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p70 lupus autoantigen binds the enhancer of the T-cell receptor β-chain gene

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ABSTRACT **The p70 (Ku) autoantigen has been described as a nonhistone nuclear protein recognized by antibodies from lupus patients. In our studies on the regulation of T-cell receptor (TCR) β-chain gene expression we have identified the p70 lupus autoantigen as a DNA-binding protein that binds the enhancer of the TCR β-chain gene. This enhancer is essential for expression of the TCR β gene. The core TCR β enhancer contains the E3 motif, which we show here is essential for enhancer activity. The protection of the E3 motif in T cells and the marked reduction in enhancer activity when the E3 motif is mutated underline its physiological importance in regulating β enhancer activity. The p70 lupus autoantigen gene was identified by screening T-cell λgt11 libraries with an E3 probe. The gene encodes a protein which binds the E3 motif in a sequence-specific manner. The identification of a 70-kDa protein as a major E3-binding protein by UV crosslinking is consistent with the conclusion that the p70 lupus autoantigen binds the β enhancer. Finally, we have shown that T-cell nuclear proteins which bind the E3 motif bear p70 (Ku) lupus autoantigenic determinants. Together these data suggest that the p70 autoantigen binds a critical motif in the β enhancer and probably regulates TCR β gene expression.**

The generation of anti-nuclear antibodies is a prominent clinical finding in systemic lupus erythematosus (SLE). The p70 lupus autoantigen is one such target of autoimmune responses in SLE and related autoimmune disorders (1–6). The production of anti-Ku (p70/p80) autoantibodies correlates strongly with particular subsets of autoimmune disease (1, 2).

The physiological function of the Ku lupus autoantigen remains unresolved. Some observations suggest the p70/p80 (Ku) heterodimer binds nonspecifically to ends of DNA (7, 8). In other studies, purified proteins that protected the target DNA motif were shown to bear Ku serological determinants (9–11), which suggests that Ku-like proteins might be sequence-specific DNA-binding proteins. However, it is not clear from these studies whether Ku is a sequence-specific DNA-binding protein or whether it just copurifies with other DNA-binding proteins.

We now show that the p70 lupus autoantigen binds to the transcriptional enhancer of the T-cell receptor (TCR) β-chain gene. The TCR β enhancer, located downstream of the $C_{\beta 2}$ constant-region gene segment, is essential for β gene rearrangement (13, 14) and expression (15, 16). The region of the β enhancer which is essential for conferring enhancer activity on a heterologous promoter has been mapped (17, 18), and multiple motifs in the β enhancer which bind nuclear proteins have been identified (refs. 17 and 18; T.F., data not shown). In this report we demonstrate that the E3 motif, also named $T_{\beta 3}$ (17) or βE4 (18) is critical for β enhancer activity. A Southwestern screen of a T-cell λgt11 library identified two cDNA clones which encode a sequence-specific E3-binding protein. The gene encoding this sequence-specific DNA-binding activity was found to be identical to the gene encoding the p70 lupus autoantigen. This delineation of the physiological function of p70 might suggest which target tissues are the source of the autoantigens in SLE.

MATERIALS AND METHODS

Cells and Transient Transfections. The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum. The TCR β enhancer (positions 440–780) fragment with a mutant E3 motif was generated by PCR. The position numbers refer to the original description (16) of the TCR β enhancer sequence. The native E3 motif sequence 5′-AGGAGTCACAACAGGATGTGGTTTGACATT-3′ was mutated to 5′-GCGGCCGCACCACTTCGTGTTGGCGGCCGC-3′. The β enhancer fragment or its mutagenized derivatives were cloned at the *Hin*dIII site upstream of the thymidine kinase promoter in the pBLCAT2 vector (24). The conditions for transient transfection and chloramphenicol acetyltransferase assays have been described (12).

