

Protein Heterogeneity in the Human Ro/SSA Ribonucleoproteins

The 52- and 60-kD Ro/SSA Autoantigens Are Encoded by Separate Genes

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Abstract

Two cDNA clones encoding the 52-kD form of a protein present in human Ro/SSA ribonucleoprotein complexes were cloned from a λ gt11 human thymocyte cDNA library. These clones reacted with lupus patient sera which had anti-52-kD Ro/SSA antibodies, and with affinity-purified anti-52-kD Ro/SSA antibodies. Moreover, affinity-purified antibodies isolated from isopropyl- β -D-thiogalactopyranoside-induced proteins of these clones reacted only with the 52-kD protein of lymphocytes in Western blots and precipitated Ro/SSA hY RNAs, confirming that the clones encode a 52-kD Ro/SSA antigen.

The cDNA contains a single open reading frame of 1,425 nucleotides and encodes a predicted 475-amino acid polypeptide with a molecular mass of 54,108 D. This protein appears unique in that both a zinc finger and leucine zipper motif are present on this protein. Surprisingly, no homology was found between the 52-kD Ro/SSA gene or protein and three published 60-kD Ro/SSA sequences. However, significant similarity of the 52-kD Ro/SSA was detected with human rfp and mouse rpt-1. These three proteins each contain similar zinc finger motifs in approximately their first 145 amino acid residues. The cDNA and the protein expressed therefrom are useful in the analysis of the structural and functional properties of this autoantigen. (*J. Clin. Invest.* 1991. 87:177-186.) **Key words:** autoimmunity • human autoantigen nucleotide sequence • systemic lupus erythematosus

Introduction

Ro/SSA is a ribonucleoprotein particle composed of a single polypeptide and one of four small RNA molecules (1-3). It is present in all mammalian cells studied but has no known function. The antigenic portions of the Ro/SSA particle reside on the polypeptide chain (4). Autoantibodies to Ro/SSA are found in the sera of 30-50% of patients with systemic lupus erythematosus (SLE)¹ and in at least 50% of patients with primary Sjogren's syndrome (3, 5). These autoantibodies occur in a higher percentage in patients with subacute cutaneous lupus erythematosus, and in essentially all neonatal lupus erythematosus patients with congenital heart block (6, 7). The presence of

anti-Ro/SSA antibodies is associated with lymphopenia, photosensitive dermatitis, pulmonary disease, and renal disease in lupus patients (8-13), suggesting a pathogenic role for these antibodies in this disease.

Recent studies have shown heterogeneity in the molecular structure of the Ro/SSA antigen. Anti-Ro/SSA antibodies from SLE patients eluted from Western blots of human lymphocyte extracts indicate that a 60- and 52-kD protein are associated with the hY Ro/SSA RNAs in nucleated cells (14, 15). In human red blood cells, Ro/SSA particles contain either a 60- or 54-kD polypeptide. Immunoprecipitation of these particles reveals that they associate with only a subset of the hY RNAs (15).

Studies from our laboratory have shown that the 60-kD Ro/SSA polypeptides in lymphocyte and red blood cell are distinct but related. Most patient autoantibodies which bind to the 60-kD Ro/SSA lymphocyte polypeptide cross-react with the 60-kD red blood cell Ro/SSA polypeptide. However, certain patients produce antibodies that recognize only one form of this protein. Similarly, antibodies binding to the lymphocyte 52-kD Ro/SSA polypeptide cross react in most but not all cases with the red blood cell 54-kD Ro/SSA protein. Thus, the four isoforms of Ro/SSA fall into two non-cross-reacting families (16).

The majority of biochemical studies of Ro/SSA polypeptides have centered around the analysis of the 60-kD containing particles. Although Western blot analyses using different patient sera and tissue extracts show no evidence for heterogeneity in the 60-kD proteins, three different sequences of the gene encoding 60-kD Ro/SSA have been reported. Two of them differ only in the region encoding the carboxy-terminal amino acid residues (17, 18); the third is entirely different from these two sequences (19). To determine the relationship between the 60- and 52-kD Ro/SSA proteins, we have cloned a gene which encodes the human 52-kD form of the Ro/SSA protein. In this study, we report the sequence of this gene and show that it is unique from the reported 60-kD Ro/SSA genes. The 52-kD Ro/SSA polypeptide is not a degradation product of the 60-kD form, consistent with the lack of immunological cross-reactivity between these proteins. Furthermore, the 52-kD Ro/SSA gene encodes both zinc finger and leucine zipper motifs, both of which have been implicated in nucleic acid and protein interactions. This study demonstrates further the considerable heterogeneity that exists in the Ro/SSA autoantigenic particles, and has important implications in the generation of an autoimmune response to Ro/SSA in patients with certain rheumatic diseases.

Methods

Adsorption of *Escherichia coli* antibodies. Sera from patients with systemic lupus erythematosus (20) and controls were adsorbed against *E. coli* after lysis with the bacteriophage vector λ gt11 to deplete naturally occurring anti-*E. coli* antibodies. Briefly, five petri dishes with 30,000-

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1. Abbreviations used in this paper: IPTG, isopropyl- β -D-thiogalactopyranoside; SLE, systemic lupus erythematosus.

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50,000 plaque-forming units (pfu) were plated with *E. coli* strain Y1090 cells on LB agar and grown for 3.5 h at 42°C. The cultures were overlaid with a single nitrocellulose filter for 3 h on one side and 2 h on the other side at 37°C. Filters were washed in TBST (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween-20), and then sequentially incubated at room temperature with a 1:100 dilution of human serum. Adsorptions were monitored by exposing a small piece of each filter with an alkaline phosphatase-conjugated, goat anti-human IgG antisera (Sigma Chemical Co., St. Louis, MO). The sections of these filters were then reacted with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) according to the ProtoBlot system (Promega Biotec, Madison, WI).

Screening a human cDNA library. A human thymocyte cDNA library cloned in the λ gt11 expression vector was obtained from Clontech Laboratories, Palo Alto, CA. 30,000 pfu per petri dish were plated with *E. coli* Y1090 cells on LB agar. The partially lysed *E. coli* lawn was overlaid for 3 h at 37°C with a nitrocellulose membrane which had previously been soaked in 10 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Filters were washed in TBST, incubated with an *E. coli*-adsorbed patient serum which contained antibodies to the 52-kD Ro/SSA protein, and screened using the alkaline phosphatase method described above. Blue-colored plaques were purified using the same patient serum.

Affinity purification of antibodies from patient serum. Antibodies were affinity-purified from two sources using either nitrocellulose membranes from (a) Western blots of polyacrylamide gel-separated human lymphocyte extracts, or (b) blots of IPTG-induced proteins from plaque purified bacteriophage clones. Lymphocytes were lysed by sonication in water and centrifuged to remove debris. Supernatants were reduced, heat-denatured, and subjected to electrophoresis on a 10% polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose membranes (21). To localize the region of the filter containing 52-kD proteins, a small section of the membrane was reacted with serum from a patient containing anti-52-kD Ro/SSA autoantibodies. Binding of patient antibodies was detected as described above. The corresponding section of the remainder of the membrane was then excised and incubated with patient serum using a modification of a method by Krohne et al. (22). Briefly, the membrane was incubated with serum for 30 min at 37°C, and the antibodies were eluted with 3 M sodium (vs. potassium) thiocyanate. The elution process was repeated five times and the resulting solution was concentrated ~50-fold using a Centriprep 30 concentrator (Amicon Division, W. R. Grace, Danvers, MA). Antibodies which bound to 60-kD Ro/SSA proteins, or proteins produced in IPTG-induced bacteriophage plaques were eluted in an identical manner.

