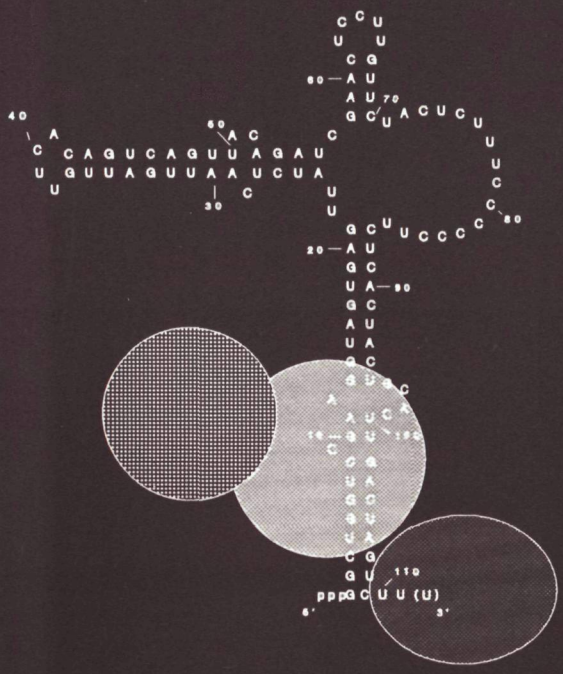
The structure and antigenicity of Ro ribonucleoprotein complexes



R.L. Slobbe

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The structure and antigenicity of Ro ribonucleoprotein complexes

een wetenschappelijke proeve op het gebied van de
Natuurwetenschappen in het bijzonder de Biochemie

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In nagedachtenis aan TT

Voor mijn ouders

Voor Marlea

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Chapter 1:
General introduction

Preface

The presence of autoantibodies in patient serum is one of the hallmarks for autoimmune diseases. In some diseases the presence of certain autoantibodies is of diagnostic significance and the detection and measurement of autoantibodies may therefore be of help in the selection of a proper treatment and management of the disease.

Alternatively, autoantibodies can be used as a tool for the characterization of the autoantigens in most cases complicated nucleic acid-protein complexes with important cellular functions. It is believed that the elucidation of the structure and function of autoantigens may help to understand some of the events causing the aberrant immune response to self components.

Outline of this thesis

In this thesis autoantibodies directed against the Ro and La ribonucleoprotein (RNP) complexes were studied. Both the presence of anti-Ro and anti-La autoantibodies in patient sera and the diagnostic relevance of their presence is discussed. In addition, the molecular composition of the Ro (SS-A) and La (SS-B) ribonucleoprotein particles is examined, as well as the interactions occurring in Ro complexes, reconstituted *in vitro*.

In **Chapter 2** an overview is given concerning both the molecular nature and the antigenicity of the Ro and La ribonucleoproteins based upon current knowledge.

To study the composition of Ro RNP complexes it is essential to be assured of the monospecificity of the antisera used in the experiments. Therefore, in **Chapter 3** a new sensitive method is described to analyze patient sera for the presence of anti-Ro and anti-La antibodies.

Chapter 4 deals with the detection of one of the components of Ro RNP complexes, the 52 kDa Ro protein, and its involvement in Ro particles *in vivo*. The presence of anti-Ro60 and anti-Ro52 antibodies is analyzed by immunoblotting and the association with RNA and intracellular localization of the Ro proteins is examined. Moreover, the evolutionary conservation of the antigens is studied by Western blotting. In the **Appendix** of this chapter the evolutionary conservation of the Ro RNAs is analyzed as well.

By various *in vitro* reconstitution techniques, the molecular composition of the Ro RNP complexes has been analyzed in **Chapter 5** and **Chapter 6**, as well as the RNA-protein and protein-protein interactions occurring in the complexes.

The results of the studies described in this thesis are summarized in **Chapter 7**.

Chapter 2:
Ro (SS-A) and La (SS-B) ribonucleoprotein complexes: Structure, function and antigenicity.

R.L. Slobbe, G.J.M. Pruijn and W.J. van Venrooij

Annales de Médecine Interne (1991) in press

Introduction

During the last decade a great deal of information concerning the Ro and La ribonucleoprotein (RNP) complexes has been obtained. For studies on the structure and function of these RNA-protein complexes autoantibodies directed against the Ro and La antigens, frequently found in patients with systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS), have been of great importance. Reversely, the analysis of the RNPs and the cloning of the antigens has been and will be of great importance for the understanding of the immunological basis of autoimmunity. In this review results concerning both the molecular composition of Ro and La RNPs and the pathogenic and diagnostic significance of the occurrence of anti-Ro and anti-La autoantibodies will be discussed.

Historical background

In 1961, Anderson and co-workers described two immunologically distinct antibody systems in sera from patients with Sjögren's syndrome, SjT and SjD (Harley, 1987). A few years later, Reichlin and coworkers demonstrated two distinct precipitin systems, designated Ro and La, in sera from patients with SLE (Clark et al., 1969; Mattioli and Reichlin, 1974). The similarity in physical and serological properties suggested that SjD corresponds to the Ro and SjT to the La antigen. Later on, Alspaugh and Tan (1975) reported two new antibody specificities in sera from patients with SS, called SS-A and SS-B, whereas Akizuki et al. (1977) detected a precipitating antibody in sera from patients with primary SS and SLE with SS, which was termed Ha. In 1979, comparison of all data showed that SS-A corresponds to the Ro (SjD) antigen and SS-B and Ha to the La (SjT) antigen (Alspaugh and Maddison, 1979).

Structure of La RNPs

La RNPs are composed of a 46.7 kDa antigenic phosphoprotein (hereafter referred to as La) associated with an RNA polymerase III transcribed RNA molecule. The RNA moiety of the complex includes (precursors of) 7S RNA, 5S rRNA, tRNA, U6 RNA and the Ro RNAs as well as some virally encoded RNAs like VA_I and VA_{II} RNAs, EBER1 and EBER2 RNAs (all RNA polymerase III transcripts) and leader RNAs of vesicular stomatitis virus and rabies virus (transcribed by virus-specific RNA polymerases) (reviewed by Pruijn et al., 1990). In addition, U1 RNA, an RNA polymerase II transcript has been reported to associate with La as well (for review see Keene et al., 1987). It has been shown that a 3'-uridine stretch common to all RNA polymerase III transcribed RNAs constitutes the La binding region of the RNA (Stefano, 1984; Matthews and Francoeur, 1984; Glickman et al., 1988). Since this 3' sequence motif is mostly lost upon maturation of the transcripts, the La protein in most cases binds to precursor-RNAs only transiently. However, the Ro RNAs and the virally encoded VA and EBER RNAs contain the La binding region in the mature RNA and, therefore, the La protein is considered to bind to these RNAs in a more stable manner.

Structure of the La protein

Complementary DNA clones encoding the complete La protein have been isolated by several groups (Chambers et al., 1988; Chan et al., 1989a). The human La protein is composed of 408 amino acids with a predicted molecular weight of 46.7 kDa and is well conserved during evolution as revealed by a high degree of amino acid conservation between human, bovine and *Xenopus laevis* La cDNA clones (Chan et al., 1989a; Moreau et al., 1990) and by the fact that La proteins from many different species are immunoreactive with human autoantibodies (Chan and Tan, 1987a). Analysis of genomic clones revealed that the La gene is split into 11 exons. The promoter region resembles that of "housekeeping" genes (i.e. it contains a CCAAT box and an SP1 binding sequence, but lacks a TATA-box), and contains a G/C rich region that is similar to a region in the H-ras gene promotor (Chambers et al., 1988).

The La protein contains an 80 amino acids domain, termed RNA recognition motif, RNA binding domain or RNP-80 motif, often found in RNA binding proteins (Bandziulis et al., 1989; Query et al., 1989; Scherly et al., 1989) and shown to be essential for RNA-binding of a number of proteins. Results reported by Chan and Tan (1987b) indicate that the RNA binding region of La is located at the amino-terminal part of the protein, consistent with the location of the RNP-80 motif. Although it is presumed that this motif is important for the binding of La to the cognate RNAs, it has been shown that additional sequences at the amino-terminus of the La protein are required for the interaction with RNA as well (Moreau et al, 1990; our unpublished results). Other structural features of La include an α-helical central domain, three so-called PEST-regions, known to be susceptible to protease digestion (Chan et al., 1989a) and two presumed ATP binding sites (Figure 1). The PEST regions may explain the susceptibility of the La protein to proteolytic degradation, often leading to characteristic degradation products of 43 and 28 kDa (Habets et al., 1983).

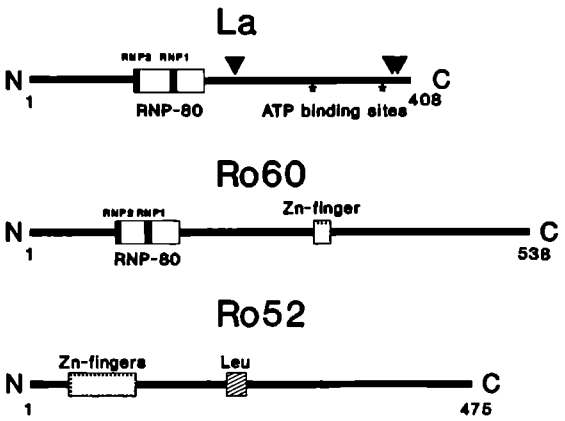

Figure 1 ***Structural features of La, Ro60 and Ro52 polypeptides.***

Structural features of human La, Ro60 and Ro52 proteins are RNP-80 RNP-80 motif, RNA recognition motif or RNA binding region), Leu leucine zipper, RNP1 and RNP2 most highly conserved sequences within the RNP-80 motif Arrowheads indicate the so-called PEST regions that are susceptible to protease digestion Numbers refer to the amino acids of the respective proteins N is amino-terminus, C carboxy-terminus

Function of La RNPs

Because of the apparent loose association of the La antigen with intracellular structures, leading to altered distributions in the cell during biochemical fractionation or fixation, the intracellular localization of the La antigen has been the subject of extensive research and confusion. However, during the past decade it has become evident that the La antigen is primarily located in the nucleus as detected by light (Hendrick et al., 1981; Habets et al., 1983; Bachmann et al., 1986) and electron microscopic techniques (Carmo-Fonseca et al., 1989). This localization and the association with newly synthesized RNA polymerase III products led to the suggestion that La participates in the transcription, maturation and/or nuclear export of the transcripts. Indeed, Gottlieb and Steitz (1989a,b) demonstrated that La is required for efficient and correct termination of RNA polymerase III transcription. Furthermore, it was published that purified La protein may function as an ATP dependent helicase able to melt RNA-DNA hybrids (Bachmann et al., 1990a), thereby resembling the prokaryotic transcription termination factor rho. However this finding has not been confirmed yet. Another putative role for La may be its involvement in nucleocytoplasmic transport of the RNA polymerase III transcripts (Bachmann et al., 1989), although it has been shown recently that La is not involved in the transport of precursor 5S rRNA from the nucleus to the cytoplasm in *Xenopus laevis* oöcytes (Guddat et al., 1990).

Structure of Ro RNPs

Ro RNPs are comprised of several Ro proteins (Table I) complexed with a subset of La associated RNAs, the RNA polymerase III transcribed Y RNAs. Since the Y RNAs are presumably not subjected to processing (Wolin and Steitz, 1983), the La binding region of these RNA molecules is present in the mature RNAs and, therefore, the La protein can be considered a more stable component of Ro RNPs (Mamula et al., 1989a; Boire and Craft, 1990). In human cells, four RNAs have been identified, termed hY1, hY3, hY4 and hY5, varying in length between 84 and 112 nucleotides (Hendrick et al., 1981). The sequences have been determined (Wolin and Steitz, 1983; Kato et al., 1982; O'Brien and Harley, 1990) and the derived secondary structures of the RNAs are shown in Figure 2. These are characterized by base-pairing of the 3' and 5' termini, thus forming an extended stem-loop structure. The lower part of the stem of all hY RNAs contains a highly conserved region which has been shown to be protected against RNAse degradation (Wolin and Steitz, 1984), presumably by bound protein(s).

Table I *Ro RNP components.*

Ro associated RNAs	
	hY1, hY3, hY4, hY5 (human)
	bY1, bY2, bY3, bY4 (bovine)
	cY1, cY3, cY4, cY5 (cavia)
	lY1, lY2, lY3 (leporine)
	rY1A, rY1B, rY2 (rat)
	mY1, mY2 (murine)
	dY1, dY2 (duck)
Ro associated proteins	
	60 kDa (=Ro60), 52 kDa (=Ro52) (ubiquitous)
	60 kDa, 54 kDa (erythrocytes)
	La

The Ro RNPs show heterogeneity at several levels. First, the total number of cellular Ro RNAs

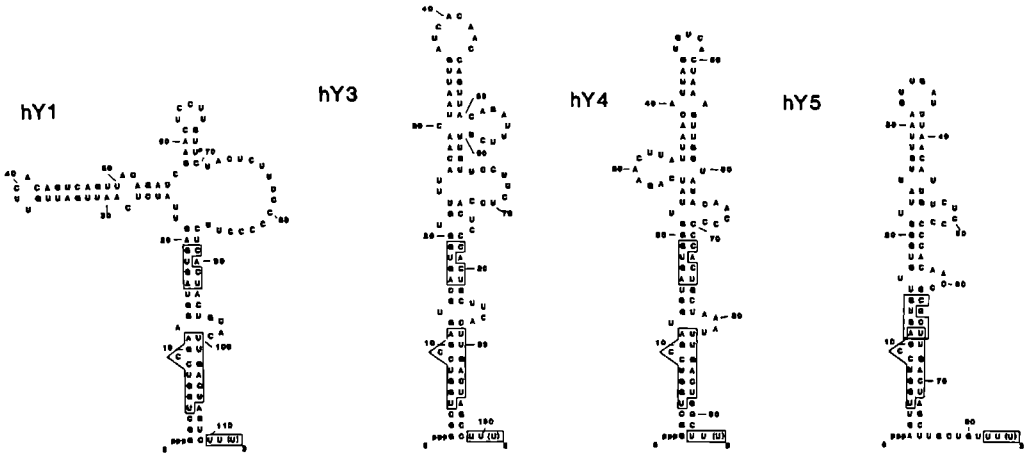

Figure 2 ***Human Ro RNAs***

Predicted secondary structures of hY1, hY3, hY4 and hY5 based upon free energy minimizations using parameters of Turner et al., 1988. The structures of hY3 and hY4 were slightly modified with minor effects on free energy of formation to maintain a constant structure of the most conserved region. At the 3' end of hY4 a uridine residue was added in order to rescue the La binding region of hY4 RNA. Most highly conserved regions are boxed Similar structures have been published by Wolin and Steitz (1983), O'Brien and Harley (1990) and Pruijn et al. (1990).

differ among species, varying from two in mouse and duck cells to four in human, bovine and guinea pig cells (see Table I)(Hendrick et al., 1981; Reddy et al., 1983; Mamula et al., 1989b; Itoh et al., 1990). An RNA of similar size as hY1 RNA has been found in all species analyzed as detected by analysis of anti-Ro immunoprecipitated RNAs, whereas Y RNAs homologous to hY1 and hY3 have been detected by Northern blotting in all mammalian cells examined (our unpublished results).

Second, differences in Ro RNP composition have been observed between cells within a species. In human red blood cells only hY1 and hY4 RNA are immunoprecipitated (Rader et al., 1989; O'Brien and Harley, 1990) in contrast to the four RNAs found in other human cells. Moreover, two antigenic Ro proteins of 60 and 54 kDa have been detected in red blood cells (Rader et al., 1989), while in other human cells 60 kDa (Ro60, antigenically distinct from the red blood cell 60 kDa protein) and 52 kDa (Ro52) antigens have been described (Ben-Chetrit et al., 1988). Additionally, a third immunoreactive 60 kDa protein has been described in Wil-2 cells (Lieu et al., 1988). This protein was shown to be homologous to a number of calcium binding proteins (McCauliffe et al., 1990) but the evidence concerning its role as a Ro RNP component, however, is as yet doubtful. In fact, in follow-up studies no evidence has been obtained to confirm the association of this 60 kDa protein with Ro RNP complexes (Dr L. Rokeach, personal communication; our unpublished results).

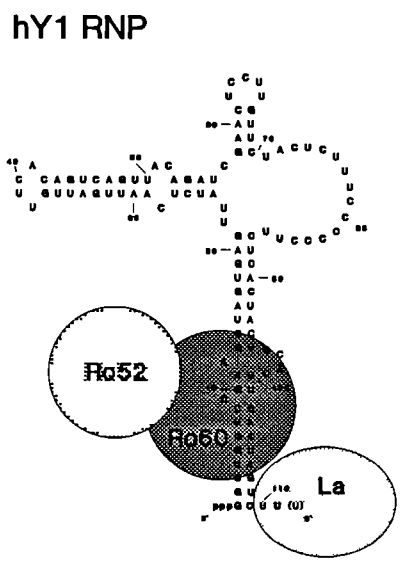

Figure 3 ***Model for the hY1 RNP.***
Secondary structure of hY1 RNA is shown as predicted by the method of Turner et al , 1988 Proteins are shown as filled circles

Third, within a cell different Ro RNPs can be distinguished based upon different physicochemical properties (Boire and Craft, 1990). The molecular weights of the Ro complexes as estimated by gel filtration were quite heterogeneous: Ro RNP complexes were found at 230 kDa (containing hY4 RNA only) and between 300 and 350 kDa (one subset of RNP particles containing hY1, hY3 and hY4 RNA and one containing hY5 RNA only). These unexpectedly high molecular weights may be explained by the existence of multimers of proteins or particles or may be due to the differential binding of yet unidentified Ro components. A further indication for the heterogeneity of Ro RNPs within the cell is the detection of a human antiserum precipitating hY5 RNA only, thus indicating that the hY5 RNP particle contains unique antigenic determinants (Boire and Craft, 1989).

Results from our laboratory have identified some of the interactions occurring in Ro RNP complexes. As summarized in Figure 3, no direct interactions between the two Ro antigens and the La antigen could be observed, indicating an independent binding of the Ro and La antigens to the hY RNA. Binding of Ro52 to hY RNA, however, was found to be dependent on the presence of Ro60. A direct RNA-independent protein-protein interaction between Ro60 and Ro52 has been identified in which a region of Ro60 between amino acid 276 and 318 was required for the binding of Ro52. Additionally, the presumed binding regions of the Ro proteins (the conserved nucleotides in the lower part of the stem of the RNA) and La (the 3' uridine stretch) on hY RNAs were indeed shown to be the sites of interaction of these proteins (Slobbe et al., submitted; Pruijn et al., manuscript in preparation).

The evolutionary conservation of the Ro antigen has been the subject of several studies. Ro RNPs have been detected immunologically in a number of species, such as man, monkey, dog, cat, guinea pig, bull, rabbit, rat, mouse and duck (Hendrick et al., 1981; Mamula et al., 1989b; Byers et al., 1990; Slobbe et al., 1991a). Since most of these authors used antisera with mixed specificities and, therefore, could not discriminate between Ro60 and Ro52, the evolutionary conservation of Ro60 and Ro52 has not been elucidated in detail. Results from our laboratory (Slobbe et al., 1991a) indicate that Ro60 is well conserved during evolution. In contrast, Ro52 could be detected immunologically in primate cells only. Whether this limited conservation of Ro52 reflects the loss of

epitopes during evolution or the absence of a related protein is as yet not clear. Hopefully, analyses by Northern blotting will answer this question in the near future.

Structure of the 60 kDa Ro protein

Two cDNAs encoding Ro60 have been isolated and characterized (Deutscher et al., 1988; Ben-Chetrit et al., 1989). Some differences exist in the 3' sequences of the coding regions of the cDNAs (the C-terminal 24 amino acids of the predicted protein sequence by Deutscher et al. are replaced by 11 amino acids in the protein deduced from the cDNA reported by Ben-Chetrit et al.) leading to molecular weights of 60.6 and 59.2 kDa, respectively. Whether this difference is caused by alternative splicing of one gene product or reflects differential expression of two genes encoding the Ro60 protein (the two cDNAs were obtained from human cells derived from different tissues) is not clear yet. The common part of the deduced Ro60 proteins contains an RNP-80 motif and a putative zinc finger (see Figure 1). Although the RNP-80 domain is thought to dominate the RNA-Ro60 interaction, it has become clear that the conformation of Ro60 is extremely important for the binding to hY RNA since small deletions at the amino- or carboxy-terminus of the protein precluded the binding to hY RNA (Pruijn et al., manuscript in preparation). The zinc finger is a cluster of cysteine and histidine residues originally described to be involved in the binding of DNA by DNA-binding proteins, but possibly involved in RNA-protein or protein-protein interactions as well (Berg, 1986; Nelissen et al, 1991). Deutscher et al. (1988) were able to reconstitute ribonucleoprotein complexes *in vitro* composed of hY1 RNA and recombinant Ro60 protein, suggesting a direct association of Ro60 with hY RNA.

Structure of the 52 kDa Ro protein

Ro52 was not detected by Western blotting until recently, because it comigrates in SDS-PAGE with the La protein. However, altering the crosslinking level of the polyacrylamide gels enables the separation of the Ro52 and La polypeptides and allows their subsequent detection by immunoblotting (Ben-Chetrit et al., 1988; Buyon et al., 1990; Slobbe et al., 1991a). Recently, two cDNA clones coding for Ro52 have been isolated and characterized (Chan et al., 1991; Itoh et al., 1991). Except for some small differences, possibly due to DNA sequencing errors, both cDNAs encode a protein with a molecular weight of 54.1 kDa. No homology was found with the Ro60 protein, but a significant homology was found with a mouse T-cell down-regulatory protein *rpt-1* and with human transforming protein *rfp*. The deduced amino acid sequence and secondary protein structure reveal a number of interesting features (Figure 1): First, a number of putative zinc finger motifs were found in the amino terminal part of the protein. The location, number and spacing of these motifs are highly conserved in the Ro52, *rfp* and *rpt-1* proteins, which may relate to a similar function for all three proteins. Second, the same region of Ro52 where the putative zinc fingers are located contains a consensus sequence for a newly identified cysteine-rich motif found in a number of DNA binding proteins (Freemont et al., 1991). However, the biological significance of this motif has not been defined yet. Third, a leucine zipper motif is present in the central part of the

Ro52 protein. Although leucine zippers were originally described in DNA binding proteins (Landschultz et al., 1988), these motifs are now known to participate in protein-protein interaction and dimer formation as well (Kouzarides and Ziff, 1988; Sassone-Corsi et al., 1988; Landschultz et al., 1989). Therefore, the putative leucine zipper of Ro52 may be important for the interaction with other proteins or for dimerization. No direct association of Ro52 with Ro RNAs has been found.

Function of Ro RNP complexes

Although the intracellular localization is not clear yet, various biological functions have been proposed for the Ro RNPs. Among these are nuclear functions such as roles in transcription, processing and transport processes, based upon a potential nuclear localization and the relation with the La antigen. Because of the observed cytoplasmic localization, some cytoplasmic functions have been suggested as well, such as translation and storage of processed mRNA. At present, it cannot be excluded that different Ro RNPs are involved in different processes and since Ro RNPs seem to be reasonably well conserved in evolution, it is likely that these RNA-protein complexes support important functions.

