

## 52-kD SS-A/Ro: Genomic Structure and Identification of an Alternatively Spliced Transcript Encoding a Novel Leucine Zipper-minus Autoantigen Expressed in Fetal and Adult Heart

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### Summary

The 52-kD SS-A/Ro protein is one of the antigenic targets strongly associated with the autoimmune response in mothers whose children have manifestations of neonatal lupus. In addition to the cDNA clone we previously reported for the full-length 52-kD SS-A/Ro protein, an interesting MOLT-4 cDNA clone, p52-2, was found to have an internal deletion of 231 nucleotides including the domain encoding the leucine zipper motif. To further investigate the nature of this deletion, genomic DNA clones were isolated from a  $\lambda$ FIXII library. The complete gene for the full-length 52-kD protein ( $\alpha$  form, 52 $\alpha$ ) spans 10 kb of DNA and is composed of seven exons. Exon 1 contains only the 5' untranslated sequence, while the translation initiation codon is located 3 kb downstream in exon 2, which also encodes the three zinc finger motifs. Exon 4 encodes amino acids 168–245, including the coiled coil/leucine zipper domain. Exon 7 is the longest and encodes the rfp-like domain and the 3' untranslated region. The cDNA p52-2 can now be accounted for as a product of alternative messenger RNA (mRNA) derived from the splicing of exon 3 to exon 5, skipping exon 4, which results in a smaller protein (52 $\beta$ ) with a predicted molecular weight of 45,000. An initial approach to identifying this alternatively spliced form in the human heart used a ribonuclease protection assay. Using an RNA probe corresponding to bases 674–964 of the full-length cDNA, two protected mRNA fragments were identified, a 290-bp fragment corresponding to expression of 52 $\alpha$  and a smaller fragment of 144 bp, the predicted size of 52 $\beta$ . Using reverse transcription followed by polymerase chain reaction, cDNAs from a 16-wk fetal heart, 24-wk heart, and adult heart were amplified with primers flanking exon 4. Two polymerase chain reaction products were observed in each tissue, one 1.0 kb likely representing 52 $\alpha$  and a second 0.78 kb, consistent with 52 $\beta$ . The 0.78-kb fragment identified in the 16-wk heart was cloned, and DNA sequencing confirmed the 52 $\beta$  type. Immunoprecipitation of *in vitro*-translated <sup>35</sup>S-labeled 52 $\beta$  form was performed to evaluate the antigenicity of this novel form of 52-kD SS-A/Ro. 26 (87%) of 30 sera tested from mothers whose children were known to have neonatal lupus immunoprecipitated the 52 $\beta$  form. In summary, we herein describe an alternatively spliced mRNA encoding a novel 52 SS-A/Ro autoantigen expressed in a variety of tissues including the fetal heart. Recognition of this 52 $\beta$  isoform suggests that antigenicity of the 52-kD SS-A/Ro protein can be independent of the putative leucine zipper domain. These findings may further elucidate the cellular function of the 52-kD SS-A/Ro antigen and its role in the development of an autoimmune disease and subsequent injury to fetal tissue.

In addition to the 60-kD SS-A/Ro and 48-kD SS-B/La proteins, another major antigenic target that characterizes the autoimmune response in mothers whose children have manifestations of neonatal lupus is the 52-kD SS-A/Ro

protein (1–4). A molecular definition and sequence motifs for this nuclear autoantigen have been provided by the isolation of cDNA clones encoding the full-length protein, which consists of 475 amino acids (5, 6). The deduced

structure contains three distinct domains. The NH<sub>2</sub>-terminal region is rich in cysteine/histidine motifs known as zinc fingers and belongs to the RING finger protein subfamily, which includes the founding members rfp (7) and rpt-1 (8), and the newly described breast cancer susceptibility gene, *BRCA1* (9). The central region contains two putative coiled coils with heptad periodicity, one of which was previously identified to be a leucine zipper. The COOH-terminal domain is similar to the corresponding domain in the rfp protein. The human 52-kD SS-A/Ro gene is located on chromosome 11 (10). Based on its homology with other closely related proteins that function in transcriptional regulation such as rpt-1, it is reasonable to speculate that the 52-kD protein has a similar role. However, the cellular function for the 52-kD SS-A/Ro is not presently defined.

Alternatively spliced messenger RNAs (mRNA)<sup>1</sup> have been described for transcription factors as well as inflammatory mediators, signal transduction components, proteins involved in the immune response, growth factor receptors, and other housekeeping proteins. It has been speculated that such mRNAs either suppress the translation of the functional counterpart or represent intermediates that can be further processed depending on the tissue (11). There is accumulating evidence for alternatively spliced forms of the nuclear autoantigens of the SS-A/Ro and SS-B/La systems. Most recently, Troster et al. described an alternative type of SS-B/La mRNA in which an alternative exon 1 (exon 1') was used (12). It was proposed that a second promoter in the previously described intron 1 regulates the transcription of exon 1'. Moreover, albeit secondary to a different splicing event than that described for SS-B/La, two versions of the 60-kD SS-A/Ro protein that diverge in their respective COOH termini have been reported (13, 14).

Insights into understanding the cellular function of the 52-kD SS-A/Ro protein, its antigenicity, and to-date unexplained involvement in the development of fetal cardiac conduction disturbances may be gained as more information on the genomic structure becomes available and potential alternatively spliced mRNAs are identified. Specifically, with regard to the pathogenesis of congenital heart block (CHB), we hypothesized the existence of an isoform in which additional or deleted sequences promote access to autoantibodies or which in turn may function to down-regulate or up-regulate expression of the putative 52-kD SS-A/Ro. Alternative splicing as a means to regulate on-off type gene expression has been described in several sex determining proteins in *Drosophila* (15). Accordingly, we herein define the genomic structure of the 52-kD SS-A/Ro protein and report the isolation of a novel alternative form in which exon 4 encoding the leucine zipper domain is deleted. With respect to the potential role of this novel anti-

gen in the development of neonatal lupus, attention is focused on expression of this 52 $\beta$  isoform in the human fetal heart and its recognition by sera from mothers of children with cardiac and cutaneous manifestations of neonatal lupus.