Immunoprecipitation of Ro/SSA proteins and hY RNAs. Affinity-purified antibodies isolated from IPTG-induced bacteriophage plaques were used to immunoprecipitate nucleic acids bound to Ro/SSA proteins from HeLa cells (23). These antibodies were bound to staphylococcal protein A-coated Sepharose CL-4B beads (Pharmacia, Inc., Piscataway, NJ). HeLa cells were lysed by sonication in the presence of 0.05% NP-40 and mixed with these beads. Bound material was eluted with 0.3 M sodium acetate and 1% SDS, and subjected to phenol/chloroform extraction. Ethanol precipitated nucleic acids were dissolved in electrophoresis sample buffer and subjected to PAGE in the presence of 7 M urea. Silver staining was used to detect nucleic acids.

Characterization and sequencing of cDNA in bacteriophage clones. DNA was extracted from bacteriophage after pronase digestion. Restriction enzyme digestions were carried out under conditions recommended by the enzyme's suppliers. DNA was subjected to electrophoresis in 0.8% agarose gels and transferred to nylon membranes (Amersham Corp., Arlington Heights, IL) by the method of Southern (24). Isolated inserts were radioactively labeled with [α -³²P]dCTP using random hexamer primers (25, 26), and hybridized to nylon membranes in cross-hybridization studies. Following hybridization, membranes were washed under high stringency conditions to 0.1 \times SSPE (15 mM NaCl, 1 mM NaH₂PO₄, 0.1 mM EDTA, pH 7.0) and 0.1% SDS at 65°C. Hybridization was detected by autoradiography.

The cDNA inserts from bacteriophage clones were purified after electrophoresis in 5% *N,N*-bis-acrylylcystamine cross-linked polyacrylamide gels (Bio-Rad Laboratories, Richmond, CA), reduction with 2-mercaptoethanol, and DEAE ion-exchange chromatography. The resulting fragments were subcloned into an *Eco*RI-digested M13mp19 bacteriophage vector (27, 28). Additional DNA from clone FI18.1 was digested with restriction enzymes chosen for their ability to produce DNA fragments which could be directly subcloned into the multiple cloning sites of M13mp19. After ligation of these inserts to the vectors with T4 DNA ligase (Bethesda Research Laboratories, Gaithersburg, MD), DNA was transformed in *E. coli* strain JM103. Single stranded DNA from M13 subclones was prepared (27) and subjected to nucleotide sequencing using the dideoxy chain-termination method (29) with T7 DNA polymerase (30) (U.S. Biochemical Corp., Cleveland, OH). Nucleotide sequences were also determined from deletion subclones produced with T4 DNA polymerase (31).

Computer analyses of nucleotide and amino acid sequences were performed using the Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin (32) on a VAX 8250 computer, and with other programs written for use on a Cray Y-MP supercomputer at the Pittsburgh Supercomputing Facility or on a IBM PC, including modification of those described by Schwindinger and Warner (33).

Results

Isolation of the 52 kD Ro/SSA cDNA clones. A human thymocyte cDNA library cloned into the bacteriophage vector λ gt11 was screened with the lupus patient serum DU after IPTG induction of the β -galactosidase gene (Fig. 1A). Autoantibodies in this serum bind to the 52- but not 60-kD Ro/SSA polypeptide in Western blots of lymphocyte extracts. Two immunoreactive clones were detected from approximately one million pfu which were screened. These clones were plaque purified and have been designated "FI18.1" and "FI19.3." The binding of

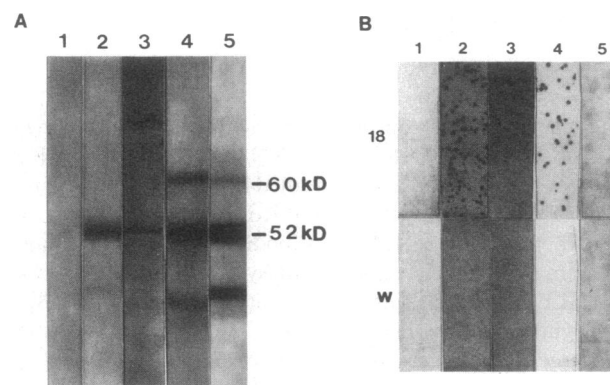


Figure 1. (A) Western blot of human lymphocyte extracts resolved by SDS-PAGE. Section of the nitrocellulose membrane to which the proteins were transferred were incubated with a normal human serum (lane 1) and patient sera DU, MO, BO, and DA (lanes 2, 3, 4, and 5, respectively). The 60- and 52-kD Ro/SSA proteins are labeled; the lower band in lanes 4 and 5 is the 45-kD breakdown product of the La/SSB protein. A summary of autoantibody specificities of these and other patients is listed in Table I. (B) Specific reaction of IPTG-induced proteins of clone FI18.1 (18) and a wild-type λ gt11 clone (W) with SLE patient and control sera. IPTG-induced proteins in bacteriophage plaques were bound to nitrocellulose membranes and incubated with a normal human serum (lane 1), patient sera DU and MO (lanes 2 and 3, respectively), affinity-purified anti-52-kD Ro/SSA antibody from patient BO (lane 4), and affinity-purified anti-60-kD Ro/SSA antibody from patient DA (lane 5).

DU antibodies to these bacteriophage clones was IPTG dependent, indicating that this reactivity was specific for cDNA-encoded epitopes, and not due to bacterial or vector-encoded antigens.

IPTG-induced proteins expressed in these clones were screened with a panel of sera from different SLE patients, with affinity-purified antibodies, as well as with normal human sera. All sera were adsorbed to remove naturally occurring anti-*E. coli* antibodies before use. The autoimmune specificity of five sera tested in this study as detected in Western blots of human lymphocyte extracts is shown in Fig. 1 A. The reactivity of IPTG-induced proteins in clone FI18.1 with these human sera is shown in the top portion of Fig. 1 B. Lane 2 shows the reactivity detected with serum DU, the serum used in the library screening. Reactivity was also detected with a patient serum (MO) which contains only anti-52-kD Ro/SSA reactivity (lane 3). No reactivity was detected with sera from healthy controls (lane 1), or with IPTG-induced λ gt11 infected *E. coli* plaques with any of these sera (bottom panels, Fig. 1 B). In all comparisons, qualitative and quantitative levels of reactivity of IPTG-induced proteins of clones FI18.1 and FI19.3 were found to be identical. A summary of all sera tested is summarized in Table I. It is clear from these experiments that autoan-

tibody reactivity with IPTG-induced recombinant proteins is absolutely correlated only with reactivity to 52-kD Ro/SSA protein. These data suggest the cDNA in these clones encode an epitope present on the 52- but not the 54- or 60-kD Ro/SSA protein.

