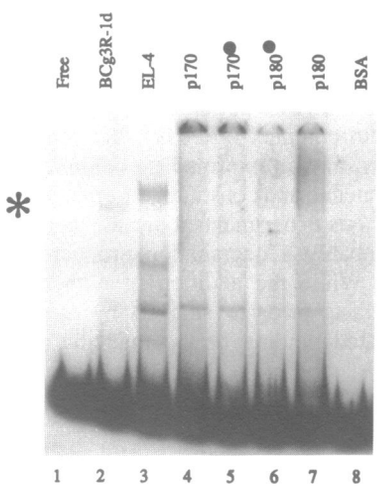

Figure 6. Purified topoisomerase II does not reconstitute the mobility shifted inducible complex. Mobility shift assays were performed with purified α (p170) and β (p180) isoforms of topoisomerase II. Lane 1 contains bf150 fragment without extract. Lanes 2 and 3 contain IL-5 + antigen induced extract from the BCg3R-1d and EL-4 extract, respectively. Topoisomerase II protein was added mixed with bovine serum albumin (BSA) such that the total protein concentration identical to that used for the crude extracts (5 μg/lane). Five μg of BSA alone was added in lane 8. The closed circles indicate lanes that contained 1 ng pdIdC as a nonspecific inhibitor. The asterisk indicates mobility of the inducible complex.

(Figure 5). An additional mobility-shifted species was also observed in the presence of high concentrations of these antibodies and it migrated more quickly than either of the other species. These data suggest that the inducible complex contains a topoisomerase II related protein.

Purified topoisomerase II does not reconstitute the IL-5 + antigen inducible mobility shifted band

To determine whether either isoform could reproduce the mobility shifted complex that we observed, we performed mobility shift

assays with purified proteins. Neither the α or β isoform reconstituted a protein complex similar to the B cell-specific inducible complex. In Figure 6, a mobility shift assay was used to compare an IL-5 + antigen induced B cell extract, an EL-4 extract, and purified α (p170) and β (p180) isoforms of topoisomerase II. Purified protein was added with or without poly dIdC DNA as a nonspecific competitor. Total protein concentrations were adjusted with bovine serum albumin (BSA) so that 5 μg of purified proteins or extract was added to each lane. Although both the α and β isoforms gave rise to a mobility shifted band approximately the size of the uninduced ubiquitous complex, protein-DNA complexes of a mobility similar to the IL-5 + antigen inducible complex could not be reproduced under any conditions tested. Nor could we reproduce this band by the addition of the α and β isoforms together as might be expected to occur if they formed a heterodimer (data not shown). Addition of purified topoisomerase II α and β to uninduced extracts, or to extracts from BCg3R-1d cells treated with IL-5 or Ag alone was also unsuccessful in producing an increase in the inducible mobility shifted complex (data not shown). Therefore, we conclude that the inducible complex does not appear to result from binding of either of the α or β isoforms alone.

DISCUSSION

Treatment of the B cell transfectant, BCg3R-1d, with IL-5 + Ag caused increased levels of two DNA-binding protein complexes that may effect immunoglobulin gene transcription. In this study, we showed that five of six polyclonal anti-sera against the known topoisomerase II isoforms effected binding or mobility of these protein complexes in mobility shift assays. These antisera were generated against peptides from both the amino and carboxyl portions of topoisomerase II, including amino acids 148−162, 195−209 and 928−942 (13). One of the affinity purified antisera raised against a peptide of topoisomerase II conserved in both isoforms produced antibody-bound, super-shifted forms of the inducible complex (Figure 3). While high levels of some of these antibodies also affected binding of the constitutive complex (Fig. 5), we were not able to consistently detect super-shifted forms of this species. Our earlier experiments suggested that these complexes may share protein components (18). These data strongly suggest that one of the protein components of the IL-5 + Ag inducible complexes is a topoisomerase II. While topoisomerase II has been purified from several species and shown to interact specifically with distinct DNA sequences, to our knowledge, this is the first report where a topoisomerase II related protein participated in an inducible, cell type-specific mobility shifted complex.