"*In Vivo*" Footprinting. The *in vivo* footprints were mapped by using the PCR linker ligation method (19) with minor changes. The oligonucleotides used for first-strand synthesis (step *i*), PCR (step *ii*), and primer extension with the labeled oligonucleotide (step *iii*) for mapping footprints in the TCR β enhancer were as follows. Coding strand: (*i*) 5′-TGGGTTCCCCTTTAGAGACCCTCCTCTTGG-3′; (*ii*) 5′-GGGGCTTGAGATGAGATATATGGGTTCCCC-3′; (*iii*) 5′-GGCTTCTGTTTCCTGTGTGACTTCT-3′. Noncoding strand: (*i*) 5′-CAGCTCCAGACACACACATTGTGAT-3′; (*ii*) 5′-CATTCCTGGGACTTTTCGGTTCCTGAAGAC-3′; (*iii*) 5′-CGGTTCCTGAAGACAATGGGGGAAGGGGTGGAAGC-3′. The oligonucleotides for the linker ligation were a 25-mer, 5′-GCGGTGACCCGGGAGATCTGAATTC-3′, and an 11-mer, 5′-GAATTCAGATC-3′. The cells were treated with 1 μl of dimethyl sulfate per 10 ml of medium. The genomic DNA from both treated and untreated cells was isolated and cleaved with piperidine, and the β enhancer was amplified as described (19).

Gel Shift Assays. Nuclear extracts from the mouse thymoma cell line EL-4 and the human T-cell line Jurkat were made as described (20). The end-labeled double-stranded oligonucleotide probes were incubated with 5–10 μg of nuclear extract and 50 ng of poly(dI·dC) in 1× Singh buffer (21). The reaction mixtures were then analyzed by electrophoresis in nondena-

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Abbreviations: SLE, systemic lupus erythrematosus; TCR, T-cell receptor; TRE, tetradecanoylphorbol acetate response element.

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turing 5% polyacrylamide gels with 1× TBE buffer (89 mM Tris base/89 mM boric acid/2 mM EDTA).

UV Crosslinking. UV crosslinking was done as described (22), with minor modifications. The four pairs of oligonucleotides used to generate the bromodeoxyuridine-substituted probes for the E3 region were as follows. The E3A coding oligonucleotide was 5′-CCAGGAGTCACAACAGGATGTGGTTTG-3′ and the E3A noncoding primer was 5′-CAAACCACAT-3′. The E3A noncoding oligonucleotide was 5′-CAAACCACATCCTGTTGTGACTCCTGG-3′ and the E3A coding primer was 5′-CCAGGAGTCA-3′. The E3B coding oligonucleotide was 5′-CAACAGGATGTGGTTTGACATTTAC-3′ and the E3B noncoding primer was 5′-GTAAATGTCA-3′. The E3B noncoding oligonucleotide was 5′-GTAAATGTCAAACCACATCCTGTTG-3′ and the E3B coding primer was 5′-CAACAGGATG-3′. The same proteins were identified with all four bromodeoxyuridine-substituted E3-region probes. About 10^6 cpm of the probe was used with ≈30 μg of nuclear extract in the binding reaction. The DNA–protein complexes were resolved in nondenaturing 5% polyacrylamide gels with 1× TBE buffer. The gels were exposed for 30 min to UV light with a Fotodyne transilluminator 5 cm away. The bands were eluted and analyzed by SDS/10% PAGE.

Screening of λgt11 Libraries and Analysis by Southwestern Blot. A Jurkat cDNA λgt11 library was screened with a multimerized E3A probe by standard methods (23). The pUC18 plasmid containing three concatemerized E3A motifs was linearized and labeled by a Klenow fill-in reaction and the insert was purified by gel electrophoresis (23). The binding specificity of the λgt11 clone was ascertained by screening with four probes whose sequences are listed in Table 1. The AP-1 and Ets motifs are from the promoter TCR $V_{\beta 2}$ variable-region gene and bind JunB/c-Fos and Ets-1 transactivators, respectively (24).