## Materials and Methods

**MOLT-4 Cell cDNA Library Screening.** In our original report (5), after obtaining a partial cDNA for the 52-kD SS-A/Ro from a HepG2 cell cDNA library using a human autoantibody, a 5' probe was generated and used to screen a MOLT-4 cell cDNA library. Two specific clones, p52FL and p52-2, were selected for further analysis. The longer cDNA (p52FL) was characterized completely and found to encode the full-length protein (5), while the shorter cDNA (p52-2) was not examined in detail. This study describes the complete sequence analysis of clone p52-2. All nucleotide positions refer to accession number M35041 unless otherwise stated (5).

**Genomic DNA Clones of 52-kD SS-A/Ro.** The full-length cDNA insert from clone p52FL (5) was labeled by the method of Feinberg and Vogelstein (16) and used for DNA hybridization screening of a human placental genomic DNA library cloned in the  $\lambda$ FIXII Vector (Stratagene, La Jolla, CA). Positive clones were plaque purified and examined by restriction enzyme analysis and Southern blotting. Restriction fragments containing the region of interest were identified and subcloned in pBluescript SK (Stratagene).

**Mapping of Transcriptional Start Sites.** The method of Hirzmann et al. (17) using 5' rapid amplification of cDNA ends (RACE) to determine the 5'-cap-G structure of mRNA was used. Human placenta 5' RACE-Ready cDNA was purchased from CLONTECH (Palo Alto, CA), and the RACE procedure was performed according to the manufacturer's instructions. The primer 5'-TCAAGCGTGCTGCTGAAGCCATTGTCAAGT-3' (complementary to nucleotide 78-107) was used in the first PCR together with the provided anchor primer. 10% of the PCR product from the first reaction was used to set up the second PCR with a nested primer 5'-TTTAGGGGGTTTGGCTTTACAGGGCAACTC-3' (complementary to nucleotide 21-50) used to substitute for the first primer. The second PCR product was analyzed by electrophoresis in a 4% agarose gel and subcloned using a cloning kit (pCR-Script; Stratagene), and nucleotide sequences were determined from 12 subclones.

**DNA Sequence Analysis.** Nucleotide sequences were determined using the dye terminator cycle sequencing and a DNA sequencer (373A; Applied Biosystems Inc., Foster City, CA). Oligonucleotide primers were synthesized with a DNA synthesizer (394; Applied Biosystems Inc.). DNA sequences were determined in both strands and compiled using an alignment program (SeqEd; Applied Biosystems Inc.).

**RNA Isolation.** Human fetal tissues were obtained after elective termination of normal pregnancy by dilatation and evacuation. This was done in accordance with the guidelines of the Institutional Review Board and after obtaining consent from the mothers. RNA was extracted as described (18) using the RNA isolation kit (Stratagene). Adult heart RNA was obtained commercially (CLONTECH).

**RNAse Protection Assays.** Plasmid p52PB containing the internal PstI-BamHI (covering bases 674-964) was constructed to generate an antisense RNA probe. The PstI-BamHI fragment was obtained by a partial PstI and BamHI digestion of full-length

<sup>1</sup>Abbreviations used in this paper: aa, amino acid; CHB, congenital heart block; LINE, long intersperse element; mRNA, messenger RNA; nt, nucleotide; RACE, rapid amplification of cDNA ends; RT, reverse transcription; SS, Sjögren's syndrome; UAS, undifferentiated autoimmune syndrome.

52-kD SS-A/Ro cDNA and subcloned into BamHI and PstI sites of pBluescript SK vector. RNase protection assays were carried out using a kit from Ambion Inc. (RPA II; Austin, TX) according to the manufacturer's directions. The antisense riboprobes were synthesized by *in vitro* transcription (19) of p52PB linearized with HindIII using T7 RNA polymerase and incorporating [<sup>32</sup>P]UTP. Each hybridization included 10–100 mg of total cellular RNA and 5 × 10<sup>7</sup> cpm riboprobe with incubations for 18 h at 42°C. All riboprobes were present in >10-fold excess relative to the cellular 52-kD SS-A/Ro mRNAs in the hybridizations. RNase A was used at 250 U/ml and RNase T1 at 10,000 U/ml. The labeled probe hybridized to complementary mRNA in the sample mixture is "protected" from ribonuclease digestion, separated on 15% polyacrylamide gels, and visualized by autoradiography.

**In Vitro Transcription and Translation.** Since p52-2 represents a partial cDNA for 52β, an expression vector containing a full-length cDNA lacking exon 4 was constructed from p52FL following the protocol described in the site-directed mutagenesis kit (Transformer; CLONTECH). The primer 5'-AAGAGAGCA-GACTGGAAG|GAGGTGATAATTGTCCTG-3' was designed with the 5' half corresponding to the 3' end of exon 3 and the 3' half to the 5' end of exon 5. After initial priming with the mutant primer to generate deletion mutants, nonmutated plasmid was inactivated by digestion with SfiI restriction enzyme, which cut within exon 4 alone (see Fig. 1). After SfiI digestion, the mutated plasmid preparation was transformed into the *Escherichia coli* DH5α. A clone p52β was selected, and DNA sequencing was used to confirm the complete in-frame deletion of exon 4. For the synthesis of full-length 52β, plasmid p52β was linearized with HindIII and used as a substrate for *in vitro* transcription using T3 RNA polymerase and translation using a rabbit reticulocyte lysate (TNT Coupled Reticulocyte Lysate System; Promega, Madison, WI) in the presence of [<sup>35</sup>S]methionine (Trans <sup>35</sup>S-label, 70% methionine and 15% cysteine, ICN Biochemicals, Inc., Costa Mesa, CA) as described (20). A second construct, which resulted in the deletion of the COOH-terminal 115 amino acid residues (equivalent to amino acids [aa] 360–475 of 52-kD SS-A/Ro), was obtained by digestion of p52β with the restriction enzyme PstI before *in vitro* transcription and translation.