Topoisomerase II subtype

Whether the topoisomerase II-like protein induced by IL-5 + Ag in our B cell transfectant is a known isoform of topoisomerase II or a previously unidentified form is unknown at this time. The antisera that were specific for the β isoform were always effective at higher dilutions than even affinity purified antibodies specific for the homologous peptide of the α isoform. We cannot formally rule out the possibility that the antisera differ in concentration or affinity despite the fact that they seem to work equally well at the same dilution in Western blots.

Some explanation is necessary to account for the fact that topoisomerase II is present in all cell types including our B cell line, yet our inducible, serologically reactive complex migrates at a mobility that is only found in B cells. Attempts to reconstitute a gel retarded species of the same mobility with either of the known murine isoforms of topoisomerase II were unsuccessful. Several possible explanations exist for these data. First, the B cell-specific mobility shifted band may be the result of a unique isoform of topoisomerase II. Although only two isoforms have been identified to date, purification schemes often yield lower molecular weight species (19,22,23). None of these protocols have used induced cells or B cells as a starting material, so new isoforms or alternatively spliced forms could exist in these cells.

Alternatively, the binding of at least one other protein besides topoisomerase II may be required to reproduce the mobility shifted complex. In fact, inhibition of topoisomerase II binding in some cases (Fig. 5), led to the appearance of a new mobility-shifted species that may represent binding of such a protein. In several instances, other proteins have copurified with topoisomerase II in such a way that a close association of the proteins has been suggested (19,24). Likewise, footprinting with *Drosophila* topoisomerase only showed a protected region of 25 bp (25), whereas the protected region spanned by our protein complex covers some 46 bp (18).

Finally, the activation state of the topoisomerase may be crucial for its ability to bind to DNA in a mobility shifted complex. Phosphorylation state and dimerization are both thought to be important for the activity of topoisomerase II (19,26). In fact, one of the proteins that is thought to be tightly associated with both *Drosophila* and murine L cell-derived topoisomerase II is a protein kinase (19,24). Activation of B cells with antigen and mitogenic stimuli is known to stimulate protein kinase activity (27,28), and may play an important role in the induced binding of our mobility shifted complex. Nothing is known about the phosphorylation state of the serologically reactive proteins in the mobility shifted complexes or of the purified α and β isoforms used for the reconstitution experiments in Figure 6. Further purification will be required to resolve these issues.

Implications for immunoglobulin transcription

Earlier studies showed that an increased abundance of the IL-5 + antigen inducible complexes correlated with increased levels of μ mRNA (18). If a topoisomerase II-like protein is part of the inducible protein complexes, one might suggest a possible mechanism for the increased μ mRNA levels in which the inducible IL-5 + Ag topoisomerase II complex may facilitate transcription processes by relieving torsional stress in the DNA. Other studies have shown that the topoisomerase II inhibitor VP-16 inhibits transcription of the murine homeobox gene *Hox-2.1* suggesting that topoisomerase II is important in the transcription of this gene (29).

We do not know whether all immunoglobulin V_H regions contain similar topoisomerase II binding sites 5′ of their promoters. Topoisomerase II consensus sequences (GTNA/TA-C/TATTNATNNA/G) (6), are present in the protein binding regions of both the bf150 and TX125 sequences. We have identified topoisomerase II consensus sites 5′ all thirteen of the available mouse V_H genes that contained enough 5′ flanking sequence (300 bp) for comparison. These topoisomerase II sites occurred within regions with the most similarity to the protein binding site that occurred within the TX125 fragment. However, it should be noted that the consensus site for topoisomerase II is fairly degenerate. The role of a topoisomerase II-like proteins in the regulation of other immunoglobulin genes will require further experimentation.

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