Fine Epitope Mapping of the Human SS-B/La Protein

Identification of a Distinct Autoepitope Homologous to a Viral Gag Polyprotein

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Abstract

To analyze the autoepitopes on the SS-B/La protein, a cDNA covering the entire region coding the protein was isolated from a human cDNA library. The cDNA was subcloned into an expression plasmid vector, pEX, to express its protein product as a fusion protein with cro-β-galactosidase in *Escherichia coli*. A recombinant pEX plasmid expressing three-fourths of the protein (amino acid 112-408) was also constructed. The antigenicities of these recombinant proteins were confirmed with a patient's serum. Their various deletion mutants were produced with exonuclease III treatment from the 3' ends of the cDNAs without changing the proper translational frame. Immunoblot analysis and enzyme-linked immunosorbent assay were used to evaluate the reactivities of the recombinant proteins with patients' sera to determine the autoepitopes. A narrow segment (amino acid 88-101) and the region where several epitopes were located (amino acid 283-338) on the SS-B/La protein were universally recognized by all the sera with anti-SS-B/La antibodies examined. An additional epitope region (amino acid 179-220) was recognized by some patients' sera. Computer analysis revealed that the most distinct autoepitope, amino acid 88-101, had a striking homology to a retroviral gag polyprotein. These findings indicate that exogenous or endogenous retroviruses may play a role in initiation of the anti-SS-B/La autoimmunity. (*J. Clin. Invest.* 1990. 85:1566–1574.) autoantibody • autoantigen • anti-SS-B/La antibody • molecular mimicry • Sjögren's syndrome

Introduction

Autoantibodies directed against various nuclear proteins of normal cells are detectable in sera from patients with systemic autoimmune diseases (1, 2). Although it seems unlikely that these antinuclear antibodies directly injure the organs, some antibodies are related with particular diseases or symptoms. Analysis of the mechanisms of autoantibody production should therefore provide important clues to the pathogenesis of autoimmune disorders. Several mechanisms of autoantibody production have been proposed by previous investiga-

tors. They include polyclonal B cell activation (3), an antigen-driven mechanism (4) and molecular mimicry (5). To determine which mechanism is predominantly involved in the production of a particular autoantibody, it is useful to analyze the numbers and region of autoepitopes recognized by patients' sera.

The SS-B/La antigen is a small nuclear ribonucleoprotein (snRNP)[1] with a molecular weight of 47 kD. Anti-SS-B/La antibodies are strongly associated with primary Sjögren's syndrome (SS), and serve as a diagnostic marker of SS. The SS-B/La protein is mainly associated with the precursor forms of RNA polymerase III transcripts, including transfer RNAs, 7S RNA, 5S RNA (7), and virus-encoded RNAs such as adenovirus-encoded RNAs (VAI and VAII) (8), Epstein-Bar virus–encoded RNAs (EBER) (9) and leader RNAs of some negative stranded viruses (10, 11). Recent studies have shown that the SS-B/La protein is essential in the biogenesis of these RNAs (7, 12). However, it remains to be clarified why autoantibodies against such an important molecule are related to SS, an autocrine exocrinopathy.

Several investigators have performed epitope mapping of the autoepitopes on the SS-B/La protein (13–17). However, their results were not able to lead to clear explanation of autoantibody production, since they were only assigned in long stretches of peptide. We report here the isolation of a full-length cDNA encoding the human SS-B/La autoantigen and its epitope mapping with deletion mutants, which revealed three antigenic regions. The most distinct and universally recognized epitope was found to be homologous to a retroviral gag polyprotein. These results raise the possibility that molecular mimicry might induce anti-SS-B/La autoimmunity.

Methods

Patient sera. Sera were obtained from patients with SS or other autoimmune diseases who were treated in the hospitals of the University of Tokyo, Tokyo Medical and Dental University, and Kanazawa Medical College. Precipitating antibodies against the SS-B/La protein were evaluated with the double immunodiffusion method using rabbit thymus extract and a standard anti-SS/La serum.

Isolation of cDNAs for the human SS-B/La protein from λgt11 expression library and pcD library. Details of isolation have been described previously (18, 19). Following the procedures of Young and Davis (20), a human fibroblast cDNA expression library constructed in the λgt11 vector was screened with serum from a patient with primary SS, containing anti-SS-B/La antibodies as an antibody probe. After screening with immunostaining, each cDNA insert of positive clones was subcloned into an expression plasmid pEX-2 (21) as described in

1. *Abbreviations used in this paper:* ExoIII, exonuclease III; snRNP, small nuclear ribonucleoprotein; SS, Sjögren's syndrome.

the next paragraph. The recombinant pEXs were induced to express fusion proteins with cro-β-galactosidase and products were evaluated by their reactions to the patient's serum. The longest insert among positive clones was named pA158 and its characteristics as a part of cDNA for the human SS-B/La protein were reported (19). For the isolation of full-length cDNAs, the screening of pcD plasmid libraries (22) constructed from fibroblasts and foreskin cells was performed with ^{32}P-labeled pA158 cDNA as a hybridization probe. The longest cDNA insert among positive clones was derived from the fibroblast library and verified as the full-length cDNA for the human SS-B/La protein.

Expression of cloned cDNA. To express the cloned cDNAs, cDNA inserts were recovered from vectors with restriction enzyme digestion (Eco RI for λgt11, Stu I and Bam HI for pcD plasmid) and subcloned into expression vector pEX plasmids at their polylinkers. To adjust the translational frame, three pEXs with different frames (pEX-1,2,3) were used for subcloning. *Escherichia coli,* POP2136 transformed by recombinant pEX, were grown in a Luria-Bertani medium overnight at 30°C. The cultures were then shifted to 42°C and incubated for 2 h to express the fusion proteins with cro-β-galactosidase. Recombinant pEXs with a correct frame were selected by examining the reactivities of their products with the patient's serum.

Affinity-purification of autoantibodies. A modified method of Olmsted (23) was used for purification of autoantibodies. Bacterial lysates with induced recombinant autoantigens were electrophoresed on polyacrylamide/SDS gels and transferred to nitrocellulose membranes. After staining with Ponceau S solution (Sigma Chemical Co., St. Louis, MO), the protein bands expected to contain fusion proteins were cut out and these pieces of membrane were saturated with PBS-0.5% skim milk (Yukijirushi, Tokyo, Japan) followed by 0.5% Tween 20 in PBS for 30 min. They were then incubated with a patient's serum diluted 1:10 in PBS-0.5% skim milk followed by washing five times with PBS. The bound antibodies were eluted with 4 ml of 0.2 M glycine-HCl, pH 2.6, containing 0.15 M NaCl and 1% BSA. After 5 min of incubation, the eluate was neutralized with 1 ml of 1 M Tris-HCl, pH 7.5.

Immunoblotting. The procedures were reported previously in detail (18). The lysates of human peripheral leukocytes or bacteria with induced proteins were separated on polyacrylamide/SDS gels and transferred to nitrocellulose membranes. These were reacted with sera or affinity-purified antibodies. The antigenic protein bands were detected with rabbit anti-human IgG antibodies then goat anti-rabbit immunoglobulin antibodies coupled with horseradish peroxidase.