Bacterial Expression of p70 Lupus Autoantigen. The cDNA encoding the C-terminal portion of p70 lupus autoantigen was cloned in-frame in the pRSET vector. For protein production the plasmids were transformed into *Escherichia coli* BL21(DE21) containing the pLysE plasmid. In this vector–host system (26) very little expression of the protein occurs before induction. This tight regulation of expression was essential because a leaky promoter expressing the C-terminal portion of p70 prematurely kills bacteria. This bacterially expressed recombinant p70 protein (after precipitation with ammonium sulfate) was used in gel shift assays with an E3A probe. The probe was incubated with 1 μl of recombinant p70 in the presence of 1 μg of poly(dI·dC), 50 mM Tris (pH 7.9), 12 mM $MgCl_2$, 1 mM EDTA, 0.2 mM dithiothreitol, and 4% (vol/vol) glycerol. The reaction mixtures were analyzed by PAGE with 0.5× TBE buffer. The control extracts from bacteria transformed with vector alone did not contain protein that bound the E3A probe.

Western Immunoblots. Extracts from the human leukemic cell line Manca (25 μg) were analyzed by Western blotting as described (27, 28). The blots were developed with an enhanced chemiluminescence reagent (ECL; Amersham).

RESULTS

The E3 Motif Is Critical for TCR β Enhancer Activity. The mapping of *in vitro* E3 footprints in both mouse and human DNA (17, 18) and the 100% conservation of the E3 motif between mouse and human (17) suggest that the E3 motif might be important for β enhancer activity. The E3 motif (Fig. 1*A*) is also known as $T_{\beta 3}$ (17) or βE4 (18). We reasoned that if the E3 motif were physiologically relevant, it would be protected "*in vivo*" in the T-cell chromatin. We used ligation-mediated PCR to map *in vivo* footprints in the TCR β enhancer in T cells. Fig. 1*B* displays the mapping of the E3 *in vivo* footprint on both strands of the murine TCR β enhancer in EL-4 cells. We therefore conclude that the E3 DNA–protein interaction occurs not only *in vitro* but also in the T-cell chromatin. The critical role of the E3 motif in regulating β enhancer activity was then determined by substitutional mutagenesis of the E3 motif in the TCR β enhancer fragment (positions 440–780). The mutation of the E3 motif decreased TCR β enhancer activity by >80% (Fig. 1*C*), allowing us to conclude that the E3 motif is critical for TCR β enhancer activity.

Identification of E3-Binding Proteins by UV Crosslinking. Having established the critical role of the E3 motif in regulating β enhancer activity, we wanted to identify the proteins which bind to this motif in a sequence-specific manner. Nuclear extracts from T cells were incubated with E3-region probes, and the DNA–protein complexes were resolved in nondenaturing gels. The E3A and E3B probes we used in these assays represent overlapping regions of the E3 footprint (Table 1). Two shifted bands were identified with both E3 probes (Fig. 2*A*). The E3A and E3B motifs crosscompeted. The sequence-specific nature of the binding to the E3 probe was then determined by competition with E3 or irrelevant motifs (Fig. 2*A*).

UV crosslinking was used to determine the molecular sizes of the proteins which comprise the two E3 DNA–protein

Table 1. Oligonucleotides used in DNA-binding assays

Name	Sequence
Wild-type E3A*	CCAGGAGTCACAACAGGATGTGGTTTG
Mutant E3A†	CCAGGAGTCACACCAAGATCTTGGTTC
E3B*	CAACAGGATGTGGTTTGACATTTAC
βE1‡	TCTCACCCCAGGTCTGGCTGTTTATCTGTAAGTAACA
E4A‡	TCTGGGTGTTTATCTGTAAGTA
$V_{\beta 2}$ −84 to −62 (AP-1 + Ets)§	TATGCTTAGTCAGTTTCCTGAGGAAGCA
$V_{\beta 2}$ −85 to −73 (AP-1)§	TATGAGCTTAGTCAGTTCA
$V_{\beta 2}$ −75 to −62 (Ets)§	TATGTTTCCTGAGGAAGCA
Collagenase TRE (AP-1)¶	AATTCATGAGTCAGATCTG

*The E3A and E3B motifs represent overlapping parts of the E3 motif in the TCR β enhancer.

†This mutant E3A motif, when substituted in the TCR β enhancer, reduces enhancer activity by 70% (17).