Prevalence of anti-Ro and anti-La autoantibodies

Autoantibodies directed against the Ro and La antigens can be found in sera from patients with a variety of autoimmune disorders (see Table II) but do not seem to be completely disease specific. The variance of the numbers mentioned in Table II can be explained partly by the use of different methods for the detection of the autoantibodies. For example, it is known that anti-Ro antibodies, in contrast to anti-La antibodies, can not be detected as sensitive by Western blotting as by counter immunoelectrophoresis (Williams et al., 1986; Meilof et al., 1990; Provost et al., 1991; van Venrooij et al., 1991). In addition, for the detection of anti-La antibodies a very sensitive RNA precipitation assay has been developed by which very low titers of anti-La antibody can be detected (Slobbe et al., 1991b). In some cases, "normal" human sera may also contain very low titers of anti-Ro antibodies (Gaither et al., 1987). High frequency presence of anti-Ro antibodies has been associated with subacute cutaneous lupus (Sontheimer et al., 1979 and 1982; Provost, 1991) and with neonatal lupus (Provost, 1983; Buyon et al., 1989; Watson et al., 1991). In neonatal lupus IgG antibody is transplacentally transferred from mother to infant and manifested in skin rash and complete congenital heart block. However, the pathogenesis of neonatal lupus may not be due to the presence of autoantibodies since many mothers with anti-Ro antibodies give birth to normal offspring.

Since both anti-Ro and anti-La antibodies are most prevalent in sera from patients with either Sjögren's syndrome or systemic lupus erythematosus, these two patient groups have been studied extensively in order to reveal possible disease associations. In Sjögren's syndrome anti-Ro and anti-La antibodies were found more frequently in patients with earlier disease onset, longer disease duration, parotid or major salivary gland enlargement and intensive lymphocytic infiltrations of the minor salivary glands (Manoussakis et al., 1986). Additionally, the presence of anti-Ro and anti-La antibodies correlated well with

Table II *The prevalence of anti-Ro and anti-La antibodies in connective tissue diseases.*

Disease[1]	Method[2] of detection	anti-Ro (%)	anti-La (%)	reference
SLE	ELISA	55	24	Maddison et al., 1985
	ID	51	6	Yamagata et al., 1986
	ID	63	12	Boey et al., 1988
	CIE	19-69	39-41	Speransky et al., 1988
	IB	17	10	De Rooij et al., 1988
	CIE, ID	35	15	Chan and Tan, 1989
	CIE	12-28	18-45	Swaak et al., 1990
	RNA	n.d.	35	Slobbe et al., 1991b
SS	ELISA	96	87	Harley et al., 1986
	CIE, ID	63	40	Manoussakis et al,1986
	ID	83	25	Yamagata et al., 1986
	IB	38	77	De Rooij et al., 1988
	CIE, ID	60	40	Chan and Tan, 1989
	RNA	n.d.	58	Slobbe et al., 1991b
RA	ELISA	28	22	Maddison et al., 1985
	ID	15	0	Yamagata et al., 1986
	RNA	n.d.	16	Slobbe et al., 1991b
Polymyositis	ELISA	18	18	Maddison et al., 1985
	ID	9	0	Yamagata et al., 1986
Controls	ELISA	5	0	Maddison et al., 1985
	ID	2	0	Yamagata et al., 1986
	ELISA	17.5	7.5	Gaither et al., 1987
	RNA	n.d.	0	Slobbe et al., 1991b

[1] SLE; systemic lupus erythematosus, SS; Sjögren's syndrome, RA; rheumatoid arthritis
[2] ID; immunodiffusion, IB; immunoblotting, CIE; counter immunoelectrophoresis, RNA; RNA precipitation

other disease manifestations such as purpura, leukopenia, lymphopenia, increased polyclonal gamma globulins and reumatoid factor titers (Harley et al., 1986). Both anti-Ro and anti-La antibody was found in the saliva of SS patients, but it is not clear whether these antibodies are produced locally or whether the presence of these autoantibodies in saliva was due to leakage from blood towards the saliva (Horsfall et al., 1989; Manoussakis et al., 1989), nor has it been demonstrated that the presence of anti-Ro or anti-La autoantibody in the saliva accounts for the "dry mouth" syndrome typical for patients with Sjögren's syndrome.

In SLE patients clinical disease manifestations associated with the presence of anti-La and anti-Ro antibodies are not identified unambiguously, although some studies in this area have been performed. Wasicek and Reichlin (1982) found a strong positive correlation of the presence of anti-Ro antibodies alone with serious renal disease. However, Maddison et al. (1988) reported a negative correlation of the presence of anti-Ro antibodies with nephritis, in agreement with Swaak et al. (1990). These latter authors also reported a positive correlation of the presence of anti-La antibodies with central

nervous system involvement. Additionaly, both Wasicek and Reichlin (1982) and Speransky et al. (1988) reported a positive correlation of the presence of both anti-Ro and anti-La antibodies in SLE patients with skin rash.

Since most of the studies mentioned above could not discriminate between anti-Ro60 and anti-Ro52 antibodies, only few information on the presence of these two autoantibodies in patient sera is available. Ben-Chetrit et al. (1990) and Slobbe et al. (1991a) reported the presence of both anti-Ro60 and anti-Ro52 antibody in SLE and SS patient sera. The presence of anti-Ro60 alone, however, was indicative for SLE, whereas the presence of anti-Ro52 alone was indicative for Sjögren's syndrome. In neonatal lupus, the predominant anti-Ro antibody response was found to be directed to the Ro52 antigen (Buyon et al., 1989), often accompanied by the presence of anti-La antibody. Clinical manifestations in SLE and SS patients associated with the presence of anti-Ro60 or anti-Ro52 antibodies have not been reported yet.

Autoepitopes on Ro and La antigens

Autoepitopes of the La protein have been described by many authors using different biochemical and molecular biological approaches (summarized in Figure 4). In 1986, Chan and co-workers described the presence of autoepitopes on two domains of the La protein obtained by limited proteolytic cleavage, designated X (29 kDa) and Y (23 kDa), later identified as the N- and C-terminal part of the La protein, respectively. The cloning of (part of) the cDNA encoding La has facilitated the characterization of B-cell epitopes considerably. By cloning of the carboxy-terminal end of La, Sturgess et al. (1988) showed that an epitope was located in a region between amino acid (aa) 306-408. Rauh et al. (1988) identified three B-cell epitopes located at the C-terminus of La between aa. 224-292, aa. 293-345 and aa. 346-380, respectively. Since in this study every anti-La antiserum tested contained antibodies to all three epitopes at a similar ratio, the suggestion was made that the production of anti-La autoantibodies is antigen driven. By analysis of the antigenicity of the complete La protein

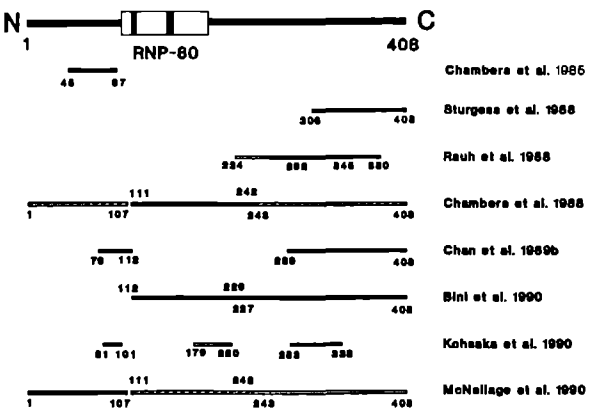

Figure 4 ***Autoepitopes of La.***
Schematic representation of autoepitopes of the La protein, as described by the authors indicated on the right. Solid bars represent major epitopes, dashed bars epitopes of intermediate importance, open bars epitopes of low significance. Numbers refer to the numbers of the amino acids of La. Asterisks mean that only partial La cDNAs were used in the determination of autoepitopes of the La protein.

using recombinant protein, an epitope in the N-terminal region of the La protein was identified (termed A: aa. 1-107) in addition to epitopes in the middle of the protein (termed C: aa. 111-242) and at the C-terminus at aa. 242-408 (termed D; Chambers et al., 1988; St. Clair et al., 1988; McNeilage et al., 1990). St. Clair et al. (1990a) showed by analysis of sequential sera that antibody responses to these different epitopes (A,C and D) vary in parallel over time both in Sjögren's syndrome and in SLE patient sera, indicating a coordinated expression of different anti-La antibody populations. In 1989, Chan and coworkers (1989b) described preliminary results concerning the presence of two autoepitopes on La, located at aa. 76-112 and at aa. 280-408. Recently, Bini et al. (1990) described two immunodominant epitopes located at aa. 112-226 and aa. 226-408, whereas Kohsaka et al. (1990) identified two major epitopes at aa. 81-101 and aa. 283-338 and a minor epitope at aa. 179-220. Up till now, no clinical manifestations of autoimmune patients could be linked to the presence of autoantibodies to specific epitopes on La (St.Clair et al., 1989), suggesting that the quantitative and qualitative aspects of the anti-La immune response are determined by factors distinct from those determining the clinical expression of disease.

Virtually nothing is known about the location and significance of autoepitopes of Ro52 and Ro60. Preliminary results indicate that autoepitopes are located along the entire length of Ro60 (Saitta et al., 1989).

Onset and perpetuation of the autoimmune response

One can only speculate on the mechanisms governing the autoimmune response to the La antigen. Generally it is believed that the autoimmune response is driven by the antigen itself, as indicated by the multiplicity of the epitopes on the antigens found in most sera, the coordinated expression of different anti-La antibody populations (St. Clair et al., 1989) and the preferred reactivity of human autoantibodies with human antigens (Reichlin et al., 1989; Reichlin and Reichlin, 1989; Provost et al., 1991). However, it is not clear whether the antigen itself is involved in the onset of the immune response. By immunization with La protein, it was shown that the immune response to La in mice is different from the autoimmune response in man (St. Clair et al., 1990b). This may be due to differences in the immune system of mice compared to man, but, alternatively, may be explained by the fact that the La protein itself is not involved in the onset of the immune response. In addition, a striking homology between an autoimmune epitope of La (between aa. 81-101) and a viral *gag* protein has been noticed (Kohsaka et al., 1990) and it has been shown that immunodominant epitopes of the HIV capsid protein are located in the regions with high similarity to epitopes found in a number of autoantigens, among which is the N-terminal epitope located between aa. 81 and 101 of La (Garry, 1990). Furthermore, McNeilage et al. (1990) detected a primary reaction against an immunodominant epitope of the La protein located at aa. 1-107 which in a later stage of the disease was followed by the appearance of other antibody classes recognizing other epitopes. This may indicate that the onset of the autoimmune response is indeed directed against the N-terminus of the La protein, which includes the epitopic region with high homology to a viral protein (Kohsaka et al., 1990). Therefore, an interesting hypothesis is

that the autoimmune response to the La protein is invoked by cross-reaction of antibodies originally directed against a viral protein (molecular mimicry).

However, other models for the onset of the immune response to the La antigen have been proposed. For example, it has been observed that the La antigen is able to translocate to the cell membrane after virus infection or UV-irradiation (Baboonian et al., 1989; Bachmann et al., 1990b; Furukawa et al., 1990). After translocation to the cell surface the La antigen might be exposed to the immune system and after binding to HLA class II proteins an autoimmune response might be induced. In this model the antigen itself (or parts of the antigen) is responsible for the onset of the immune response.

Perspectives

As will have become clear, in spite of the fact that a great deal of knowledge has become available in the last decade, a lot of intriguing questions still remain to be answered. We have to know more about the structure, function and antigenicity of the Ro and La RNP complexes to be able to answer more fundamental questions about the molecular basis of autoimmunity. It is expected that the molecular cloning of the RNP components will provide further tools for tackling these problems and that further research will help us to understand better the molecular basis of autoimmunity.

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Chapter 3:
The use of adenovirus infected HeLa cells for the detection of low titer autoantibodies

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The use of adenovirus infected HeLa cells for the detection of low titer autoantibodies

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Abstract

Upon infection of HeLa cells with adenovirus type 5 the cellular La protein becomes predominantly associated with the virally encoded RNA polymerase III products VA_I and VA_{II}, while most of the host RNA polymerase II (e.g. U1, U2, U4, U5 and mRNA) and RNA polymerase III transcription (e.g. U6 and pre-tRNAs) is stopped. Interestingly the cellular Ro RNAs, RNA polymerase III products as well, are still being transcribed and assembled into ribonucleoprotein complexes containing the Ro (SS-A) antigen.
Using a [^{32}P] pulse-chase labelled, adenovirus infected HeLa cellular extract as a source of antigen, anti-La (SS-B) and anti-Ro (SS-A) antibodies can be detected simultaneously using an immunoprecipitation assay. It is shown that this method is more sensitive in detecting anti-La antibodies then counter immunoelectrophoresis and immunoblotting. Because of the sensitivity of the method, the percentage of sera from patients suffering from rheumatic diseases positive for anti-La antibody is raised significantly, especially in patients with systemic lupus erythematosus.

Introduction*

The La protein, also called SS-B antigen (Alspaugh et al., 1979, Provost, 1979, Lerner et al., 1981a), is a highly conserved 47 kDa phosphoprotein. Mammalian cells contain approximately $2 * 10^7$ copies of this protein. La is predominantly localized in the cell nucleus and forms tight complexes with newly synthesized RNA polymerase III transcripts (for review, see Pruyn et al., 1990). Some abundant viral small RNA transcripts are also known to be associated with the La protein, among which the VA I and VA II RNAs of adenovirus (Lerner et al., 1981a, Rosa et al., 1981, Francoeur et al., 1982), the EBER 1 and EBER 2 RNAs of Epstein-Barr virus (Lerner et al., 1981b) and the leader RNAs of vesicular stomatitis virus (Wilusz et al., 1983) and rabies virus (Kurilla et al., 1984).

The Ro (SS-A) ribonucleoprotein (RNP) particle in human cells consists of at least two antigenic proteins of 60 and 52 kDa complexed with four RNAs, termed hY1, hY3, hY4 and hY5. These RNAs are known to be transcribed by RNA polymerase III and are therefore associated with the La protein as well. The exact molecular composition, cellular localization and function of the Ro ribonucleoprotein complexes is still unknown (Pruyn et al., 1990).

For some reason, the La and Ro proteins can become targets of the immune system in several autoimmune syndromes, often leading to the occurrence of both anti-La and anti-Ro autoantibodies in patients (for review, see Tan, 1989). Many studies have pointed to the diagnostic value of anti-La and anti-Ro autoantibodies in serum of patients suffering from connective tissue diseases. In these studies up to 40% of Sjögren's syndrome (SS) patients, 10-15% of systemic lupus erythematosus patients (SLE)(de Rooij et al., 1988, Chan et al., 1989), 31% of undifferentiated connective tissue disease (UCTD) patients and 6% of rheumatoid arthritis (RA) patients (de Rooij et al, 1988) were found to have anti-La antibodies, whereas about 60% of SS, 35% of SLE (Chan et al, 1989), 11% of UCTD and 2% of RA (de Rooij et al., 1988) patients were positive for anti-Ro antibodies. However, these figures are mainly based upon the detection of antibodies by conventional immunological methods such as immunoblotting, counter immunoelectrophoresis (CIE) and ELISA, that all have their respective pro's and contra's. Immunoblotting is known for its sensitivity but detection may be negatively influenced due to the disruption of conformational epitopes. Counter immunoelectrophoresis is very simple but not as sensitive and very dependent on the quality and source of antigen used in the assay. The ELISA method is very rapid, sensitive and easy to handle, but can be used only when purified antigens are available.

Since monoclonal antibodies directed against the Ro and La proteins are still

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*Abbreviations: **RNP:** ribonucleoprotein **snRNA:** small nuclear RNA **SS:** Sjögren's syndrome **UCTD:** undifferentiated connective tissue disease **RA:** rheumatoid arthritis **CIE:** counter immunoelectrophoresis **VA RNA:** adenovirus associated RNA **m_3G:** N^2,N^2,N^7-trimethylguanosine

relatively rare, human sera containing autoantibodies against these components are widely used in studies on the function, composition and localization of Ro and La ribonucleoprotein complexes. For this type of study it is essential to be assured of the monospecificity of the human autoantibody. Using adenovirus infected HeLa cell extracts as the source of antigen in an immunoprecipitation assay, the VA RNAs precipitated by patient sera are highly indicative for the presence of anti-La antibodies, whereas the Ro RNAs, precipitated by anti-Ro antibodies, can be demonstrated simultaneously. Due to the large amount of VA RNA produced in these cells and the extreme sensitivity of detecting the precipitated [^{32}P]-labelled VA RNAs, this method is much more sensitive than immunoblotting and counter immunoelectrophoresis. Using this method we were able to detect low concentrations of anti-La antibody, that seem to be frequently present in sera from patients with systemic lupus erythematosus.

Materials and methods

Human sera

Sera were obtained from patients with connective tissue diseases such as systemic lupus erythematosus (SLE), rheumatoïd arthritis (RA), Sjögren's syndrome (SS) and undifferentiated connective tissue disease (UCTD), all diagnosed according to established criteria as described before (de Rooij et al., 1988).

Infection and labelling of HeLa cells

HeLa S3 monolayer cells were infected with adenovirus type 5 at approximately 100 pfu/cell in Eagles minimal essential medium (EMEM) supplemented with 1 mM arginine at about 60% confluency. After 16 h of infection cells were labelled for various periods of time with [^{32}P]-orthophosphate (1mCi/10 ml medium) in EMEM supplemented with 5% dialysed fetal calf serum and when indicated chased for 7 h in non-radioactive EMEM with 5% fetal calf serum.

Preparation of cellular extract

The infected and labelled cells were washed once with PBS and harvested in NE-4 buffer (10mM Tris-Cl pH7.4, 1mM $MgCl_2$, 100mM NaCl) at 4.0 * 10^6 cells/ml. After sonication of the cells (Branson sonifier; output 3, duty cycle 50%, pulsed, 2 * 1 min.) the suspension was centrifuged at 13000g for 10 min at 4 °C. The supernatant was stored at -70 °C in aliquots.

RNA precipitation

Protein A Sepharose (PAS) beads were prepared for RNA precipitation by rotating 250μl 10% PAS with 20μl serum in 400μl IPP (10mM Tris-Cl pH 8, 0.5M NaCl, 0.1% Tween 20, 0.1% NP40) for 1 h followed by washing three times with IPP. The PAS beads coated with antibody were subsequently rotated with 125 μl cellular extract in 400 μl IPP for 2h at 4 °C. After washing 5 times with 1 ml IPP the PAS beads were resuspended in 350 μl NET-2 buffer (150mM NaCl, 50mM Tris-Cl pH7.4, 0.05%

NP40) containing 0.25% SDS and phenol extracted. After 20 min. RNAs were ethanol precipitated using 5μg carrier tRNA and analysed on a 10% polyacrylamide-8M Urea gel.

Preparation of HeLa S3 cytoplasmic extract and immunoblotting

HeLa S3 suspension cells were harvested and washed twice with NKM (125mM NaCl, 5mM KCl, 1.5mM $MgCl_2$) and once with hypotonic Hepes buffer (10mM Hepes pH 7.9, 10mM KCl, 1.5mM $MgCl_2$, 0.5mM DTT). After resuspending the cells in 2 volumes of hypotonic Hepes buffer and incubation on ice for 20 min. the cell suspension was homogenized by 10 strokes in a dounce-homogenizer (B-pestle). After addition of 0.1 volume of isotonisation buffer (0.3M Hepes pH 7.9, 1.4 M KCl, 0.03 M $MgCl_2$) the extract was centrifuged for 10 min. at 13000g and used for immunoblotting (Towbin et al., 1976).

Counter immunoelectrophoresis and immunodiffusion

Counter immunoelectrophoresis (CIE) was carried out essentially as described before (Kurata et al., 1976). Lyophilized rabbit thymus extract (Pelfreez Biologicals, Rogers, Arkansa, USA) was used as source of antigen for the determination of anti-La and human spleen extract , prepared as described before (Clark et al., 1969), for the detection of anti-Ro antibodies. All antigens were made to a final protein concentration of 5 mg/ml. Reference human sera, monospecific for anti-Ro and anti-La antibodies were obtained from CDC, Atlanta, Georgia, USA.

Results

Ro, La and U RNA synthesis after adenovirus infection of Hela cells

When [^{32}P]-orthophosphate labelled Hela cells are used as antigen source, the Ro RNAs, termed hY1- hY5, the La RNAs, like pre-5S and pre-tRNAs and the U RNAs can be precipitated by patients sera with antibodies directed against their respective antigens (figure 1, panels A,B and C, lanes 1). To examine the turnover and stability of these ribonucleoprotein (RNP) complexes, pulse-chase labelling of HeLa cells with [^{32}P]-orthophosphate was performed. For the Ro RNP complex, the RNAs need about 5-7h to associate fully with the Ro (SS-A) antigens (figure 1, panel A, lanes 2 and 3), as do the U RNAs with the Sm antigen (figure 1, panel B, lanes 2 and 3). The La RNAs like pre-5S and pre-tRNAs become associated to the La (SS-B) antigen directly after transcription and are then gradually released (figure 1, panel C, lanes 2 and 3).

After infection of HeLa cells with adenovirus for 16h, the Ro RNAs are still transcribed and become associated with the Ro (SS-A) antigen (figure 1, panel A, lane 4), in contrast to the U RNAs, which are no longer precipitable by Sm sera (figure 1, panel B, lane 4) or by anti-m_3G-cap antibodies (data not shown). The virally encoded VA RNAs become associated with the La (SS-B) antigen with a concomitant decrease of La-host RNA complexation (figure 1, panel C, lane 4). Pulse-chase labelling of infected HeLa cells reveal that the stability and turnover of the Ro RNP complex is not markedly changed after adenovirus infection (figure 1, panel A, lanes 5 and 6), whereas the binding of VA

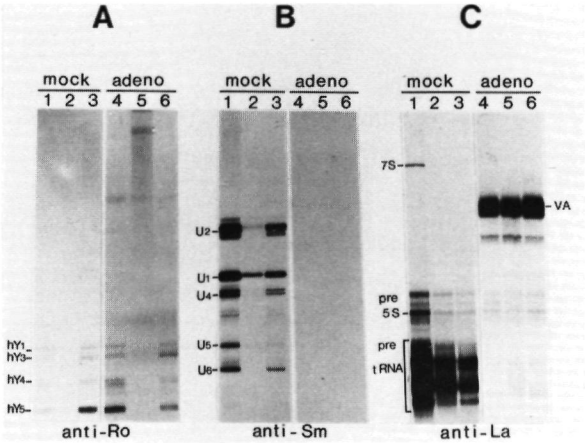

Fig.1. Precipitation of labeled RNA in uninfected and adenovirus infected HeLa cells.
RNA precipitation patterns of uninfected (marked mock;lanes 1-3) or adenovirus infected (marked adeno;lanes 4-6) [^{32}P]-orthophosphate labeled HeLa cellular extracts. In **panel A** a monospecific anti-Ro (SS-A) serum, in **panel B** an anti-Sm serum and in **panel C** a monospecific anti-La (SS-B) serum was used. In lanes 1 and 4 cells were labeled for 9h, in lanes 2 and 5 cells were labeled for 2h and in lanes 3 and 6 cells were pulse labeled for 2h and chased for 7h with non-radioactive medium.

RNA to the La protein is quite stable (figure 1, panel C, lanes 5 and 6).

The use of RNA precipitation for the simultaneous detection of anti-Ro (SS-A) and anti-La (SS-B) antibodies using adenovirus infected HeLa cellular extracts

Because anti-Ro (SS-A) and anti-La (SS-B) antibodies are often found together in patient sera, a system to test sera for anti-Ro and anti-La activity was set up. Since the VA RNAs in adenovirus infected cells become strongly associated with the La (SS-B) antigen (Francoeur et al., 1982), a [^{32}P]-orthophosphate labelled adenovirus infected HeLa cellular extract was used for immunoprecipitation.

As shown in figure 2, panel A, both monospecific anti-Ro and monospecific anti-La sera precipitate their cognate RNAs (lanes 2 and 3), but when a serum is used that contains both anti-Ro and anti-La antibodies, the Ro RNAs, precipitated by the anti-Ro antibodies, are not easily detected because of their comigration with the pre-tRNAs, that are precipitated by the anti-La antibodies (lane 4).