Construction of expression plasmids with deleted cDNAs. To construct a cDNA lacking one-fourth of the 5' terminal (this cDNA is hereafter referred to as FSBB), a full-length cDNA (referred to as FSBF) was cut with the authentic Bgl II site and its 3' part was subcloned into pEX plasmids in three different translational frames. The recombinant pEX with the correct frame was selected using the patient's serum. Restriction enzymes and exonuclease III (ExoIII; Takara, Kyoto, Japan) were used for the modification and deletion of the 3' terminal of the cDNAs, essentially by the methods of Henikoff (24) and Putney (25). The plasmids with full-length cDNAs were digested with restriction enzyme Sal I and filled in 3' recessed ends with alpha-phosphorothioate deoxynucleotides (Promega Biotek, Madison, WI) and Klenow DNA polymerase (Takara). Thereafter, they were digested with restriction enzyme Bam HI. The other plasmid, FSBB, was digested with Pst I and Bam HI. After deletion with ExoIII, end repair with mung bean nuclease (Takara) and Klenow DNA polymerase was performed before ligation with T4 DNA ligase (Takara). *E. coli* hosts were transformed with the ligated vectors. Plasmids containing cDNAs of different length, were obtained by changing the duration of ExoIII treatment.

DNA sequencing. The isolated cDNA inserts and deleted inserts were subcloned into bluescript plasmid vectors (Strategene, La Jolla, CA) to be sequenced. Their sequences were determined by Sanger's dideoxy method (26) using modified T7 DNA polymerase (USB, Cleveland, OH). To sequence the full-length cDNA in bluescripts, they

were also deleted with ExoIII. The sequences of DNAs and deduced amino acids were analyzed using SDC-GENETYX Genetic Information Processing Software (Software Developing Co., Tokyo, Japan), the protein sequence database of the National Biomedical Research Foundation (NBRF) and SWISS-PROT protein sequence database of European Molecular Biology Laboratory (EMBL).

Enzyme-linked immunosorbent assay (ELISA). After induction of the fusion protein in *E. coli,* bacteria were harvested by centrifugation from 500-ml cultures and lysed in 20 ml of lysozyme solution [1 mg/ml Lysozyme (Wako, Osaka, Japan), 8% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris-HCl, pH 8.0] with sonication. After centrifugation, pellets were suspended in 20 ml of DNase buffer [5 g/ml DNase I (Sigma Chemical Co.)], 150 mM NaCl, 10 mM MnCl$_2$, 50 mM Tris-HCl, pH 7.6] for 2 h at 37°C followed by sonication to fragmentate the bacterial DNAs. After centrifugation, the pellets were washed twice with 20 ml of the solution containing 0.5% Triton X-100, 10 mM EDTA, 50 mM NaCl and 50 mM Tris-HCl, pH 7.6. Finally, the purified pellets were solubilized by adding 4 ml of 8 M urea and 10 mM DTT in PBS. Each well of multititer plates was coated with 3 μg/150 μl of the purified protein in carbonate buffer, pH 9.6, followed by blocking with 0.5% skim milk and 0.05% Tween 20 in PBS. Human sera were preincubated with bacterial lysate containing nonrecombinant pEX products. Serially diluted sera with 1% skim milk in PBS and, subsequently, peroxidase-coupled protein A were applied to the plate. Bound antibodies were detected enzymatically with *o*-phenylenediamine as the substrate, using a microplate-photometer at 492 nm. The quantity of coated proteins was evaluated by applying rabbit anti-β-galactosidase. Binding units defined as the reciprocal of the serum dilution that produced an OD_{492} of 1.0 were used to quantitate reactivity.
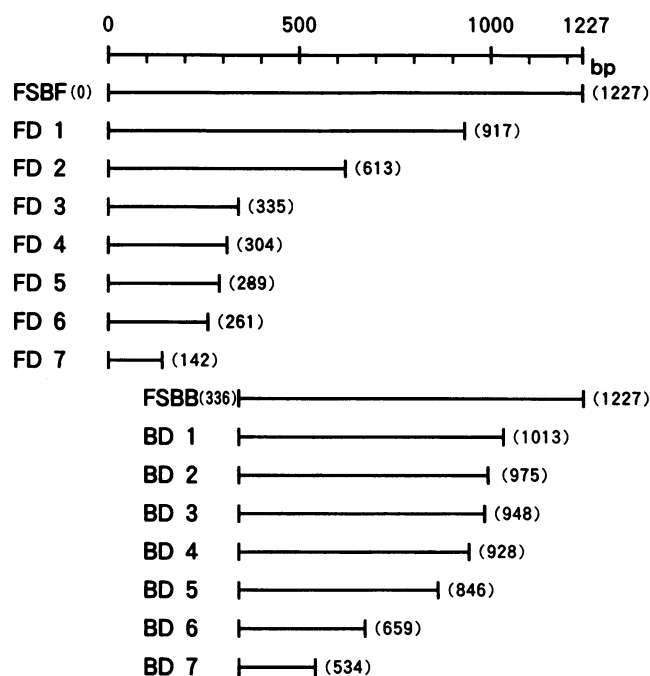
Results

Isolation of full-length cDNAs encoding the human SS-B/La protein. Using serum from a patient (T.S.) which contains precipitating antibodies against the SS-B/La protein, λgt11 human fibroblast cDNA expression library was screened. The longest cDNA insert (1.5 kb) was named pA158 clone and analyses of its product in pEX expression vector was previously reported (19). To obtain a full-length cDNA, pcD plasmid libraries constructed from human fibroblasts and foreskin cells were screened with labeled pA158 cDNA. After screening of each library, the longest cDNA (1.8 kb) was derived from fibroblast library and named FSBF clone. This was subcloned into pEX plasmids, and a recombinant clone with a correct translational frame was selected by immunostaining with the patient's serum. The protein induced using FSBF (named FSBF·EX) and the vector-produced protein (pEX) were separated by 8% SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted to nitrocellulose membranes. The anti-pEX or anti-FSBF·EX antibodies was purified by affinity-purification with these membranes. In the immunoblot analysis of human peripheral leukocyte proteins fractionated in 12.5% SDS-PAGE, a standard anti-SS-B/La serum (a serum from patient T.S.) and anti-FSBF·EX antibodies recognized an identical polypeptide with a molecular weight of $\sim$ 50 kD (Fig. 1). For further characterization, human peripheral leukocyte lysate was incubated and precipitated with anti-FSBF·EX antibodies. After phenol extraction of the precipitates, the remaining nucleic acids were fractionated in urea-PAGE. Serum from the patient and anti-FSBF·EX antibodies precipitated RNAs that are known to be associated with the SS-B/La protein, e.g., precursors of 5S RNA and transfer RNAs (data not shown). The nucleotide sequence of FSBF cDNA determined by Sanger's method matched the pre-
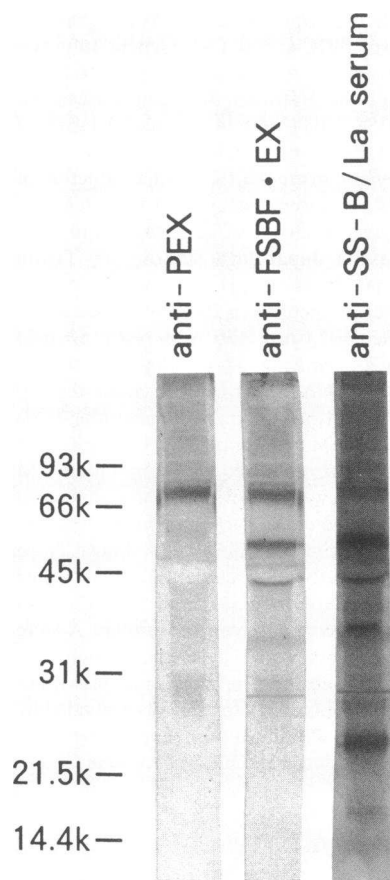
Figure 1. Immunoblot analysis of affinity-purified antibodies against the recombinant proteins. A full-length cDNA encod ing the SS-B/La protein (FSBF) was subcloned into an expression plasmid vector, pEX. The recombinant fusion proteins, FSBF·EX and PEX, produced by the pEX vector as a control, were used for affinity-purification of the anti-FSBF·EX and anti-PEX antibodies from a patient's serum. Human peripheral leukocyte proteins were fractionated in 12.5% polyacrylamide/SDS gel and electroblotted onto nitrocellulose filters. They were then stained with anti-PEX antibodies, anti-FSBF·EX antibodies and a standard anti-SS-B/La serum.