**DNA Amplification Method.** First-strand synthesis was accomplished using 1 μg of poly(A<sup>+</sup>) RNA containing 40 U RNasin (Promega) in the 3' rapid amplification of cDNA ends (3' RACE) system (GIBCO BRL, Gaithersburg, MD) as described by the supplier. This cDNA was used for both 3' RACE and reverse transcription (RT)-PCR. PCR was performed using a cyclor (TC9600; Perkin-Elmer, Oberlingen, Germany). The 50-μl assay in 1× Taq buffer contained 2 U Taq polymerase (GIBCO BRL), 2.5 mM MgCl<sub>2</sub>, 200 mM of each dNTP, 20 pmol of each primer, and 100 ng of cDNA. Amplification was started by heating for 2 min to 94°C. 30 cycles followed, each consisting of 1 min at 94°C, 2 min at 55°C, and 1.5 min at 72°C. The temperature was then held for 10 min at 72°C and cooled down to 4°C. 6 μl of the respective product was mixed with 1.5 μl of sample buffer (50% glycerol, 0.25% bromophenol blue in H<sub>2</sub>O) and applied to a 1.5–1.8% agarose gel in Tris-borate EDTA buffer for analysis. For determination of the molecular weights of the PCR products, 250 ng of λX174 DNA HaeIII digest (GIBCO BRL) was run in parallel. The gels were subsequently stained with ethidium bromide, and photographs were taken (film 667; Polaroid Corp., Cambridge, MA).

3' RACE amplifications were performed for 30 cycles using a primer spanning bases 423–443 (5'-TATGTGCCAGTCTCG-

GAAAC-3') located upstream of the sequence encoding the leucine zipper of the 52-kD SS-A/Ro. Amplification products were purified (MicroSpin S-400 HR Columns; Pharmacia Biotech Inc., Piscataway, NJ). 2 μl of purified product was amplified by an additional 30 cycles using a nested primer covering bases 609–628 (5'-AATCTAGGATTCACGCAGAG-3'). Both amplifications were done in combination with the Universal Amplification Primer of the 3' RACE kit.

RT-PCR was performed using the same reaction conditions with the sense primer spanning bases 423–443 as used in 3' RACE, and an antisense primer covering bases 1377–1396 (5'-GGCACATTCAGAGAAGGAGT-3'). After the first 30 cycles, the amplification products were run on a 1.5% Tris-acetate EDTA gel. The lower 0.78-kb band was purified and used as a template in a second round of amplification with the same primers. Selected amplification products were cloned using the TA cloning kit (Invitrogen, San Diego, CA) as described by the manufacturer, and DNA sequencing was used to confirm their identity.

**Patient Sera.** Sera from 32 women whose children had neonatal lupus (30 CHB, 2 cutaneous manifestations) were tested in immunoprecipitation of the *in vitro*-translated radiolabeled 52β form and the COOH-terminal truncated fragment (two mothers were only tested with the deletion mutant and one mother was only tested with the 52β form). Of these women, 8 had Sjögren's syndrome (SS), 12 had SLE, 4 had an undifferentiated autoimmune syndrome (UAS), and 9 were completely asymptomatic. All sera were previously demonstrated to have antibodies to the 52-kD SS-A/Ro (defined by ELISA or SDS-immunoblot).

Patients classified as SLE met the criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) (21). Six of the patients considered to have SS had symptomatic dry eyes and/or dry mouth, plus ophthalmological confirmation of keratoconjunctivitis sicca (22) and/or a positive salivary gland biopsy according to the recommendations of T. E. Daniels (23). One mother whose child had neonatal lupus was categorized as having SS but had dry eyes and mouth without objective criteria, and one mother, also best categorized as SS, had only parotid swelling as an adult, which required treatment with glucocorticoids. The sera from patients with SS and SLE were selected because they contained anti-52-kD SS-A/Ro reactivity and were from mothers whose children had neonatal lupus and thus do not represent a random sample of patients with SS and SLE. Patients were considered to have UAS if they had symptoms but did not meet criteria for the diagnosis of SLE, SS, or rheumatoid arthritis.

Of the 32 neonatal lupus sera tested, 26 (81%) had been previously evaluated in an analysis of antigenic regions of the 52-kD SS-A/Ro (20). 14 tested were previously known to react with *Staphylococcus aureus* V8 protease digestion fragments of the 52-kD SS-A/Ro, aa1–291, aa1–151, and aa1–78 (type II reactivity). 11 sera tested were previously known to react only with fragment aa1–291 and did not immunoprecipitate the more NH<sub>2</sub>-terminal fragments aa1–151 or aa1–78 (type I reactivity). One serum further evaluated in this study was marginally positive for anti-52-kD SS-A/Ro antibodies by ELISA but did not immunoprecipitate the radiolabeled protein. Six of the maternal sera had not been previously tested by immunoprecipitation or reported.