‡The βE1 motif (18) completely overlaps the E4A motif in the TCR β enhancer and is an effective competitor for proteins which bind the E4A motif. The single nucleotide difference between the E4A and βE1 motifs is based on two published murine β enhancer sequences (16, 18). This nucleotide difference does not interfere with the binding of proteins to the E4 motif (data not shown) and may represent allelic differences.

§The AP-1 and Ets motifs from the TCR $V_{\beta 2}$ promoter bind Jun/Fos and Ets transactivators, respectively (24).

¶The collagenase tetradecanoylphorbol acetate response element (TRE) motif binds Jun/Fos (24, 25).

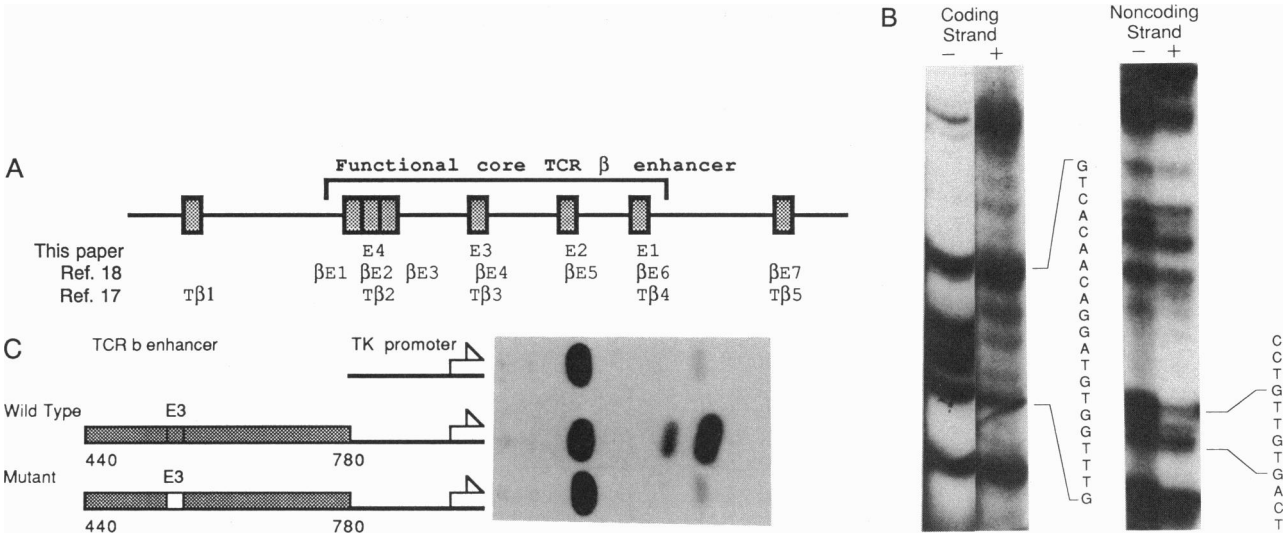

FIG. 1. The E3 motif is critical for TCR β enhancer activity. (*A*) Map of the TCR β enhancer. The four motifs we identified are termed E1–E4. The synonyms used to describe these footprints in two other studies (17, 18) are also listed. (*B*) Mapping of the E3 *in vivo* footprint on both strands of the TCR β enhancer by linker-mediated PCR (19). Cells were treated with dimethyl sulfate in tissue culture ("*in vivo*") (lanes +) or genomic DNA obtained from untreated cells was treated with dimethyl sulfate *in vitro* (lanes −). The TCR β enhancer region after piperidine cleavage was amplified as described. (*C*) Effect of mutating the E3 motif on the enhancer activity of the TCR β enhancer. The enhancer activity of the wild-type and mutated TCR β enhancer fragment (positions 440–780) was read off the thymidine kinase promoter in the pBLCAT2 vector. The chloramphenicol acetyltransferase activity expressed was assessed by incubation of bacterial extract with [^{14}C]chloramphenicol, followed by TLC separation and autoradiographic visualization of chloramphenicol and its acetylated products.

complexes. The upper shifted band is essentially an ≈70-kDa protein, while the lower shifted band is an ≈85-kDa protein (Fig. 2*B*). Binding of these two proteins to the bromodeoxyuridine-substituted probes was shown to be sequence-specific in competition experiments with E3 and irrelevant oligonucleotides (data not shown).