Figure 2. Schematic diagram of a full-length cDNA (FSBF) encoding the SS-B/La protein and its deletion mutants used for immunoblot analysis and ELISA. FSBF was enzymatically manipulated to make deletion mutants FD1-FD7 with exonuclease III, and FSBB was constructed using Bgl II restriction enzyme. FSBB was also deleted to make BD1-BD7. The pEX expression vectors containing them were induced to express hybrid proteins with cro-β-galactosidase.

viously published one (17, 27). It begins −2 bp from the initiation codon and covers the entire region coding the SS-B/La protein.

Fine epitope mapping of the SS-B/La protein using deleted cDNAs. To identify the autoepitopes on the human SS-B/La protein, the recombinant pEX plasmid with FSBF cDNA was deleted enzymatically from the 3' end of the cDNA fragment by ExoIII treatment. Because this procedure does not change the proper translational frames of the cro-β-galactosidase gene and the inserted cDNAs, the resultant deletion mutants are capable of expressing the truncated proteins in the correct translational frame. The inserts of the representative deletion mutants (FD1-FD6) used for epitope mapping are shown in Fig. 2. They were all induced to express the truncated SS-B/La autoantigens as fusion proteins in bacteria, and the products were separated on polyacrylamide/SDS gels (Fig. 3 *A*). Staining with Coomassie brilliant blue showed the recombinant products were equally large in amount and their molecular weights depended on the length of cDNA inserts. Their reactivity with sera containing anti-SS-B/La antibodies was determined by immunostaining. As shown in Fig. 3 *A* and Table I, the most intensive binding of antibodies was found in FSBF. In all sera examined, reactivities dropped dramatically between the FD5 and FD6 products. FD5 reacted less than FD4. These findings indicated that one of the major autoepitopes is located in the area between the COOH-terminals of the FD4 and FD6 products (named Ept1, Fig. 4). Moreover, since the difference between FD5 and FD4 is only four amino acids, this epitope seems to reside in the vicinity of the COOH-terminal of the FD5 product.

That the reactivity of FSBF was superior to those of FD1 and FD2 in most sera suggested the existence of another distinct autoepitope at the COOH-terminal portion of the SS-B/La protein. Therefore, to exclude the influence of Ept1 antigenicity, the FSBF cDNA insert was cut at the Bgl II restriction site (335 bp from the initiation codon), and its 3' part (named FSBB) was subcloned into pEX vectors. The recombinant pEX with FSBB in the proper translational frame was selected by the same serum. As expected, its product was found to react with all the sera containing anti-SS-B/La autoantibodies. To localize other epitopes, deletion mutants (BD1-BD7), derived from FSBB, were generated in the same way as FSBF (Fig. 2). The reactivities of the products with patients' sera were also examined (Fig. 3 *B* and Table I). In all sera, decreased reactivity was mainly found from BD1 to BD5. This area between the COOH-terminal of BD1 and BD5 products was named Ept2 (Fig. 4). However, there were no common segments where the intensities of staining changed among the eleven sera tested. The change was found between BD1 and BD2 in sera from patients 2, 10, and 11. The reactivity in sera from the patients 8, 10, and 11 was altered between BD2 and BD3; in sera from patients 1-9, and 11 between BD3 and BD4; and in sera from patients 1, 3, 5-7, and 9 between BD4 and BD5. Each serum had more than two regions that changed in intensity of staining, but nevertheless, we could not identify any distinct area in this region that was recognized by all the sera. Sera from patients 2, 4-9, and 10 reacted with BD5 and BD6 products. The intensity of staining with BD5 and BD6 products was equal in every serum and no serum reacted with BD7. This supported the presence of the third epitope region.
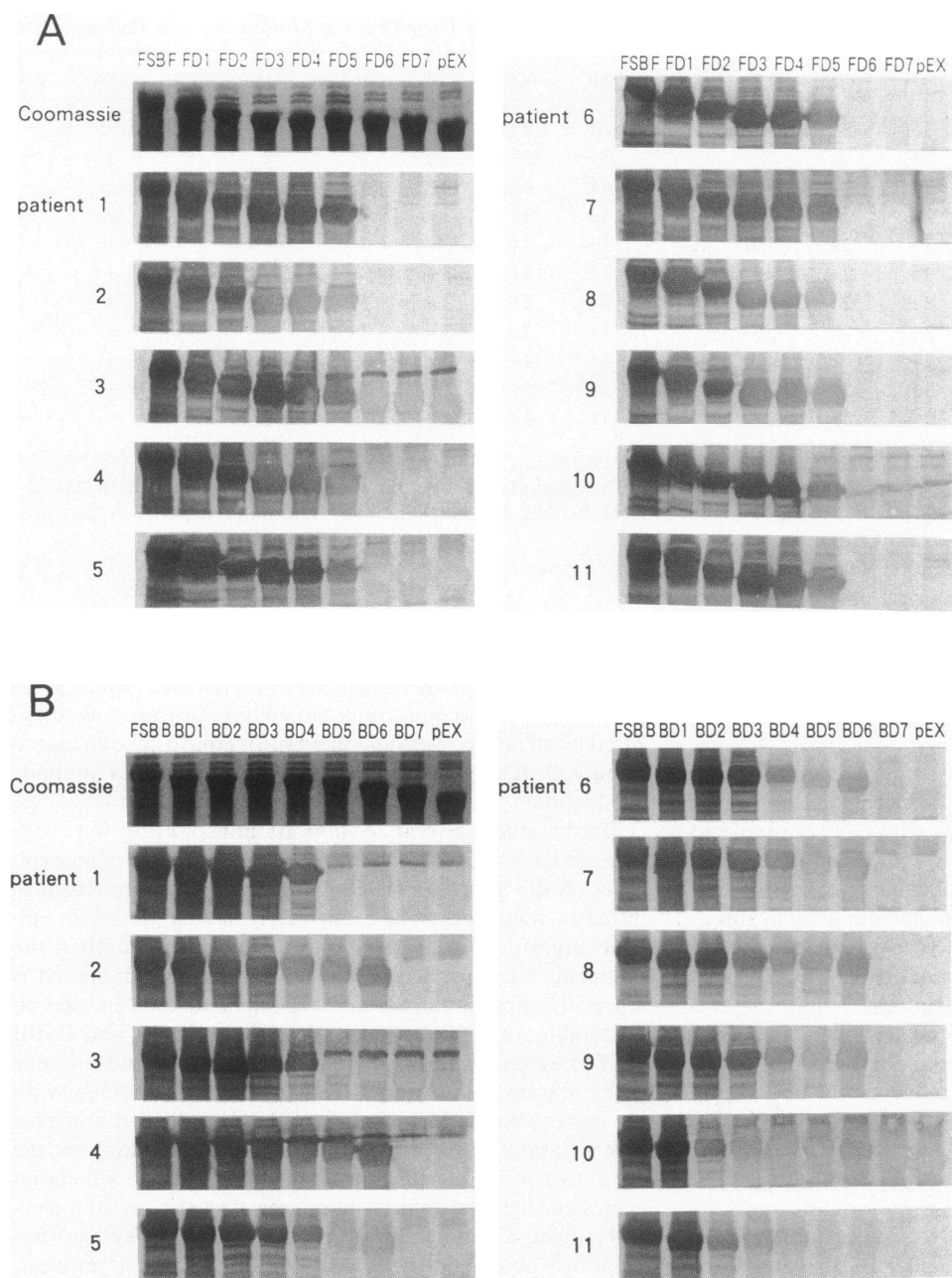
Figure 3. Antigenicities of the protein encoded by FSBF and its deletion mutants. The recombinant autoantigens (*A:* FSBF, FD1-FD7; *B:* FSBB, BD1-BD7) and the vector-produced protein (pEX) were separated in 8% polyacrylamide/SDS gels. One of each was stained with Coomassie brilliant blue and the others were transferred onto nitrocellulose filters. Immunostaining with 11 sera from patients containing anti-SS-B/La antibodies was performed.