It is possible that antibodies in the sera used above were binding to epitopes in these clones not detected in Western blots. In order to test this possibility, antibodies from either BO or DA were eluted (22) from the 45-, 52-, and 60-kD regions of Western blots of human lymphocyte extracts. Antibodies which were affinity purified from the 52-kD region of human lymphocyte extracts bound to the IPTG-induced proteins in this clone (Fig. 1 B, lane 4). However, affinity purified anti-60-kD antibodies failed to bind to either clone (lane 5), strongly suggesting that the cDNA encoded epitopes are present on 52- but not 60-kD Ro/SSA polypeptides. Antibodies eluted from the 45-kD La/SSB area of the Western blot also failed to bind to proteins expressed in these clones (data not shown).

IPTG-induced proteins of clones FI18.1, FI19.3, and wild-type λ gt11 were blotted to nitrocellulose filters. These filters were exposed to *E. coli*-adsorbed DU serum, and bound antibodies were eluted with 3 M NaSCN. Fig. 2 shows the results of Western immunoblotting of human lymphocyte extracts with these affinity-purified antibodies. DU serum antibodies bind to the 52-kD form of Ro/SSA (lane 1). Affinity-purified antibodies from this patient which were eluted from IPTG-induced clones FI18.1 and FI19.3 reacted specifically with a 52-kD lymphocyte protein (lanes 2 and 3, respectively). Antibodies eluted from IPTG-induced λ gt11 plaques failed to bind with this protein demonstrating the clonal specificity of this reaction (lane 4). The most parsimonious explanation for these findings

Table I. Sera Tested

Patient	Ro/SSA specificity*			Other autoantibodies [‡]	Clone reactivity [§]
	52	54	60		
TU	-	-	+	nRNP	-
DV	-	-	+	nRNP	-
VQ	-	-	+	nRNP	-
WS	-	-	+	nRNP	-
NE	+	+	+	-	+
ST	+	+	+	-	+
DS	+	+	+	-	+
CH	+	+	+	-	+
TR	+	+	+	La/SSB	+
BO	+	+	+	La/SSB	+
BM	+	+	+	La/SSB	+
DA	+	+	+	La/SSB	+
ME	+	+	+	La/SSB	+
DU	+	+	-	-	+
MO	+	-	-	-	+
NHS [†]	-	-	-	-	-

* The size of the Ro/SSA polypeptide detected in Western blots using different patient sera are shown. The 52- and 60-kD forms of the Ro/SSA proteins are detected in extracts of human lymphocytes. The 54-kD Ro/SSA protein is detected in extracts of human red blood cells. +, detection of an antibody which binds to the particular antigen; -, indicates no antibody to that antigen was detected.

[‡] Antibodies to other ribonucleoprotein complexes include those which bind to La/SSB and nRNP. The presence of these antibodies has been detected either by Ouchterlony immunodiffusion, Western blot analyses, or ELISA.

[§] Antibodies were tested against IPTG-induced proteins expressed by clone FI18.1.

^{||} Reacts with the 60-kD Ro/SSA in red blood cells but not with the 60-kD Ro/SSA in nucleated cells.

[†] NHS, normal human serum. No autoantibodies to the specificities tested were detected in sera from four healthy individuals.

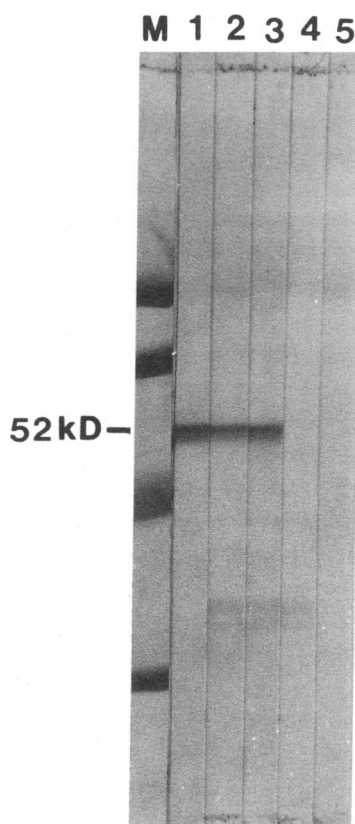


Figure 2. Characterization of affinity-purified antibodies isolated from IPTG-induced proteins of clones FI18.1 and FI19.3. Human lymphocyte extracts were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Protein molecular mass markers are shown in lane M (Pharmacia, Inc.) from high to low molecular mass: phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD). The Western blot was developed with patient DU serum (lane 1), affinity purified antibodies isolated from IPTG-induced proteins of clones FI18.1, FI19.3 and a wild-type λ gt11 clone (lanes 2-4, respectively), and normal human serum (lane 5).

is that a similar epitope exists on the 52-kD Ro/SSA protein and the cDNA-encoded protein. To further test this hypothesis, this experiment was repeated using serum from patient DA which contains not only anti-52-kD Ro/SSA antibodies, but also anti-60-kD Ro/SSA antibodies and anti-La/SSB antibodies. Antibodies eluted from clone FI18.1 reacted only with a 52-kD protein (data not shown); no anti-60-kD Ro/SSA or anti-La/SSB activity was detected, confirming the specificity of antibodies eluted from such membranes.

If this cDNA encodes the 52-kD Ro/SSA protein, a lysate of this clone should specifically block the anti-52- but not the anti-60-kD Ro/SSA reactivity detected on Western blots of human lymphocyte extracts. *E. coli* infected with either clone FI18.1 or λ gt11 wild-type bacteriophage were grown in 1 liter of culture with IPTG to maximal optical density. The cells were washed, resuspended in 10 ml of water, and lysed with lysozyme and sonication. These lysates were treated with 12.5 U of DNAase I to reduce viscosity and subsequently used as blocking agents in Western blots of human lymphocytes treated with sera containing both anti-52- and anti-60-kD Ro/SSA antibodies. Blocking of the anti-52- but not anti-60-kD Ro/SSA antibodies was observed only with lysates from the recombinant clone indicating a lack of cross-reactivity between the recombinant protein and the 60-kD form of Ro/SSA.

To determine if the 52-kD protein detected in the previous experiment is present in a Ro/SSA ribonucleoprotein particle, affinity-purified antibodies from clones FI18.1 and FI19.3 were used to immunoprecipitate particles from HeLa cells. After phenol extraction to remove proteins from the immunoprecipitated material, denaturing PAGE was performed to characterize nucleic acids that might be present in the samples. Fig. 3 shows that all hY Ro/SSA RNAs were found only when particles were immunoprecipitated with affinity-purified antibodies from clones FI18.1 and FI19.3. Small RNAs associated with La/SSB were not precipitated by these affinity purified antibodies, and no hY RNAs were observed using antibodies which were affinity-purified from wild-type λ gt11 transduced *E. coli* cells or serum from a healthy control. These results suggest that the 52-kD proteins detected on Western blots are proteins present in Ro/SSA particles and that the cDNA inserts of clone FI18.1 and FI19.3 encode at least a portion of this protein.