Because of the stability of the binding of VA RNA to the La protein, the relatively fast turnover of the pre-tRNA to La protein binding and the slow association of the Ro RNAs with the Ro antigen, infected HeLa cells were pulse labelled for 2h and subsequently chased for 7h in nonradioactive medium (figure 2, panel B). Monospecific anti-Ro and anti-La sera still precipitate the Ro RNAs and VA RNAs respectively (lanes 2 and 3) and, moreover, a serum containing both anti-Ro and anti-La antibodies precipitates both VA RNA and the Ro RNAs, the latter not being masked any more by other precipitated RNAs (lane 4). Therefore in our further experiments, an adenovirus infected HeLa

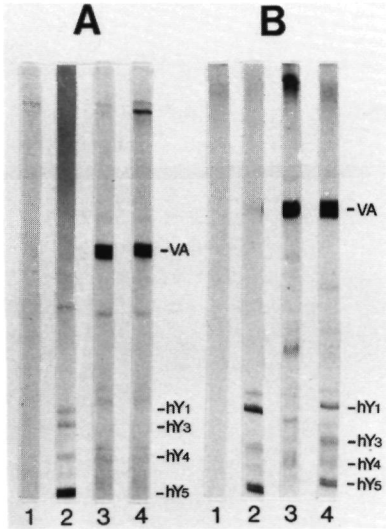

Fig.2. The effect of pulse-chase labeling on RNA precipitation in uninfected and adenovirus infected HeLa cells.
RNA precipitation patterns, using normal human serum (lanes 1), monospecific anti-Ro serum (lanes 2), monospecific anti-La (lanes 3) or an anti-Ro/La serum (lanes 4). In **panel A** HeLa cells were infected with adenovirus for 16h, labeled with [^{32}P] -orthophosphate for 7h and then used as antigen source. In **panel B** HeLa cells were infected with adenovirus for 16h, labeled for 2h with [^{32}P] -orthophosphate, chased for an additional 7h with non-radioactive medium and then used as a source of antigen.

cellular extract, pulse labelled for 2h with [^{32}P]-orthophosphate and chased for 7h with non-radioactive medium, was used to determine the presence of both anti-Ro and anti-La antibodies in patient sera.

To establish the sensitivity of this method for the detection of anti-La antibodies, we analysed a number of sera by counter immunoelectrophoresis (CIE) and immunoblotting and compared the presence of anti-La antibodies demonstrated by these techniques with the results of the VA RNA precipitation method. As shown in table 1, 132 randomly selected sera of patients suffering from different connective tissue diseases were tested for the presence of anti-La antibodies. Of these 132 sera, 23 were positive for anti-La activity in CIE and 24 sera were found positive by immunoblotting. Strikingly, by using VA RNA precipitation 22 additional sera were found to contain anti-La antibodies. This demonstrates the greater sensitivity of the VA RNA precipitation method for the detection of low levels of anti-La antibody. To ascertain that indeed anti-La antibodies were responsible for the precipitation of the VA RNA, uninfected [^{32}P]-labelled HeLa cellular extract was immunoprecipitated with some antisera positive for anti-La antibody in the VA RNA precipitation assay, but negative by

Table I: Comparison of the sensitivity of counter immuno electrophoresis (CIE) and immunodiffusion (ID) with immunoblotting (IB) and VA RNA precipitation for the detection of anti-La antibodies.

	n	IB positive		VA RNA positive	
		n	(%)	n	(%)
CIE negative sera	109	1	(1)	22	(20)
CIE positive sera	23	23	(100)	23	(100)
ID negative sera	11	n.t.		5	(46)
ID positive sera	12	n.t.		12	(100)

n= number of patient sera
n.t. = not tested

Table II: *Percentage of anti-La positive patient sera.*

diagnosis	n	CIE/IB positive %	VA RNA positive %
SS	46	48	58
-primary	20	n.t.	85
-secondary	26	n.t.	35
SLE	95	13	35
RA	105	8	16
UCTD	44	21	36
controls	11	0	0

n= number of patient sera
n.t. = not tested

other techniques. All of these sera precipitated the characteristic La RNAs like pre-5S, pre-tRNA and 7S RNA (data not shown), indicating that precipitation of VA RNA in adenovirus infected HeLa cell extracts is caused by the presence of anti-La antibodies in a serum and not by any other serum specificity. To compare the sensitivity of immunodiffusion with VA RNA precipitation, a relatively small number of sera (n=23) was tested for anti-La activity. Twelve sera were found positive for anti-La (SS-B) by immunodiffusion and 5 more by VA RNA precipitation (Table I).

To substantiate the use of VA RNA precipitation for the characterization of sera shown to be positive only for anti-Ro antibodies in CIE, we tested 19 of such "Ro monospecific" sera by immunoblotting and VA RNA precipitation. Eleven of these 19 sera were found positive for anti-La antibodies by the VA RNA method, but were negative by immunoblotting as well as by CIE (Table I). All 19 sera were positive for anti-Ro antibodies as indicated by the precipitation of Ro RNAs. This result not only corroborates the extreme sensitivity of the VA RNA precipitation method for the detection of anti-La antibodies but underlines also the reliability of the method for the demonstration of anti-Ro antibodies.

The presence of anti-La antibodies in CTD patients

It is known that anti-Ro and anti-La antibodies occur predominantly in sera from patients with SLE and/or Sjögren's syndrome (SS). To investigate whether the presence of low levels of anti-La antibodies is related to one of these disorders, we classified the sera tested by VA RNA precipitation, CIE and immunoblotting according to diagnosis. As can be concluded from Table II, the presence of previously undetected low-titer anti-La antibody levels enhances the number of anti-La positive sera in every group of patients, but especially in SLE patients. When SS sera are divided in their respective serological subgroups, anti-La antibodies are most frequently found in sera from primary SS patients (85%).

Discussion

In this paper we show that after adenovirus infection of HeLa cells the synthesis of most small RNAs is stopped. An exception is the synthesis of the Ro RNAs that

proceeds at seemingly normal rates. The Ro RNAs still associate with the Ro antigen while the La antigen becomes predominantly associated with virally encoded VA RNA transcripts. A selective labeling procedure was then developed to allow the simultaneous and sensitive detection of anti-Ro and anti-La autoantibodies.

To detect the presence of autoantibodies in sera from patients suffering from connective tissue diseases many methods are routinely employed. Immunodiffusion and counter immunoelectrophoresis are highly specific but relatively insensitive since precipitating antibodies can only be demonstrated at an optimal antigen/antibody ratio. Furthermore, immunodiffusion and CIE are sometimes difficult to interpret when sera are used that contain multiple specificities (Williams et a., 1986). Immunoblotting is probably the most sensitive technique used routinely at the moment, but the method is not so easy to perform and has the possible disadvantage of not demonstrating antibodies that recognize conformational epitopes. Immunoprecipitation is an even more sensitive technique, highly specific but very time consuming. However, when it is essential to know whether anti-Ro and anti-La antibodies are present, a precipitation assay should be used that gives an unambiguous answer, i.e. precipitates a well defined complex, as for example the VA RNA-La protein complex.

The precipitation assay described here is more sensitive for the detection of anti-La antibodies than all other methods described previously due to the large amount of VA RNA synthesized in adenovirus-infected HeLa cells, its specific interaction with the cellular La protein and the high specific activity that can be obtained by [^{32}P]-labelling of adenovirus-infected HeLa cells. A further advantage of the method is that anti-Ro antibodies, often present in anti-La sera, can be detected simultaneously by the precipitation of the Ro RNAs hY1-hY5 when the infected and labeled cells are chased with non-radioactive medium. Although the La protein is also capable of binding to the Ro RNAs, the majority of the Ro RNAs are specifically precipitated by the anti-Ro and not by the anti-La antibody. The explanation for such a behaviour may be that the La protein only transiently associates with the Ro RNAs (Hendrick et al, 1981, and figure 1C, lanes 1-3), so that these RNAs will not be present in a La precipitation pattern when a chased cell extract is used.

Our results clearly show the sensitivity of the VA RNA precipitation method for the demonstration of low concentrations of anti-La antibodies in patient sera. Compared to counter immunoelectrophoresis, 22 out of 109 CIE negative sera precipitated VA RNA while only 1 was found positive for anti-La antibodies by immunoblotting (Table 1). All of the sera found to be positive by CIE and immunoblotting for anti-Ro and anti-La antibodies were also positive for both activities in the VA RNA precipitation assay.

When differentiated according to diagnosis, anti-La antibodies were found at a higher incidence in all classes of connective tissue disease patients then hitherto has been described. For RA (16%) and UCTD (36%) this increase seemed relatively insignificant, when compared with data from previous studies (Maddison et al., 1985; Harley et al, 1986; De Rooij et al., 1988), but for SLE patients the incidence of anti-La antibodies is more strikingly influenced (35% positive as compared to 10-15% mentioned before (De Rooij et al., 1988; Chan et al., 1989). When Sjögren's syndrome patients are subdivided

in subclasses, anti-La antibodies are primarily found in primary SS (85%) and to a minor extent in secondary SS (35%). For both primary and secondary SS these figures agree well with those published in previous reports (Akizuki et al., 1976; Kassan et al, 1977; Isenberg et al., 1982; Venables et al., 1983). However, it is clear that the higher sensitivity of detection almost automatically leads to a lowered specificity for anti-La antibody as a disease marker for SS and SLE (see Table II).

For studies on the complexity and function of Ro and La ribonucleoproteins monospecific sera are indispensable. Since good monoclonal antibodies directed against these complexes are relatively rare, monospecific human sera are a useful alternative. However, this study clearly shows that counter immunoelectrophoresis, immunodiffusion and immunoblotting techniques are not sufficient to select such sera. In fact, monospecific anti-Ro and monospecific anti-La sera proved to be more rare than hitherto was expected.

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Chapter 4:
The detection and occurrence of the 60 and 52 kDa Ro (SS-A) antigens and of autoantibodies against these proteins

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The detection and occurrence of the 60 and 52 kDa Ro (SS-A) antigens and of autoantibodies against these proteins.

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Abstract

The simultaneous detection of anti-La, anti-60 kDa Ro and anti-52 kDa Ro antibodies by immunoblotting is greatly improved by changing the crosslinking level in the gel to an acrylamide/bisacrylamide ratio of 19:1. Using this method for the analysis of a number of SLE and Sjögren's syndrome patient sera it was observed that antibody to the 52 kDa Ro protein without anti-60 kDa Ro antibody was restricted to Sjögren's syndrome patients (9/26), whereas antibody to the 60 kDa Ro protein without contaminating anti-52 kDa Ro antibody was only found in SLE patients (4/38). Moreover, in Sjögren's syndrome patient sera anti-Ro antibody was found only in combination with anti-La antibody (20/26), whereas in SLE patient sera anti-Ro antibody could be found without detectable anti-La specificity (8/38). Double immunofluorescence microscopy revealed that the 52 kDa Ro and the 60 kDa Ro proteins co-localize in the cytoplasm as well as in the nucleus, whereas immunoprecipitation of [^{32}P]-labelled HeLa cell extract with monospecific anti-52 kDa Ro and anti-60 kDa Ro sera showed that both proteins are associated with the Ro RNAs. These data suggest the presence of both the 52 kDa and the 60 kDa Ro proteins in the same ribonucleoprotein complexes. To study the evolutionary conservation of the 52 kDa Ro, the 60 kDa Ro and the La proteins, extracts of cell lines derived from various mammalian species were analysed on Western blots using monospecific human antibodies. In contrast to the 60 kDa Ro and the La antigens which are well conserved in evolution, the 52 kDa Ro antigen could be detected in primate cells only by this immunological approach. The implications of the selective reactivity of anti-52 kDa Ro antibodies with primate 52 kDa Ro antigen is discussed.

Introduction

In patients with autoimmune disorders protein antigens, often associated with RNA in ribonucleoprotein (RNP) complexes, become targets of the immune system leading to the occurrence of autoantibodies. In systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS) these autoantigenic targets include the Ro (SS-A) and La (SS-B) RNP complexes. Many studies have pointed to the diagnostic value of antibodies to the Ro and La antigens, as these have frequencies of 60% and 40% respectively in SS and 35% and 15% respectively in SLE patient sera (reviewed by Chan and Tan, 1989a).

The La RNP, predominantly localized in the cell nucleus, consists of a 46.7 kDa antigenic protein which binds non-covalently to RNA polymerase III transcribed RNAs (e.g. pre-5S, pre-tRNAs and Ro RNAs). The La protein binds to the uridine stretch at the 3' end of the transcripts and functions as an RNA polymerase III transcription termination factor (reviewed by Pruijn et al., 1990).

In human cells, the Ro RNP complexes consist of four RNAs, termed hY1,hY3,hY4 and hY5, each complexed to a single 60 kDa antigenic protein (Ro60). Complementary DNAs encoding the Ro60 protein have been cloned (Deutscher et al., 1988; Ben-Chetrit et al.,1989). Recombinant Ro60 was shown to be antigenic and could be reconstituted *in vitro* with hY1 RNA. Recently, another putative component of the Ro RNPs, the 52 kDa Ro protein (Ro52), was identified (Ben-Chetrit et al.,1988). Apart from Ro60 and Ro52 three more proteins have been designated Ro proteins. Two of these, with molecular weigths of 60 kDa and 54 kDa, were found in erythrocytes only and thus might be tissue specific Ro components (Rader et al.,1989a). Both of these proteins were antigenically distinct from the Ro60 and Ro52 polypeptides discussed above, but the 60 kDa proteins from red blood cells and lymphocytes as well as the 54 and 52 kDa proteins from these cells were shown to be related. Another putative Ro protein, the human analogue of calreticulin (McCauliffe et al., 1990) has been cloned recently but its behaviour as a Ro component is as yet not clear.

The evolutionary conservation of the Ro and La proteins has been the subject of several studies. Originally, the Ro antigen was described in human and mouse cells (Hendrick et al., 1981). Later, Harmon et al. (1984) described the absence of Ro antigen in mouse, rat, rabbit, hamster and chicken cells and the presence of Ro complexes in human, monkey, dog and guinea pig cells, whereas Mamula et al. (1989) could demonstrate Ro RNPs in human, bovine, rabbit and duck cells. Recently, the red blood cell and lymphocyte Ro antigens have been described in human, baboon, guinea pig, dog, bovine and mouse cells (Byers et al., 1990), although in this study an antiserum with mixed Ro60, Ro52 and La specificities was used, which seriously hampers the interpretation of the Western blots.

In this study a gel system was designed to separate Ro60, Ro52 and La in order to detect anti-Ro and anti-La antibodies in patient sera on Western blots. RNA precipitation and immunofluorescence was performed to confirm the co-existence of Ro60 and Ro52 in the same RNP complexes and finally a panel of cell lines from different mammalian species was analyzed in order to study the evolutionary conservation of the Ro and La antigens.

Materials and methods

Cell extracts and immunoblotting

Cytoplasmic extract of Raji cells was prepared as described before (Ben-Chetrit et al., 1988) and used for routine immunoblotting (2.5 mg protein per gel; dimensions 150x150x1.5mm) as described by Habets et al. (1985), using 10% SDS-PAGE gels (Laemmli, 1970) with modified acrylamide to bisacrylamide ratios as indicated in the legend of Figure 1. Strips of these blots were blocked with PT-buffer (PBS-0.5% Tween20) containing 3% bovine serum albumin (BSA) for 30 min and incubated with antiserum in PT-1% BSA, diluted 1/100, for 2h. After 3 washes with PT, the strips were incubated with [^{125}I]-protein A (Amersham, UK) in PT with 1% BSA (1 μCi per 100 ml) for 2h. After extensive washing with PT (3x) and water (3x) the strips were autoradiographed for various periods using Kodak XAR5 films.

To establish the monospecificity of the antisera, Western blots were prepared containing bacterially expressed Ro60 and La proteins (unpublished results) and incubated as described before (Habets et al., 1985).

HeLa cells were labelled overnight at a density of 0.5 x 10^6 cells/ml with [^{32}P]-orthophosphate (0.5 mCi/ml). Cytoplasmic extracts were prepared as described above and used for immunoprecipitation as described by Slobbe et al. (1991).

Western blots of cell extracts originating from cell lines of different species were obtained as follows: Approximately 40*10^6 cells were harvested and washed once with PBS pH7.0. After resuspending the cells in 500 μl RSB^{110} (10mM Tris-HCl pH7.4, 110mM NaCl, 1.5mM $MgCl_2$ containing 0.5mM Phenylmethylsulfonyl chloride [PMSC]), cells were lysed by repeated freeze-thawing. After lysis cell extracts were incubated with 250 μg DNAse I and, after removing cell debris by centrifugation, the protein content was determined using the method of Lowry et al. (1951) using BSA (Sigma, USA) as a standard. About 50μg of protein was loaded per lane of the SDS-PAGE gel and blotted as described above.

Antisera

All patients were diagnosed according to established criteria (de Rooij et al., 1988): All patients with SLE were positive on 4 or more of the revised criteria of the Amerian Rheumatism Association (Tan et al., 1982) and all SS patients were positive for xerophtalmia, xerostomia and/or salivary gland infiltration (Alexander et al., 1982).

For the detection of antigens on Western blots several patient sera were used: O60 and O12 (containing anti-Ro60, anti-Ro52 and anti-La antibody), H142 (containing only anti-Ro60 antibody), Jo7 (containing only anti-Ro52 antibody), W10 (containing anti-La and anti-Ro52 antibody) and reference serum Ge (courtesy of Prof. E.M. Tan, containing antibodies to both Ro60 and Ro52). In addition, a polyclonal rabbit anti-Ro60 antiserum, prepared by immunizing a rabbit with recombinant Ro60 protein (unpublished results), was used for the double immunofluorescence microscopy. Antibodies to Ro60 and La peptides were affinity purified using bacterially expressed antigens coupled to CNBr-activated Sepharose 4B (Pharmacia, Sweden) according to protocols supplied by the manufacturer.

Immunofluorescence microscopy

Double immunofluorescence microscopy using a human monospecific anti-Ro52 serum and a monospecific polyclonal rabbit anti-Ro60 serum was performed on human HEp-2 cells. First, the cells were fixed with methanol for 5 minutes (-20°C), rinsed with acetone (-20°C) and air dried. The fixed cells were incubated with the monospecific human anti-Ro52 serum (diluted 1/50 with PBS) for 30 min followed by incubation with FITC labelled goat anti-human second antibody for 30 min. Subsequently, the incubated cells were stained with the affinity purified rabbit anti-Ro60 polyclonal antiserum (diluted 1/50 with PBS) for 30 min, followed by incubation with TRITC labeled swine anti-rabbit second antibody for 30 min. Second antibodies (Dakopatts, Denmark) were used at a dilution of 1/50 in PBS. All possible combinations of antibody control incubations were included and found to be negative.

Results

Separation of Ro60, Ro52 and La polypeptides in SDS-PAGE

Since the discovery of the 52 kDa Ro protein as a major Ro autoantigen, several gel systems have been used to detect Ro52 on immunoblots. Using a Ro52 reference serum containing antibodies to both Ro60 and Ro52 but not to the La protein we were able to detect Ro52 in our usual gel system, migrating at the same position as the La protein (Figure 1, lanes 5 and 7, middle panel). Since anti-La and anti-Ro antibodies frequently concur in patient sera, an alteration in the antigen separation was required for the detection of both anti-Ro and anti-La autoantibodies in patient sera by this method. Changing the crosslinking percentage in the gel by varying the ratio of acrylamide to bisacrylamide from 39:1 to either 79:1 (left panel) or 19:1 (right panel) revealed that, while La and Ro60 do not change migration relative to each other, the relative position of Ro52 is markedly affected. At a ratio of 79:1, Ro52 migrates slower than the La protein (Figure 1, left panel, lanes 4,5 and 7),

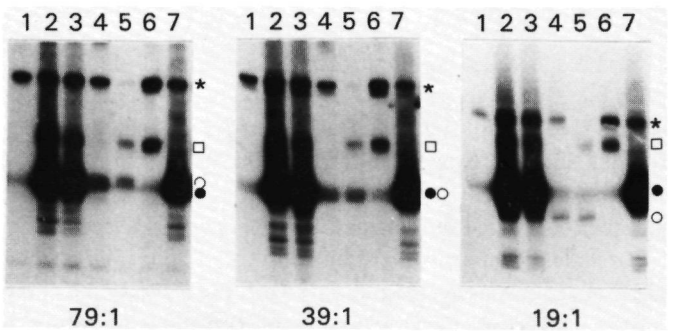

Fig.1. Western blots of SDS-PAGE gels with different acrylamide to bisacrylamide ratios
In the left panel an acrylamide to bisacrylamide ratio of 79:1 was used, in the middle a ratio of 39:1 and on the right 19:1. Lanes 1: normal human serum; lanes 2 and 3: sera with anti-Ro60, anti-Ro52 and anti-La antibodies (O60 and O12 respectively); lanes 4: anti-Ro52 antiserum (Jo7); lanes 5: reference serum Ge (anti-Ro60 and anti-Ro53; lanes 6: anti-Ro60 antiserum (H142); lanes 7: serum containing anti-La and anti-Ro52 antibodies (W10). The positions of the Ro60, Ro52 and La proteins on the blots are indicated by □, ○ and ● respectively. Background band typically present in Raji cell extracts is marked by an asterisk.

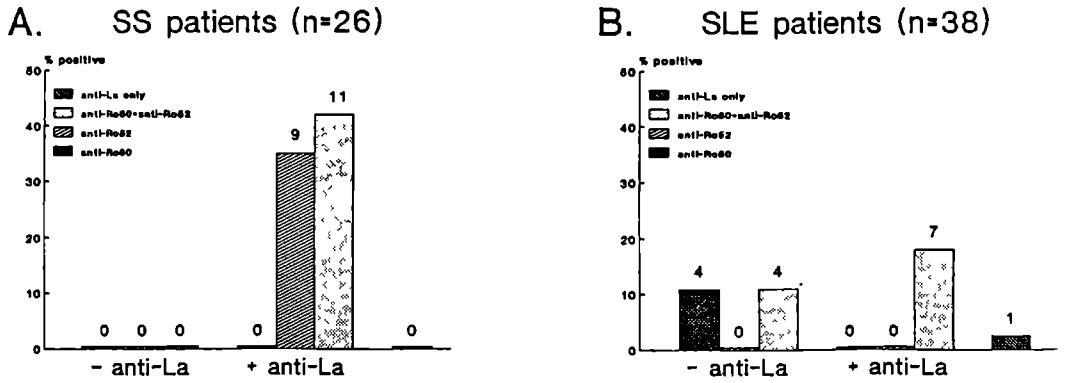

Fig 2. The occurrence of anti-Ro and anti-La antibodies in SS and SLE patient sera.
panel A: The occurrence of anti-Ro and anti-La antibodies in sera from patients with Sjögren's syndrome. **panel B:** The presence of anti-Ro and anti-La antibodies in sera from patients with systemic lupus erythematosus. Numbers in the figure indicate the number of positive patient sera. The patterns are as indicated.

at a ratio of 39:1 it comigrates with the La protein, while at a ratio of 19:1 Ro52 migrates faster than La (Figure 1, right panel, lanes 4,5 and 7). The separation of Ro52 from La and Ro60 is best in the latter gel system, enabling the detection of antibodies to all three proteins simultaneously (Figure 1, lanes 2, 3 and 6; compare left and right panel). Consequently, this system was used in the experiments described below.

Detection of anti-Ro60, anti-Ro52 and anti-La antibodies in autoimmune patient sera

Using the immunoblotting system described above, a number of patient sera were analysed for the presence of anti-Ro60, anti-Ro52 and anti-La antibodies. As summarized in Figure 2, sera from patients with SLE and Sjögren's syndrome often contain antibodies to the Ro and La antigenic proteins. However, anti-Ro52 antibody without anti-Ro60 antibody was found only in sera from patients with Sjögren's syndrome (9/26; 34%), whereas anti-Ro60 antibody without concomitant anti-Ro52 antibody was demonstrated only in SLE sera (4/38; 10%). Moreover, anti-Ro antibody without anti-La antibody was detected in SLE patients only (8/38; 21%), whereas in all anti-Ro positive sera from patients with Sjögren's syndrome anti-La antibody was detected as well (20/26; 77%). Sera from healthy controls did not contain detectable levels of anti-Ro60, anti-Ro52 or anti-La antibodies.