We named the peptide between the COOH-terminals of BD6 and BD7 products Ept3 but it was recognized only by some of the patients' sera.

ELISA using deletion mutants. To substantiate that all sera from patients with anti-SS-B/La autoantibody predominantly react with Ept1 and Ept2, sera from patients with SS or other systemic autoimmune diseases containing anti-SS-B/La precipitating antibodies were assayed using the products FD4, FD6, BD1, and BD5 and non-recombinant pEX as a control. Wells of multititer plates were coated with an equal quantity of each deleted recombinant SS-B/La protein, and patients' sera were diluted serially from 1:100 to $1:3^7 \times 100$. Assays were performed in duplicate, and in order to eliminate the effect of antibodies against bacterial components or induced β-galactosidase, the final data were obtained by subtraction of values against the pEX protein from those against the recombinant

proteins. Two representative examples of the quantitation of antibodies against them are shown in Fig. 5. Both showed significantly decreased reactivity between FD4 and FD6 and between BD1 and BD5. The sera shown in Fig. 5 *B* still reacted with BD5, implying this serum contains antibodies against epitopes in the Ept3 area. We tested 28 anti-SS-B/La positive sera and the antibody binding units of each serum against each protein are shown in Table II. These data showed that all sera with anti-SS-B/La antibodies reacted intensively with FD4 and BD1, but never with FD6. Reactions displayed as less than 100 binding units indicate an eventual lack of reaction, i.e., OD_{492} < 0.3 in 1:100 dilution (see Fig. 5A). Therefore, even sera from patients 3, 10, 13, 16, 22, and 24 (binding units for FD4 < 200) reacted far more intensively with FD4 than with FD6. This was confirmed using immunoblot analysis (data not shown). 22 of 28 sera were found to react with BD5 even though their

Table I. Summary of the Antigenicities of Recombinant Proteins Produced by FSBF and Their Deletion Mutants against Patients' Sera

Pt	FF	FD1	FD2	FD3	FD4	FD5	FD6	FD7	FB	BD1	BD2	BD3	BD4	BD5	BD6	BD7
1	4+	3+	3+	3+	3+	2+	−	−	3+	3+	3+	3+	2+	−	−	−
2	4+	3+	3+	2+	2+	+	−	−	3+	3+	2+	2+	+	+	+	−
3	4+	3+	3+	3+	3+	2+	−	−	4+	4+	4+	4+	3+	−	−	−
4	4+	3+	3+	2+	2+	+	−	−	3+	3+	3+	3+	2+	2+	2+	−
5	4+	3+	3+	3+	3+	2+	−	−	3+	3+	3+	3+	2+	+	+	−
6	4+	3+	3+	3+	3+	2+	−	−	3+	3+	3+	3+	2+	+	+	−
7	4+	3+	3+	3+	3+	2+	−	−	3+	3+	3+	3+	2+	+	+	−
8	4+	3+	3+	2+	2+	+	−	−	3+	3+	3+	2+	+	+	+	−
9	4+	3+	3+	2+	2+	+	−	−	4+	4+	4+	3+	2+	+	+	−
10	4+	3+	3+	3+	3+	2+	−	−	3+	3+	2+	+	+	+	+	−
11	4+	3+	3+	3+	3+	2+	−	−	3+	3+	2+	+	−	−	−	−

The intensity of the bound antibodies were arbitrarily graded from − to 4+. The dilution of each serum was as follows: Patient 1 (1:10,000), 2 (1:40,000), 3 (1:1,000), 4 (1:80,000), 5 (1:80,000), 6 (1:5,000), 7 (1:80,000), 8 (1:40,000), 9 (1:80,000), 10 (1:1,000), 11 (1:40,000) Pt, patient number, FF, FSBF, FB, FSBB.

reactivities with BD5 were weaker than those with BD1. This suggests all sera recognize predominantly the epitopes in Ept1 and Ept2. It is also suggested that an additional autoepitope, Ept3, which is reactive with only some of sera, is located in the middle of the SS-B/La protein.

Computer analysis of the amino acid sequence of epitope regions. The amino acid sequences deduced for Ept1, Ept2, and Ept3 were analyzed for homology with DNA/amino acid analyzing software. After comparison with other amino acid sequences in the protein sequence databases of NBRF and EMBL, Ept1 was found to have a striking homology to amino acids 32 to 39 of the feline sarcoma virus gag leader protein (28, 29) (Fig. 6). In the COOH-terminal part of Ept1, six of eight amino acids were identical, and the remaining two amino acids and the two flanking amino acids were analogous biochemically. No homology was found for Ept2 or Ept3. There was no homology among the sequences of Ept1, Ept2, and Ept3.