Cloning of a Gene Encoding an E3-Binding Protein in a λgt11 Screen of a Jurkat T-Cell Library. To identify the genes encoding the proteins which bind to the E3 motif in a sequence-specific manner, a Jurkat λgt11 library was screened with a multimerized E3 probe. Out of ≈5 × 10^6 clones screened, 2 clones bound the E3A probe in a sequence-specific manner. The protein encoded by these clones bound the E3A and the E4A motifs but not the AP-1 (positions −85 to −73) or Ets (−75 to −62) motifs (Fig. 3*A*). The λ lysogenic extracts of only the positive clone bound the E3A probe in Southwestern blot assays (data not shown). Sequence analysis revealed that the two clones encoding the E3-binding proteins were identical to the gene encoding the p70 lupus autoantigen (29, 30). One of the E3-binding p70 clones encoded amino acids 111–222 fragment of the lupus p70 autoantigen. This DNA-binding region of the p70 lupus autoantigen represents the C-terminal half of the molecule. This region contains a leucine zipper-like motif with an adjacent basic region, similar to that found in other leucine zipper-containing transcription factors (31).

The C-terminal portion (amino acids 111–222) of the p70 lupus autoantigen was expressed in *E. coli*, and the recombinant protein was tested in gel shift assays. p70 bound the E3A probe, and formation of this labeled DNA–protein complex was blocked by competition with nonradioactive E3A or βE1 (completely overlapping the E4A motif) motif but not $V_{\beta 2}$ AP-1 (−85 to −73) or $V_{\beta 2}$ Ets (−75 to −62) motif (Fig. 3*B*). The mutant E3A motif, unlike the wild-type E3A motif, failed to compete. When this mutant E3A motif was substituted in the wild-type β enhancer, enhancer activity was reduced by 70% (17). This result supports the conclusion that p70 lupus autoantigen binds DNA in a sequence-specific manner and probably is a positive modulator of β enhancer activity. The ability of p70 to bind apparently unrelated sequences like E3A and E4A suggests that the p70 lupus autoantigen binds a degenerate motif in a sequence-specific manner.

Monospecific Ku (p70/p80) Autoantigenic Determinants Are Present on E3-Binding Proteins. Identification of the p70 lupus autoantigen as an E3-binding protein was consistent with our earlier identification of a 70-kDa E3-binding protein in nuclear extracts. It was therefore important to use a monospecific anti-Ku (p70/p80) autoantiserum to determine whether the p70 lupus autoantigen is the major protein which binds the E3 motif. If this were true, addition of this antibody to gel shift assays with end-labeled DNA probes should supershift E3 DNA–protein complexes but not irrelevant DNA–protein complexes such as TRE–Jun/Fos. The monospecific nature of the Ku antiserum for p70 and p80 lupus autoantigens was determined by Western analysis of human cell-line extracts. This Ku antibody bound to two proteins of 70 and 85 kDa (Fig. 4*A*). In gel shift assays using an E3 probe with Jurkat extracts this antibody (1:1000 dilution) bound to the DNA–protein complexes but not to naked DNA alone (Fig. 4*B*). In a control experiment with a collagenase TRE probe (25) which binds Jun/Fos heterodimers, this antibody did not bind the DNA–protein complex or the naked TRE probe (Fig. 4*B*). These data demonstrate that a majority of the E3-binding proteins in T cells bear Ku autoantigenic determinants. We conclude that the p70 gene encodes a protein which binds a critical motif in the TCR β enhancer.

DISCUSSION

We have shown that the p70 lupus autoantigen binds DNA in a sequence-specific manner. The p70 autoantigen binds the E3 motif in the TCR β enhancer. This conclusion is based on a Southwestern screen of a T-cell prokaryotic expression library with an E3 probe. Recombinant p70 lupus autoantigen expressed in bacteria was shown to bind the E3 motif in a sequence-specific manner. A mutant E3 motif was a less effective competitor than the wild-type E3 motif. This mutant E3 motif has been shown to markedly reduce functional enhancer activity. Finally, Ku monospecific antiserum supershifted E3 DNA–protein complexes but not TRE DNA–protein complexes or the labeled E3 and TRE probes.

