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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 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 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. It is likely that they stabilize the correct conformation of the RNP 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 (1–4). 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 (1, 5, 6). However, in human red blood cells immunoreactive Ro proteins of 60 kDa (distinct from Ro60) and 54 kDa have been detected (7). 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 (8, 9). 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 strech at the 3' terminus of the newly transcribed RNA (10–13). 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 (14, 15).

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 (2, 18, 19). 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 (5).

Antibodies against Ro (both Ro60 and Ro52) and La, which are often present in sera from autoimmune patients suffering from Sjögren's syndrome or systemic lupus erythematosus (SLE), have been very helpful for the identification and characterization of Ro and La RNPs (16). 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 (9, 17).

cDNAs encoding Ro60, Ro52 and the La protein have been isolated and analysed. 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 (20, 21). An RNP motif encoding region was also found in La (22, 23) while two putative zinc fingers are present in Ro52 (24, 25).

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 (15). Indirect evidence indicates that one Ro60 polypeptide binds to one Y RNA (5). The results of Ouchterlony analyses, immunoprecipitations and immunofluorescence studies suggest that all three proteins, Ro60, Ro52 and La, can be associated simultaneously with a single Y RNA (6, 9, 14). 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 (15). It therefore is most likely that other, as yet unidentified Ro proteins exist.

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. 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. Our data show 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.

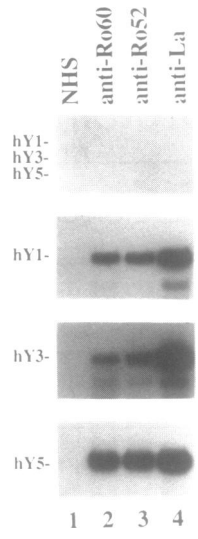

Figure 1. Association of HeLa S100 proteins with hY RNAs produced *in vitro*. Radioactively labelled hY1, hY3 or hY5 RNA, transcribed by T7 RNA polymerase using HindIII-digested pHY1, pHY3 or pHY5 as template, was incubated in HeLa S100 extract. The association of Ro60, Ro52 and La polypeptides with these RNAs was then analysed by immunoprecipitation with normal human control serum (lanes 1), monospecific anti-Ro60 antibody (lanes 2), monospecific anti-Ro52 antibody (lanes 3) and monospecific anti-La antibody (lanes 4). Precipitated RNA was visualized by denaturing polyacrylamide gel electrophoresis followed by autoradiography. The upper panel illustrates the inability of these antisera to precipitate the RNAs (which were added as a mixture) in the absence of HeLa S100 proteins.

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′ and 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′-CTAAGC-TTAAAACAGCAAGCTAGTCAA-3′) followed by PCR in the presence of two hY5 specific primers: 5′-CTGAATTCA-GTTGGTCCGAGTGTTGTGG-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.* (26).

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 (20). From the 11 cDNA clones that were isolated only one 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 (20) and La (22) cDNAs except for the termini (see Fig. 3).

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.* (27). Biotinylated and [^{32}P]-labelled 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 were produced 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 (27). Translation of this mutant results in a truncated La protein where the N-terminal 111 amino acids are replaced by Met-Gly.