Discussion

After the isolation of a full-length cDNA for the human SS-B/La protein (FSBF), Ept1 (amino acid 88-101), and Ept2 (amino acid 283-338) on the SS-B/La protein were found to contain distinct autoepitopes by making several deletion mutants to produce truncated proteins. Ept1 was defined as the area between the COOH-terminals of FD4 and FD6, and Ept2 as that between the COOH-terminals of BD1 and BD5 (Fig. 4). Antibody binding with FD4, BD1 and BD5 in ELISA disclosed that all sera from patients containing anti-SS-B/La precipitating antibodies recognized both Ept1 and Ept2. All sera that reacted potently to FD4 have no reactivity to FD6. Moreover, the intensity of antibody binding to FD5 was weaker than that to FD4, whereas the amino acid sequence difference between them is only four amino acids. These facts suggested the COOH-terminal of FD5 might be located just on the epitope. In contrast, Ept2 is a long stretch of peptides consisting of 56 amino acids. Although this area was reactive with all patients' sera, there seems to be no universal epitope. It is likely that Ept1 is a single distinct autoepitope, even though not yet proved, whereas Ept2 consists of several antigenic epitopes, the

reactivities of which differ from patient to patient. In addition, not a few sera reacted with epitopes in Ept3. However, this region was not equally recognized by all patients' sera. Taking these facts into account, only the epitope in Ept1 was recognized by all positive sera and therefore seems to have an association with the mechanisms triggering anti-SS-B/La antibody production.

In this study, several deletion mutants of FSBF were constructed enzymatically and used for the assessment of autoepitopes on the SS-B/La protein. In general, when proteins produced by fragments from a long cDNA are employed for epitope mapping, it needs to be determined whether the fragments are expressed in correct translational frames. It is especially important in the case of a fragment that expresses no detectable antigen. In our experiments, FSBF and FSBB cDNAs were first subcloned into expression plasmids in three different translational frames. As expected, only the clone with the correct translational frame produced a distinct antigenic protein, and it was selected for further analyses. However, the other two series of clones with incorrect frames sometimes expressed slight antigenicity in an unexpected range of molecular weight, a finding also observed in the λgt11 system (unpublished observation). The reason for this is not yet clear. Judging from the molecular weight and the amount of weakly stained recombinant protein, it could be speculated that a portion of the frame after the authentic stop codon is translated, probably because of the strong expression system in E. coli. To avoid making deletion mutants with an incorrect translational frame, we mainly used ExoIII treatment at the 3' end of the gene, and many deletion mutants were constructed without changing their frames. However, with this procedure it is difficult to determine the precise NH2-terminal boundary of the epitope and to identify the minor epitopes residing on the COOH-terminal side of the major epitopes. It is also possible that the epitope is discontinuous and the identified short peptide is a part of it. Therefore, to identify the precise range of the peptide as an autoepitope, cDNAs deleted from the NH2-terminal by other manipulations or synthetic peptides could be useful. These systems, however, do not always work properly. Thus, the method used in this study seems sufficient to determine the location of the major epitopes on the autoantigens.
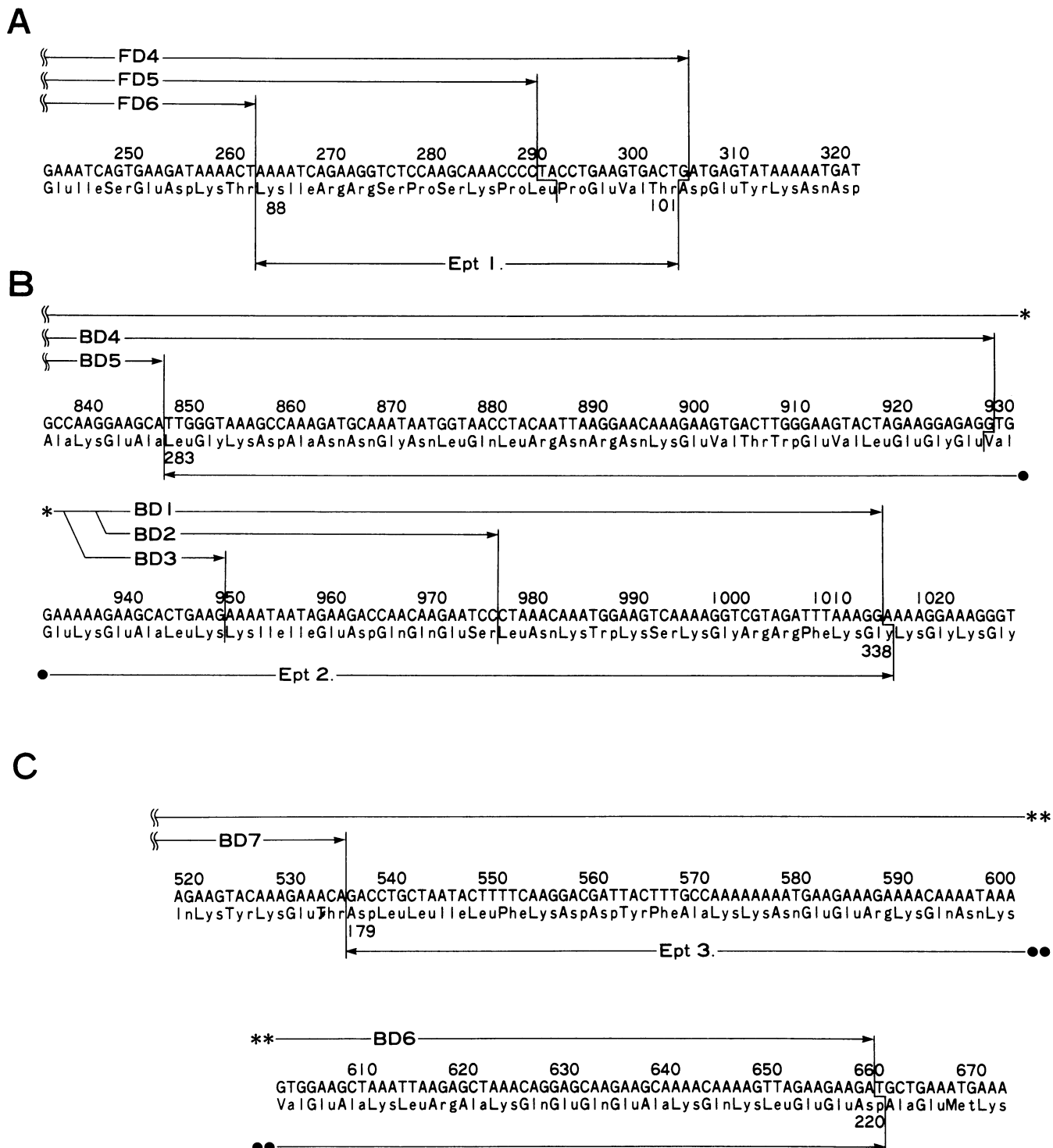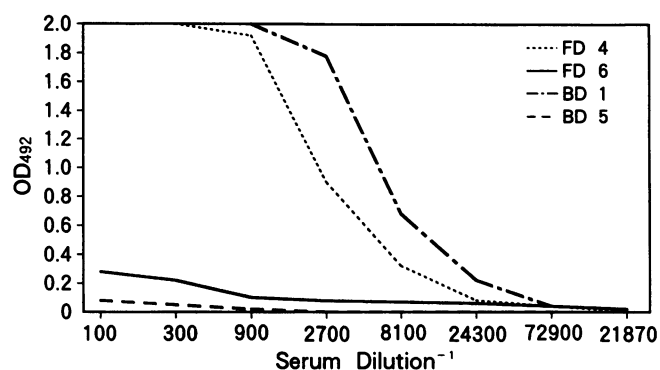
A