Characterization of monospecific antisera

To obtain antibodies monospecific for either the Ro60 or the La antigen, specific antibodies were immunoaffinity purified using recombinant antigen coupled to CNBr-activated Sepharose 4B yielding monospecific anti-La and anti-Ro60 antibodies from patients and monospecific rabbit anti-Ro60 antibody. For anti-Ro52 antibody a serum was used that did not contain detectable levels of anti-Ro60 or anti-La antibody on immunoblot. To remove possible trace amounts of anti-Ro60 antibody, this serum was passed repeatedly over a Ro60-column, that had been shown to be able to remove anti-Ro60 antibodies from an anti-Ro60/La serum.

The resulting monospecific sera were checked for their monospecificity in various ways: first, Western blot analysis of these monospecific sera did not show the presence of

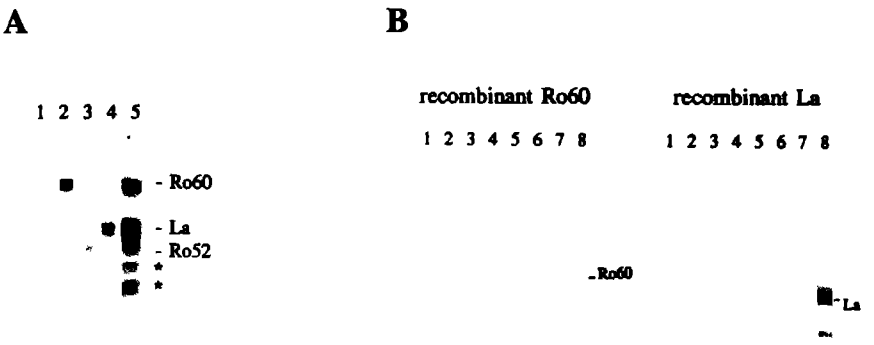

Fig.3. Evidence for the monospecificity of the anti-Ro60, anti-Ro52 and anti-La antibody.
panel A: Western blot of HeLa cytoplasmic extract incubated with monospecific anti-Ro60 (lane 2), anti-Ro52 (lane 3) or anti-La (lane 4) serum. Lane 5 contains a reference serum with antibodies to Ro52, Ro60 and La (O60), lane 1 normal human serum. Degradation products of La are indicated by asterisks. **panel B:** Western blots of recombinant Ro60 (left) and recombinant La (right) protein. In lane 1 total protein is stained with Ponceau S (Sigma Diagnostics, USA), in lanes 2 normal human serum, in lanes 3 rabbit anti-Ro60 antibody, in lanes 4 human affinity purified anti-Ro60 antibody, in lanes 5 human affinity purified anti-La antibody, in lanes 6 human monospecific anti-Ro52 antibody, in lanes 7 anti-Ro (SS-A) and in lanes 8 anti-La (SS-B) antisera were used (CDC, Atlanta, USA).

any contaminating antibody (Figure 3, panel A); second, using Western blots containing large amounts of bacterially expressed recombinant Ro60 or La polypeptides revealed that only the monospecific anti-Ro60 and the monospecific anti-La reacted with the respective antigens (Figure 3, panel B); third, the absence of low levels of anti-La antibody in the anti-Ro sera was tested by the immunoprecipitation of VA RNA from adenovirus infected cell extracts (Slobbe et al., 1991) and none of the purified anti-Ro antibodies precipitated VA RNA (results not shown).

RNA precipitation and double immunofluorescence microscopy using monospecific anti-Ro52 and anti-Ro60 antisera

RNA precipitation from [^{32}P]-labelled HeLa cell extract with monospecific anti-Ro52 and monospecific anti-Ro60 antibody showed that both anti-Ro52 and anti-Ro60 antibody precipitated the characteristic Ro RNAs (Figure 4, panel A, lanes 5 and 6). As controls RNA precipitations performed with normal human serum (lane 2), anti-Ro (SS-A) CDC reference serum (lane 3), affinity purified rabbit anti-Ro60 antibody (lane 4), anti-La CDC reference serum (lane 7) and affinity purified anti-La serum (lane 8) are included.

We used indirect immunofluorescence to determine the intracellular localization of the Ro60 and Ro52 proteins at a light microscopic level. Antibodies to either the Ro60 or the Ro52 protein yielded a diffuse cytoplasmic staining next to a strong punctuated nuclear staining, while the nucleoli were negative. Double immunofluorescence using monospecific rabbit polyclonal anti-Ro60 antibodies and monospecific anti-Ro52 patient antibodies showed

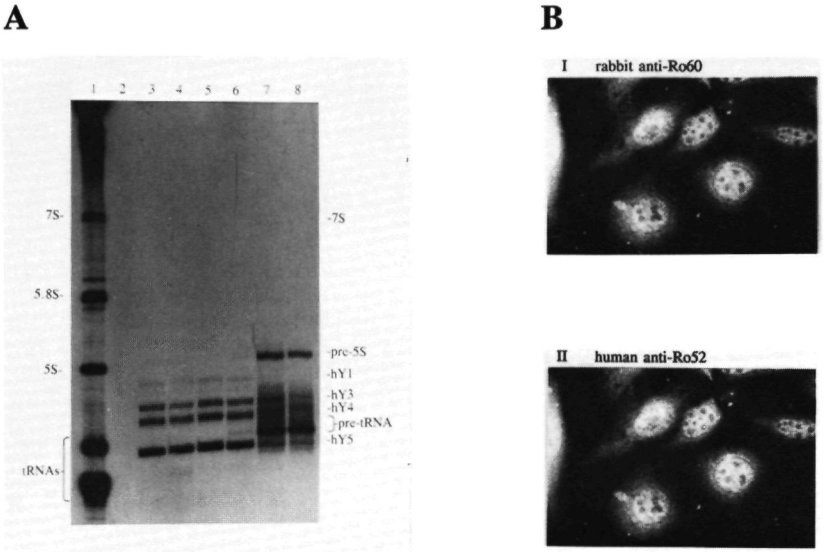

Fig.4. Evidence for the association of Ro60 and Ro52 in Ro RNP complexes.
panel A: RNA precipitation of HeLa [^{32}P]-labelled cellular extract immunoprecipitated with normal human serum (lane 2), anti-Ro (SS-A) reference serum (CDC, Atlanta, USA; lane 3), monospecific polyclonal rabbit anti-Ro60 antibody (lane 4), affinity purified monospecific human anti-Ro60 antibody (lane 5), monospecific anti-Ro52 antiserum (lane 6), anti-La (SS-B) reference serum (CDC, Atlanta, USA; lane 7) and affinity purified monospecific anti-La antibody (lane 8). In lane 1 total [^{32}P]-labelled RNA is shown. **panel B:** Double immunofluorescence microscopy of HEp-2 cells incubated with rabbit anti-Ro60 antibody and human anti-Ro52 antiserum. In panel I, TRITC labelled anti-rabbit antibody (anti-Ro60), in panel II FITC labelled anti-human antibody (anti-Ro52) is shown. Magnification 100x.

that both staining patterns were identical, suggesting the co-localization of both proteins (see Figure 4, panel B).

Detection of Ro60, Ro52 and the La protein in extracts of tissue culture cells originating from different species

To study the evolutionary conservation of Ro60, Ro52 and the La protein, a panel of mammalian cell line extracts was immunoblotted with the monospecific sera described above.

As shown in Figure 5, Ro60 and the La protein are well conserved in evolution as indicated by the apparent conservation of epitopes, although reactivity of the antigens and their apparent molecular weights seem to differ among species. Despite the use of PMSC as protease inhibitor, some proteolytic degradation was observed, especially of the La protein, which is very susceptible to proteolytic degradation (Habets et al., 1983; Chan et al., 1989b). This resulted in La-degradation products of 43 and 28 kDa molecular weight (marked by an asterisk in Figure 5). In Vero cell extracts the apparently high protease activity has caused degradation of La and some degradation of Ro60 as well. In contrast to the conservation of Ro60 and the La polypeptide, Ro52 could be detected only in cell lines originating from

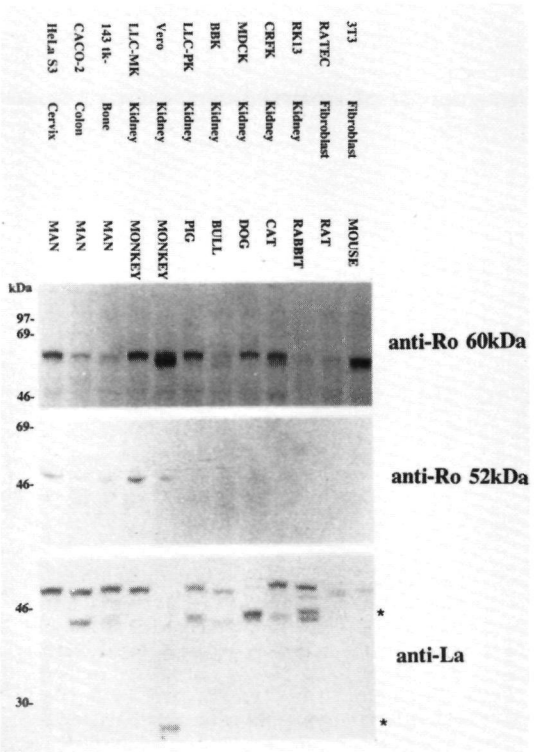

Fig.5. Immunological conservation of Ro60, Ro52 and La.
Identical Western blots of cell extracts originating from cell lines of different species (as indicated on top) were incubated with monospecific patient antibody. The upper panel was incubated with monospecific anti-Ro60 antibody, the middle panel with monospecific anti-Ro52 antiserum and the lower panel with monospecific anti-La antibody. Molecular weight markers are indicated on the left, degradation products of the La protein on the right by an asterisk. The positive reactions with bull rabbit and rat cell extracts were weak and may not reproduce well.

primates, which indicates that either the antigenic determinants of Ro52 have altered during evolution or that a protein related to Ro52 is absent in mammals other than primates. An extract of chimpanzee liver tissue also contained all three proteins (results not shown).

Discussion

The use of a modified gel system to detect unambiguously the Ro52 antigen on Western blots is described. While this work was in progress, other investigators reported similar observations (Buyon et al.,1990) and suggested conformational effects in the presence of SDS causing the aberrant migration of the 52 kDa Ro protein on Laemmli gels. Structural studies of Ro52 have to be awaited to gain insight into the phenomena causing this deviant behaviour.

Using our modified gel system for the detection of anti-Ro (SS-A) and anti-La (SS-B) autoantibodies in patient sera we established that anti-Ro52 and anti-Ro60 antibodies are often found in patients with SLE or Sjögren's syndrome (SS) (15/38 for SLE and 20/26 for SS patients). Our results confirm recent data from Ben-Chetrit et al. (1990) that sera containing anti-Ro52 but not anti-Ro60 antibodies seem to be restricted to SS patients, whereas anti-

Ro60 without anti-Ro52 antibodies could only be found in SLE patients (see Figure 2). Additionally, in all sera from patients with Sjögren's syndrome containing anti-Ro antibody, anti-La antibody could be detected as well. In contrast, anti-Ro antisera without anti-La antibody were found only in the SLE patient group. This latter finding confirms results of previous reports (Wasicek and Reichlin, 1982; Rader et al., 1989b). Therefore, based upon these results, it is likely that the presence of anti-Ro60 antibody without anti-La antibody in autoimmune patient sera is indicative for SLE, whereas the presence of anti-Ro52 and anti-La antibody without anti-Ro60 antibody is restricted to sera from patients with Sjögren's syndrome.

To confirm the association of Ro52 with Ro RNP complexes, RNA precipitations with monospecific anti-Ro52 or anti-Ro60 antibody were carried out. The results showed that monospecific antibodies to both antigens were able to precipitate the characteristic Ro RNAs. Since the Ro RNA precipitation patterns do not seem to differ in Ro60 and Ro52 precipitation it is concluded that both Ro60 and Ro52 are associated with all four Ro RNAs. This renders it unlikely that differences observed in Ro RNP particles (Boire and Craft, 1990) are caused by differential binding of Ro52 or Ro60 to the Ro RNAs. Moreover, double immunofluorescence microscopy showed that the Ro52 and Ro60 antigens co-localize in the nucleus as well as in the cytoplasm, indicating an intimate relation between these proteins.

The evolutionary conservation of Ro60, Ro52 and the La protein was studied by incubating Western blots of cell extracts from cell lines originating from different species with monospecific polyclonal antisera. This method may have the disadvantage of not demonstrating antigens whose antigenic determinants have altered during evolution, but the polyclonality of the patient sera used still allows the detection of proteins containing only a limited number of conserved epitopes. The Western blots shown in Figure 5 reveal that the antigenic properties of Ro60 and La seem to be well conserved during evolution, since proteins of comparable molecular weight are recognized by the antisera in each cell line tested. In the case of the La protein it is noteworthy that degradation products of approximately 43 and 28 kDa, previously identified in human cell extracts (Habets et al., 1983; Chan et al., 1989b) are also found in other species, indicating the conservation of regions in the protein that are susceptible to protease digestion. The apparent molecular weights of both Ro60 and La seem to differ slightly among species, possibly caused by structural differences or by proteolytic degradation. Additionally, the intensity of reacting antigen seems to vary among species. This may be due to variations in abundance of the antigens and/or antibody reactivity. Some variation in the levels of reacting antigen may be caused by tissue specific expression of the antigenic proteins. It has been reported that isoforms of the Ro proteins with different antigenicity exist in human red blood cells as compared to lymphocytes (Rader et al, 1989a). Therefore, it cannot be excluded that the antibody used in this study has a different affinity for some tissue specific isoforms of Ro. For example, it is known that rabbit thymus extracts contain little or no Ro (SS-A) complexes detectable with human antisera (Reichlin and Reichlin, 1989), whereas we find a weak reactivity with anti-Ro60 antibodies in rabbit kidney cells (Figure 4).
Interestingly, the Ro52 protein could only be demonstrated unambiguously in human and monkey cell lines. Whether the lack of reactivity of the anti-Ro52 antibody in mammalian

cells other than primate cells reflects the absence of a related protein is not entirely clear. Although these experiments could be reproduced several times and the same result was obtained using three different patient sera, it still seems possible that only major epitopes of Ro52 have altered during evolution and that a related, but antigenically distinct, protein is present in other mammalian species. The availability of DNA probes for screening different species for the expression of this antigen on Northern blots might answer this question. Still, the reactivity of patient antibodies with primate Ro52 only is rather unexpected since most autoantibodies have been reported to react with antigens of different species. For instance, reactivity of autoantibodies to the Sm antigen has been found in a broad range of vertebrates and in Drosophila (Mount et al., 1981), autoantibodies to the Jo-1 antigen (histidyl-tRNA synthetase) react with human, bovine, rat and rabbit protein (Nishikai et al., 1980, Mathews and Bernstein, 1983, Yang et al.,1984), anti-nucleolar 34 kDa fibrillarin antibodies recognize the human, mouse and Drosophila nucleoli (Lischwe et al., 1985), and anti-La antibodies are known to be reactive with proteins of comparable molecular weights in a number of mammalian species (this study) and with Xenopus laevis La antigen (Moreau et al., 1990). Species-specific recognition of an autoantigen has been reported only for the anti-Ku autoantibody (Mimori et al., 1981), that does react with human and bovine tissue but not with mouse liver. If Ro52 indeed is a primate specific autoantigen, then it would support the idea that the human autoantigen itself functions as a trigger in the onset and perpetuation of the immuneresponse.

Finally, since Ro52 could not be detected in mammals other than primates and anti-Ro (SS-A) sera frequently contain antibodies to this protein, it is evident that one should be careful in choosing cell extracts for performing counter-immunoelectrophoresis, ELISA or Western blotting for the screening of patient sera.

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Appendix:

The evolutionary conservation of Ro RNAs

R.L.Slobbe, P.A.E.T.M. Wingens, G.J.M. Pruijn and W.J. van Venrooij

The results presented in Chapter 3 show that the 60 kDa Ro antigen is reasonably well conserved in evolution as indicated by the immunological reactivity of proteins of similar size in mammalian cells other than human cells. The 52 kDa protein, however, could be detected immunologically in primate cells only. This suggests that either the autoepitopes of the 52 kDa Ro protein have altered in evolution and that a similar, but immunologically non-reactive, protein is present in non-primate cells or that a protein related to Ro52 is absent in non-primates.

Because of this difference in evolutionary conservation between the 60 kDa and 52 kDa Ro antigens and the fact that they are both present in the same ribonucleoprotein complex in human cells, we analyzed the evolutionary conservation of Ro RNAs in mammalian cells as well. Previous studies have identified Ro RNAs in human, bovine, cavia, leporine, rat, murine and duck cells via immunoprecipitation of Ro RNP complexes (Hendrick et al., 1981; Reddy et al., 1983; Itoh et al., 1990; Mamula et al., 1990). In these studies the number of Ro RNAs varied between two (in murine and duck cells) and four (in human, bovine and guinea pig cells), but the identity of the RNAs of these species was not elucidated. The only report on the identity of Ro RNAs in other species shows that in murine cells two Ro RNAs exist (referred to as mY1 and mY2), that are homologous to hY1 and hY3, respectively (Wolin and Steitz, 1983).

Total RNA was isolated from cells of different mammalian species by two subsequent phenol extraction procedures (Sambrook et al., 1989). The isolated RNAs were separated by polyacrylamide-urea gel electrophoresis, electroblotted onto Hybond N membrane and subsequently hybridized with antisense hY1, hY3 and hY5 RNA probes to detect the presence of RNAs homologous to hY1, hY3 or hY5, respectively.

As shown in Figure A1 and summarized in Table AI, both antisense hY1 and antisense hY3 RNA probes hybridized with RNAs of similar size in RNA preparations of all mammalian species examined. This indicates that RNAs homologous to hY1 and hY3 are present in all of these cells and that the Y1 and Y3 RNAs are well conserved in evolution. It is noteworthy that upon hybridization with antisense hY1 RNA often two strongly hybridizing bands were found, probably corresponding to hY1 and a degradation product thereof, previously described as hY2 (Hendrick et al., 1981; Wolin and Steitz, 1983). In spite of the stringent conditions used during hybridization both antisense hY1 and hY3 probes hybridized to the other Y RNAs, albeit at a significantly lower level.

Antisense hY5 RNA hybridized very specifically with RNA of similar size in all examined RNA preparations of mammalian species, except for cow, mouse, rat and hamster RNA. These results suggest that Y5 RNA is not universally present.

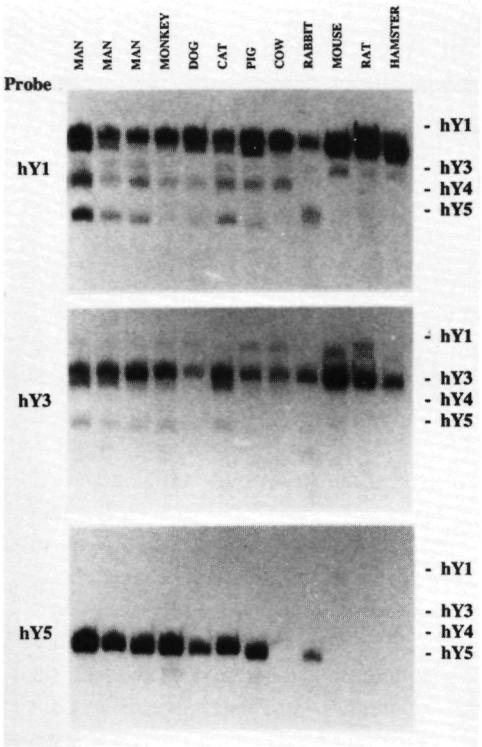

Fig.A1. The evolutionary conservation of Y RNAs
Total cellular RNAs were isolated from cells originating from different mammalian species as indicated on top of the Figure, separated on a 10% polyacrylamide-urea gel and electroblotted onto a Hybond N membrane. This Northern blot was subsequently hybridized with equal amounts of anti-sense hY1, hY3 and hY5 RNA probes of identical specific activity (conditions: 6x SSC, 68 °C), as indicated on the left.

Table AI The presence of Y RNAs in different mammalian species

species	Y1	Y3	Y4*	Y5
human	+	+	+	+
monkey	+	+	+	+
dog	+	+	+	+
cat	+	+	+	+
pig	+	+	+	+
cow	+	+	+	−
rabbit	+	+	+	+
mouse	+	+	−	−
rat	+	+	−	−
hamster	+	+	−	−

* evidence for the presence of Y4 homologues is derived from the presence of an RNA of similar length as hY4 RNA, cross-hybridizing with the antisense hY1 RNA probe and not hybridizing with the anti-sense hY3 and hY5 probes as determined by superimposing the autoradiograms.

conclusion concerning the presence of hY4 homologues can be drawn from these blots. However, since the antisense hY1 RNA probe cross-hybridized with all four Ro RNAs in human cells (including hY4 RNA), it is tempting to presume that the presence of bands cross-hybridizing with the hY1 RNA probe, but not hybridizing with the anti-sense hY3 or hY5 probes (as determined by superimposing the autoradiograms) and of similar length as hY4, are indicative for the presence of Y4 RNA. If these assumptions are allowed, then it seems that a Y4 RNA homologue is present in all mammalian species but mouse, rat and hamster.

Cellular extracts of Xenopus laevis, moth, bean, tomato, potato, tobacco and Saccharomyces cerevisiae were also analyzed for the presence of Ro RNAs. Y1, Y3 and Y5 RNAs could not be detected in these species under stringent hybridization conditions, indicating that Y RNAs are probably not present.

Generally, the data presented here are in agreement with previous reports, although some deviations were found. In rabbit cells, an RNA homologous to but migrating slightly faster than hY5 was detected, whereas others described the presence of only three larger RNAs, approximately of the size of hY1, hY3 and hY4. Additionally, four Ro RNAs have

been described in bovine cells, whereas in Figure A1 only three Ro RNAs, homologous to hY1, hY3 and (probably) hY4 could be identified. An explanation for these deviating results may be the fact that in other studies anti-Ro immunoprecipitated RNA was analyzed, whereas here total RNA has been examined. Another cause might be possible degradation of Y RNAs; for example, Y1 RNA may be degraded to Y2 RNA.

The possibility that the analysis of total RNA may differ from the analysis of anti-Ro immunoprecipitated RNA is supported by another observation. The analysis of the presence of Ro RNAs in total RNA preparations can be interpreted quantitatively, since the blot in Figure A1 is incubated with equal amounts of anti-sense RNA probes of similar specific activity. In addition, hybridization of this blot with a mixture of equal amounts of anti-sense hY1, hY3 and hY5 RNA probes of similar specific activities yielded identical results (results not shown). As a result, it seems that Y1 and Y5 RNAs are present in equal amounts in mammalian cells, whereas Y3 RNA is less prominent. This seems to conflict with the data presented in Chapter 4, Figure 4A. In RNA preparations obtained by immunoprecipitation of [^{32}P]-orthophosphate labelled HeLa cell-extracts with anti-Ro antisera, hY5 RNA from appears to be much more abundant than hY1 and hY3 RNA. Therefore, since hY1 RNA is relatively more abundant in total RNA preparations than in anti-Ro immunoprecipitated RNA, it might be possible that part of the hY1 RNA molecules are not complexed with the Ro antigens.