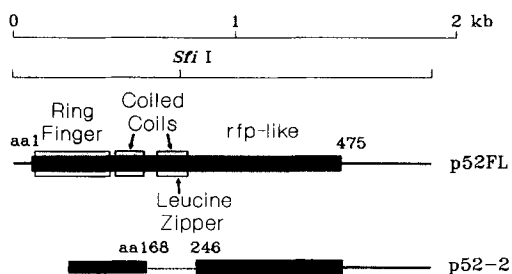
**Immunoprecipitation.** Immunoprecipitation of the *in vitro* translation products was performed as described (20). Briefly, 1 μl of patient's serum was mixed with 50 μl 50% protein A Sepharose, 100 μl 10-mg/ml BSA, 200 μl reaction buffer (150 mM NaCl, 4 mM EDTA, 50 mM Tris-HCl, pH 7.4, 0.5% NP-

40, 0.5% sodium deoxycholate, and 0.1% SDS), and 1  $\mu$ l of the labeled *in vitro* 52 $\beta$  translation product. For evaluation of the deletion mutant, 10  $\mu$ l of sera and 10  $\mu$ l of the radiolabeled protein were used. The reaction mixtures, in 1.5-ml Eppendorf tubes, were rotated at 4°C for 1–3 h. The beads were then washed four times with chilled reaction buffer, eluted, boiled with reducing SDS sample buffer, and then run on 15% high-ratio monomer: cross-linker acrylamide gels overnight at 7.5 mA until the bromophenol blue dye front ran off (24). The gel was then stained with Coomassie blue dye and destained to visualize molecular weight markers and to ensure uniformity of immunoglobulin heavy and light chains precipitated in each reaction, dried with heat and vacuum, and placed with film at –70°C.

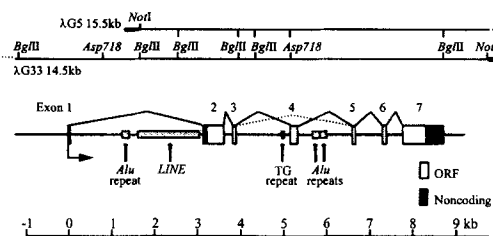
## Results

**52-kD cDNA Variant.** During the screening of the MOLT-4 cell cDNA library to obtain the full-length cDNA for the 52-kD SS-A/Ro as described in our original report (5), clone p52-2 with a 1.4-kb insert was found also to be specific in the DNA hybridization reaction with the 52-kD cDNA probe. Initial DNA sequence determinations showed that the 5' end of p52-2 started at nucleotide position 257 (full-length cDNA 1.9 kb; reference 5), while the 3' end matched perfectly those of other cDNAs for 52-kD SS-A/Ro. By comparison with the full-length cDNA, it was apparent that there might be an internal deletion of ~243 nucleotides [nt] (1.9 kb – 1.4 kb – 257nt = 243nt) in the p52-2 cDNA. As the complete nucleotide sequence was determined for p52-2, a deletion of 231nt was confirmed corresponding to a loss of 77 amino acid residues (aa168–245) spanning the leucine zipper region (Fig. 1). This deletion suggested that an alternative mRNA might exist, and thus we proceeded to characterize the gene structure for the 52-kD protein.

**Genomic Structure of the 52-kD SS-A/Ro.** Southern blot analysis of human DNAs from several sources demonstrated that there was probably only one gene for the 52-kD protein (5). Two genomic clones, G5 and G33, were obtained from a human placenta DNA library using DNA hybridization with a labeled cDNA probe. As depicted in Figs. 2 and 3, the complete gene for the 52-kD protein spans 10 kb of DNA and is composed of seven exons. The 5' untranslated



**Figure 1.** Identification of an internal deletion in a MOLT-4 cell cDNA for the 52-kD SS-A/Ro. Compared with the sequence of the full-length cDNA p52FL, 231 bp (equivalent to 77 aa) were found to be absent in p52-2. Solid boxes represent the open-reading frame, and the numbers are the amino acid with respect to the full-length protein.



**Figure 2.** Human gene for the 52-kD SS-A/Ro protein. Two  $\lambda$ FIXII clones, G5 and G33, initially selected by DNA hybridization, were found to be overlapping as shown schematically. The 52-kD SS-A/Ro gene spans 10 kb of DNA and is composed of seven exons. The translational initiation and stop codons are located in exon 2 and 7, respectively. The alternative form 52 $\beta$  is derived from splicing of exon 3 to exon 5, skipping exon 4 containing the coiled coil/leucine zipper domain. Repetitive sequence elements such as LINE, Alu elements, and simple repeat of the T and G nucleotides (TG repeat) are indicated as they are often found in introns.

sequence is split between exon 1 and 2, separated by a 3-kb intron. The translation initiation codon is located in exon 2, which encodes the three cysteine-histidine zinc fingers. Exon 4 encodes aa168–245, including the leucine zipper. Exons 3, 5, and 6 are short exons, while exon 7 is the longest and encodes both the rfp-like domain and the 3' untranslated region. Three types of repetitive elements were found within the gene for the 52-kD protein. Repetitive sequences such as the long intersperse element (LINE) and Alu sequences were found in introns, as indicated in Fig. 2. A dinucleotide TG repeat was also detected upstream of exon 4. With the exception of a G nucleotide equivalent to position 239 of the cDNA, no differences were found between the sequence of these exons and the cDNA previously identified (5).

A partial gene structure of the 52-kD SS-A/Ro containing the last six exons has been recently published (25). This study did not address the first 36nt (part of exon 1) included in our original cDNA (5). Although the gene structures reported by Tsugu et al. (25) and herein are concordant in general, there is a major difference in the location of exon 1, which is ~3 kb upstream of exon 2 in clone G33. To confirm that exon 1 is a functional exon and to determine the transcriptional start site, 5' RACE was used to obtain sequences upstream of the published noncoding sequence (5). Only one single band of 118 bp (including the sequence of the anchor primer) was identified in the second PCR product. 12 subclones containing inserts were sequenced; in four clones transcription starts at nucleotide position 1 (Fig. 3, first arrow); in one clone at position 2 (however, missing the 5'-cap-G), one at position 5 (second arrow), and six clones at position 11 (third arrow). The sequence data are consistent with the agarose gel analysis above, which demonstrated a single band; thus, transcription begins in a relatively restricted region. The identification of exon 1 as delineated herein is significant, since in a recent report (25) the promoter region would have been located >3 kb downstream in intron 1. Further analysis of the promoter activity for the 52-kD SS-A/Ro gene will be provided in a separate report.