FD4
FD5
FD6

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      250       260       270       280       290       300       310       320
GAAATCAGTGAAGATAAAACTAAAATCAGAAGGTCTCCAAGCAAACCCCTACCTGAAGTGACTGATGAGTATAAAAATGAT
GluIleSerGluAspLysThrLysIleArgArgSerProSerLysProLeuProGluValThrAspGluTyrLysAsnAsp
                     88                                 101
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Ept 1.

B

BD4
BD5

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      840       850       860       870       880       890       900       910       920      930
GCCAAGGAAGCATTGGGTAAAGCCAAAGATGCAAATAATGGTAACCTACAATTAAGGAACAAAGAAGTGACTTGGGAAGTACTAGAAGGAGAGGTG
AlaLysGluAlaLeuGlyLysAspAlaAsnAsnGlyAsnLeuGlnLeuArgAsnArgAsnLysGluValThrTrpGluValLeuGluGlyGluVal
            283
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BD1
BD2
BD3

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      940       950       960       970       980       990      1000      1010      1020
GAAAAAGAAGCACTGAAGAAAAATAATAGAAGACCAACAAGAATCCCTAAACAAATGGAAGTCAAAAGGTCGTAGATTTAAAGGAAAAAGGAAAGGGT
GluLysGluAlaLeuLysLysIleIleGluAspGlnGlnGluSerLeuAsnLysTrpLysSerLysGlyArgArgPheLysGlyLysGlyLysGly
                                                                                         338
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Ept 2.

C

BD7

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      520       530       540       550       560       570       580       590       600
AGAAGTACAAAGAAACAGACCTGCTAATACTTTTCAAGGACGATTACTTTGCCAAAAAAAATGAAGAAAGAAAACAAAATAAA
InLysTyrLysGluThrAspLeuLeuIleLeuPheLysAspAspTyrPheAlaLysLysAsnGluGluArgLysGlnAsnLys
                   179
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Ept 3.

BD6

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      610       620       630       640       650       660       670
GTGGAAGCTAAATTAAGAGCTAAACAGGAGCAAGAAGCAAAACAAAAGTTAGAAGAAGATGCTGAAATGAAA
ValGluAlaLysLeuArgAlaLysGlnGluGlnGluAlaLysGlnLysLeuGluGluAspAlaGluMetLys
                                                      220
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Figure 4. Nucleotide and amino acid sequences around the three autoepitope areas on the SS-B/La protein. Ept1 is located between the COOH-terminals of FD4 and FD6. Ept2 is between BD1 and BD5. Ept3 is between BD6 and BD7. This figure shows the 3' end nucleotide sequence of each deletion mutant surrounding Ept1 (*A*), Ept2 (*B*), and Ept3 (*C*). The amino acids that were deduced are also shown.

The pEX vectors that we used have certain advantages. The pEXs inserted by cDNAs express cro-β-galactosidase hybrid protein in inclusion bodies, the low solubility of which protects the products against proteolysis. Moreover, the products of deletion mutants have naturally diverse molecular weights. Since the non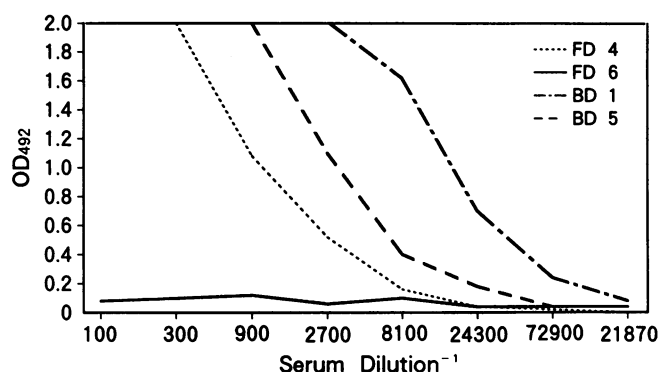-recombinant pEX product, β-galactosidase, has a much larger molecular weight than cDNA products, various deletion mutants are able to be analyzed under nearly the same condition without considering the difference in molecular weights.

Epitope mapping of the recombinant human SS-B/La protein has been reported by several investigators. Sturgess et al.

A

Graph A: Y-axis OD492 (0 to 2.0), X-axis Serum Dilution⁻¹ (100, 300, 900, 2700, 8100, 24300, 72900, 21870). Legend: FD 4, FD 6, BD 1, BD 5.

B

Graph B: Y-axis OD492 (0 to 2.0), X-axis Serum Dilution⁻¹ (100, 300, 900, 2700, 8100, 24300, 72900, 21870). Legend: FD 4, FD 6, BD 1, BD 5.

Figure 5. Quantitative analysis of anti-SS-B/La antibodies binding to the products encoded by deleted cDNAs. Multititer wells were coated with fusion proteins produced by recombinant pEXs (FD4, FD6, BD1, BD5). Sera from patients proved to have anti-SS-B/La antibodies were diluted with 1% skim milk at 1:100 to $1:3^7 \times 100$ and applied to the wells. The final data were calculated by subtraction of the value against the pEX protein from that against recombinant proteins. Sera from patients 1 (*A*) and 2 (*B*) reacted potently to the proteins produced by FD4 and BD1, while neither reacted to FD6. However, serum from patient 2 still reacted to BD5 less intensively than to BD1.

Table II. Antibodies Binding against the Hybrid Proteins Expressed by FD4, FD6, BD1, and BD5

Patient No.	Binding units			
	FD4	FD6	BD1	BD5
1	2,400	<100	6,000	<100
2	1,200	<100	16,800	2,900
3	110	<100	1,800	<100
4	7,300	<100	35,000	6,900
5	9,900	<100	34,500	1,400
6	930	<100	3,100	190
7	6,400	<100	17,400	960
8	1,900	<100	6,900	3,300
9	930	<100	2,100	690
10	130	<100	220	<100
11	2,200	<100	6,400	730
12	5,400	<100	48,100	14,000
13	150	<100	1,000	320
14	550	<100	1,000	800
15	580	<100	2,400	<100
16	100	<100	580	150
17	2,500	<100	14,000	7,800
18	1,600	<100	3,800	<100
19	1,800	<100	420	410
20	200	<100	3,700	1,600
21	2,500	<100	1,100	260
22	100	<100	1,100	390
23	2,800	<100	17,100	12,800
24	100	<100	100	<100
25	230	<100	600	150
26	3,000	<100	11,500	6,400
27	1,000	<100	4,900	2,600
28	270	<100	860	500
Healthy donors	<100	<100	<100	<100