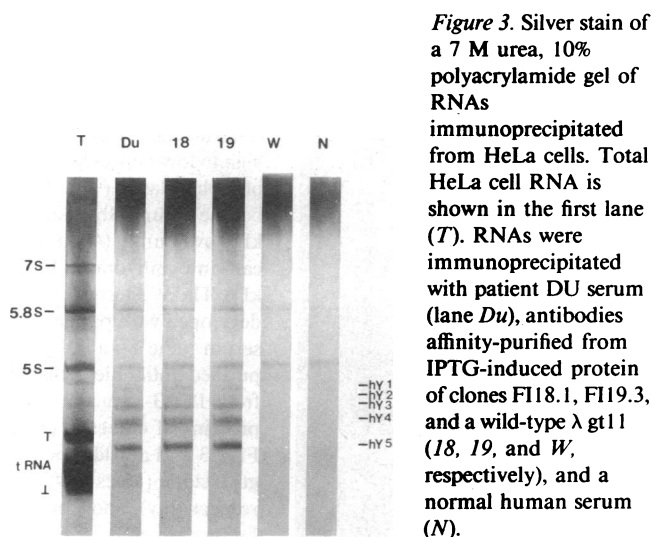


Figure 3. Silver stain of a 7 M urea, 10% polyacrylamide gel of RNAs immunoprecipitated from HeLa cells. Total HeLa cell RNA is shown in the first lane (T). RNAs were immunoprecipitated with patient DU serum (lane Du), antibodies affinity-purified from IPTG-induced protein of clones FI18.1, FI19.3, and a wild-type λ gt11 (18, 19, and W, respectively), and a normal human serum (N).

Characterization of the bacterially expressed Ro/SSA proteins. IPTG-induced proteins in clones FI18.1 and FI19.3 were further examined. Lysogens were constructed for each clone in *E. coli* strain Y1089. After IPTG induction, crude bacterial lysates were resolved in 7.5% SDS-polyacrylamide gels, transferred to nitrocellulose filters, and developed with either a mouse anti- β -galactosidase monoclonal antibody, DU patient serum, affinity-purified anti-52-kD Ro/SSA antibodies, or normal human sera. If the cDNA in clones FI18.1 or FI19.3 encoded a full-length polypeptide, a 168-kD β -galactosidase fusion protein was predicted when screened with either DU serum or the mouse anti- β -galactosidase antibody. Fig. 4 shows that bands close in size to the native β -galactosidase, and its degradation products, were detected with mouse anti- β -galactosidase antibodies. When using DU serum, a 48–50-kD band was unique to clones FI18.1 and FI19.3. A faint band \sim 160 kD specific to these clones was detected only with patient antibodies. The lack of reactivity of a similarly sized faint band detected with anti- β -galactosidase antibodies makes it questionable as to whether this weak band is a blotting artifact or an example of a β -galactosidase fusion protein which is produced and rapidly cleaved in these cells to yield two polypeptides, one approximately the size of β -galactosidase and the other just slightly smaller than the expected size of the 52-kD Ro/SSA protein. Anti-52-kD Ro/SSA affinity-purified antibodies eluted from Western blots of lymphocyte extracts bound to the this 50-kD protein expressed in these bacteria. Furthermore, antibodies eluted from the 50-kD protein expressed in bacteria bound specifically to a 52-kD lymphocyte protein and immunoprecipitated the Ro/SSA hY RNAs (not shown). All of these findings are consistent with the cDNA insert in each clone encoding the 52-kD Ro/SSA protein.

Sequence analysis of the 52-kD Ro/SSA cDNA clones. Bacteriophage DNA from clones FI18.1 and FI19.3 was digested with *EcoRI*, subjected to electrophoresis, and stained with ethidium bromide to determine the size of the cDNA inserts in

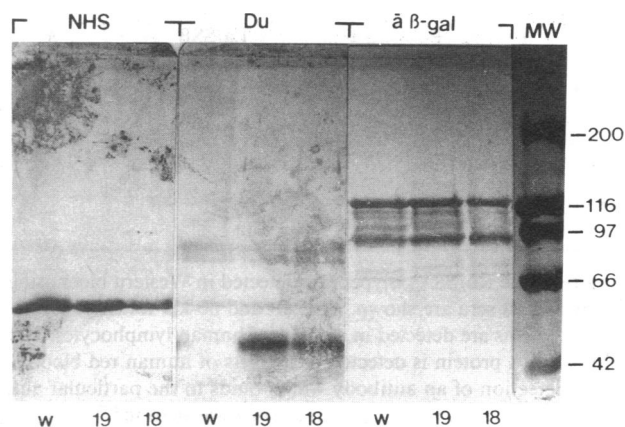


Figure 4. Western blot analysis of proteins expressed in *E. coli* lysogens. Lysogens from clone FI18.1, clone FI19.3, and a wild-type λ gt11 clone, designated 18, 19, and W, respectively at the bottom of the figure are shown following separation in a 7.5% SDS-polyacrylamide gel and transfer to a nitrocellulose membrane. The membrane was probed with normal human serum (NHS), DU patient serum, or a mouse anti- β -galactosidase antibody ($\bar{\alpha}$ β -gal). Sizes of protein molecular mass markers in kilodaltons are shown to the right of the figure.