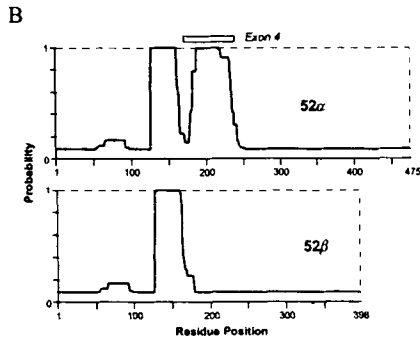


**A**

Exon	Size (bp)	5' Splice donor	Intron	Size (bp)	3' Splice acceptor
1	52	<b>GTAAAG</b> gtgagt	1	3173	cttacacagactttctcatattctctttccctttttcttctctccag <b>CCAAAC</b>
2	457	<b>TACCAAG</b> gtgagg	2	252	ccctattacttggttgcatgggggacgaataagctgtctttctctgcag <b>GAGAAG</b>
3	96	<b>TGCAAG</b> gtaaga	3	1123	cttgaggagattttcataaaaaccacctctcccttcccttgc <b>acctaag</b> <b>AANAACA</b>
4	231	<b>CTGCAG</b> gtgaga	4	1306	agtggcgatccagcaatccagaacttcttctgtctcttttctctccag <b>GAGGTG</b>
5	23	<b>GGAAAG</b> gtaagg	5	710	ggtttgggataggataggagacaggatctcaaaactctctttcccccag <b>CACTGA</b>
6	101	<b>GTGCAG</b> gtgagg	6	303	ccttagtgaactcccccatgcaaggctgactgtgtctctctctctgcag <b>TCCACA</b>
7	946				

Donor: AAG gtaagt  
C g

Acceptor: ccccccccncag  
tttttttt



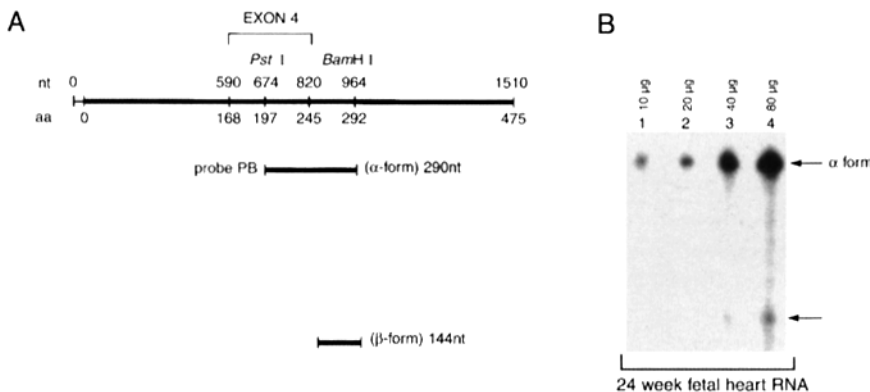
**Figure 4.** 52 $\beta$  generated from alternative splicing. (A) Summary of exons, introns, and splicing sites for the 52-kD SS-A/Ro gene. With the exception of four nucleotides (*bold type*) at the 3' splice of intron 3, sequences for 5' and 3' splice sites are in strong agreement with known consensus sequences. (B) Prediction of coiled coils in 52-kD SS-A/Ro. The second coiled coil corresponding to exon 4 alone is absent in 52 $\beta$ .

a transcript of 1.0 kb, corresponding to the full-length 52 $\alpha$  form, and a second of 0.78 kb, the expected size for the 52 $\beta$  form (Fig. 6, lanes 1–3). Interestingly, the larger band was the predominant 52Ro isoform identified in the adult heart and 24-wk heart, while the two forms were of equal intensity in two of three 16-wk hearts. Increased expression of the 52 $\beta$  relative to 52 $\alpha$  mRNA was observed in one of the two 14-wk hearts. The 0.78-kb fragment identified in one of the 16-wk hearts was gel purified and was used as a template for a second amplification with the same primers. This amplified product was cloned, and DNA sequencing unambiguously confirmed the identity of the 52 $\beta$  form as outlined in the genomic sequence shown in Fig. 3.

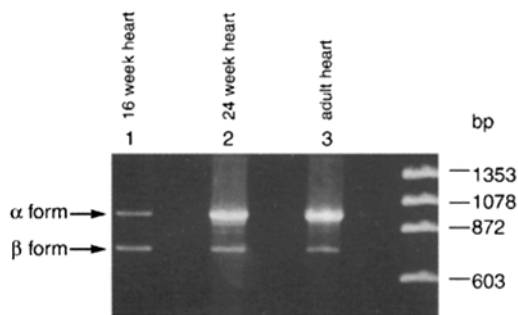
**3' End of the 52-kD SS-A/Ro mRNA.** Since the full-length 52-kD SS-A/Ro protein is highly homologous to a cellular homologue of a human transforming protein, a fusion of the NH<sub>2</sub>-terminal zinc finger domain of rfp and COOH-terminal domain deriving from a tyrosine kinase (28), we sought to determine whether the sequence of the 52 $\alpha$  form in the fetal heart diverged from the sequence published for other tissues. Our strategy was to clone flanking 3' regions that might be derived from such a putative gene fusion using 3' RACE and RNA from a 16-wk fetal