28 sera from patients with precipitating anti-SS-B/La autoantibodies were assayed by ELISA. Binding units are defined as the reciprocal of the serum dilution producing an OD_{492} of 1.0. Sera from healthy donors did not react with the recombinant autoantigens. Less than 100 binding units indicates an eventual lack of binding, i.e., OD_{492} values were below 0.3 in 1:100 dilution.
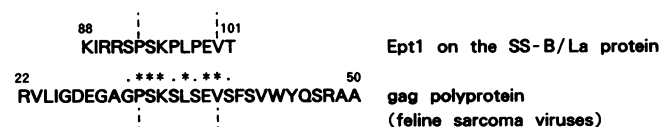
(14), using cDNA encoding a part of the SS-B/La protein, reported the 103 amino acid COOH-terminal fragment as the only autoepitope. They failed to find antigenicity in Ept1 and the region of Ept3. They did not succeed in expressing any of the 5' parts of cDNA fragments and did not ensure the expression of the protein in the correct translational frame. Rauh et al. (15) used a non-full-length cDNA divided into many fragments by various restriction enzymes. They also used the pEX vector system for expression. They revealed at least three segments (amino acids 224-292, 293-335, 336-380) on the SS-B/La protein that contained autoepitopes. This result is compatible with ours, although their assignments were rather broad and, most importantly, their cDNA did not cover the Ept1 segment. St. Clair et al. (16) used three non-overlapping cDNAs covering almost the whole protein coding region and expressed them in the λgt11 system. They reported that antigenic sites resided in a middle fragment (amino acid 111-242) of the SS-B/La protein. It was the first study in which a nearly full-length cDNA had been engaged. However, there are some

questionable points. First, the hybrid protein, reported to contain the amino acid 1-107 fragment, had a higher molecular weight in polyacrylamide/SDS gels than those containing longer segments of the SS-B/La protein. Next, in their ELISA assessment, the amino acid 111-408 fragment was more anti-

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88            101
   KIRRSPSKPLPEVT                Ept1  on  the  SS-B/La  protein
22        .***.*.**.        50
RVLIGDEGAGPSKSLSEVSFSVWYQSRAA   gag  polyprotein
                                (feline  sarcoma  viruses)
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Figure 6. Homologous amino acid sequence of Ept1 on the SS-B/La protein and feline sarcoma virus gag polyprotein. Upper sequence represents amino acid 88-101 in Ept1 on the SS-B/La protein. Lower sequence represents amino acid 22-50 in the gag polyprotein. Asterisks indicate amino acids that are identical in the two peptides. One dot indicates those that are analogous in their biochemical characteristics.

genic with patients' sera than the 111-242 fragment, while the 242-408 fragment had little antigenicity. As we described before, λgt11 with cDNA in incorrect translational frames could produce small amounts of proteins with unexpectedly high molecular weights even if the induced major hybrid proteins with lower molecular weights had no antigenicity. Therefore it is possible that the amino acid 1-107 and 242-408 fragments described in their paper were produced by expression in incorrect translational frames. The different assessment of Ept1 and Ept2, located in amino acid 1-107 and 242-408 fragments, respectively, might be resulted from the incorrect expression or from different population of the patient sera.

Comparison of Ept1, Ept2, and Ept3 sequences with reported amino acid sequences in databases disclosed a high degree of homology between the amino acid 93-100, the COOH-terminal part of Ept1, and the amino acid 32-39 of feline sarcoma virus gag polyprotein. Between unrelated proteins, the probability of matching six amino acids from eight amino acids is calculated to be 4.4×10^{-7}. Moreover, two different amino acids within this homologous region and flanking amino acids are conservative changes. The probability of having such changes is extremely low. The amino acid 32-39 resides in the leader peptide of the gag polyprotein. Besides, as we discussed, the COOH-terminal part of Ept1 seemed to be the antigenic site. It is likely that the autoepitope on Ept1 has actual homology to the gag polyprotein of the viruses or similar unknown viruses. Retrovirus infections are known to cause a broad range of chronic systemic diseases. They can be associated with oncogenesis or the pathogenesis of adult immunodeficiency syndrome. Quary and Keene (30) reported previously that part of the 70-kD protein of U1 snRNP was homologous to retroviral p30^{gag} protein and they were immunologically cross-reactive. Maul et al. (31) recently reported that one epitope on another autoantigen, human DNA topoisomerase I was homologous to the other region of retrovirus p30^{gag} protein. Even though the homologous epitopes have not shown to be the most distinct epitope, these reports suggested that retroviral infection might cause autoimmunity against some nucleoproteins and play a role on pathogenesis of autoimmune diseases. In Sjögren's syndrome, environmental factors, especially viral infections have been thought to play an important role in the initiation of the disease (32-34). In our study, we demonstrated that the region containing peptide homologous to a viral gag protein was universally recognized as the most distinct autoepitope. This leads us to the following speculations on the initiation of anti-SS-B/La autoimmunity. First, infection with exogenous retroviruses or expression of endogenous retroviruses that have structures similar to feline sarcoma viruses evoke an immune response against their gag proteins. Next, a certain expanded B cell repertoire against the gag protein could react with Ept1 on the SS-B/La protein via cross-reactivity, and these B cells could present a part of the SS-B/La molecule to a host immune system as an immunogenic peptide, as suggested by Lanzavecchia (35). Alternatively, considering the nature of a leader protein which is likely to be within the cells, this homologous region could elicit T cell responses against the self SS-B/La protein. Thereafter the other parts of the SS-B/La protein with potential immunogenicity might begin to be recognized. Since antibody production against these additional epitopes seems to be defined not only by the biochemical character of the peptide but also the genetic background of the individual, they are not necessarily recognized equally by all patients' sera. Indeed, reactivity against the epitopes located on Ept2 and Ept3 varied according to the individual patient's serum, while Ept1 was reactive with all sera. Efforts to examine the cross-reactivity of the gag protein with the Ept1 peptide are in progress.

However, these evaluations should be carefully performed. Synthetic oligopeptides do not always represent the antigenicities of the real molecules. In addition, we suspect that feline sarcoma virus might not be the casative agent of Sjögren's syndrome. Another unknown virus having a similar structure could be the candidate. Thus we are now trying to express the large region of the gag leader protein in *E. coli* and to exchange some amino acids by site directed mutagenesis.

References

1. Tan, E. M. 1982. Autoantibodies to nuclear antigens (ANA): their immunobiology and medicine. *Adv. Immunol.* 33:167–240.

2. Tan, E. M. 1989. Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. *Adv. Immunol.* 44:93–151.

3. Hang, L., J. H. Slack, C. Amundson, S. Izui, A. N. Theofilopoulos, and F. Dixon. 1983. Induction of murine autoimmune disease by chronic polyclonal B cell activation. *J. Exp. Med.* 157:874–883.

4. Tan, E. M., E. K. L. Chan, K. F. Sullivan, and R. L. Rubin. 1988. Antinuclear antibodies (ANAs): diagnostically specific immune markers and clues toward the understanding of systemic autoimmunity. *Clin. Immunol. Immunopathol.* 47:121–141.