Taken together, parts of the Ro RNP complexes seem to be well conserved in mammals, i.e. Ro60 and La antigens and Y1 and Y3 RNAs, whereas the Ro52 antigen and Y4 and Y5 RNA seem to be present in only a subset of mammals. The implications of this limited conservation of (parts of) the Ro RNP complexes for the function of the particles are not clear yet. In this respect, it is possible that some Ro RNPs (including Y1 and Y3 RNA, support important functions in all mammals, whereas other Ro RNP complexes (containing Y4 and Y5 RNA) may be involved in species specific functions.

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Chapter 5:
Analysis of protein-RNA interactions within Ro ribonucleoprotein complexes

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Abstract

The interactions between Ro and La proteins and hY RNAs have been analysed. The binding site for the 60 kDa Ro protein on hY RNAs is shown to be the terminal part of the base paired stem structure, which contains the most highly conserved sequence among hY RNAs. The bulged C-residue within this region of hY RNAs plays an important role in the recognition by this protein. The same regions of hY RNAs are essential for the association of the 52 kDa Ro protein with the RNAs, strongly suggesting that the 60 kDa Ro protein is required for the 52 kDa Ro protein to bind, presumably via protein-protein interactions, to Ro RNPs. The binding site for the La protein on hY RNAs is shown to be the oligouridylate stretch near the 3'-end of the RNAs, which is also recognized when a few additional nucleotides flank this motif at the 3'-side. Additional sequence elements in hY3 and hY5, but not in hY1, are bound by the La protein as well. Deletion mutagenesis showed that the RNP motif, previously identified in many ribonucleoprotein (RNP) proteins and in some cases shown to be almost sufficient for the interaction with RNA, of both the 60 kDa Ro and the La protein are not sufficient for the interaction with hY RNAs. Substantial parts of these proteins flanking the RNP motif are needed as well. These results suggest that parts of these proteins outside the RNP motif stabilize the correct conformation of this motif for RNA binding.

Introduction

Ro RNPs are composed of several proteins complexed with the RNA polymerase III transcribed Y RNAs. In human cells four different Y RNAs, called hY1, hY3, hY4 and hY5, all about 100 nucleotides in length, have been identified while in other species two to four Y RNAs have been found, always one of them being of hY1-size (Hendrick et al., 1981; Wolin and Steitz, 1983; Mamula et al., 1989a; Pruijn et al., 1990). In most human cells at least three proteins are associated with hY RNAs: the 60 kDa Ro protein (Ro60), the 52 kDa Ro protein (Ro52) and the La protein (Hendrick et al., 1981; Wolin and Steitz, 1984; Ben-Chetrit et al., 1988). However, in human red blood cells immunoreactive Ro proteins of 60 kDa (distinct from Ro60) and 54 kDa have been detected (Rader et al., 1989). Using an anti-Ro antiserum a third human immunoreactive 60 kDa protein has been purified from Wil-2 cells (Lieu et al., 1988). Proteins immunologically related to Ro60 and La have been found in several other mammalian species while Ro52 could only be detected in extracts from primate cells (Hoch et al., 1984; Slobbe et al., 1991).

Ro RNPs can be considered a subset of La RNPs. After termination of RNA polymerase III transcription, a step which only occurs accurately and efficiently in the presence of the La protein, this protein is associated with the oligouridylate stretch at the 3' terminus of the newly transcribed RNA (Stefano, 1984; Mathews and Francoeur, 1984; Gottlieb and Steitz, 1989a, 1989b). The interaction of the La protein with most RNA polymerase III products is lost upon maturation of the 3' end of the RNA. However, mature Y RNAs still contain a complete La binding site and a stable association of La with these Ro RNPs has been demonstrated (Mamula et al., 1989b; Boire and Craft, 1990).

Antibodies against Ro (both Ro60 and Ro52) and La, which are often present in sera from autoimmune patients, especially those suffering from Sjögren's syndrome and systemic lupus erythematosus (SLE), have been very helpful for the identification and characterization of Ro and La RNPs (Tan, 1989). Since these sera often contain antibodies against two or even three of these Ro and La proteins and since Ro52 comigrates with La in usual gel systems, a modification of the gel system is required for accurate characterization of autoimmune sera by Western blotting (Buyon et al., 1990; Slobbe et al., 1991).

The sequences of hY1, hY3, hY4 and hY5 have been determined and their predicted secondary structures are characterized by base-pairing of the 5' and 3' termini (Kato et al., 1982; Wolin and Steitz, 1983; O'Brien and Harley, 1990). The terminal part of the base-paired stem contains the most conserved nucleotides and this region was shown to be protected, presumably by bound proteins, against RNase degradation (Wolin and Steitz, 1984).

Complementary DNAs encoding Ro60, Ro52, La and the Wil-2 60 kDa protein have been isolated and analyzed. Two, except for the 3' end, almost identical cDNAs encoding Ro60 have been isolated and are characterized by the presence of an RNP motif and a putative zinc finger (Deutscher et al., 1988; Ben-Chetrit et al., 1989). An RNP

motif encoding region was also found in La (cDNA) (Chambers et al., 1988; Chan et al., 1989) while two putative zinc fingers are present in Ro52 (cDNA) (Chan et al., 1991; Itoh et al., 1991). The Wil-2 60 kDa protein cDNA encodes an acidic, calcium binding protein that contains an endoplasmic reticulum retention sequence and that is homologous to murine calreticulin (McCauliffe et al., 1990).

Biochemical fractionation of human Ro RNPs has shown that, within a cell, different Ro RNPs with distinctive physicochemical properties can be distinguished and that Ro RNPs consist of their constituent RNA plus more than one polypeptide (Boire and Craft, 1990). Indirect evidence indicates that one Ro60 polypeptide binds to one Y RNA (Wolin and Steitz, 1984). The results of Ouchterlony analyses, immunoprecipi-tations and immunofluorescence studies suggest that all three proteins, Ro60, Ro52 and La, can be associated simultaneously with a single Y RNA (Ben-Chetrit et al., 1988; Mamula et al., 1989b; Slobbe et al., 1991). However, the apparent molecular weights of the fractionated Ro RNPs in gel filtration, 230-350 kDa, can not be explained by the presence of only one copy of these proteins in such a particle (Boire and Craft, 1990).

In this paper we have analysed the interaction of Ro60, Ro52 and La with hY RNAs *in vitro*. Our results confirm previous data and unambiguously show that Ro60 and La bind directly to their presumed binding sites on hY RNAs. Additionally, La is shown to be able to interact with other sequences of hY3 and hY5. The analyses of the binding of HeLa S100 proteins to hY RNAs further strongly suggest that Ro52 requires Ro60 for its association with hY RNAs. Evidence for a direct protein-protein interaction between Ro60 and Ro52 will be documented in a separate paper (Slobbe et al., submitted). Here we also analysed the binding of *in vitro* expressed recombinant Ro60 and La as well as of deletion mutants of both proteins to hY RNA was analysed. The results suggest that the RNP motif of both Ro60 and La is not sufficient for specific RNA binding. Other sequence motifs may be involved and/or, especially in case of Ro60, a correct conformation of the RNA-binding domain may be critically dependent on other parts of the protein.

Materials and Methods

Cloning of hY RNAs

pHY1 and pHY3 were constructed as follows: Via PCR the coding sequences of the hY1 RNA and hY3 RNA genes were isolated out of a human genomic fragment harbouring both genes (a kind gift of Dr J.A. Steitz) using the following primers:

for hY1 5'-CTGAATTCGGCTGGTCCGAAGGTAGTGA-3' and
5'-CTAAGCTTAAAAGACTAGTCAAGTGCAGT-3'

for hY3 5'-CTGAATTCGGCTGGTCCGAGTGCAGT-3' and
5'-CTAAGCTTAAAAGGCTAGTCAAGTGAAGC-3'.

A comparable fragment for hY5 was obtained by reverse transcription of a hY RNA enriched HeLa RNA preparation using a primer complementary to the 3'-end of hY5 (5'-CTAAGCTTAAAACAGCAAGCTAGTCAA-3') followed by PCR in the presence of two hY5 specific primers: 5'-CTGAATTCAGTTGGTCCGAGTGTTGTGG-3' and the hY5-

primer mentioned above. PCR products were digested with EcoRI and HindIII and cloned into the EcoRI and HindIII sites of pGEM-3Zf(+).
Mutant hY RNAs were obtained by three approaches: (i) hybridization of the oligonucleotide complementary to the 3'-end of the hY RNA to the respective hY RNA transcribed in vitro followed by incubation in HeLa S100 extract (see: In vitro RNA-S100 protein binding assay); (ii) Linearization of template DNA for in vitro transcription of hY RNA by restriction enzymes recognizing sites in the hY RNA encoding sequence (hY1: SpeI; hY5: AluI); (iii) Site directed mutagenesis via PCR using alternative primers containing single-nucleotide mutations and the hY5 RNA-pGEM-3Zf(+) construct described above as template. The predicted secondary structures of the hY RNAs produced in vitro and mutants thereof were obtained using the algorithms of Turner et al. (1988).

cDNA cloning

A human placental λgt11 cDNA library was screened by hybridization with a 5'-end [^{32}P]-labeled oligonucleotide (5'-CATCAGGGCGCCACTTCAGTTTCCCTC-3') derived from a previously published Ro60 cDNA (Deutscher et al., 1988). From the 11 cDNA clones that were isolated only one (Ro4-7) contained the complete coding sequence as established by dideoxy sequencing. The other clones contained partial cDNAs encoding C-terminal parts of the protein, e.g. clones Ro7-4 and Ro3-1, which lack nucleotides encoding the N-terminal 53 and 274 amino acids, respectively.

A cDNA containing the complete coding sequence of the La protein was isolated out of a human teratocarcinoma cDNA library via an anti-La specific autoimmune patient serum.

After recloning the cDNA inserts in pGEM-3Zf(+) sequence analysis revealed that these were identical to previously published Ro60 (Deutscher *et al.*, 1988) and La (Chambers et al., 1988) cDNAs except for the termini (see Fig. 3).

S100 extracts

HeLa S100 extracts were prepared as previously described (Slobbe et al., submitted for publication).

In vitro transcription/translation

In vitro transcription by T7 RNA polymerase and *in vitro* translation of the T7-mRNAs were performed as described by Scherly et al. (1989). Biotinylated and [^{32}P]-labeled Y and U RNAs were prepared by linearization of the template-plasmids with HindIII (unless indicated otherwise) and in vitro transcription in the presence of Biotin-11-UTP or [α-^{32}P]ATP, respectively. Wild type and truncated Ro60 and La proteins were produced by translation of mRNA in wheat germ extract produced by T7 RNA polymerase transcription of template DNA after linearization with XbaI (Ro60wt, Ro7-4, Ro3-1, Ro60ΔA and Ro60ΔB), TaqI (Ro60ΔC530), SacI (Ro60ΔC524), TaqI (Ro60ΔC486), KpnI (Ro60ΔC419), PstI (Ro60ΔC395), HindIII (Lawt and LaΔN112), BstEII (LaΔC293), XbaI (LaΔC226), AluI (LaΔC202), DdeI (LaΔC164), ScaI

(LaΔC136). With the help of the partial Ro60 cDNAs (lacking sequences that encode N-terminal parts of the protein: Ro7-4 and Ro3-1) we could demonstrate (see Figure 5B) that the most prominent polypeptides migrating faster than Ro60 in an SDS-polyacrylamide gel of *in vitro* translated protein were produced via internal start-codon usage and thus correspond to N-terminal deletion mutants. In Ro60ΔA and Ro60ΔB internal deletions were created by digestion with AccI and BglII, respectively, and religation. A construct for the production of N-terminally deleted La (LaΔN112) was obtained by ligating the 1.3 kbp BglII-HindIII fragment of La-pGEM-3Zf(+) into the BamHI and HindIII sites of A2/3 (Scherly et al., 1989). Translation of this mutant results in a truncated La protein where the N-terminal 111 amino acids are replaced by Met-Gly.

In vitro RNA-S100 protein binding assay

The analysis of the binding of HeLa S100 proteins to [^{32}P]-labeled in vitro produced hY RNAs was performed as previously described (Slobbe et al., submitted for publication).

In vitro transcribed RNA-recombinant protein binding assay

The analysis of the interaction of recombinant Ro60, La and mutants of both proteins was performed essentially as described by Scherly et al. (1989). After incubation of the [^{35}S]-labelled recombinant proteins with biotinylated RNA, RNA-protein complexes were precipitated with streptavidin-agarose. Precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis.

Results

Interaction of HeLa S100 proteins with hY RNAs

In order to be able to produce the individual hY RNAs *in vitro* we cloned the hY RNA genes in the vector pGEM3-Zf(+). By PCR techniques using hY1 and hY3 specific primers the hY1 and hY3 genes were isolated out of the genomic clone described by Wolin and Steitz (1983). The hY5 cDNA was obtained by reverse transciption of a Ro RNA enriched HeLa cell RNA preparation using an hY5 specific primer (based upon the hY5 RNA sequence determined by Kato et al., 1982) followed by PCR in the presence of two hY5 specific primers. In addition to hY specific sequences, primer sequences contained restriction enzyme recognition sequences that allowed cloning in the EcoRI and HindIII sites of the pGEM3-Zf(+). Due to this cloning procedure T7 RNA polymerase transcription of the cloned genes produces wild type hY RNAs with 10 additional nucleotides (nts) linked to the 5'-ends and 5 additional nts linked to the 3'-ends. Computerized secondary structure predictions for these extended hY RNA molecules showed that predicted wild type structures (Wolin and Steitz, 1983; Pruijn et al., 1990) are not affected by the extensions. Analysis of these *in vitro* produced hY RNAs on denaturing polyacrylamide gels demonstrated that these transcripts were of the expected size (hY1:128 nts; hY3:117 nts; hY5:99 nts; data not shown, see also Fig 2A).

The individual hY RNA transcripts (^{32}P-labelled) described above were incubated

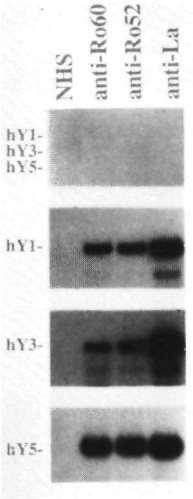

Fig.1. Association of HeLa S100 proteins with hY RNAs produced in vitro.
Radioactively labelled hY1, hY3 or hY5 RNA, *in vitro* transcribed by T7 RNA polymerase, was incubated with HeLa S100 extract. Subsequently, the association of the Ro and La antigens with the RNAs was analyzed by immunoprecipitation with mono-specific anti-Ro60, anti-Ro52 or anti-La antibody or with normal human control serum. The precipitated RNA was examined by denaturing polyacrylamide gel electrophoresis followed by autoradiography. The upper panel illustrates the inability of these antisera to precipitate the Y RNAs (added as a mixture) in the absence of HeLa S100 proteins.

analyzed by immunoprecipitation with either anti-Ro60, anti-Ro52 or anti-La specific antibodies. The specificity of these antibodies has been established before (Slobbe *et al.*, 1991). This approach allows the separate analysis of the association of the various proteins with the RNAs. The results in Figure 1 show that all three proteins, Ro60, Ro52 and La are able to assemble with all hY RNA transcripts under these conditions. The specificity of this assay for the analysis of reconstituted Ro RNPs is illustrated by the lack of precipitated hY RNAs when control antibodies are used and when the S100 extract is absent (Figure 1). It should be noted that these results do not necessarily mean that a direct interaction of each of these proteins with the RNAs occurs.

Interaction of HeLa S100 proteins with hY RNAs mutants

Hybridization of an oligonucleotide complementary to the 3'-end of an *in vitro* expressed hY RNA to this RNA prior to the incubation in S100 extract leads to truncation of the RNA due to the action of endogenous RNAseH in the S100 extract (Fig. 2B, panel a). The efficiency of truncation varies between hY1, hY3 and hY5. Analysis of these truncated hY RNAs on denaturing polyacrylamide gels showed that the 3'-ends of the truncated hY RNAs are positioned approximately at hY1 nt 98, hY3 nt 87 and hY5 nt 71 (wild type hY RNA numbering, see Fig. 2A). Immunoprecipitation of these truncated hY RNAs via the associated proteins and the respective antibodies described above revealed that both Ro60 and Ro52 binding to all hY RNAs is abolished by 3'-truncation of the RNAs (Fig. 2B, panels b and c, lanes 1-6). The strong signals in lanes 4 (truncated hY3) correspond to precipitation of non-truncated wild type hY3. These results are completely in agreement with the most highly conserved hY RNA stem structure being the primary binding site for Ro antigen(s) as indicated by the RNAse protection experiments of Wolin and Steitz (1984). Immunoprecipitation with anti-La antibodies, however, showed that, although truncation of hY1 interferes with La binding, La is still able to bind to truncated hY3 and hY5, albeit less efficiently. Obviously, La does not (only) bind to the presumed binding site (the 3'-oligouridine stretch) but is also able to interact with other parts of hY3 and hY5 (Figure 2B, panel d). For hY1 and hY5 RNA these results were confirmed using 3'-truncated RNAs generated by restriction enzyme digestion of transcription-template DNA: SpeI for hY1 leading to a 3'-end at position 107; AluI for hY5 leading to

A.

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                                10        20        30        40        50        60        70        80        90       100       110
hY1   gggcgaauucGGCUGGGUCCGAAGGUAGUGAGUUAUCUCAAUUGAUUGUUCACAGUCAGUUACAGAUCGAACUCCUUGUUCUACUCUUUCCCCCCUUCUCACUACUGCACUUGACUAGCCUUUuaagcu
hY3   gggcgaauucGGCUGGGUCCGAGUGCAGUGGUGUUUACAACUAAUUGAUCACAACCAGUUACAGAUUUCUUGUUCCUUCUCCACUCCCACUGUGCUUCACUUGACUAGCCUUUuaagcu
hY5   gggcgaauucAGUUGGGUCCGAGUGUUGUGGGUUAUUGUUAAGUUGAUUUAACAUUGUCUCCCCCCACAACCGCGCUUGACUAGCUUGCUGUUUuaagcu
hY5a  gggcgaauucAuUgGGUCCGGgGUUGUGGGUUAUUGUUAAGUUGAUUUAACAUUGUCUCCCCCCACAACCGCGCUUGgCUAGCUUGCUGUUUuaagcu
hY5c  gggcgaauucAGUUGGGUCCGAuUuUUGUGGGUUAUUGUUAAGUUGAUUUAACAUUGUCUCCCCCCACAACCGCGCUUGACUAGCUUGCUGUUUuaagcu
hY5e  gggcgaauucAGUgGGGUCCGAuUGUUG-GGGUUAUUGUUAAGUUGAUUUAACAUUGUCUCCCCCCACAACCGCGCUUGACUuGCUUGCUGUUUuaagcu
hY5ΔC9 gggcgaauucAGUUGGGUC-GAGUGUUGUGGGUUAUUGUUAAGUUGAUUUAACAUUGUCUCCCCCCACAACCGCGCUUGACUAGCUUGCUGUUUuaagcu
hY5A9 gggcgaauucAGUUGGUCaGAGUGUUGUGGGUUAUUGUUAAGUUGAUUUAACAUUGUCUCCCCCCACAACCGCGCUUGACUAGCUUGCUGUUUuaagcu
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B.

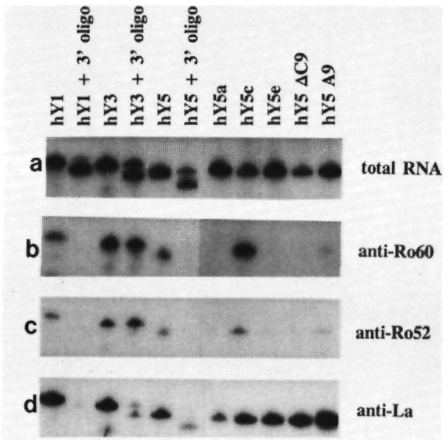

Fig.2. Interaction of HeLa S100 Ro60, Ro52 and La with mutated hY RNAs.

Panel A: The sequences of the transcripts used in the RNA-binding assay are listed. Nucleotides corresponding to wild type hY RNAs are in upper case, nucleotides derived from vector/linker sequences in lower case. Numbering is according to wild type hY RNAs (every tenth nucleotide is underscored). The most highly conserved regions are shaded. The 3' ends of the oligonucleotide/RNase H truncated RNAs are indicated by dashed underlining, the 3' ends of hY1*SpeI and hY5*AluI by an asterisk. Mutated nucleotides in the hY5 point mutants are indicated by smaller capitals or by dashes (single nucleotide deletions). **Panel B:** Analysis of the association of Ro60, Ro52 or La with hY RNA mutants, as indicated on top of the figure. The analysis was performed as described in the legend of Figure 1. Panel a shows the RNAs after incubation with HeLa S1200 extract. The other panels show immunoprecipi-tation results of the RNAs after incubation with HeLa S100 extract by monospecific anti-Ro60 (panel b), anti-Ro52 (panel c) or anti-La (panel d) antibody. The RNAs in the lanes marked hY1 + 3' oligo, hY3 + 3' oligo and hY5 + 3' oligo were hybridized to oligonucleotides complementary to the 3' ends of hY1, hY3 and hY5, respectively, before incubation with HeLa S100 extract and thus are (partially) truncated at the 3' end.

a 3'-end at position 73 (results not shown).

During the cloning procedure of the hY5 RNA cDNA several clones were obtained that contain 2 to 5 point-mutations in comparison with the wild type hY5 RNA sequence. The sequence of these hY5 RNA mutants, called hY5a, hY5c and hY5e is depicted in Figure 2A. Both hY5a and hY5e contain mutations within the presumed Ro-protein binding site, while the presumed La binding site in none of these mutants is changed. Two additional hY5 point-mutants were constructed in order to investigate whether the bulged-C residue, which is part of the presumed Ro-protein binding site, is involved in Ro-protein binding. The bulged-C was either deleted (hY5ΔC9) or altered into an A-residue (hY5A9) via PCR using mutant-primers. Secondary structure predictions for all these mutants revealed that the wild type hY5 structure was not severely affected by

the mutations. All mentioned hY5 mutants are efficiently transcribed in vitro as illustrated in Figure 2B, panel a.

The interaction of Ro60, Ro52 and La with the hY5 RNA point-mutants was analyzed using the immunoprecipitation assay described above. The results, shown in Figure 2B, indicate that mutations in the presumed Ro-protein binding site all abolish or at least decrease the association with both Ro60 and Ro52. Most interestingly, a deletion of the bulged C-residue completely eliminated Ro-protein association while alteration of the bulged C into a bulged A still allows assembly, albeit at a lower efficiency. These results confirm that the most highly conserved stem structure of hY RNAs including the bulged C-residue is an important determinant for Ro-protein binding. As expected, since neither of the hY5 point-mutants contains nucleotide changes within the presumed La binding site, La interacted efficiently with all of these mutants (Fig. 2B, panel d).

Interaction of recombinant Ro60 and La with hY RNAs

In order to be able to study the macromolecular interactions between Ro RNP components in more detail we isolated cDNAs encoding Ro60 and La out of human placental and human teratocarcinomal cDNA libraries. DNA-sequencing showed that these cDNAs were identical to those published by Deutscher et al. (1988) for Ro60 and Chambers et al. (1988) for La except for the 5' and 3' termini (see Figure 3). Coding sequences are identical to those previously published; the 3' non-coding sequences are identical (La) or truncated (Ro60) while the 5' non-coding sequences are extended in both cases. Remarkably, the 5' non-coding sequence of the Ro60 cDNA clone is distinct from the Ro60 cDNAs published before (Deutscher et al., 1988; Ben-Chetrit et al., 1989): only the first 18 base pairs flanking the ATG start codon are identical.