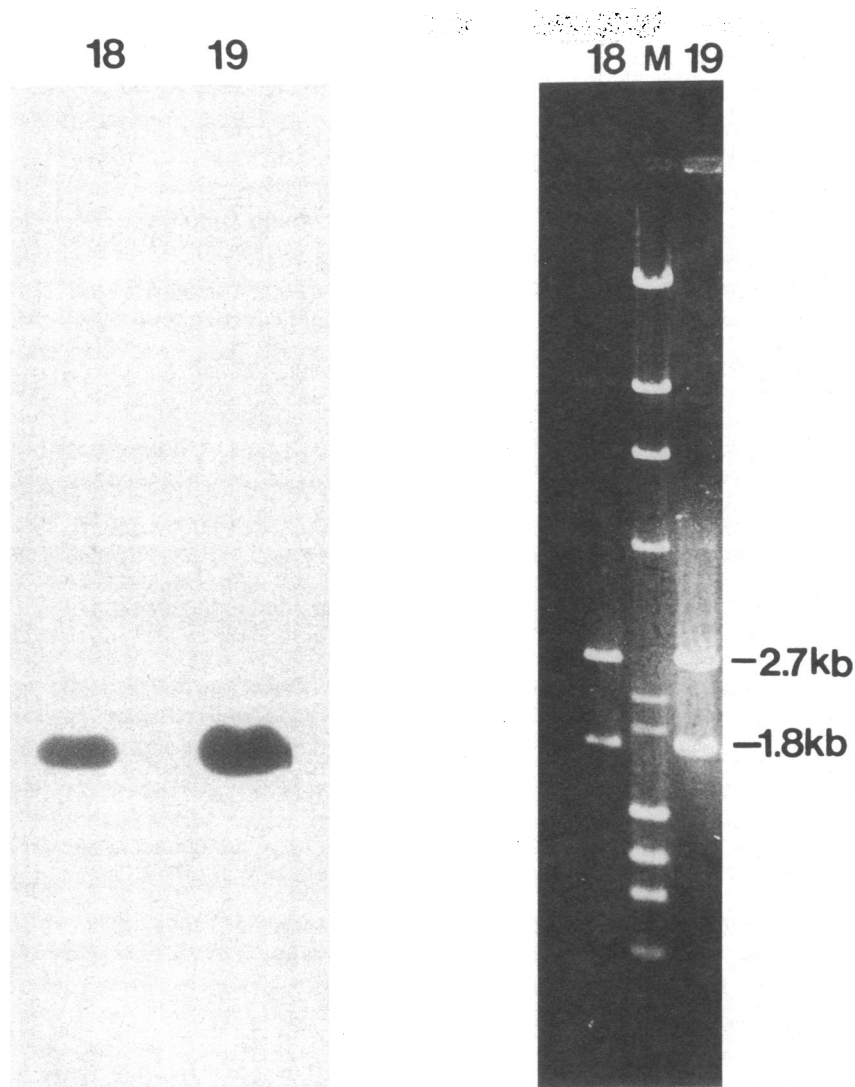


Figure 5. Southern blot hybridization of isolated cDNAs. cDNAs from clones FI18.1 (18) and FI19.3 (19) were subcloned into the pUC19 vector, digested with *EcoRI*, and electrophoresed in a 0.8% agarose gel (right). DNA was transferred to a nylon membrane (24) and hybridized to radiolabeled clone FI18.1 cDNA. Hybridization was detected with autoradiography (left).

these clones. A single 1.8-kb insert was present in each clone. Inserts from both clones were isolated and subcloned into the plasmid pUC19 (28). Similarity in the nucleotide sequence of these cDNAs was demonstrated in the following ways. First, DNA from each plasmid subclone was digested with *EcoRI*, subjected to electrophoresis in a 0.8% agarose gel, and transferred to a nylon membrane using the method of Southern (24). The isolated 1.8-kb insert from clone FI18.1 was radiolabeled (25, 26) and hybridized to this Southern blot. Fig. 5 shows cross hybridization between this probe and the insert from each clone. Secondly, products of restriction enzyme digestions of each insert with seven restriction enzymes were subjected to electrophoresis in polyacrylamide gels. Ethidium bromide staining of these fragments revealed no apparent differences between these clones (data not shown).

The FI18.1 and FI19.3 cDNAs were each subcloned into the M13mp19 bacteriophage vector (28) and their nucleotide sequence was determined using the method of Sanger et al. (29). No differences in the terminal sequences of FI18.1 and FI19.3 were detected. The complete nucleotide sequence of clone FI18.1 was determined from both strands and is shown in Fig. 6. A single open reading frame of 1,425 nucleotides extends from a methionine codon (ATG) at position 39 to a

stop codon (TGA) at position 1464. The nucleotide sequence adjacent to this initiation codon is consistent with an optimal eukaryotic consensus sequence for initiation of translation (34, 35). The coding region is followed by a 3' untranslated region characterized by numerous stop codons in all reading frames and a putative polyadenylation signal (AATAAA) at position 1827. If the *EcoRI* site at the 5' end of this cDNA was ligated to the *EcoRI* site of the *lacZ* gene of λ gt11, the 52-kD Ro/SSA cDNA would be out of frame with β -galactosidase. Therefore, we hypothesize that a small number of additional bases were present at the 5' end of the intact cDNA which produced a single open reading frame in the original λ gt11 clone, and that the 5' *EcoRI* site of this clone most likely represents an internal *EcoRI* site in the cDNA. Attempts to find a longer cDNA for this gene in our laboratory have proven to be unsuccessful.

The predicted amino acid sequence encoded by this cDNA is also shown in Fig. 6. This sequence encodes a 475-amino acid polypeptide with a predicted molecular mass of 54,108 D. 57 basic and 66 acidic amino acid residues were found, predicting a net negative charge for this protein. This protein possesses two motifs which have been associated with nucleic acid and protein interactions. Multiple zinc finger motifs are located in the amino-terminal end of the polypeptide between residues 16