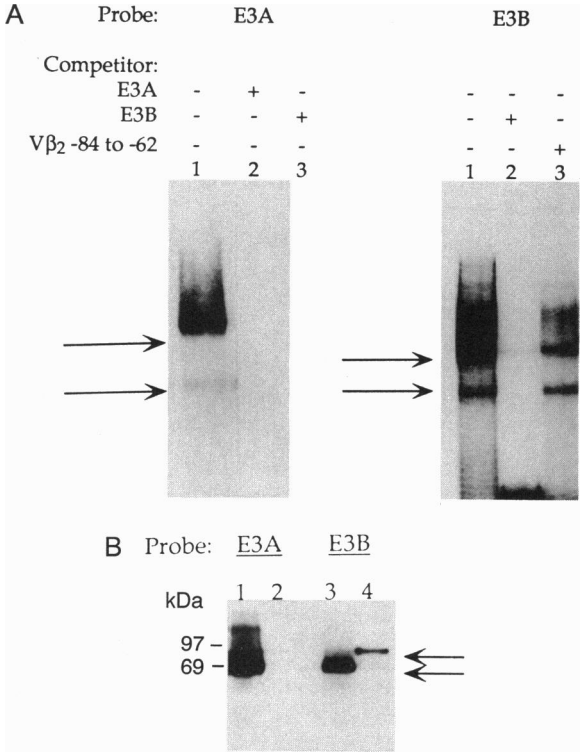

FIG. 2. Identification of nuclear proteins which bind the E3 motif. (*A*) The E3 motif binds to T-cell nuclear proteins in a sequence-specific manner. The E3A probe was incubated with EL-4 (murine T-cell line) nuclear extracts in the presence or absence of competitor as indicated. Gel shift assays showed that the E3A and E3B oligonucleotides competed effectively with the E3A probe. In gel shift assays with an E3B probe, the −84 to −62 oligonucleotide from the TCR $V_{\beta 2}$ promoter did not compete. It did decrease some nonspecific binding. Identical results were obtained in competition experiments with an E3A probe (data not shown). The JunB/c-Fos heterodimer and Ets-2 transactivator bind the −84 to −62 oligonucleotide from the TCR $V_{\beta 2}$ promoter (24) at two nonoverlapping motifs. Arrows identify the two DNA–protein complexes. (*B*) Identification of E3-binding proteins by UV crosslinking. The ^{32}P-labeled E3A and E3B probes were substituted with bromodeoxyuridine in the noncoding strand. The DNA–protein complexes were first resolved in nondenaturating gels, before the UV-crosslinked complexes were run in SDS/polyacrylamide gels. Lane 1, upper shifted band with E3A probe; lane 2, lower shifted band with E3A probe; lane 3, upper shifted band with E3B probe; lane 4, lower shifted band with E3B probe. The same proteins were detectable when the coding or noncoding strands of either the E3A or E3B probes were substituted with bromodeoxyuridine. Arrows identify the two proteins which bind the E3 motif.