The cDNAs obtained were cloned into the EcoRI site of pGEM3-Zf(+). T7 RNA polymerase transcription and subsequent translation of the transcripts in wheat germ extract in the presence of ^{35}S-Methionine resulted in radiolabeled proteins of the expected molecular weights (Ro60: 60 kDa; La: 47 kDa). Both the size of the obtained proteins and immunoprecipitation experiments using reference anti-Ro and anti-La antisera (results

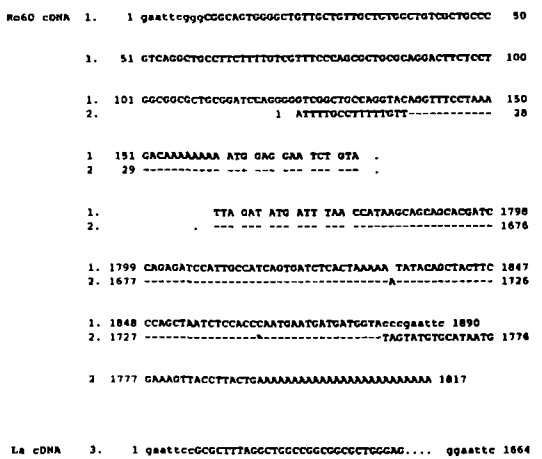

Fig.3. Structure of the cDNAs encoding Ro60 and La.
The sequence of our Ro60 cDNA clone (1.) is compared with that of a previously published Ro60 cDNA (Deutscher et al., 1988; 2.). Similarly, the sequence of our La cDNA clone (3.) is compared with that of Chambers et al. (1988) (4.). Identical nucleotides are indicated by dashes. Central cDNA parts indicated by dots are completely identical. In the Ro60 cDNAs triplets mark the coding region.

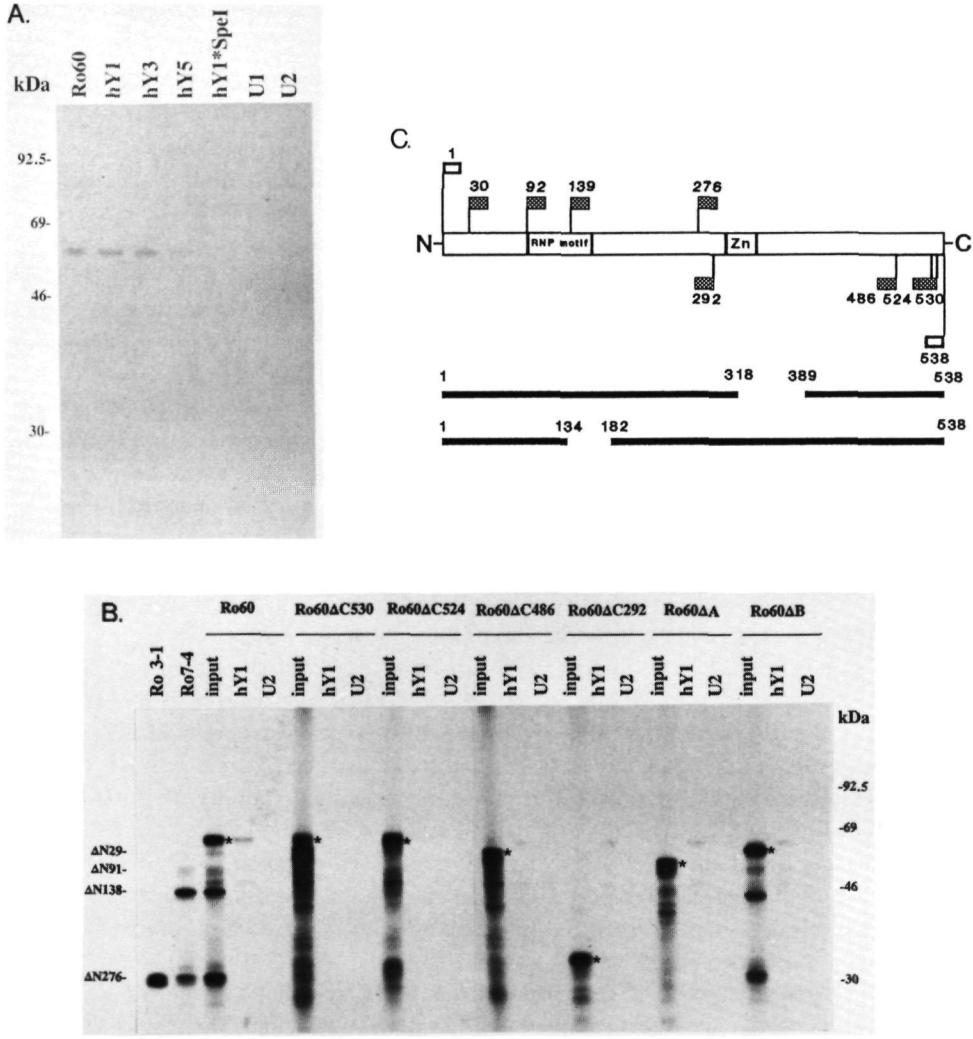

Fig.4. Analysis of the interaction of recombinant Ro60 with hY RNA.
Recombinant Ro60 was translated in wheat germ extract in the presence of L-[^{35}S]-methionine, incubated with biotinylated RNA and precipitated with streptavidin-agarose. Precipitated proteins were analyzed by SDS-PAGE followed by autoradiography. **Panel A:** Wild type Ro60 was incubated with biotinylated RNA as indicated on top of the panel. The lane marked Ro60 contains 25% of the input protein. **Panel B:** RNA binding of Ro60 deletion mutants. In the lanes marked Ro3-1 and Ro7-4 translates obtained by translation of mRNA derived from Ro60 cDNA clones Ro3-1 and Ro7-4, respectively, are shown. The position of N-terminal Ro60 deletion mutants is indicated on the left. The binding of Ro60 (or Ro60 deletion mutants) to hY1 and U2 RNA was analyzed. Each set of three lanes contains 25% of input protein (marked input), protein bound by hY1 RNA (marked hY1) and protein bound by U2 RNA (marked U2). The positions of C-terminal and internal deletion mutants are marked by asterisks. Wild type Ro60 was added as an internal positive control to the deletion mutant analyses, except for the analyses of Ro60ΔC530 and Ro60ΔC524. The band at about 45 kDa co-occurring with RNA-bound Ro60 is due to Ro60 degradation after the RNA binding assay. **Panel C:** Structure of the Ro 60 deletion mutants and summary of the RNA-binding analyses. The Ro60 protein (538 amino acids) is drawn schematically. The positions of the RNP-80 motif and the putative zinc finger are indicated. The first and last amino acid of N- and C-terminal deletion mutants are indicated by flags. The numbers refer to the position of these amino acids in the wild type protein. White flags indicate the ability to bind hY1 RNA; Grey flags indicate lack of RNA binding. The structure of the internal deletion mutants Ro60ΔA and Ro60ΔB (both unable to bind RNA) is illustrated by the black bars.

not shown) confirm that our cDNAs encode genuine Ro60 and La polypeptides, respectively.

In order to study the interaction of recombinant proteins with the hY RNAs we employed the assay described by Scherly et al. (1989). In short, biotinylated RNA is incubated with radiolabeled protein translated *in vitro* and protein-RNA complexes are precipitated with streptavidin-agarose. Finally, precipitated protein is analyzed by SDS-PAGE. The results in Figure 4A show that Ro60 specifically interacts with hY1, hY3 and hY5 RNA and not with control RNAs (U1, U2) nor with the the 3' truncated hY1*SpeI mutant. Thus, the binding specificity of recombinant Ro60 is identical to what was observed when HeLa S100 was used as protein source.

We next wanted to analyze which parts of Ro60 are involved in RNA binding. As has been documented by Deutscher et al. (1988) and Ben-Chetrit et al. (1989) at the primary sequence level two (potentially nucleic acid binding) motifs can be discerned. One of these, the RNP motif (Scherly et al., 1990), has been found in many RNA-binding proteins and, in some cases, has been shown to be directly involved in the interaction with U RNAs (Scherly et al., 1990; Nagai et al., 1990). The second motif strongly resembles a so-called zinc-finger, which is involved in the interaction of a number of DNA-binding proteins with DNA (Berg, 1990).

Several Ro60 deletion mutants were produced *in vitro* and the binding of these mutants to hY1 and U2 RNA (as a control) was assayed as described above. When mutant was clearly separated from wild type Ro60 (all except Ro60ΔC530 and Ro60ΔC524) full length Ro60 was included in the assays as an internal positive control. The results in Figure 4B show that all deletions eliminated the capacity of Ro60 to bind specifically to hY1 RNA. Even an N-terminal deletion of 29 amino acids or a C-terminal deletion of only 8 amino acids resulted in the loss of binding (Figure 4C). Since it is not very likely that both extreme termini of Ro60 are directly involved in the interaction with RNA, these results suggest that the presence of both termini is a prerequisite for maintaining a conformational structure that is indispensible for RNA binding.

The approach used to study the interaction of recombinant La with hY RNA was analogous to the one described above for the Ro60-RNA interaction. The results shown in Figure 5A show that La specifically interacts with hY1, hY3, hY5 and U1 RNA but not with hY1*SpeI nor with U2 RNA. These data indicate that the RNA-binding specificity of recombinant La in this assay reflects the specificity of native La in S100 extracts.

hY1 and U2 RNA were selected for the analyses of the RNA-binding capacity of La deletion mutants, in order to study which parts of La are essential for the interaction with RNA. At the primary sequence level an RNP motif has been found in La as well (Figure 5C; Chambers et al., 1988). Several La deletion mutants were produced *in vitro* and the effects of these deletions on RNA-binding of La were analyzed (Figure 5B). C-terminal deletion-mutants containing an intact RNP domain were all able to bind hY1 RNA, but deletions affecting the RNP motif eliminated its RNA-binding capacity. However, the result of the N-terminal deletion mutant shows that the presence of the RNP motif alone is not sufficient for RNA binding of La. These results strongly suggest

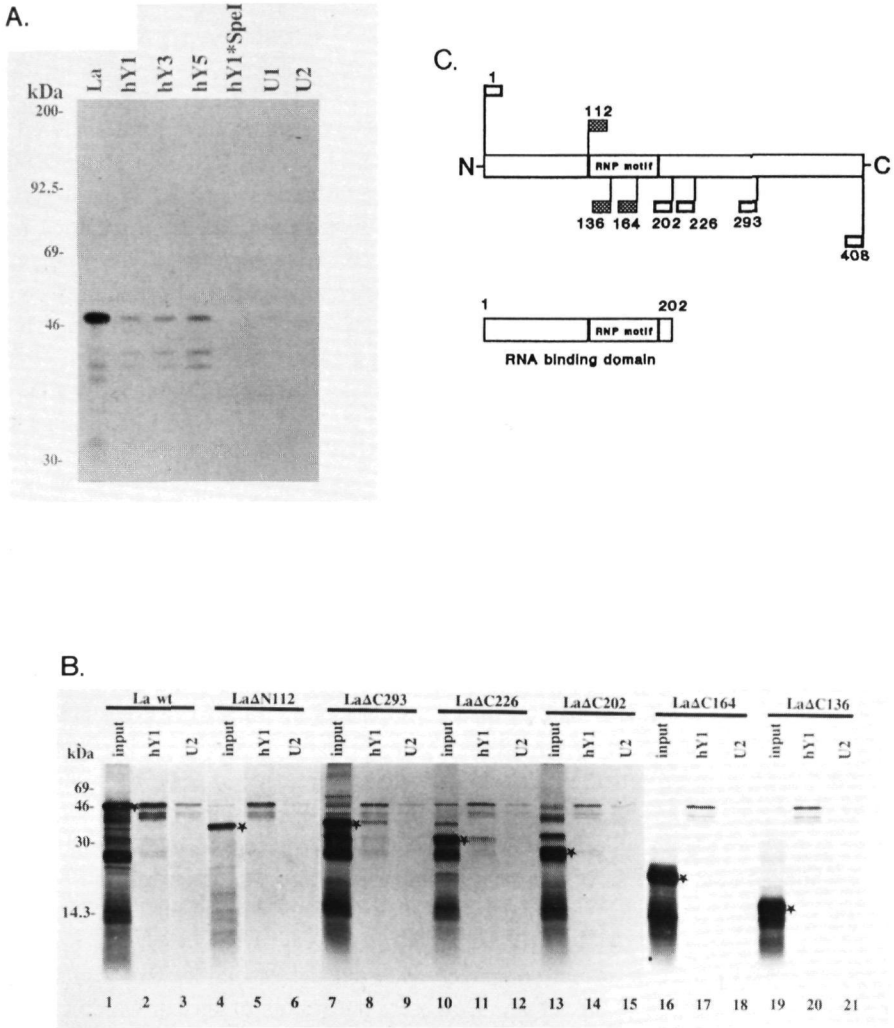

Fig.5. Analysis of the interaction of recombinant La with hY RNA.
Recombinant La was translated in wheat germ extract in the presence of L-[^{35}S]-methionine, incubated with biotinylated RNA and precipitated with streptavidin-agarose. Precipitated proteins were analyzed by SDS-PAGE followed by autoradiography. **Panel A:** Wild type La (lane marked La contains 25% of input protein) was incubated with the RNAs as indicated on top of the panel. Polypeptides migrating faster than La represent degradation products of La. **Panel B:** RNA-binding of La deletion mutants. Each set of three lanes contains 10% of input protein (marked input), protein bound by hY1 RNA (marked hY1) and protein bound by U2 RNA (marked U2). The positions of N- and C-terminal deletion mutants are indicated by asterisks. Wild type La was added as a positive internal control to the deletion mutant analyses. **Panel C:** Structure of La deletion mutants and summary of RNA-binding analyses. The La protein (408 amino acids) is drawn schematically. The position of the RNP-80 motif is indicated. The first and last amino acid of N- and C-terminal La deletion mutants, respectively, are indicated by flags. The numbers refer to the position of these amino acids in the wild type protein. White flags indicate the ability to bind hY1 RNA; Grey flags indicate lack of RNA binding. The region of La required for RNA-binding is shown below.

that the minimal RNA-binding region of La consists of the RNP motif and the adjacent part of the N-terminus of the protein (Figure 5C).

Discussion

RNA-protein interactions occurring within Ro RNP particles have been analyzed. Reconstitution of Ro RNPs by incubation of *in vitro* transcribed hY RNAs in HeLa S100 extract followed by immunoprecipitation with monospecific antisera proved to be a very specific and sensitive way to analyze the effects of RNA mutations on the association of the individual proteins with these particles. In order to study regions of the proteins involved in RNA-binding in more detail we isolated and cloned cDNAs encoding Ro60 and La and used these for *in vitro* expression of the corresponding proteins. The interaction of these proteins and mutants thereof with biotinylated hY RNAs was analyzed by precipitation with streptavidin-linked agarose beads as described by Scherly et al. (1989). The results of both approaches unambiguously show that Ro60 and La directly bind to hY RNAs.

The RNA binding site for Ro60 and La

Previous RNase protection experiments (Wolin and Steitz, 1984) have shown that the most highly conserved region of the human Y RNAs is protected against degradation, presumably by bound Ro antigenic protein(s). In agreement with data from Deutscher et al. (1988), who showed that recombinant Ro60 is able to bind hY1 RNA, the results of our reconstitution experiments, using either HeLa S100 extract as protein source or *in vitro* expressed recombinant Ro60, indicate that Ro60 is the protein that directly binds to the most highly conserved region of hY RNAs. Moreover, the bulged C-residue present in this region seems to be an important determinant for this interaction since both deletion and alteration of the bulged C severely affected binding. The importance of bulge structures for the interactions of proteins with RNA has been found in other systems as well. For example, the interaction of the bacteriophage R17 coat protein with the RNA is dependent on the presence of a single bulged A residue (Wu and Uhlenbeck, 1987) while a bulge structure in HIV-1 TAR RNA is required for binding of the viral Tat protein (Roy et al., 1990). Our results do not completely rule out the possibility that also Ro52 interacts with the same region of hY RNAs. However, the results of further experiments, to be published elsewhere (Slobbe et al., submitted for publication), show that Ro52 is completely dependent on the presence of Ro60 for its association with hY RNAs and that a direct protein-protein interaction between Ro60 and Ro52 can be observed, even in the absence of RNA. We conclude that Ro60 directly interacts with the most highly conserved region of hY RNAs.

The lack of interaction of both S100- and recombinant Ro60 with the hY1 mutant terminating at nucleotide 107 (hY1*SpeI) is somewhat puzzling because the complete conserved region of the Ro60 binding site is present and because hY2, a truncated version of hY1 occurring *in vivo* which terminates between nts 103 and 107, is precipitable from cell extracts with anti-Ro60 antibodies (Hendrick et al., 1981: Wolin and Steitz, 1983).

The most plausible explanation for this discrepancy is that hY1 might be cleaved in cell(extract)s while it is bound by Ro60, thereby not affecting the binding of the protein. Alternatively, the secondary/tertiary structure of the truncated hY1 RNA made *in vitro* might be such that it does not allow Ro60 to bind.

The La protein is generally believed to bind to the common sequence motif present in La-associated RNAs, a short stretch of uridylate residues at the 3' terminus of the RNA. Indeed, evidence for this motif being the site of interaction with the La protein has been obtained (Stefano, 1984; Mathews and Francoeur, 1984). Since none of our *in vitro* transcribed RNAs contains an oligouridylate stretch positioned at the 3'-end of the molecule, our data show that La is also able to interact with RNAs lacking this motif. Nevertheless, most La binding RNA molecules analyzed do contain an oligouridylate stretch near the 3' terminus and deletion of these nucleotides in hY1 impaired La binding, suggesting that this sequence motif should not necessarily be located at the extreme 3'-end of the RNA molecule for La to bind. At present we are investigating whether La binds with different affinities to oligouridylate stretches at the 3' end of the molecule compared to those flanked on both sides by other nucleotides. That the La binding site might be even less stringently defined is illustrated by the binding of La to truncated hY3 and hY5 RNAs lacking the U_4 stretch near the 3' end. At present, it is not known whether La in these cases recognizes other internal oligouridylate stretches (U_3 and U_2 stretches are present in hY3 and hY5, but also in hY1) or whether completely divergent sequences are bound. Interestingly, La does also interact with U1 RNA both *in vitro* (Fig. 5A) and *in vivo* (Madore et al., 1984).

Regions of Ro60 and La required for RNA binding

Both Ro60 and La contain an amino acid sequence motif, mostly referred to as RNP motif, RNA binding domain or RNA recognition motif, often found in RNA binding proteins (Bandziulis et al., 1989; Query et al., 1989; Scherly et al., 1990). In several cases, e.g. poly(A) binding protein (Sachs et al., 1987), U1-70K (Query et al., 1989), U1-A (Scherly et al., 1989) and U2-B" (Scherly et al., 1990), this RNP motif with only a few flanking amino acids has been shown to be sufficient for specific RNA binding. In this paper we have shown that for hY RNA binding of Ro60 as well as La much larger parts of the RNP motif flanking sequences are required. While for La C-terminal deletions up to the RNP motif, which ranges from amino acid 112 to amino acid 187, are allowed all deletions in Ro60, containing the RNP motif from amino acid 92 to amino acid 161, abolished its capacity to bind RNA. These observations might be explained by two phenomena: (i) In addition to the RNP motif other amino acid motifs might be directly involved in and absolutely required for the interaction with RNA. (ii) Other parts of the protein, while not having a direct role in the interaction with RNA, might be required for the RNP motif to fold properly. Although the putative zinc finger, a motif found to be important for the interaction of a number of DNA-binding proteins with DNA, of Ro60 is a good candidate for a motif being also directly involved in RNA-binding we do not favour this hypothesis since deletions not affecting either the zinc finger or the RNP motif still abolished RNA binding and because the zinc finger region of

Ro60 is already directly involved in the interaction with Ro52 (Slobbe et al., submitted).

The Ro60 protein binds a region of hY RNAs which is predicted to adopt a primarily double-stranded structure. In this respect Ro60 deviates from other RNP containing RNA-binding proteins, because poly(A) binding protein, U1-70k, U1-A, U2-B" and La all have been shown to interact with at least partially single-stranded regions of the respective RNAs (Stefano, 1984; Sachs et al., 1987; Query et al., 1989; Scherly et al., 1989 and 1990).

Several reports have shown evidence for the heterogeneity of Ro RNPs (for review see Pruijn et al., 1990). The data presented in this paper and the data presented by Slobbe et al. (submitted), indicating that Ro60, Ro52 as well as La are able to associate with all hY RNAs (a similar binding of both Ro60 and La to the recently sequenced hY4 (O'Brien and Harley, 1990) may be predicted based upon sequence homology), can not or only partially explain differences in protein composition of the individual Ro RNPs. The putative binding of a second La polypeptide to hY3 and hY5 RNP but not to hY1 RNP may be responsible for heterogeneity but does not reflect the apparent differences in molecular weight of Ro particles in gel filtration nor their different behaviour during biochemical fractionation (Boire and Craft, 1990). Possibly other, yet unidentified proteins are associated with Ro RNPs as well. Interestingly, a third conserved sequence motif has been identified within the hY RNAs (Pruijn et al., 1990). Although a weak interaction of Ro52 with this motif can not be excluded yet, it might be the recognition site of another protein as well. Since the position of this motif relative to the motif determining Ro60 binding differs in hY5 compared to the other hY RNAs (in hY5 both motifs are overlapping), this might explain heterogeneity to some extent. The identification of an hY5 RNP specific epitope (Boire and Craft, 1989) might be easily explained by the binding of an hY5 RNP-specific protein, bearing the epitope, to this particle. The assays described in this paper to study the Ro RNP structure already have (Slobbe et al., submitted) and will be helpful to study the Ro RNP structure in more detail and to unravel differences in the molecular composition of the various Ro RNPs.

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Chapter 6:
Analysis of protein-protein interactions within Ro RNP complexes

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submitted for publication

Analysis of protein-protein interactions within Ro RNP complexes

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Abstract

The human Y RNAs, small RNAs with an unknown function, are complexed with at least three proteins: the 60 kDa Ro protein (Ro60), the 52 kDa Ro protein (Ro52) and the La protein (La). In this study we examined the intermolecular interactions between the components of these so-called Ro ribonucleoprotein (Ro RNP) complexes.

Incubation of [^{32}P]-labelled hY1 RNA in HeLa S100 extract allows the reconstitution of Ro RNP complexes, which was analyzed by immunoprecipitation with monospecific antisera. By immunodepletion of HeLa S100 extracts for either Ro60, Ro52 or La, followed by suppletion with recombinant Ro60 or La, it was demonstrated that both Ro60 and La bind to hY1 RNA directly without being influenced by one of the other proteins. However, binding of Ro52 to hY1 required the presence of Ro60, indicating the association of Ro52 with hY1 RNA via Ro60.

To further define the protein-protein interaction between Ro60 and Ro52, [^{35}S]-labelled Ro60 deletion mutants were incubated in HeLa S100 extract followed by immunoprecipitation with monospecific anti-Ro52 antibody. This approach allowed the identification of a region of Ro60 (amino acids 276-318) responsible for binding to Ro52. The protein-protein interaction between Ro60 and Ro52 was found to be RNA independent, since reconstitution of Ro60-Ro52 complexes in micrococcal nuclease treated HeLa S100 extracts yielded identical results. The RNA independency of the interaction between Ro60 and Ro52 is further supported by the fact that Ro60 deletion mutants, not capable of binding to hY RNA, still are able to bind to Ro52.

Introduction

Autoantibodies to the Ro (SS-A) and the La (SS-B) ribonucleoprotein complexes (RNPs) are found in sera from patients with systemic lupus erythematosus and Sjögren's syndrome (reviewed by Chan and Tan, 1989). Such patient autoantibodies have been of great value in the identification and characterization of these RNA-protein complexes.

La RNPs are composed of a single RNA polymerase III transcript, that is bound through its 3'-uridine stretch to the 46.7 kDa La protein (La). This protein is highly conserved among mammalian species and contains the antigenic determinants of the complex. La belongs to a family of RNA binding proteins sharing an 80 amino acids domain, referred to as RNA recognition motif, RNA binding domain or, more neutrally, RNP-80 motif (Bandziulis et al., 1989; Query et al., 1989; Scherly et al., 1989). The La protein functions as an RNA polymerase III transcription termination factor (Gottlieb and Steitz, 1989a,b) and is predominantly localized in the nucleus (for review see Pruijn et al., 1990).