1 GAATTCGGGCACACTGCTGTTTAAACGGCACACTTGACAATGGCTTCAGCAGCACGCTTGACAATGATGTGGGAGGAGGTCACATGCCCT
 MetAlaSerAlaAlaArgLeuThrMetMetTrpGluGluValThrCysPro 17
 90 ATCTGCCTGGACCCCTTCGTGGAGCCTGTGAGCATCGAGTGTGGCCACAGCTTCTGCCAGGAATGCATCTCTCAGGTTGGGAAAGGTGGG
 IleCysLeuAspProPheValGluProValSerIleGluCysGlyHisSerPheCysGlnGluCysIleSerGlnValGlyLysGlyGly 47
 180 GGCAGCGTCTGTCTGTGTGCCGGCAGCGCTTCTGCTCAAGAATCTCCGGCCCAATCGACAGCTAGCCAACATGGTGAACAACCTTAAA
 GlySerValCysProValCysArgGlnArgPheLeuLeuLysAsnLeuArgProAsnArgGlnLeuAlaAsnMetValAsnAsnLeuLys 77
 270 GAAATCAGCCAGGAGGCCAGAGAGGGGCACACAGGGGGAACGGTGTGCAGTGCATGGAGAGAGACTTCACCTGTTCTGTGAGAAAGATGGG
 GluIleSerGlnGluAlaArgGluGlyThrGlnGlyGluArgCysAlaValHisGlyGluArgLeuHisLeuPheCysGluLysAspGly 107
 360 AAGGCCCTTTGCTGGGTATGTGCCAGTCTCGGAAACACCGTGACCACGCCATGGTCCCTCTTGAAGGAGGCTGCACAGGAGTACCAGGAG
 LysAlaLeuCysTrpValCysAlaGlnSerArgLysHisArgAspHisAlaMetValProLeuGluGluAlaAlaGlnGluTyrGlnGlu 137
 450 AAGCTCCAGGTGGCATTAGGGGAACTGAGAAGAAACAGGAGTGGCTGAGAAGTTGGAAGTGGAAATTGCAATAAAGAGAGCAGACTGG
 LysLeuGlnValAlaLeuGlyGluLeuArgArgLysGlnGluLeuAlaGluLysLeuGluValGluIleAlaIleLysArgAlaAspTrp 167
 540 AAGAAAACAGTGGAAACACAGAAATCTAGGATTCACGCAGAGTTTGTGCAGCAAAAAAATCTCTGTTGAAGAACAACAGAGGCAGCTG
 LysLysThrValGluThrGlnLysSerArgIleHisAlaGluPheValGlnGlnLysAsnPheLeuValGluGluGluGlnArgGlnLeu 197
 630 CAGGAGCTGGAGAAGGATGAGAGGGAGCAGCTGAGAATCTGGGGGAGAAAGAGGCCAAGCTGGCCAGCAGAGCCAGGCCCTACAGGAG
 GlnGluLeuGluLysAspGluArgGluGlnLeuArgIleLeuGlyGluLysGluAlaLysLeuAlaGlnGlnSerGlnAlaLeuGlnGlu 227
 720 CTCATCTCAGAGCTAGATCGAAGGTGCCACAGCTCAGCACTGGAAGTGTGCAGGAGGTGATAATTGCTCTGAAAAGGAGTGAGTCTGG
 LeuIleSerGluLeuAspArgArgCysHisSerSerAlaLeuGluLeuLeuGlnGluValIleIleValLeuGluArgSerGluSerTrp 257
 810 AACCTGAAGGACCTGGATATTACCTCTCCAGAACTCAGGAGTGTGTGCCATGTGCCAGGGCTGAAGAAGATGCTGAGGACATGTGCAGTC
 AsnLeuLysAspLeuAspIleThrSerProGluLeuArgSerValCysHisValProGlyLeuLysLysMetLeuArgThrCysAlaVal 287
 900 CACATCACTCTGGATCCAGACACAGCCAATCCGTGGCTGATACTTTCAGAAGATCGGAGACAAGTGAAGGCTTGGAGACACCAGCAGAGC
 HisIleThrLeuAspProAspThrAlaAsnProTrpLeuIleLeuSerGluAspArgArgGlnValArgLeuGlyAspThrGlnGlnSer 317
 990 ATACCTGGAATGAAGAGAGATTGATAGTTATCCTATGGTCTGGTGCCAGCACTTTCCTCTGAAAACATTACTGGGAGGTAGAT
 IleProGlyAsnGluGluArgPheAspSerTyrProMetValLeuGlyAlaGlnHisPheHisSerGlyLysHisTyrTrpGluValAsp 347
 1,080 GTGACAGGAAAGGAGGCTGGGACCTGGGTGTCTGCAGAGACTCTGTGCGCAGGAAGGGGCACTTTTGTCTAGTCCAAGAGTGGCTTC
 ValThrGlyLysGluAlaTrpAspLeuGlyValCysArgAspSerValArgArgLysGlyHisPheLeuLeuSerSerLysSerGlyPhe 377
 1,170 TGGACAATTTGGTTGTGGAACAAACAAAAATATGAGGCTGGCACCTACCCAGACTCCCTCCACCTCAGGTGCTCCATGCCAAGTT
 TrpThrIleTrpLeuTrpAsnLysGlnLysTyrGluAlaGlyThrTyrProGlnThrProLeuHisLeuGlnValProProCysGlnVal 407
 1,260 GGGATTTTCTGGACTATGAGGCTGGCATGTCTCCTTCTACAACATCACTGACCATGGCTCCCTCATCTACTCCTTCTCTGAATGTGCC
 GlyIlePheLeuAspTyrGluAlaGlyMetValSerPheTyrAsnIleThrAspHisGlySerLeuIleTyrSerPheSerGluCysAla 437
 1,350 TTTACAGGACCTCTCGGCCCTTCTCAGTCTGGTTCAATGATGGAGGAAAAACACAGCCCTCTAACCTCTGTCCACTGAATATT
 PheThrGlyProLeuArgProPhePheSerProGlyPheAsnAspGlyGlyLysAsnThrAlaProLeuThrLeuCysProLeuAsnIle 467
 1,440 GGATCACAAGGATCCACTGACTATTGATGGCTTCTCTGACACTGCCACTCTCCCATTTGGCACCCTTCTCAGCCACAAACCCTGCCT
 GlySerGlnGlySerThrAspTyr *
 1,530 CTTTTCCCATGAACTCTGAACCACCTTTGTCTCTGCAGAGGCATCCGGATCCAGCAAGCGAGCTTTAGCAGGAAGTCACTTCACCAT
 1,620 CAACATTCCTGCCCAGATGGCTTTGTGATCCCTCCAGTGAAGCAGCCTCCTTATATTGGCCAAACTCATCTTGATCAACCAAAAAC
 1,710 ATGTTTCTGCCTTCTTTATGGACTTAAGTTTTTTTTTCTCTCTCCATCTCTAGGATGTCGTCTTTGGTGAGATCTCTATTATATCTT
 1,800 GTATGGTTTGCAAAAGGGCTTCTAAAAATAAAAACCCGAATTC

Figure 6. The nucleotide and predicted amino acid sequence of the 52-kD Ro/SSA cDNA. The amino acid sequence was predicted from the nucleotide sequence of clone FI18.1 cDNA. Nucleotide sequence positions are numbered to the left of the figure, while amino acid sequence positions are numbered to the right. The positions of the zinc finger and leucine zipper motifs are described in the text. This sequence has been added to the GenBank nucleic acid sequence database, Los Alamos National Laboratory, NM, and has been assigned accession number M34551.

and 123. The amino acid sequence CXXC occurs four times, HXXC occurs twice, whereas CXXH and HXXH each occur once (where "X" denotes any amino acid). The pairing of the

cysteine and histidine residues in these putative metal binding structures is unclear. For example, Cys-36 may form a zinc finger pair either with His-33 or Cys-39. Similar sequences

have been observed in DNA and RNA binding proteins (36–39), and heterogeneity in the motif structure has been reported (40). A leucine zipper structure is also present in the 52-kD Ro/SSA protein between amino acids 211 and 232. The heptad repeat of leucine residues occurs three times in this 22-amino acid region. 12 of the 18 amino acids between these leucine residues are either uncharged polar residues, or charged amino acids, characteristic of other leucine zippers (41). Another leucine residue is present 14 amino acids in the NH₂-terminal direction from Leu-211, although glutamic acid rather than leucine occurs at position 204. Again, a disproportionately high number of charged and glutamine residues occur in this region, suggesting that residues 197–210 may also represent a portion of this protein's leucine zipper. Leucine zippers are proposed to exist in α -helical conformations which allow pairing between DNA-binding proteins (41, 42).

Predictions of hydrophobicity by the method of Kyte and Doolittle (43) and secondary structure by the method of Chou and Fasman (44) suggest a highly hydrophobic α -helical region in the first 24 amino acids, an extended α -helical hydrophilic region between amino acids 130 and 225, and a hydrophobic carboxy-terminal 75 amino acids. Consistent with this prediction, the leucine zipper motif of the 52-kD Ro/SSA protein occurs in a predicted α -helical region. A potential N-linked glycosylation site is located at amino acid 422. Although we are not aware of any data on whether Ro/SSA proteins are glycosylated, the absence of this posttranslational modification in *E. coli* suggests that glycosylation is not required for antigenic recognition by serum autoantibodies in the panel of lupus patients studied here.

A search through the Genbank (release 60.0) and EMBL (release 19.0) nucleic acid sequence data banks revealed only two sequences with moderate similarity to the 52-kD cDNA sequence. The first 428 nucleotides in the coding region of the 52-kD Ro/SSA gene are 62% identical to the 5' portion of the mouse rpt-1 gene, whose product is involved in the downregulation of the IL-2 receptor (45). 54% of the nucleotides of this

same region of the 52-kD Ro/SSA gene are identical to the 5' portion of the human rfp gene (46). (Two gaps were inserted in each comparison to optimize the alignment of these sequences.) Furthermore, the last 411 bases of the 52-kD Ro/SSA coding region are 64% identical with the 3' portion of the coding region of the human rfp gene (after the introduction of three gaps to optimize sequence alignments). Little similarity was found in the middle portion of the coding regions of the latter two genes.