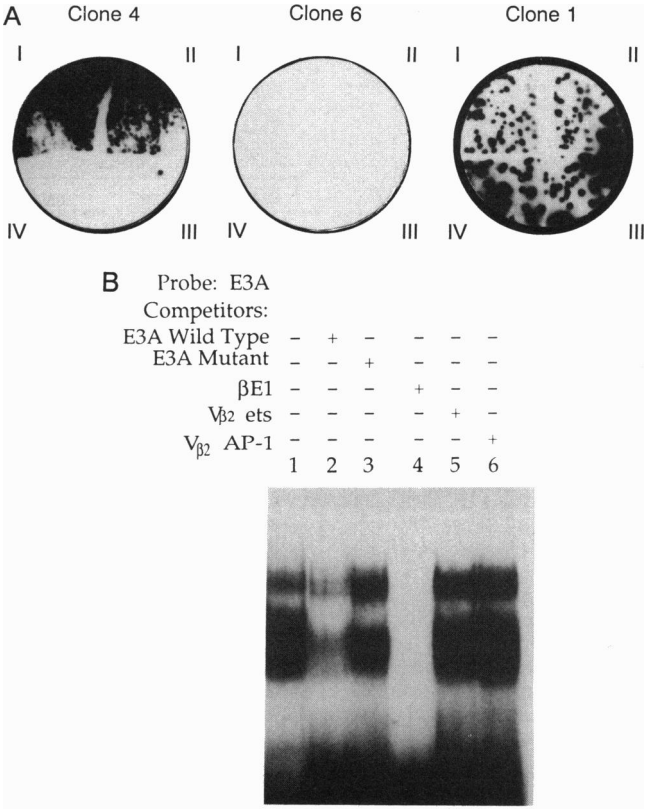

FIG. 3. The p70 lupus autoantigen binds the E3A motif in a sequence-specific manner. (*A*) Sequence-specific binding of the p70 λgt11 clone to the multimerized E3A probe. The three clones analyzed included the positive clone 4; clone 6, which does not bind DNA; and clone 1, which binds DNA in a sequence-nonspecific manner. The four probes included the TCR β enhancer E3A (probe I) and E4A (probe II) motifs, and the TCR $V_{\beta 2}$ promoter AP-1 (probe III), and Ets (probe IV) binding motifs (24). (*B*) The bacterially expressed recombinant C-terminal portion (amino acids 111–222) of the p70 lupus autoantigen was analyzed in gel shift assays with the E3A probe. A panel of nonradioactive excess (400 ng) oligonucleotides was tested for the ability to compete with the E3A probe. The competitors included wild-type E3A (lane 2), mutant E3A (lane 3), βE1 (lane 4), and $V_{\beta 2}$ Ets (lane 5) and AP-1 (lane 6) motifs. This E3A mutation in the β enhancer reduces enhancer activity by 70% (17). The βE1 oligonucleotide (18) completely overlaps the β enhancer E4A motif.

The p70 lupus autoantigen is associated with another polypeptide called the p80 lupus autoantigen (apparent molecular mass, 80–89 kDa) (1–4, 29, 30, 32, 33). The ability of the E3 motif to bind a 70-kDa and an 85-kDa protein suggests that the p80 lupus autoantigen might also bind to the E3 motif. The ability of lupus Ku-monospecific antiserum to bind most of the E3 DNA–protein complexes is consistent with this reasoning. It is possible that the p80 lupus autoantigen does not renature after denaturation and therefore was not clonable by the λgt11 strategy. It is important to mention that the majority of the 70- and 85-kDa E3-bound proteins form distinguishable DNA–protein complexes (i.e., form distinct shifted bands). This suggests that a simple heterodimer with a 1:1 stoichiometry between these two polypeptides does not explain its ability to bind DNA in a sequence-specific manner. The fact that we were able to clone the p70 from a λgt11 library suggests that the p70 lupus autoantigen is able to bind DNA as a monomer or homodimer. It is possible that heterodimers (p70 with p80) with more complex stoichiometry may also bind DNA in a sequence-specific manner.

The p70 lupus autoantigen is expressed in human liver, thymus, thyroid, and spleen (4, 30, 33). In one study no transcripts were found in human brain, muscle, and liver (33). This tissue distribution suggests that the tissue distribution of p70 is not sufficient to explain the lymphoid specificity of the β enhancer. The p70 lupus autoantigen must interact with other transactivators while regulating β enhancer activity.