Although the function of the Ro RNPs still is a mystery, several studies on the structure of Ro complexes have been performed. In human cells four Ro RNAs have been described (hY1, hY3, hY4 and hY5), varying in length between 84 and 112 nucleotides (for review see Pruijn et al., 1990). The hY RNAs have been sequenced and secondary structures have been predicted (Kato et al., 1982; Wolin and Steitz, 1983;the O'Brien and Harley, 1990; Pruijn et al., 1990). The lower part of the characteristic stem structure structure of all Y RNAs contains a highly conserved region, which was shown to be protected against RNAse degradation presumably by bound protein(s) (Wolin and Steitz, 1984). The protein content of the Ro RNP complexes consists of at least two Ro proteins with molecular weights of 60 kDa (Ro60) and 52 kDa (Ro52), respectively. Since the RNAs are transcribed by RNA polymerase III, they become associated with the La protein as well (for review see Pruijn et al., 1990). Complementary DNAs containing the complete coding sequence of Ro60 have been isolated (Deutscher et al., 1988; Ben-Chetrit et al., 1989) and the recombinant Ro60 protein was shown to associate with hY1 RNA *in vitro* (Deutscher et al., 1988). Ro60 is also a member of the RNA binding protein family sharing an RNP-80 motif. Recently, cDNA clones encoding Ro52 have been described as well (Chan et al., 1991; Itoh et al., 1991). An RNP-80 motif has not been found in Ro52 and, although affinity purified anti-Ro52 antibody was able to precipitate the characteristic hY RNAs, no direct interactions between Ro52 and the Ro RNAs were identified. Apart from these two main antigenic Ro proteins, other putative Ro proteins have been described. Two of these, with molecular weights of 60 and 54 kDa, are found in red blood cells only and thus might be tissue specific Ro components (Rader et al., 1989). Another putative Ro component, the human analogue of calreticulin (McCauliffe et al., 1990), has been described, but its involvement in the complexity of Ro RNPs is as yet not clear.

To gain insight into the structure of the Ro RNP complexes, a series of experiments were performed which showed that only Ro60 and La bind directly to hY1 RNA and that Ro52 binds indirectly to hY1 RNA via Ro60.

Materials and methods

Cloning and expression of Ro60 and La in E. coli

Full length cDNAs coding for Ro60 and La were obtained by screening a human placenta λgt11 library with specific oligonucleotides (Ro60) or a human teratocarcinoma λgt11 library with a patient autoimmune serum (Pruijn et al., manuscript in preparation). The Ro60 coding sequence was identical to the one published by Deutscher et al. (1988), whereas the sequence of La was identical to the one published by Chambers et al. (1988). The cDNAs encoding Ro60 and La were mutated in order to clone the complete coding sequences in vector pET8c (Studier et al., 1990) by PCR using mutated oligonucleotides without changing the amino acid sequence of both proteins. The resulting product (see Figure 1) was ligated in vector pET8c and transfected into E. coli strain BL21 (DE3) pLysS.

The transformed bacteria were grown until an OD_{550} of 1.0 in LB medium (10g Bactotryptone, 5g Yeast extract and 10g NaCl per liter with 200 μg/ml ampicilline and 30 μg/ml chloroamphenicol) was reached. Subsequently, protein expression was induced by adding isopropylthiogalactoside (IPTG; final concentration 0.5 mM) to the medium. After 3h of induction the bacteria were harvested and either dissolved in 1/10 volume of SDS-sample buffer (2% Sodium dodecyl sulphate/ 5% ß-mercaptoethanol/ 10% glycerol/ 0.005% bromo-phenolblue/50 mM Tris HCL pH6.8) for gel analyses or lysed in 1/10 volume of PBS by repeated freezing and thawing for suppletion studies.

In vitro transcription and translation

For *in vitro* transcription and translation of Ro60, the cDNA encoding Ro60 was ligated in vector pGEM3z-f(+) in order to produce mRNA by T7 RNA polymerase transcription. For the transcription of hY1 RNA the coding sequence of the hY1 gene (Wolin and Steitz, 1983) was amplified by PCR techniques and ligated into vector pGEM3z-f(+). T7 RNA polymerase transcription of this construct yields hY1 RNA with 10 additional nucleotides at the 5' end and 5 nucleotides at the 3' end. The predicted secondary structure of this transcript is identical to the secondary structure of the wild type hY1 RNA and, as will be shown below, Ro60, Ro52 and La are able to bind specifically to this RNA (Figure 3A).

In vitro transcription was performed as follows: To produce mRNA for *in vitro* translation 2 μg of linearized template was incubated at 37 °C for 60 min in a total volume of 50 μl containing: 40 mM Tris-Cl pH 7.9, 6 mM $MgCl_2$, 10 mM spermidine-HCL, 10 mM NaCl, 2 mM dithiothreitol (DTT), 0.1 mg/ml BSA, 60 μM GpppG, 1 mM ATP, UTP, CTP and GTP, 60 U RNAse inhibitor (Promega) and 30 U T7 RNA polymerase (Promega). In order to produce [^{32}P]-labelled hY1 RNA 0.5 μg template DNA was transcribed in a total volume of 20 μl (same buffer as described above) with 20 μCi α[^{32}P]-UTP (Amersham, UK, 3000 Ci/mmol), 0.12 mM UTP and 1 mM ATP, GTP and CTP. After transcription unincorporated nucleotides were removed by a Sephadex G50 spin column, the RNA was phenol extracted, ethanol precipitated and dissolved in 10 μl (mRNA) or 100 μl ([^{32}P]-hY1 RNA) sterile water.

In vitro translation was performed by incubating 1 μl *in vitro* transcribed mRNA for 60 min at 25 °C in a total volume of 30 μl containing 15 μl Wheat Germ Extract (WGE, Amersham, UK), 120 mM potassium acetate, 67 μM of 19 amino acids mixture minus methionine and 30 μCi L-[^{35}S]-methionine (1200 Ci/mmol, Amersham, UK).

Antibody purification

Antisera monospecific for Ro60 or La polypeptides were isolated by immuno-affinity chromatography using bacterially expressed antigens. A monospecific anti-Ro52 patient antibody (Jo7) was passed over a recombinant Ro60 column to remove possible trace amounts of anti-Ro60 antibody. The resulting antibody preparations were checked for their monospecificity on Western blots containing HeLa cytoplasmic extract, on Western blots containing recombinant Ro60 or La and by immunoprecipitation of [^{32}P]-labelled cellular extracts, as described earlier (Slobbe et al.,1991a and b). For the identification of recombinant Ro60 and La well characterized autoimmune patient sera were used (references were obtained from the Center for Disease Control, Atlanta, Georgia, USA).

Preparation of extracts

For the binding of HeLa proteins to *in vitro* transcribed [^{32}P]-hY1 RNA we used HeLa S100 extract as protein source: Approximately 2*10^9 HeLa S3 suspension cells were washed twice with PBS pH 7.2 and resuspended in S100 buffer (25 mM Tris-Cl, pH 7.4, 0.1 mM EDTA, 0.25 mM DTT, 0.5 mM PMSC, 0.1 M KCl, 0.01% Nonidet-P40 and 20% glycerol) at 1*10^8 cells/ml. After 15 strokes with a Dounce homogenizer the suspension was centrifuged at 1000g for 10 min. The pellet was again centrifuged at 25000g for 20 min and the additional small amount of supernatant was carefully removed. The combined supernatants were then centrifuged at 108,000g for 60 min and the resulting S100 extract was stored in aliquots at -70 °C.

HeLa S100 extract was immunodepleted by five consecutive rounds of immunoprecipitation with monospecific antisera bound to protein A-agarose in the presence of 300 mM sodium chloride. The efficiency of depletion was monitored by Western blotting (see Figure 3B).

Binding assays

Reconstitution of Ro RNP complexes was performed by adding 1 μl [^{32}P]-hY1 RNA (approximately 30,000 cpm) to 15 μl S100 extract in a total volume of 25 μl, containing 2.5 mM $MgCl_2$, 150 mM NaCl and 5 μg tRNA. After 30 min of incubation at 20 °C, reconstituted Ro RNP complexes were immunoprecipitated with monospecific anti-Ro60, anti-Ro52 or anti-La antibody bound to protein A-agarose in the presence of 150 mM NaCl and the precipitated RNA was analyzed by gel analysis as described before (Slobbe et al.,1991b).

To elaborate the protein-protein interaction between Ro60 and Ro52, WGE translated [^{35}S]-labelled Ro60 deletion mutants were incubated in HeLa S100 extract as described above and immunoprecipitated by monospecific anti-Ro52 antibody in the

presence of 150 mM NaCl. After immunoprecipitation, Ro52 bound [^{35}S]-labeled Ro60 deletion mutants were analyzed by SDS-polyacryl-amide gel electrophoresis. After electrophoresis the gel was fixed, treated with Amplify (Amersham, UK), dried and autoradiographed using Kodak XAR5 film at -70 °C.

Results

Expression of Ro60 and La polypeptides

Complementary DNAs containing the complete coding sequence of Ro60 and La were mutated at the 5' border of the coding region, creating a NcoI restriction site, and in the 3'-non-coding sequence, creating a BamHI and HindIII site, respectively. These changes facilitated cloning of the cDNAs in vector pET8c (Studier et al.,1990) for expression of the respective non-fusion proteins in E. coli (see Figure 1).

Ro60:

```
              M  E  E  S                 I  *
5'-ACAAAAAACCATGGAGGAATCTG--1598 nt--ATTTAAGCATAAGCAGCAGCAGGATCCC-3'
```

La:

```
              M  A  E  N                 D  Q  *
5'-GATAGCCGCCATGGCTGAAAATG--1205 nt--GACCAGTAGTTTAGTAAACCAAGCTTTT-3'
```

Fig.1. Mutations in the cDNAs encoding Ro60 and La.
Full length cDNAs coding for Ro60 and La were obtained as described in Materials and Methods. Mutations in the cDNAs generated by PCR are underlined, restriction sites created by these mutations are printed bold. The protein sequences are shown above the DNA sequence. Asterisk refers to termination codon.

After induction of recombinant protein expression and lysis of the bacteria the expression of Ro60 and La was analyzed on an SDS-polyacrylamide gel (Figure 2A). The antigenicity of the recombinant proteins was confirmed on Western blots using well characterized anti-Ro60 (Figure 2B, left panel) and anti-La (Figure 2B, right panel) autoimmune sera.

Ro RNP reconstitution in HeLa S100 extract

To analyze RNA-protein interactions in Ro RNP complexes *in vitro*, T7 RNA polymerase transcribed [^{32}P]-labelled hY1 RNA was incubated in HeLa S100 extract. Subsequent immunoprecipitation with monospecific antibody in the presence of 150 mM NaCl allows the identification of proteins that are complexed with the RNA. The results of such a reconstitution assay show that monospecific anti-Ro60, anti-Ro52 and anti-La antibodies are able to precipitate the Ro RNP complexes formed during incubation (Figure 3A). The efficiency of reconstitution and subsequent immunoprecipitation generally varied between 5 and 10% of the input [^{32}P]-hY1 RNA when anti-Ro60 and anti-Ro52 antibody

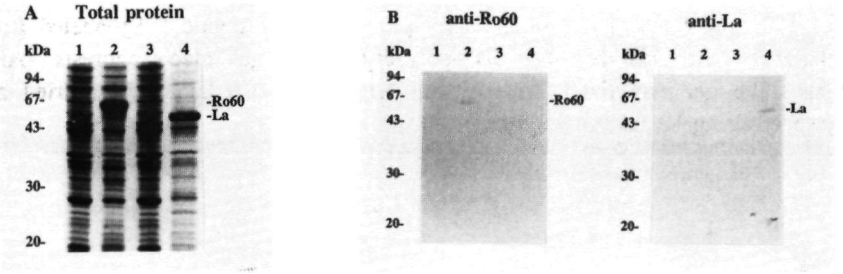

Fig.2. Induction of Ro60 and La expression in Escherichia coli.
panel A: Coomassie Brilliant Blue stained polyacrylamide gel containing extracts of non-induced (lane 1) and IPTG-induced bacteria (lane 2) transformed with Ro60 pET8c. In lane 3 extract of non-induced and in lane 4 extract of induced *E. coli* transformed with La pET8c was loaded. Bands corresponding to recombinant Ro60 or La are indicated on the right, the position of markers is shown on the left. **panel B:** Immunoblots of gels identical to the gel shown in panel A, incubated with anti-Ro (left) or anti-La (right) reference serum.

and between 15 and 20% when anti-La antibody was used.

Ro RNP reconstitution in immunodepleted HeLa S100 extracts

HeLa S100 extracts were depleted for Ro60, Ro52 or La by the procedure described in Materials and Methods. The efficiency of immunodepletion was tested by immunoblotting using a serum containing antibodies to Ro60, Ro52 and La (Figure 3B). An extract was used only when no residual antigen could be detected on the Western blot. Immunodepletion with normal human serum did not have any effect (lane 1).

Subsequently, the extracts immunodepleted for either Ro60, Ro52 or La were used for the reconstitution of Ro RNP complexes with *in vitro* transcribed [^{32}P]-labelled hY1 RNA. The "depletion"-procedure of HeLa S100 extract with normal human serum did not have any influence on the binding of Ro and La proteins to hY1 RNA (results identical to those shown in Figure 3A). The removal of Ro60 from HeLa S100 extract not only resulted in the loss of the formation of anti-Ro60 antibody precipitable Ro RNP complexes, but hY1 RNA-precipitation via anti-Ro52 antibody was abolished as well, in spite of the fact that Ro52 is still present in the extract (Figure 3C, lanes 2 and 3, left panel). The binding of La to hY1 RNA is not influenced by the absence of Ro60 (Figure 3C, lane 4, left panel).

Depletion of Ro52 from HeLa S100 extract of course abolished its binding to hY1 RNA (figure 3C, middle panel, lane 3) but did not interfere with the binding of either Ro60 or La to hY1 RNA (Figure 3C, middle panel, lanes 2 and 4). Similarly, depletion of La from HeLa S100 extract precluded the formation of La-hY1 RNA complexes (Figure 3C, right panel, lane 4), but did not influence the binding of either Ro60 or Ro52 to hY1 RNA (Figure 3C, lanes 2 and 3, right panel).

These findings suggest that (i) Ro60 binding to hY1 RNA is independent of both La and Ro52, (ii) for Ro52 binding to hY1 the presence of Ro60 is required and (iii) the binding of La to hY1 RNA is not dependent on the presence of the Ro proteins.

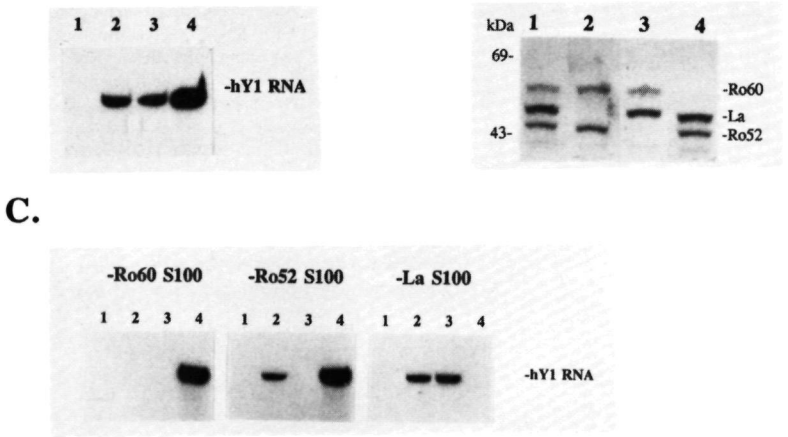

Fig.3. Analysis of Ro RNP complexes reconstituted in HeLa S100 extracts.
panel A: [^{32}P]-labelled *in vitro* transcribed hY1 RNA was incubated in HeLa S100 extract and subsequently immunoprecipitated with normal human serum (lane 1), anti-Ro60 (lane 2), anti-Ro52 (lane 3) or anti-La antibody (lane 4). Total immunoprecipitated RNA was analyzed on a 10% polyacrylamide-urea gel and autoradiographed. **panel B:** After immunodepletion of HeLa S100 extracts with monospecific anti-La (lane 2), anti-Ro52 (lane 3) or anti-Ro60 (lane 4) antibody the extracts were analyzed via immunoblotting using a reference serum containing antibodies to Ro60, Ro52 and La. Control S100 extract immunodepleted with normal human serum is shown in lane 1. Positions of Ro60, Ro52 and La are indicated on the right. **panel C:** Immunodepleted HeLa S100 extracts as indicated on top of each panel were used for the reconstitution of Ro RNP complexes with [^{32}P]-labelled hY1 RNA. The RNPs were immunoprecipitated with either normal human serum (lanes 1), anti-Ro60 (lanes 2), anti-Ro52 (lanes 3) or anti-La (lanes 4) antibody and the precipitated RNA was analyzed on a denaturing polyacrylamide-urea gel.

Supplementation of immunodepleted HeLa S100 extracts with recombinant Ro60 and La

To further define the requirement for Ro60 of Ro52 to bind to hY1 RNA, bacterially expressed Ro60 was added to Ro60-depleted HeLa S100 extract. As shown in Figure 4A, addition of recombinant Ro60 restored both binding of Ro60 and binding of Ro52 to hY1 RNA (right panel, lanes 2 and 3). Addition of control bacterial extract, containing neither Ro60 nor La recombinant protein, had no effect (left panel). The addition of recombinant Ro60 had no effect on the binding of La to hY1 RNA (Figure 4A, compare lanes 4).

To control the specificity of the reconstitution, recombinant Ro60 was added to Ro52-depleted S100 as well (Figure 4B, right panel). As expected, there was no effect on the efficiency of La or Ro52 binding (lanes 3 and 4), but a slightly diminished precipitation of hY1 by anti-Ro60 antibodies was reproducibly observed (Figure 4B, lanes 2, right vs. left panel). This can be explained by the presence of excess Ro60, only partially complexed to hY1 RNA and competing for the anti-Ro60 antibodies.

Addition of recombinant La to La-depleted HeLa S100 extract restored the La-

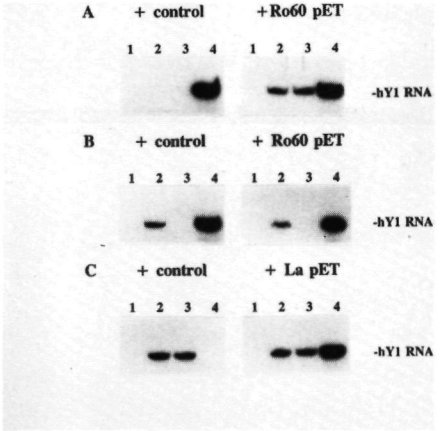

Fig.4. Supplementation of immunodepleted HeLa S100 extracts with recombinant Ro60 and La. Reconstitution of Ro RNP complexes was performed with immunodepleted HeLa S100 extracts and [^{32}P]-hY1 RNA, supplemented with bacterial extracts containing recombinant Ro60 or La protein or with control bacterial extracts as indicated on top of each panel. In **panel A** Ro60-depleted S100 was used, in **panel B** Ro52 depleted S100 and in **panel C** La depleted S100 extract. In lanes 1 normal human serum precipitations, in lanes 2 immunoprecipitations with anti-Ro60 antibody, in lanes 3 anti-Ro52 antibody precipitations and in lanes 4 anti-La antibody immunoprecipitations were performed. After reconstitution and immunoprecipitation, total precipitated RNA was analyzed on a polyacrylamide-urea gel.

hY1 RNA interaction without affecting Ro60 or Ro52 binding (Figure 4C, lanes 4, left vs. right panel).

The results described above corroborate the earlier finding that the association of Ro52 with hY1 RNA depends on the presence of Ro60, whereas the binding of both Ro60 and La to hY1 RNA is not influenced by the presence of one of the other proteins. This suggests that Ro60 either induces a conformational change of the RNP thereby enabling Ro52 to associate with the complex or that Ro52 binds directly to Ro60 in Ro RNP complexes.

Binding of recombinant Ro60 to Ro52 in HeLa S100 extract

To substantiate the presumed interaction between Ro60 and Ro52 in Ro RNP complexes, *in vitro* translated [^{35}S]-methionine labelled Ro60 protein was incubated with HeLa S100 extract and immunoprecipitated by anti-Ro52 antibody in the presence of 150 mM NaCl (Figure 5A, lane 1). As a control a similar precipitation was performed in the absence of HeLa S100 extract (Figure 5A, lane 2). The results show that Ro60 can be precipitated by anti-Ro52 antibodies only in the presence of factors (i.e. Ro52) contained in the S100 extract.

To test whether the Ro52-Ro60 interaction is dependent on the presence of RNA, HeLa S100 extract was pre-treated with micrococcal nuclease. After this treatment no hY RNAs could be detected any more when a Northern blot of this nuclease treated extract was probed with anti-sense hY1, hY3 or hY5 RNA (data not shown). However, *in vitro* translated Ro60 was still precipitated efficiently by anti-Ro52 antibodies (figure 5A, lane 3), suggesting that the protein-protein interaction between Ro60 and Ro52 does not require the presence of intact hY RNAs.