Searches for similarities between the predicted amino acid sequence of the 52-kD Ro/SSA protein and other protein sequences in the NBRF database (release 21.0) using the algorithm of Wilbur and Gipman (47) showed a significant match only with the predicted sequence of the human rfp transforming protein. The regions of similarity correspond to those identified above in the DNA. Alignment of these sequences reveals 42% identity in the NH₂-terminal portions of these proteins, and 51% identity in their COOH-terminal regions. Using the method of Gribskov and Burgess (48), the NH₂-terminal residues of these proteins are 84% similar, while the COOH-terminal amino acids are 86% similar. A comparison of the predicted amino acid sequences of the Ro/SSA 52-kD protein and the mouse rpt-1 protein using the method of Smith and Waterman (49) also revealed 47% identity (84% similarity) between the amino acids encoded by the 5' portions of the coding regions described above.

The NH₂-terminal portions of these three proteins contain zinc finger motifs in approximately their first 145 amino acid residues. Conservation of both amino acid sequence and spacing of these motifs was found (Fig. 7). His-100 of the 52-kD Ro/SSA sequence is absent in the other two proteins, suggesting that these residues may not form a zinc finger element. Because of the similarity between these zinc finger motifs, an additional search was made for similarities between the zinc finger region of the 52-kD Ro/SSA polypeptide and nine other proteins with zinc finger motifs. Of six comparisons with mammalian zinc finger containing proteins, similarity of the

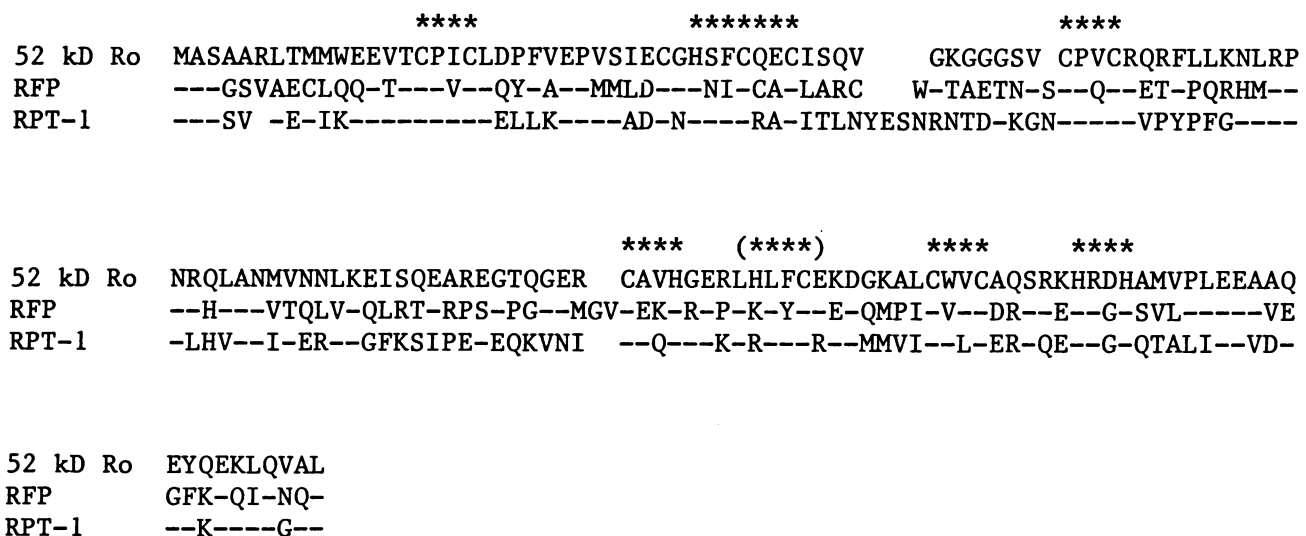


Figure 7. Alignment of the amino-terminal portions of three proteins containing conserved zinc finger motifs. The first 143-amino acid residues of clone FI18.1 and 147 residues of the human rfp and mouse rpt-1 protein are shown. Asterisks indicate locations of potential zinc fingers. Spaces indicate positions where gaps were introduced to optimize alignment of the sequences. Dashes indicate identity to the residues of the 52-KD Ro/SSA protein.

Ro/SSA zinc finger region was found only with the *rfp* and *rpt-1* proteins. Neither of two *Drosophila* nor one *Xenopus* zinc finger containing protein were similar to the Ro/SSA 52-kD zinc finger region. The spacing and amino acid conservation of this region between these three proteins suggests that they are evolutionarily and perhaps functionally related.

Interestingly, no significant homology was found between the 52-kD Ro/SSA gene or protein and the three other sequences of the 60-kD form of Ro/SSA. Two of the sequences for the 60-kD Ro/SSA gene differ only in their 3' end (17, 18). A third 60-kD Ro/SSA sequence shows no similarity to either of these sequences or to the 52-kD sequence reported here (19). Neither RNP-1 nor RNP-2 consensus binding sequences occur in the 52-kD Ro/SSA protein (50). It is clear from these data that the gene encoding the 52-kD Ro/SSA polypeptide is unique from any of the 60-kD Ro/SSA genes, and that the former polypeptide is not a transcriptionally or posttranslationally modified product of the 60-kD gene.

Discussion

Two apparently identical human cDNA clones encoding the 52-kD Ro/SSA autoantigen were isolated from a λ gt11 expression library using an SLE patient serum which contains antibodies reactive with the 52-kD Ro/SSA protein. This gene encodes a protein with two classes of nucleic acid binding structures, a zinc finger and a leucine zipper. Although fusion proteins with different DNA binding motifs have been described, they are the result of chromosomal translocations of different genes, and are apparently limited in expression to malignant cells (51, 52). Ro/SSA, on the other hand, is expressed in all cells examined to date. Therefore, it appears that the presence of these two nucleic acid binding motifs is unique to Ro/SSA.

The immunological identity of the antigens encoded by these cDNAs was established in several ways. First, IPTG-induced proteins in these clones reacted with affinity-purified antibodies from the 52-kD band of lymphocyte extracts, but not with those isolated from the 60-kD Ro/SSA band. Secondly, antibodies affinity-purified from these clones specifically recognized a 52-kD polypeptide in Western blots and immunoprecipitated all the Ro/SSA-associated hY RNAs in HeLa cells. The cDNA inserts from these two clones cross-hybridize, and their end terminal sequences are identical. These experiments demonstrate that cDNA FI18.1 and FI19.3 encode the 52-kD Ro/SSA antigen.

Because the amino-terminal sequence of the 52-kD protein has not been determined, and the size of the full-length protein is closer to that of the 54-kD Ro/SSA protein, a formal possibility exists that this cDNA may encode the 54-kD form of the Ro/SSA protein, or a unique 60-kD form of Ro/SSA. Three points argue against such an interpretation. First, the only common property of the sera reactive with the IPTG-induced proteins in these clones is that they have autoantibodies which bind to the 52-kD band in Western blots. In fact, autoantibodies in one patient's serum (MO) recognize only the 52-kD Ro/SSA protein. Secondly, affinity-purified antibodies from IPTG-induced proteins in these clones bind to the 52- and not the 60-kD band in Western blots of lymphocyte extracts. Finally, the 54-kD Ro/SSA protein has been detected only in red blood cells (15) and their immediate nucleated precursors (Y.