It has been suggested that p70 and p80 lupus autoantigens form a 1:1 heterodimer which binds nonspecifically to ends of DNA (7, 8). In contrast, in our study the majority of the 70- and 80-kDa E3-bound proteins form distinct shifted bands. This observation suggests that the 70- and 80-kDa proteins bind the E3 motif as monomers or homodimers. It is reasonable to suggest that the Ku antigens have a high nonspecific DNA-binding ability (as p70/p80 heterodimers) in addition to the sequence-specific interaction (of the p70 monomer or homodimer) we have described here. Comparison of the motifs we have shown to bind (or not to bind) the p70 lupus autoantigen (Table 1) suggests that the p70 lupus autoantigen recognizes a degenerate motif, albeit in a sequence-specific

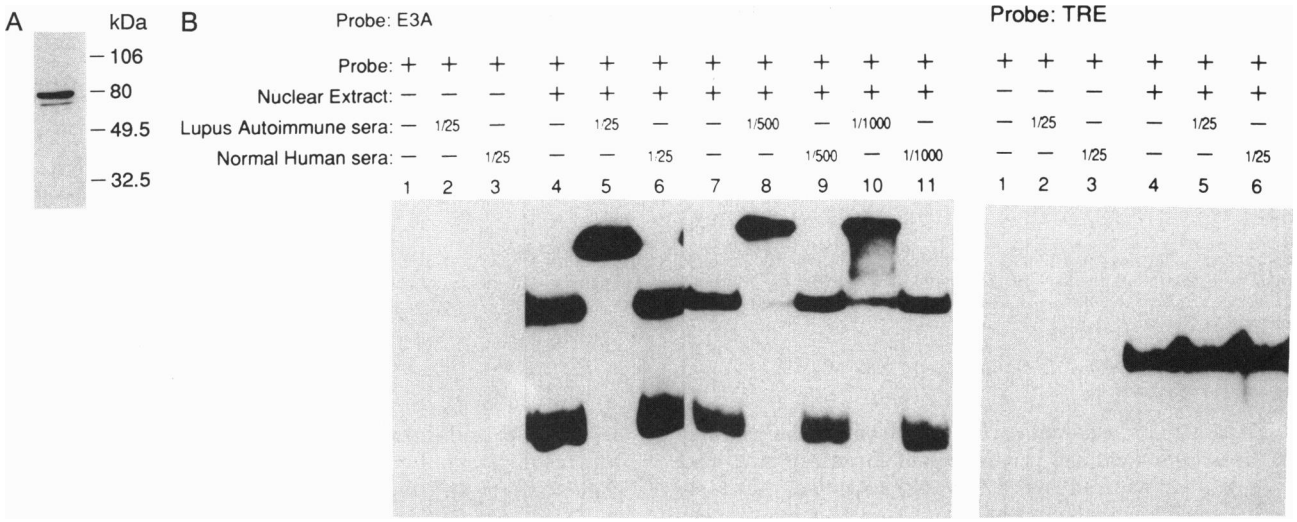

FIG. 4. The majority of the E3A-binding proteins in human T cells bear Ku (p70/p80) autoantigenic determinants. (*A*) The anti-Ku autoantiserum is monospecific for p70/p80 polypeptides as determined by Western immunoblot analysis of human lymphoid cell extracts. This lupus anti-Ku autoantiserum has a higher titer for p80 than for p70. (*B*) Monospecific anti-Ku autoantiserum supershifts the E3A DNA–protein complex but not TRE DNA–protein complexes. The labeled probes were incubated with Jurkat (human T-cell line) extracts before the Ku autoantibodies or the normal human antibodies were added. In control lanes the antibodies were also tested for their ability to bind naked DNA (labeled probe). Neither of these antibodies bound naked DNA.

manner. Other proteins which have high nonspecific DNA-binding activity in addition to a sequence-specific binding ability have been described (34). The ability of the same protein to be involved in two types of interactions with DNA suggests that p70 could be involved in regulating both transcription and replication, a suggestion which is consistent with observations with other transcription factors (35).

The conclusion that the p70 lupus autoantigen binds DNA in a sequence-specific manner and regulates transcription is consistent with the observation that purified proteins expressing Ku determinants protect transcriptionally relevant motifs (9–11). Transcription of the human U1 promoter is also abrogated by depletion of molecules bearing Ku determinants and restored by addition of proteins bearing Ku determinants (11). In this paper we show that the p70 lupus autoantigen can bind DNA in a sequence-specific manner. The inability of an E3 mutant that reduces β enhancer activity to bind to p70 suggests a probable functional role for p70 in TCR β expression.

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