Binding of Ro60 deletion mutants to Ro52

For the characterization of the region in the Ro60 protein responsible for the interaction with Ro52, several N- and C-terminal Ro60 deletion mutants were used. Their structures are schematically shown in figure 5C. After *in vitro* transcription of the

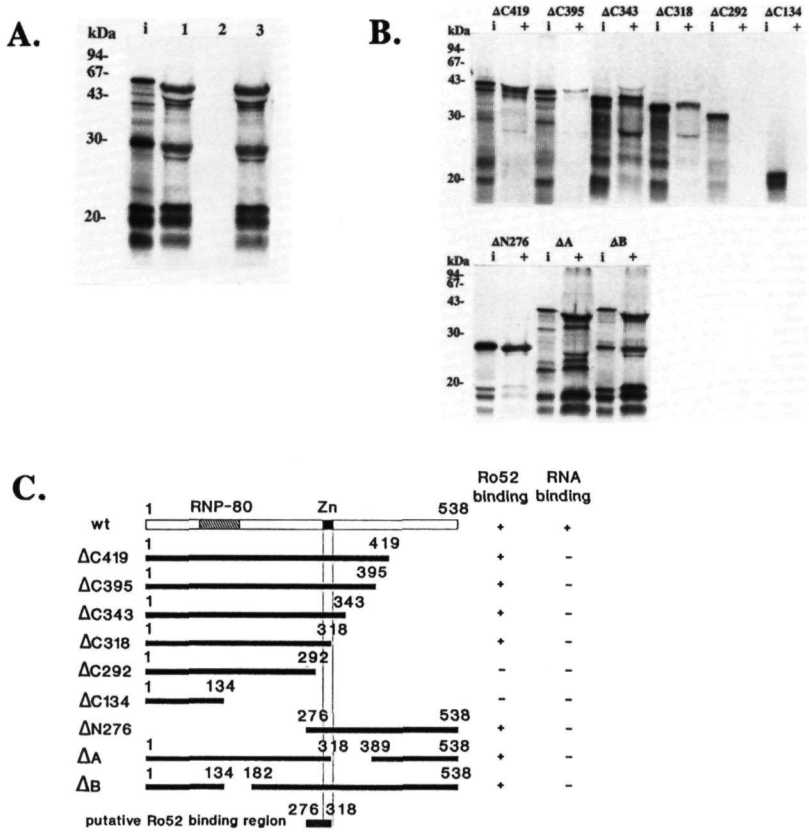

Fig.5. Protein-protein interaction between Ro52 and recombinant Ro60.
panel A: *In vitro* transcribed and wheat germ extract translated Ro60 protein was incubated with HeLa S100 extract and immunoprecipitated by monospecific anti-Ro52 antibody (lane 1). In lane 2 no HeLa S100 extract was added as a control. In lane 3, micrococcal nuclease treated HeLa S100 extract was used. Total immunoprecipitated protein was analyzed on an SDS-polyacrylamide gel. Input [^{35}S]-labelled protein is shown in the lane marked i. **panel B:** Immunoprecipitation of Ro60 deletion mutants with anti-Ro52 antibody after incubation with HeLa S100 extract. Numbers on top of the lanes refer to the deletions as indicated in panel C. In the lanes marked i total input [^{35}S]-labelled protein was loaded, in the lanes marked + the immunoprecipitated proteins are shown. Molecular weight markers are indicated on the left. **Panel C:** Schematic representation of Ro60 deletion mutants. Structural features of Ro60 such as RNP-80 motif (RNA binding domain or RNA recognition motif, dashed area) and Zn (putative Zn-finger, filled bar) are indicated. Numbers on top of the bars refer to the numbers of the first or last amino acids. Results, as shown in panel B for the binding of Ro52 to Ro60, are indicated on the right. The putative Ro52 binding domain is indicated below, ranging from amino acid 276 to amino acid 318.

truncated cDNAs and subsequent translation of the resulting mRNAs in the presence of [^{35}S]-methionine, similar types of experiments were performed as described above. Results obtained by this method are shown in figure 5B and summarized in figure 5C. Most N- and C-terminal Ro60 deletion mutants bind the Ro52 protein with a similar efficiency as wild type Ro60. However, Ro60ΔC292 and Ro60ΔC134 deletion mutants

are not capable of binding to Ro52. This suggests that a domain in Ro60, spanning from amino acid 276 to amino acid 318, is required for the stable association of Ro60 with Ro52. This domain contains two cysteine residues of a putative zinc finger, suggesting a possible role for these cysteines in the protein-protein interaction between Ro52 and Ro60. In another series of experiments the RNA binding ability of the Ro60 deletion mutants was examined (summarized in Figure 5C). Although wild type Ro60 was able to bind to hY RNA, deletions at the N- and C-terminus of Ro60 completely prevented binding to hY RNA (results not shown; Pruijn et al., manuscript in preparation). The fact that some of these non-RNA binding Ro60 deletion mutants are still able to bind to Ro52 is another indication for the RNA independency of the protein-protein interaction between both Ro proteins.

Discussion

Several studies have shown that *in vitro* reconstitution of RNA and proteins into RNPs is a reliable and informative way to analyze the RNA-protein and protein-protein interactions within ribonucleoprotein complexes (Hamm et al., 1987; Patton et al., 1987; Kleinschmidt et al., 1989).

This report describes the use of an *in vitro* system based on the incubation of [^{32}P]-labelled hY1 RNA in HeLa S100 extract, that allows the specific reconstitution of Ro RNP complexes. Our first analyses of RNA-protein interactions within the reconstituted particle (Pruijn et al., manuscript in preparation) confirm earlier data of Wolin and Steitz (1984) concerning the binding region of Ro60 on the hY RNAs and observations about the binding region of La on RNA polymerase III transcripts (Stefano, 1984). In this paper we examined the association of Ro52 with Ro RNPs in more detail and were able to identify a direct interaction between the Ro60 and Ro52 antigens.

Immunodepletion of HeLa S100 extracts either for Ro60, Ro52 or La followed by suppletion with recombinant Ro60 or recombinant La demonstrated that Ro60 and La associate with Ro RNP complexes without being influenced by the presence or absence of one of the other known protein constituents. This proves the independent binding of Ro60 and La to hY1 RNA as has been suggested recently (Boire and Craft, 1990). Although Ro60 and La seem to bind to hY1 RNA directly, confirming previous reports (Wolin and Steitz, 1984; Deutscher et al., 1988; Moreau et al., 1990), Ro52 was only able to associate with hY1 RNA when Ro60 was present in the extract. This suggests that either Ro52 binds to Ro60 or that binding of Ro60 induces a conformational change in hY1 RNA enabling Ro52 to assemble with the RNP complex. Our results indicate that the first assumption is true.

To further define the interaction between Ro60 and Ro52, [^{35}S]-labelled Ro60 was incubated in HeLa S100 extract followed by immunoprecipitation with monospecific anti-Ro52 antibody. By this method Ro52 bound [^{35}S]-Ro60 can be precipitated specifically and analyzed. The protein-protein interaction between Ro60 and Ro52 proved to be RNA independent, since reconstitution of Ro60-Ro52 complexes in hY RNA free HeLa S100 extract was equally efficient. Furthermore, Ro60 deletion mutants, not able to

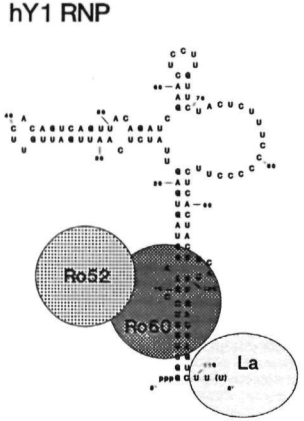

Fig.6. Model for the structure of the hY1 RNP.
The sequence of hY1 RNA is identical to the sequence published by Wolin and Steitz (1983). Secondary structure of hY1 RNA is shown as predicted using parameters of Turner et al. (1988) based upon free enrgy minimizations. A similar though somewhat different structure has been published by Wolin and Steitz (1983). The binding regions of the Ro60 and La polypeptides have been suggested by Wolin and Steitz (1984) and Stefano (1984), respectively, and were confirmed in our laboratory (Pruijn et al., submitted).

bind hY1 RNA (summarized in Figure 5c, data from Pruijn et al., submitted) are still able to bind to Ro52, corroborating the RNA-independency of the Ro60-Ro52 interaction.

By performing reconstitution studies with several N- and C-terminal Ro60 deletion mutants a domain in Ro60, responsible for binding to Ro52, was identified. In this domain, ranging from amino acid 276 to amino acid 318, two cysteine residues are located which are part of a putative zinc finger motif. Since Ro52 has three putative zinc fingers located in the N-terminal part of the protein (Chan et al., 1991) and zinc fingers possibly involved in protein-protein interactions have been described (see for example Nelissen et al, 1991), it will be interesting to know whether the zinc finger motifs of Ro52 indeed are involved in the interaction with Ro60.

Based upon the results described in this paper, it is possible to draw a model of the hY1 RNP complex (Figure 6). In this model Ro60 binds to the lower part of the stem structure of hY1 RNA (as uggested by Wolin and Steitz (1984) and confirmed by Prtuijn et al., submitted), the Ro52 protein binds to the Ro60 protein and is probably not in contact with the RNA. The La antigen binds to the 3' uridine residues (Stefano, 1984) and this association is not influenced by the binding of the Ro proteins.

Although in this study only hY1 RNP complexes were reconstituted, there is no reason to assume that the interactions in other hY RNPs concerning the Ro and La antigens will be very different. Immunoprecipitation of *in vivo* labelled Ro RNP particles with either monospecific anti-Ro60 or anti-Ro52 antibody yield identical RNA patterns (Slobbe et al, 1991b), indicating that both antigens are contained in the same subset of hY RNPs. However, it is possible that Ro60, Ro52 and La are the common or "core" Ro RNP proteins and that other yet unknown proteins may be part of subsets of Ro RNP complexes. For example, it has been reported that at least three different groups of Ro RNPs with different physicochemical properties and molecular weights of about 230 kDa (hY4) and 300-350 kDa (hY1-4, hY5) exist in human cells (Boire and Craft, 1990). The high molecular weights of these complexes may be caused by the existence of multimers of proteins or particles or by the binding of yet unidentified Ro RNP components. In addition, human sera have been described that precipitate the hY5 RNP particle only

(Boire and Craft, 1989) indicating the presence of a unique antigenic determinant on this subset of Ro complexes.

Further characterization of the complexity of Ro RNP complexes both *in vivo* and *in vitro* will help to elucidate the molecular composition of the Ro particles and may give clues to the function(s) of these ribonucleoprotein complexes.

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Chapter 7:
Survey and Summary

Survey and Summary

For some yet unknown reason patients with rheumatic diseases develop antibodies against self-components. These so-called autoantibodies frequently target proteins that are complexed with RNAs in ribonucleoprotein particles. It is obvious that such autoantibodies can be of great use in the characterization of the antigenic complexes and the determination of their cellular function.

In this thesis autoantibodies frequently found in sera from patients with systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS) were used to study the molecular composition and antigenicity of one subclass of RNA-protein particles, the Ro RNP complexes. These complexes consist of a small RNA molecule (in human cells four Ro RNAs have been identified, referred to as hY1, hY3, hY4 and hY5) complexed with at least three proteins, the La protein and two Ro proteins of 60kDa (Ro60) and 52 kDa (Ro52). No function has been found yet for these relatively abundant RNA-protein particles.

Since most patient sera contain multiple antibody specificities (anti-Ro and anti-La antibodies mostly are found together in patient sera) and good monoclonal antibodies directed against the Ro and La antigens are rare, a sensitive assay had to be developed to test patient sera for the presence of anti-Ro and anti-La antibodies in order to find monospecific sera that could be used in our studies. Therefore, in **Chapter 3** a method for the analysis of patient sera is described based upon the precipitation of [^{32}P]-labelled RNA from adenovirus infected HeLa cells. By this test anti-La antibodies can be demonstrated in very low titers, whereas anti-Ro antibodies can be detected simultaneously. It was shown that patient sera presumed to be monospecific for anti-Ro antibodies by other techniques frequently contain trace amounts of anti-La antibody. Furthermore, in this chapter it was shown that the synthesis of Ro RNAs proceeds at normal levels after adenovirus infection, whereas other cellular RNAs like the U RNAs are no longer transcribed. Since most cellular processes become arrested after adenovirus infection, this could mean that the Ro RNPs have an essential function for the propagation of the virus and have to be synthesized continuously in order to fulfil this function.

In **Chapter 4** the antigenicity of the different components in Ro RNP complexes was investigated. In order to detect Ro52 by immunoblotting the crosslinking level of the SDS-PAGE gelsystem was lowered to an acrylamide to bisacrylamide ratio of 19 to 1 to enable the separation of Ro52 from La. This allowed the simultaneous detection of anti-Ro60, anti-Ro52 and anti-La antibodies in a patient serum. By analysis of sera from patients with SLE and SS it was found that anti-Ro60 antibody without anti-Ro52 or La antibodies is indicative for SLE, whereas the presence of anti-Ro52 and anti-La antibodies without anti-Ro60 antibody is of diagnostic value for SS. In addition, the association of Ro52 in Ro RNP complexes was studied *in vivo*. It appeared that both Ro60 and Ro52 bind to hY1, hY3, hY4 and hY5 RNA and co-localize in the cytoplasm as well as in the nucleus as determined by double immunofluorescence techniques, indicating a close relationship between these two proteins. Furthermore, the evolutionary conservation of the Ro and La proteins was studied by immunoblotting. Although Ro60 and La seem to

be well conserved during evolution, Ro52 could be detected immunologically in primate cells only. At this moment, it is not clear whether the selective reactivity of anti-Ro52 antibodies with primate antigen reflects the alteration of the antigenic determinants of Ro52 during evolution or is due to the absence of a related protein in non-primates. In the **Appendix** of this chapter the evolutionary conservation of the Ro RNAs is studied by incubating Northern blots with antisense Y RNA probes. Although Y1 and Y3 RNAs seem to be generally present in all mamalian species studied, Y5 RNA seems to be absent in rodent and bovine cells. This may reflect possible different cellular functions of the various Ro RNP particles.

For the determination of the function of the Ro particles it is essential to understand the composition and complexity of the RNPs. To elucidate (part of) the interactions in Ro RNP particles a number of the components of these complexes were cloned, i.e. the cDNAs encoding Ro60 and La and the sequences coding for hY1, hY3 and hY5, as described in **Chapter 5**. By using various *in vitro* reconstitution techniques it was demonstrated that the conformation of Ro60 presumably is important for the interaction with Y RNA, since no N- or C-terminal deletions of the protein were allowed for RNA binding. For binding of La to Y RNA it was shown that the RNP-80 motif and sequences at the N-terminus of this motif were required. In addition, the regions on the RNAs that interact with the Ro and La antigens were analyzed. It was shown that the among Y RNAs highly conserved nucleotides at the lower part of the stem determine the interaction with both Ro proteins, indicating that Ro60 and Ro52 either bind to the RNA in the same region or that Ro52 needs Ro60 for binding, whereas the 3' end of the RNAs is involved in the binding of La.

In order to identify possible protein-protein interactions in Ro RNP complexes, in **Chapter 6** HeLa cell extracts were prepared from which one of the proteins involved in Ro RNP complexes had been removed by immunodepletion. Subsequent reconstitution of Ro RNP complexes revealed that the absence of the Ro proteins had no effect on the binding of La to hY1 RNA and vice versa. However, the absence of Ro60 completely prevented Ro52 from binding to hY1 RNA. Supplementation of recombinant Ro60 in Ro60 depleted extract restored both the binding of Ro60 and Ro52 to hY1 RNA, indicating that the presence of Ro60 is essential for the binding of Ro52 to Ro RNP complexes. In addition, a direct RNA-independent protein-protein interaction between Ro60 and Ro52 was identified. For this interaction a region of Ro60 between amino acids 276 and 318, which contains two cysteine residues of a putative zinc finger structure, is important.

Although in this thesis many questions remain unanswered it is evident that the elucidation of the molecular interactions in Ro RNP complexes *in vitro* may contribute to the understanding of both the structure and function(s) of the Ro particles *in vivo*. Furthermore, unraveling the complexity of Ro RNP particles may aid to a better understanding of the molecular basis of autoimmunity.

Samenvatting

Om nog onopgehelderde redenen maken patienten met rheumatische aandoeningen antilichamen tegen lichaamseigen componenten. Deze zogenaamde auto-antilichamen zijn vaak gericht tegen eiwitten die gebonden zijn aan RNA in eiwit-RNA (RNP) complexen. Het is duidelijk dat deze antilichamen van grote waarde kunnen zijn voor de karakterisatie van de antigenen en voor de bepaling van hun functie in de cel.

In dit proefschrift worden auto-antilichamen, vaak voorkomend in sera van patienten met systemische lupus erythematosus (SLE) en Sjögren's syndroom (SS), gebruikt ter bestudering van de samenstelling en antigeniciteit van een subklasse van eiwit-RNA partikels, de Ro RNP complexen. Deze complexen bestaan uit een klein RNA molekuul (in humane cellen zijn vier RNAs geïdentificeerd, genoemd hY1, hY3, hY4 en hY5) gebonden aan tenminste drie eiwitten: het La eiwit en twee Ro eiwitten van respektievelijk 60 kDa (Ro60) en 52 kDa (Ro52). Tot nu toe is van deze in de cel relatief veel voorkomende RNA-eiwit partikels nog geen functie bekend.

Aangezien de meeste patienten-sera antilichamen bevatten gericht tegen meerdere antigenen (anti-Ro en anti-La antilichamen komen meestal samen voor in patienten-sera) en goede monoklonale antilichamen gericht tegen de Ro of La antigenen zeldzaam zijn, is een gevoelige test ontwikkeld om patienten-sera te testen op de aanwezigheid van anti-Ro en anti-La antilichamen teneinde monospecifieke antisera te vinden die gebruikt kunnen worden in andere studies. In **Hoofdstuk 3** wordt een methode beschreven voor de analyse van patienten-sera gebaseerd op de immunoprecipitatie van [^{32}P]-gelabeld RNA uit adenovirus-geïnfekteerde HeLa-cellen. Door deze test kunnen zeer lage titers anti-La antilichamen maar ook anti-Ro antilichamen gedetecteerd worden. In dit hoofdstuk wordt aangetoond dat patienten-sera waarvan op grond van andere technieken aangenomen werd dat deze monospecifiek waren voor anti-Ro antilichamen toch vaak zeer kleine hoeveelheden anti-La antilichamen bevatten. Verder wordt in dit hoofdstuk beschreven dat de Ro RNAs na adenovirus-infektie nog steeds worden aangemaakt, terwijl andere RNAs zoals bijvoorbeeld de U RNAs niet langer getranscribeerd worden. Aangezien een groot aantal cellulaire processen worden stilgelegd na adenovirus-infektie betekent dit dat de Ro RNPs waarschijnlijk ook een belangrijke funktie hebben bij de aanmaak van nieuwe virus-partikels en continu gesynthetiseerd dienen te worden om deze funktie uit te kunnen voeren.

In **Hoofdstuk 4** is de antigeniciteit van de verschillende componenten van Ro RNP complexen onderzocht. Om Ro52 te detecteren met behulp van immunoblotting en het te kunnen scheiden van het La antigen is het niveau van cross-linking in het SDS-PAGE gelsystem verlaagd naar een acrylamide: bisacrylamide verhouding van 19: 1. Dit maakte een gelijktijdige detectie van anti-Ro60, anti-Ro52 en anti-La antilichamen in patienten-sera mogelijk. Na analyse van een aantal sera van SLE en SS patienten bleek

dat de aanwezigheid van anti-Ro60 zonder anti-Ro52 of anti-La antilichamen in een patienten-serum een sterke aanwijzing is voor SLE, terwijl aan de aanwezigheid van anti-Ro52 en anti-La zonder anti-Ro60 antilichamen de diagnose SS gekoppeld kon worden. Bovendien is de associatie van Ro52 met Ro RNP complexen *in vivo* bekeken. Het bleek dat Ro60 en Ro52 beide binden aan hY1, hY3, hY4 en hY5 RNAs en co-lokaliseren in cytoplasma en kern wanneer dat bepaald werd met behulp van de dubbele immunofluorescentie techniek, wat duidt op een nauwe verwantschap tussen beide eiwitten. Verder is de evolutionaire conservering van de Ro en La eiwitten bestudeerd met behulp van immunoblotting. Alhoewel Ro60 en La goed geconserveerd lijken te zijn in de evolutie, kon Ro52 allen maar aangetoond worden in cellen van primaten. Het is nu nog niet duidelijk of de selektieve reaktie van anti-Ro52 antilichamen met primaat antigenen wordt veroorzaakt door de evolutie van de antigene determinanten van Ro52 of door de afwezigheid van een verwant eiwit in niet-primaten. In de **Appendix** van dit hoofdstuk is de evolutionaire conservering van de Ro RNAs bestudeerd door het incuberen van Northern blots met anti-sense Y RNAs. Alhoewel Y1 en Y3 RNAs in alle zoogdieren aantoonbaar waren, lijkt het Y5 RNA afwezig te zijn in knaagdier en in runder cellen. Het lijkt dan ook mogelijk dat de verschillende Ro RNP partikels een verschillende cellulaire functie hebben.

Voor de bepaling van de funktie van de Ro RNP partikels is het van groot belang om de complexiteit en samenstelling van de RNPs te ontrafelen. Om (een gedeelte van) de interakties in Ro RNP partikels op te helderen zijn een aantal componenten van de complexen gekloneerd, namelijk de cDNAs coderend voor Ro60 en La en de coderende sequenties van de hY1, hY3 en hY5 genen, zoals beschreven in **Hoofdstuk 5**. Door gebruik te maken van diverse *in vitro* reconstitutie technieken kon aangetoond worden dat de conformatie van Ro60 waarschijnlijk belangrijk is voor de interaktie met Y RNA, aangezien kleine N- of C-terminale deleties de binding van deze Ro60 deletie mutanten aan het RNA verhinderden. Voor de binding van La aan Y RNA bleken het RNP-80 motief en sequenties N-terminaal hiervan essentieel te zijn. Bovendien zijn in dit hoofdstuk de regio's op het RNA gekarakteriseerd die een interaktie aangaan met de Ro en La antigenen. De bij Y RNAs sterk geconserveerde nucleotiden onder aan de stamstruktuur bleken belangrijk voor de interaktie met beide Ro eiwitten. Dit duidt erop dat ofwel Ro60 en Ro52 beide binden aan het RNA op dezelfde plaats ofwel dat Ro52 Ro60 nodig heeft voor binding. Het 3' einde van de RNAs bleek betrokken te zijn bij de binding van La.

Om mogelijke eiwit-eiwit interakties in Ro RNP complexen aan te tonen zijn in **Hoofdstuk 6** HeLa extracten gemaakt waaruit één van de Ro RNP eiwitten is verwijderd door immunodepletie. Reconstitutie van Ro RNP complexen in zulke extracten toonde aan dat de afwezigheid van Ro eiwitten geen effect heeft op de binding van La aan hY1 RNA en vice versa. Echter, de afwezigheid van Ro60 verhinderde de binding van Ro52 aan Y RNA. Aanvulling van Ro60-gedepleteerd extract met recombinant Ro60 herstelde zowel

de binding van Ro60 als van Ro52 aan hY1 RNA, wat een aanwijzing is voor het feit dat de aanwezigheid van Ro60 essentieel is voor de binding van Ro52 aan hY1 RNA. Bovendien is een direkte, RNA-onafhankelijke eiwit-eiwit interaktie aangetoond tussen Ro60 en Ro52. Een gebied van het Ro60-molecuul tussen aminozuur 276 en 318, welke twee cysteine residuen van een mogelijke zink finger bevat, blijkt belangrijk te zijn voor deze Ro60/Ro52 interactie.

Alhoewel in dit proefschrift veel vragen onbeantwoord blijven is het toch duidelijk dat de opheldering van de molekulaire interakties in Ro RNP complexen *in vitro* kan helpen bij het begrijpen van zowel de struktuur als de funktie(s) van Ro partikels *in vivo*. Bovendien kan de opheldering van de complexiteit van Ro RNP partikels bijdragen tot een beter begrip van de molekulaire basis van autoimmuniteit.

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Curriculum vitae

Rob Slobbe werd op 21 mei 1964 geboren in 's-Hertogenbosch. In 1982 werd, na het behalen van het gymnasium-ß diploma aan het Jeroen Bosch College te 's-Hertogenbosch, begonnen met de studie Scheikunde aan de Katholieke Universiteit Nijmegen. In juli 1983 werd het propedeutisch examen Scheikunde met goed gevolg afgelegd. De doctoraalstudie omvatte het Hoofdvak Biochemie (Drs. F. Pieper; Prof. Dr. H. Bloemendal) en het Bijvak Chemische Microbiologie (Dr. W. v/d Wijngaarden; Prof. Dr. Ir. Vogels). Tijdens zijn studie verleende hij twee maal assistentie bij het praktisch biochemie onderwijs voor 2e en 3e jaars scheikunde studenten en is het praktisch onderwijs microbiologie voor 2e jaars biologie studenten begeleid. Het doctoraal diploma Scheikunde werd in maart 1987 behaald.

Van 1 april 1987 tot 1 april 1991 is hij als OIO met financiele steun van N.W.O. (werkgemeenschap Scheikundig Onderzoek Nederland) werkzaam geweest op het Laboratorium voor Biochemie, waar het in dit proefschrift beschreven onderzoek is uitgevoerd in de werkgroep van Dr. W.J. van Venrooij. Tijdens de promotieperiode is het diploma C-deskundigheid stralingshygiene behaald, is een bijdrage geleverd aan het praktisch biochemie onderwijs aan 2e jaars chemie studenten en zijn een viertal hoofd- en bijvak studenten Scheikunde en Biologie (mede) begeleid. Naast de jaarlijkse SON-vergaderingen in Lunteren zijn de congressen "The structure and function of eukaryotic RNP" in Heidelberg (oktober 1987) en in Delphi (april 1990) bezocht.

Sinds 1 augustus 1991 is hij werkzaam op de afdeling Moleculaire Celbiologie van de Rijksuniversiteit Limburg.

Op 30 november 1990 is hij getrouwd met Marlea van Drunen.