heart. mRNA was reverse transcribed using a “hybrid” primer consisting of oligo (dT) residues linked to a unique 17-base oligonucleotide (“adapter”) primer. Amplification of the resulting cDNA for the first 30 cycles used the Universal Amplification Primer, which binds to each cDNA at its 3' end, and a gene-specific primer (sense primer spanning bases 423–443 in the 52 $\alpha$  form), which would amplify a fragment inclusive of exon 4. The 1.5-kb PCR product was then purified and used in a second amplification with a nested primer spanning bases 609–628. The resulting PCR product of 1.3 kb, the predicted size of the 52 $\alpha$  form, was sequenced. The data demonstrated that the 52-kD SS-A/Ro protein expressed in the 16-wk heart is identical in sequence to that obtained from a cDNA library of HepG2 (hepatocellular carcinoma) and MOLT-4 (T cell lymphoblastoma) (5).

**The 52 $\beta$  Form is Antigenic.** The plasmid p52 $\beta$  containing the cDNA for the 52 $\beta$  form was used to evaluate the efficiency of protein translation in a rabbit reticulocyte system. The antigenicity of this alternatively spliced 52-kD SS-A/Ro was examined by immunoprecipitation of the in vitro–translated <sup>35</sup>S-labeled protein. As shown in the representative autoradiograph in Fig. 7 A, sera previously dem-



**Figure 5.** RNase protection of 52-kD SS-A/Ro RNA. (A) Schematic diagram of RNA probe and predicted fragments corresponding to the 52 $\alpha$  and  $\beta$  forms. (B) RNase protection of 10–80  $\mu$ g RNA (lanes 1–4) from a 24-wk fetal heart probed with RNA spanning bases 674–964. In addition to the predominant protected fragment of 290nt corresponding to 52 $\alpha$  detected in all four lanes, a 144nt fragment is seen in lanes 3 and 4. All RNA hybridizations were incubated with 50 U RNase A and 2,000 U RNase T1.

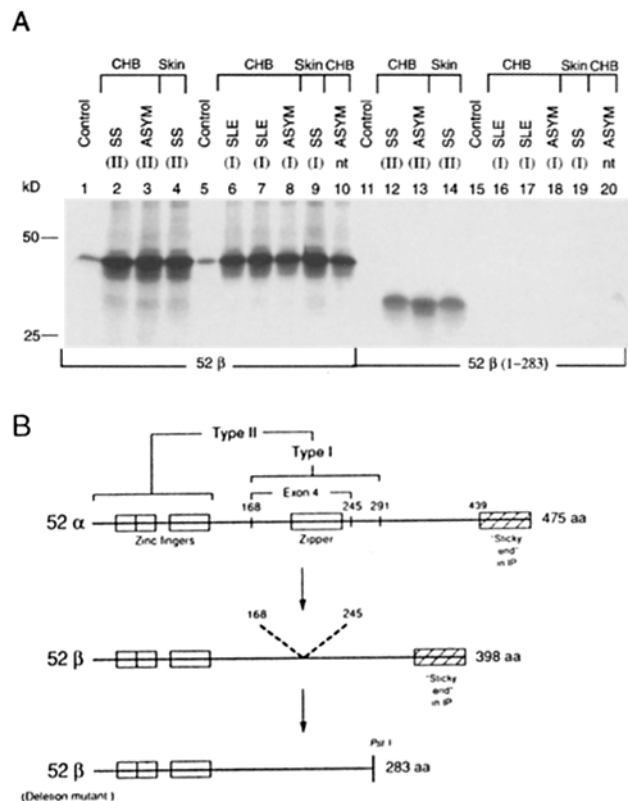


**Figure 6.** RT-PCR analysis of 52-kD SS-A/Ro mRNA. Using the sense primer spanning bases 423–443 and an antisense primer covering bases 1377–1936 of the known sequence for 52-kD SS-A/Ro, two fragments corresponding to the 52 $\alpha$  and 52 $\beta$  were amplified from cDNA of a 16-wk fetal heart, 24-wk fetal heart, and adult heart. The 0.78-kb fragment in the 16-wk fetal heart was subcloned, and nucleotide sequencing confirmed that it represented 52 $\beta$  type mRNA.

**Table 1.** Immunoprecipitation of the <sup>35</sup>S-radiolabeled 52 $\beta$  Form and COOH-terminal Truncated Fragment

Disease	52 $\beta$ (aa1–398)	52 $\beta$ (aa1–283)
SS (n = 8)		
Type I	1 (1)	0 (1)
Type II	3 (3)	4 (4)
Not tested	2 (2)	2 (2)
No IP	0 (1)	0 (1)
SLE (n = 11)		
Type I	6 (6)	2 (6)
Type II	2 (3)	3 (4)
Not tested	0 (1)	0 (1)
UAS (n = 4)		
Type I	0 (0)	0 (0)
Type II	3 (3)	2 (3)
Not tested	1 (1)	0 (0)
Asymptomatic (n = 9)		
Type I	4 (4)	0 (4)
Type II	3 (3)	3 (3)
Not tested	1 (2)	0 (2)
Totals	26 (30) = 87%	16 (31) = 52%

All sera tested were from mothers whose children had manifestations of neonatal lupus. Type I sera (n = 11) recognized the central region of the 52-kD SS-A/Ro protein including the region encoded by exon 4. Type II sera (n = 14) recognized the central region as well as the NH<sub>2</sub>-terminal region aa1–78 as discussed in Materials and Methods and in reference 20. Six sera not previously tested in immunoprecipitation but which recognized recombinant 52-kD SS-A/Ro by ELISA or immunoblot of MOLT-4 are designated “not tested.” Serum from one patient with SS was weakly positive with the recombinant 52-kD SS-A by ELISA but was unreactive by immunoprecipitation, here noted as “no IP.” Two sera (one SS, one SLE) were only tested with the COOH deletion fragment. One sera (UAS) was only tested with the 52 $\beta$  form. Numbers represent those positive of the total number tested.