Itoh, unpublished results). Posttranslational cleavage of hydrophobic leader sequences in eukaryotic proteins is common. The predicted amino acid sequence of a recently reported 60-kD Ro/SSA protein includes 17 amino acids which are absent on the mature polypeptide (19). The 52-kD Ro/SSA protein reported here has a predicted amino-terminal hydrophobic region of ~ 24 amino acids (43). If these residues are removed in the formation of a mature 52-kD Ro/SSA polypeptide, a 452-amino acid protein of 51,513 D is predicted. Amino acid sequencing of purified 52- and 54-kD Ro/SSA polypeptides, or the eventual cloning of a gene which encodes the 54-kD protein will be necessary to define the structural relationship between these two polypeptides and their posttranslational processing.

Since many of the lupus patients who produce anti-Ro/SSA antibodies also make anti-La/SSB antibodies, the possibility that the cDNA described here encodes the La/SSB protein was also considered. We have ruled out this possibility in four ways. An absolute positive correlation was found between the presence of anti-52-kD Ro/SSA antibodies as detected by Western blot analyses and the ability of those patients' antibodies to react with IPTG-induced proteins of the recombinant clones. No association between the presence of anti-La/SSB antibodies and the specificity for this clone's proteins was observed. Second, anti-La/SSB antibodies affinity purified from the 45 kD region of a Western blot failed to bind to these clones. Third, affinity purified antibodies from these clones precipitated the Ro/SSA hY RNAs, but not any of the small RNAs associated with La/SSB, and finally, the nucleotide sequence of this clone is unrelated to that of La/SSB.

The zinc finger motifs found in the 52-kD Ro/SSA protein are characteristic of proteins which bind nucleic acids (36–39). The 52-kD Ro/SSA gene product is the first of which we are aware that contains both of these structures. It is of interest that the location, number, and spacing of the zinc fingers are so highly conserved in the 52-kD Ro/SSA, *rfp*, and *rpt-1* proteins, but not in a number of other proteins containing zinc finger motifs. A portion of the *rfp* gene, designated "ret," was first identified after transformation of NIH 3T3 cells with segments of human DNA (53). Cloning of the gene from human cells indicated a portion of this gene undergoes translocation, and that this gene is expressed at high levels in mouse testis, embryos, and a variety of human and rodent tumor cell lines (46, 54). The function of the *rfp* gene product has not been identified. The *rpt-1* protein has down-regulatory activity on the receptors for interleukin 2 in mice (45). While soluble IL-2 receptor levels have been reported to be abnormal in SLE patients (55–57), the mechanisms of regulating IL-2 receptor levels by this gene product have yet to be elucidated, and therefore, are unable to currently shed light on the function of the Ro/SSA molecule.

While hY RNAs immunoprecipitate with 52- and 60-kD Ro/SSA proteins, no information is available on whether the Ro/SSA ribonucleoprotein complexes bind to DNA, although the presence of Ro/SSA in both the nucleus and cytoplasm may suggest a role for binding to DNA as well as RNA (58, 59). However, several lines of evidence indicate that Ro/SSA particles are associated with one other ribonucleoprotein complex. La/SSB has been found to be physically associated with Ro/SSA complexes in Ouchterlony immunodiffusion assays (60). Immunoprecipitation of Ro/SSA particles with certain sera lacking anti-La/SSB specificity, as well as antibodies to hY5 containing Ro/SSA particles have each been shown to precipi-

tate La/SSB proteins (61, 62). In addition, a monoclonal anti-La/SSB antibody has been reported which binds to Ro/SSA particles (63). It has not been determined if the dual heptad repeat of leucine residues in La/SSB is responsible for these protein's interaction (64). While both Ro/SSA and La/SSB particles have also been found in 45S endoribonuclease VII-RNP complexes, the sites for interactions between subunits in these complexes remain undefined (65).

It is now clear that the 52-kD form of Ro/SSA is not a differential transcriptional or posttranslational product of the 60-kD Ro/SSA molecule. These proteins are encoded by distinct gene sequences. The fact that no cross-reaction has been observed with affinity-purified antibodies that recognize these proteins is consistent with this finding (16). Three reports now exist for genes which apparently encode the 60-kD form of the Ro/SSA protein. Differences between the two most similar 60-kD Ro/SSA sequences may represent tissue specific isoforms, differential exon splicing, unusual allelic variants, or cloning artifacts (17, 18). The relationship between these and the third 60-kD Ro/SSA gene is not clear (19). However, the common characteristic of all these Ro/SSA proteins is their ability to physically associate with the small hY RNAs. Surprisingly, no similarity in the DNA or amino acid sequences was found between any of these 60-kD Ro/SSA clones and the 52-kD Ro/SSA clone reported here. The deduced amino acid sequence of the 52-kD protein lacks similarity to other RNA binding proteins. While the RNP-binding consensus sequences defined by Dreyfus et al. (50) are not present in the 52-kD Ro/SSA antigen, they are also missing in one of the 60-kD Ro/SSA sequence, the D protein of the Sm autoantigen, and in certain viral RNA binding proteins (19, 66, 67). The heterogeneity of these sequences (50) may indicate either that other sequences serving the same function exist but are currently unrecognized, or that the hY RNA binding of Ro/SSA proteins is dependent on a particular tertiary configuration.

While the function of Ro/SSA is unknown, it is clear that considerable heterogeneity exists in these ribonucleoprotein complexes. At least two isoforms are present in nucleated and red blood cells, and tissue specific differences in Ro/SSA proteins have recently been identified (Itoh et al., manuscript submitted for publication). While the common feature of these proteins is their ability to bind hY RNAs, the number and form of these RNA molecules also varies between cells (15). Accordingly, functional differences in these complexes would not be unexpected. The physical heterogeneity of these Ro/SSA particles may, in part, explain the heterogeneity in the immune response to these complexes in patients with SLE and Sjogren's syndrome. Autoantibodies to Ro/SSA are associated with particular HLA serotypes and gene markers in these patients (11, 68-71). We have recently reported T cell receptor restriction fragment length proteins which are associated with the presence of anti-Ro/SSA antibodies (72). Since the antigenic portion of these complexes resides in the polypeptide, differences in the presence of particular anti-Ro/SSA antibodies between patients may be based on the ability of that patient's immune system to process and recognize epitopes which are unique to each form of the Ro/SSA polypeptides. Given the complexity of this antigenic system, a reevaluation of polymorphisms of the immune response genes of patients with anti-Ro/SSA antibodies is in order. The ability to purify different recombinant Ro/SSA proteins and use them in immunogenetic analyses may clarify the role of such genes in the generation of an im-

mune response to Ro/SSA ribonucleoprotein complexes in patients with SLE and Sjogren's syndrome.

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