5. Oldstone, M. B. A. 1987. Molecular mimicry and autoimmune disease. *Cell.* 50:819–820.

6. Harley, J. B., E. L. Alexander, W. B. Bias, O. F. Fox, T. T. Provost, M. Rejchlin, H. Yamagata, and F. C. Arnett. 1986. Anti-Ro(SS-B) and anti-La (SS-B) in patients with Sjogren's syndrome. *Arthritis Rheum.* 29:196–206.

7. Rinke, J., and J. A. Steitz. 1982. Precursor molecules of both human 5S ribosomal RNA and transfer RNAs are bound by a cellular protein reactive with anti-La lupus antibodies. *Cell.* 29:149–159.

8. Lerner, M. R., J. A. Boyle, J. A. Hardin, and J. A. Steitz. 1981. Two novel class of small ribonucleoproteins detected by antibodies associated with lupus erythematosus. *Science (Wash. DC.)* 211:400–402.

9. Lerner, M. R., N. C. Andrews, G. Miller, and J. A. Steitz. 1981. Two small RNAs encoded by Epstein-Barr virus and complexed with protein are precipitated by antibodies from patients with systemic lupus erythematosus. *Proc. Natl. Acad. Sci. USA.* 78:805–809.

10. Kurilla, M. G., and J. D. Keene. 1983. The leader RNA of vesicular stomatitis virus is bound by a cellular protein reactive with anti-La lupus antibodies. *Cell.* 34:837–845.

11. Wilusz, J., and J. D. Keene. 1984. Interactions of plus and minus strand leader RNAs of the New Jersey serotype of vesicular stomatitis virus with the cellular La protein. *Virology.* 135:65–73.

12. Keene, J. D., S. L. Duetscher, D. J. Kenan, and A. Kelekar. 1987. Nature of the La and Ro RNPs. *Mol. Biol. Rep.* 12:235–238.

13. Chan, E. K. L., A. Francoeur, and E. M. Tan. 1986. Epitopes, structural domains, and asymmetry of amino acid residues in SS-B/La nuclear protein. *J. Immunol.* 136:3744–3749.

14. Sturgess, A. D., M. G. Peterson, L. J. McNeilage, S. Whittingham, and R. L. Coppel. 1988. Characteristics and epitope mapping of a cloned human autoantigen La. *J. Immunol.* 140:3212–3218.

15. Rauh, A. J. G., H. Hornig, and R. Luhrmann. 1988. At least three distinct B cell epitopes reside in the C-terminal half of La protein, as determined by a recombinant DNA approach. *Eur. J. Immunol.* 18:2049–2057.

16. St. Clair, E. W., D. S. Pisetsky, C. F. Reich, and J. D. Keene. 1988. Analysis of autoantibody binding to different regions of the human La antigen expressed in recombinant fusion proteins. *J. Immunol.* 141:4173–4180.

17. Chambers, J. C., D. Kenan, B. J. Martin, and J. D. Keene. 1988. Genomic structure and amino acid sequence domains of the human La autoantigen. *J. Biol. Chem.* 263:18043–18051.

18. Yamamoto, K., H. Miura, Y. Moroi, S. Yoshinoya, M. Goto, K. Nishioka, and T. Miyamoto. 1988. Isolation and characterization of a complementary DNA expressing human U1 small nuclear ribonucleoprotein C polypeptide. *J. Immunol.* 140:311–317.

19. Kohsaka, H., K. Yamamoto, H. Fujii, N. Miyasaka, H. Miura, Y. Tanaka, K. Nishioka, and T. Miyamoto. 1989. Molecular cloning of cDNAs expressing SS-B/La protein. *J. Autoimmunity.* 2(Suppl.):353–357.

20. Young, R. A., and R. W. Davis. 1983. Yeast RNA polymerase II genes: Isolation with antibody probes. *Science (Wash. DC).* 222:778–782.

21. Stanley, K. K., and J. P. Luzio. 1984. Construction of a new family of high efficiency bacterial expression vectors: identification of cDNA clones coding for human liver proteins. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1429–1434.

22. Okayama, H., and P. Berg. 1983. A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. *Mol. Cell. Biol.* 3:280–289.

23. Olmsted, J. B. 1981. Affinity purification of antibodies from diazotized paper blots of heterogenous protein samples. *J. Biol. Chem.* 256:11955–11957.

24. Henikoff, S. 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. 1984. *Gene.* 28:351–359.

25. Putney, S. D., S. J. Benkovic, and P. R. Schimmel. 1981. A DNA fragment with an α-phosphorothioate nucleotide at one end is asymmetrically blocked from digestion by exonuclease III and can be replicated in vivo. *Proc. Natl. Acad. Sci. USA.* 78:7350–7354.

26. Sanger, F. 1981. Determination of nucleotide sequence in DNA. *Science (Wash. DC).* 214:1205–1210.

27. Chan, E. K. L., K. F. Sullivan, and E. M. Tan. 1989. Ribonucleoprotein SS-B/La belongs to a protein family with consensus sequence for RNA-binding. *Nucleic Acids Res.* 17:2233–2244.

28. Besmer, P., J. E. Murphy, P. C. George, F. Qiu, P. J. Bergold, L. Ledermen, H. W. Snyder, Jr., D. Brodeur, E. E. Zuckerman, and W. D. Hardy. 1986. A new acute transforming feline retrovirus and relationship of its oncogene v-kit with the protein kinase gene family. *Nature (Lond.).* 320:415–420.

29. Hampe, A., M. Gobet, J. Even, C. J. Sherr, and F. Galibert. 1983. Nucleotide sequences of feline sarcoma virus long terminal repeats and 5' leaders show extensive homology to those of other mammalian retroviruses. *J. Virol.* 45:466–472.

30. Query, C. C., and J. D. Keene. 1987. A human autoimmune protein associated with U1 RNA contains a region of homology that is cross-reactive with retroviral p30gag antigen. *Cell.* 51:211–220.

31. Maul, G. G., S. A. Jimenez, E. Riggs, and D. Ziemnicka-Kotula. 1989. Determination of an epitope of the diffuse systemic sclerosis marker antigen DNA topoisomerase I. *Proc. Natl. Acad. Sci. USA.* 86:8492–8496.

32. Whittingham, S., J. McNeilage, and I. R. Mackay. 1985. Primary Sjögren's syndrome after infectious mononucleosis. *Ann. Intern. Med.* 102:490–493.

33. Yamaoka, K., N. Miyasaka, and K. Yamamoto. 1988. Possible involvement of Epstein-Barr virus in polyclonal B cell activation in Sjögren's syndrome. *Arthritis & Rheum.* 31:1014–1021.

34. Green, J. E., S. H. Hinrichs, J. Vogel, and G. Jay. 1989. Exocrinopathy resembling Sjögren's syndrome in HTLV-1 tax transgenic mice. *Nature (Lond.).* 341:72–74.

35. Lanzavecchia, A. 1988. Clonal sketches of the immune response. *EMBO (Eur. Mol. Biochem. Organ.) J.* 7:2945–2951.