**Figure 7.** Representative immunoprecipitations of <sup>35</sup>S-radiolabeled 52 $\beta$  SS-A/Ro and COOH-terminal truncated fragment by varied sera. (A) Immunoprecipitations with either the 52 $\beta$  form (lanes 1–10) or the truncated fragment (lanes 12–20) were performed as described in Materials and Methods. The sera used in lanes 1–10 are identical to those used in lanes 11–20, respectively. The clinical assignment is given above each lane: SS, ASYM (asymptomatic), or SLE. Control, two different sera selected from six healthy donors tested. The manifestations of the mother’s affected child, CHB or skin, are noted. (I), Type I reactivity; (II), type II reactivity; nt, not tested previously by immunoprecipitation. (B) Schematic representation of the 52 $\alpha$ , 52 $\beta$ , and 52 $\beta$  deletion mutant. The sticky end, present in both the 52 $\alpha$  and  $\beta$  forms, was previously defined as aa439–475 in 52 $\alpha$  (20). The 52 $\beta$  mutant, in which the COOH terminus (aa360–475 of 52 $\alpha$ ) is deleted by digestion of p52 $\beta$  with the restriction enzyme PstI, does not contain the sticky end. As previously reported, type I anti-52 responses are characterized by recognition of the central region of 52 $\alpha$ . Type II anti-52 responses are characterized by recognition of the central region of the 52 $\alpha$  form as well as the NH<sub>2</sub>-terminal region (20).

onstrated to recognize the 52 $\alpha$  form by ELISA and/or immunoprecipitation also recognized the 52 $\beta$  form. 26 (87%) of 30 sera tested from mothers whose children were known to have neonatal lupus immunoprecipitated the 52 $\beta$  form. This result was unexpected. 11 of the 26 immunoprecipitating sera were previously shown to be reactive with aa1–291 and not aa1–78 or 1–151, implying that in these sera the epitope recognized was within a region on the 52 $\alpha$  form encoded by exon 4 and absent in the 52 $\beta$  form (Fig. 7 A, lanes 6–9, type I reactivity).

As had been observed with immunoprecipitations of 52 $\alpha$  (20, 29), minor background reactivity was noted with control sera (Fig. 7 A, lanes 1 and 5) that increased in parallel with the quantity of sera used. Our earlier experiments

demonstrated that a "sticky" region of the 52 $\alpha$  form mapped to aa439–475 (20). Therefore, sera from the affected mothers were also tested for reactivity with a PstI restriction digest of the 52 $\beta$  form in which the COOH-terminal 115aa were deleted (Fig. 7 B). As expected, sera that had previously been shown to react with both the central region of the 52 $\alpha$  form as well as the NH<sub>2</sub> terminus immunoprecipitated this PstI digest (Fig. 7 A, lanes 12–14). Specifically, 12 (86%) of the 14 type II responders immunoprecipitated the 52 $\beta$  deletion fragment (the two unreactive sera were the weakest type II responders previously identified). In contrast, of 11 antisera that were reactive solely with the central region, only 2 (18%) were reactive with the deletion fragment, and both were substantially weaker than the reactivity observed with the type II sera. A summary of the reactivity segregated by disease and previously defined epitope specificity is provided in Table 1. Immunoreactivity with the 52 $\beta$  form suggests that antigenicity of the 52-kD SS-A/Ro protein is not dependent on the putative leucine zipper domain.

## Discussion

The 52-kD SS-A/Ro protein is expressed in a variety of human cells and tissues and, because of its sequence motifs, is likely to have an important function in transcriptional activity. An antibody response to the 52-kD SS-A/Ro protein has been nearly universally demonstrated either by immunoprecipitation, ELISA, or immunoblot in mothers whose children have manifestations of neonatal lupus (1–4). Isolation of a cDNA clone encoding the 52-kD SS-A/Ro protein was accomplished using the sera from one of these mothers. The molecular characterization of the 52-kD SS-A/Ro protein is now extended by further analysis of the complete genomic structure and the description of an alternative transcript.

In addition to the cDNA clones originally reported (5), an interesting MOLT-4 cDNA clone 52-2 was found to have an internal deletion of 231nt including the domain encoding the leucine zipper motif. The calculated masses for the full-length 52-kD SS-A/Ro (52 $\alpha$ ) and the alternative form (52 $\beta$ ) are 54,000 and 45,000, respectively. To investigate the nature of the deletion found in clone 52-2, a full-length human 52-kD SS-A/Ro cDNA was used to isolate clones from a  $\lambda$ FIXII genomic library. The 52-kD SS-A/Ro gene is comprised of seven exons and covers 10 kb of DNA. The alternative form of the 52-kD mRNA results from splicing of exon 3 to exon 5, skipping exon 4. DNA sequencing unambiguously demonstrates mRNA expression of the 52 $\beta$  form in the human fetal heart. Data obtained with RNase protection assay and PCR suggest that this form is present in other tissues, including the adult heart.

The identification of an alternatively spliced form of the 52-kD SS-A/Ro that lacks a potentially functional domain common to DNA binding proteins, nuclear transforming proteins, and transcriptional regulatory proteins is of potential biologic interest. The internal deletion of aa168–245,

which includes the leucine zipper, may affect protein–protein interaction and subsequent DNA binding, molecular functions ascribed to proteins with this putative motif (30). Since the 52 $\beta$  form includes the NH<sub>2</sub>-terminal zinc finger domains, it would be expected to retain the same DNA/RNA-binding function as that predicted for the 52 $\alpha$  form. Accordingly, the 52 $\beta$  form could function as a competitive inhibitor of the 52-kD SS-A/Ro protein, since both would compete for the same putative DNA/RNA-binding site.

Although there is no direct evidence that the newly described 52 $\beta$  form is important in the pathogenesis of neonatal lupus, several requisite conditions for its potential involvement have been met. First, a 52 $\beta$  form mRNA transcript is expressed in the fetal heart, particularly during the window of presumed vulnerability, 16–24 wk of gestational age (31). Further analysis will reveal whether there is overexpression of this alternative form relative to the full-length form in fetal versus adult heart tissue. Second, the  $\beta$  form is translated, at least in an *in vitro* system, and is antigenic, immunoprecipitated by >80% of numerous sera from mothers whose children have neonatal lupus.

It is of particular interest that the majority of mothers whose children have neonatal lupus react with this novel 52 $\beta$  form, since in previous studies (20) approximately half of these antisera were found to react only with the central region of the 52 $\alpha$  form, deleted in the  $\beta$  form. As demonstrated with the full-length 52 $\alpha$  form, there was background stickiness noted with normal human sera, which complicates the interpretation of true reactivity (20, 29). However, reactivity with sera from affected mothers was clearly greater than that observed with controls. These observations suggest that exposure of a novel epitope may have occurred by altered folding of the protein due to deletion of the central coiled coil region.

To completely eliminate the background stickiness previously attributed to aa439–475 of the full-length 52-kD SS-A/Ro protein, a truncated form of 52 $\beta$  in which the terminal 115aa were deleted was used in immunoprecipitation. As expected, all but two antisera that previously demonstrated reactivity with the NH<sub>2</sub>-terminal region of the full-length 52 $\alpha$  form immunoprecipitated this 52 $\beta$  COOH-terminal deletion mutant. In contrast, only rare sera that were apparently dependent on the central region of the 52 $\alpha$  form for reactivity immunoprecipitated the 52 $\beta$  mutant. These data support several conclusions. Exposure of a novel immunodominant epitope in the 52 $\beta$  form may be dependent on the COOH terminus. The NH<sub>2</sub>-terminal region spanning the zinc finger domains constitutes a restricted epitope in both 52 isoforms. The 52-kD SS-A/Ro protein can certainly be recognized in the absence of the putative leucine zipper domain or adjacent NH<sub>2</sub>-terminal amino acid residues 197–207, a region previously reported to be required for recognition by antisera from 21 patients with SLE (32).

At the present time, there is no definitive proof of *in vivo* translation of the 52 $\beta$  protein from this newly described alternative transcript. However, the mRNA is efficiently translated in a rabbit reticulocyte system. The eval-



uation of protein expression on SDS-immunoblot is complicated, since reactivity with the 48-kD SS-B/La and its 43-kD degradation product might obscure reactivity with the newly described 52 $\beta$  form, predicted to be 45 kD. To eliminate any confusion with SS-B/La, several sera devoid of 48-kD SS-B/La antibodies as measured by ELISA were evaluated on SDS-immunoblot. Intriguingly, these sera did not recognize any bands below the 52-kD protein (data not shown). This observation suggests that, unlike the 52 $\alpha$  form, reactivity with the 52 $\beta$  form may be dependent on native structure as reported for the 60-kD SS-A/Ro protein (33, 34). Nevertheless, on immunoblot of transfected Hep-2 cells overexpressing the 52 $\beta$  form, rabbit anti-52-kD SS-A/Ro sera clearly reacted with a band at 45 kD not detected in untransfected cells (data not shown). We have not attempted labeling of cells with  $^{35}\text{S}$ , since difficulty with this method has been noted for the 52 $\alpha$  form as it has a long half-life relative to most of the common autoantigens (20).

An additional finding of this study was that the 52-kD SS-A/Ro ( $\alpha$  form) expressed in the fetal heart was identical to that previously published in other tissues. The entire sequence for the full-length 52-kD SSA/Ro protein is highly homologous to the rfp protein, a cellular homologue of a human transforming protein, ret (7). The latter is a fusion protein comprised of the NH<sub>2</sub>-terminal zinc finger domain of rfp and a COOH-terminal domain deriving from a tyrosine kinase (28). The protein rfp is expressed in a variety of tumor cells and embryonic tissues. Inasmuch as the ret

fusion protein has been shown to be a surface membrane protein, one plausible mechanism for the zinc finger of the 52-kD SSA/Ro protein to be expressed on the cell surface might be a DNA recombination event between the 52-kD protein gene and a tyrosine kinase gene as it did in the case of the *ret* oncogene. Our strategy was to clone flanking 3' regions that might be derived from such a putative gene fusion using 3' RACE, which generates cDNAs by using PCR to amplify copies of the region between a single point in the transcript and the 3' end. Accessibility of the putative 52 $\alpha$  form in the fetal heart to maternal autoantibodies is as yet not explained by tissue-specific sequences.

In summary, a novel isoform of the 52-kD SS-A/Ro protein is described in which the central leucine zipper motif representing one of the two coiled coils has been deleted. In vitro-translated protein is recognized by antisera that also recognize the full-length 52 $\alpha$  form, notably the majority of mothers whose children have manifestations of neonatal lupus. Further work will be needed to examine the expression of these two forms in affected hearts when they are available to determine the relative importance of 52 $\alpha$  or 52 $\beta$  in the putative relationship of anti-52-kD autoantibodies to CHB. It is anticipated that the alternatively spliced form will provide insight into the function of the 52-kD SS-A/Ro and its intermolecular relationship to the 60-kD SS-A/Ro. In turn, this should facilitate a better understanding of the immune response to the SS-A/Ro autoantigens and its role in the development of neonatal lupus.

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