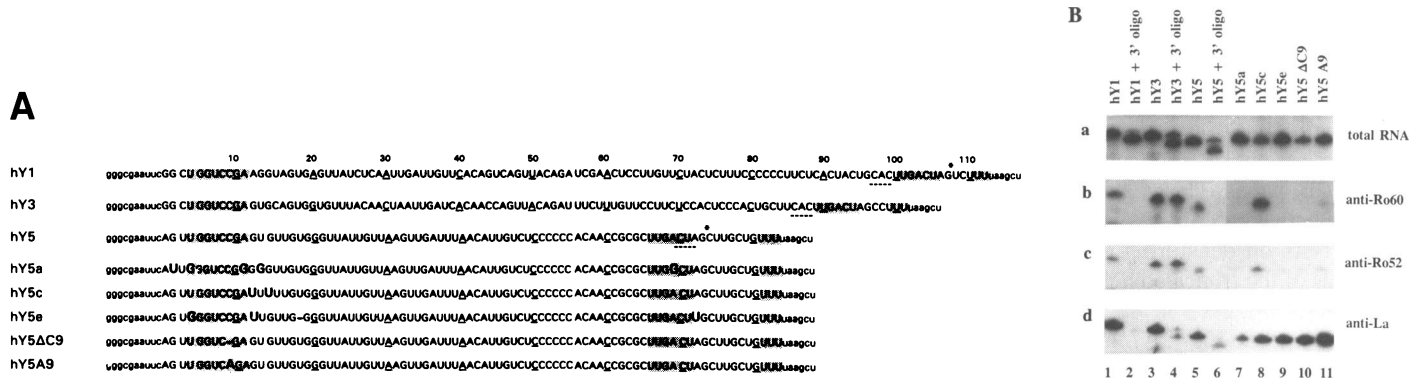

Figure 2. Interaction of Ro60, Ro52 and La from HeLa S100 with mutated hY RNAs. **(A)** The sequences of the transcripts used in the RNA-binding assays are listed. Nucleotides corresponding to wild type hY RNAs are in upper case, nucleotides derived from vector/linker sequences in lower case. Numbering is according to wild type hY RNAs (every tenth nucleotide is underscored). The most highly conserved regions are shaded. 3′-ends of oligonucleotide/RNaseH truncated RNAs are indicated by dashed underlining, 3′-ends of hY1*SpeI and hY5*AluI by an asterisk. Mutated nucleotides in the hY5 point mutants are indicated by larger capitals or by dashes (single nucleotide deletions). **(B)** Analysis of the association of Ro60, Ro52 and La with hY RNA mutants. The analysis was performed as described in the legend to Fig. 1. Panel a shows the RNAs after incubation in HeLa S100. Lanes 1, 3 and 5 contain wild type hY1, hY3 and hY5 RNA, respectively. The RNAs in lanes 2, 4 and 6 were hybridized to oligonucleotides complementary to the 3′-ends of hY1, hY3 and hY5, respectively, before incubation in S100 extract and thus are (partially) truncated. Lanes 7–11 contain hY5a, hY5c, hY5e, hY5ΔC9 and hY5A9, respectively. The other panels show immunoprecipitation of the reconstituted RNPs by monospecific anti-Ro60 (panel b), anti-Ro52 (panel c) and anti-La (panel d) antibody.

In vitro RNA-S100 protein binding assay

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

In vitro 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.* (27). After incubation of the ^{35}S-labelled recombinant proteins with biotinylated RNA, RNA-protein complexes were precipitated with streptavidin-agarose. Precipitated proteins were analysed by SDS-polyacrylamide gel electrophoresis.

RESULTS

Interaction of HeLa S100 proteins with hY RNAs

Individual hY RNAs were produced *in vitro* as described in the Materials and Methods section. As a result of the 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, however, showed that predicted wild type structures (2, 4) 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; results not shown, see also Fig. 2A).

The individual hY RNA transcripts (^{32}P-labelled) were incubated in HeLa S100 extract. The association of proteins with these RNAs was then analysed by immunoprecipitation with either anti-Ro60, anti-Ro52 or anti-La specific antibodies. The monospecificity of these antibodies has been established before (9). 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. Anti-Ro60 and anti-Ro52 precipitated 5–10% of input RNA, anti-La precipitated about 20% of input RNA. 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 or 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.

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Ro60 cDNA  1.    1 gaattcgggCGGCAGTGGGGCTGTTGCTGTTGCTGTGGCTGTCGCTGCCC   50

           1.   51 GTCAGGCTGCCTTCTTTTGTCGTTTCCCAGCGCTGCGCAGGACTTCTCCT  100

           1.  101 GGCGGCGCTGCGGATCCAGGGGGTCGGCTGCCAGGTACAGGTTTCCTAAA  150
           2.                          1  ATTTTGCCTTTTTGTT----------   28

           1.  151 GACAAAAAAAA ATG GAG GAA TCT GTA  .  .  .  .  .
           2.   29 ----------- --- --- --- --- ---  .  .  .  .  .

           1.          .  .  TTA GAT ATG ATT TAA CCATAAGCAGCAGCACGATC 1798
           2.          .  .  --- --- --- --- --- --------------------- 1676

           1. 1799 CAGAGATCCATTGCCATCAGTGATCTCACTAAAAA TATACAGCTACTTC 1847
           2. 1677 ----------------------------------A--------------- 1726

           1. 1848 CCAGCTAATCTCCACCCAATGAATGATGATGGTAcccgaattc 1890
           2. 1727 ---------------------------------TAGTATGTGCATAATG 1776

           2. 1777 GAAAGTTACCTTACTGAAAAAAAAAAAAAAAAAAAAAAAAAA 1817


La cDNA    3.    1 gaattccGCGCTTTAGGCTGGCCGGCGGCGCTGGGAG......ggaattc 1664
           4.                          1 -----------------..... 1631
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Figure 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 (2.) (Ref. 20). Similarly, the sequence of our La cDNA clone (3.) is compared with that of Chambers *et al.* (22) (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.

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, lanes 2, 4 and 6). 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. 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, hereafter indicated by hY1*SpeI; AluI for hY5 leading to a 3′-end at position 73, hereafter indicated by hY5*AluI (results not shown). 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 previously by the RNAse protection experiments of Wolin and Steitz (5). 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 bind

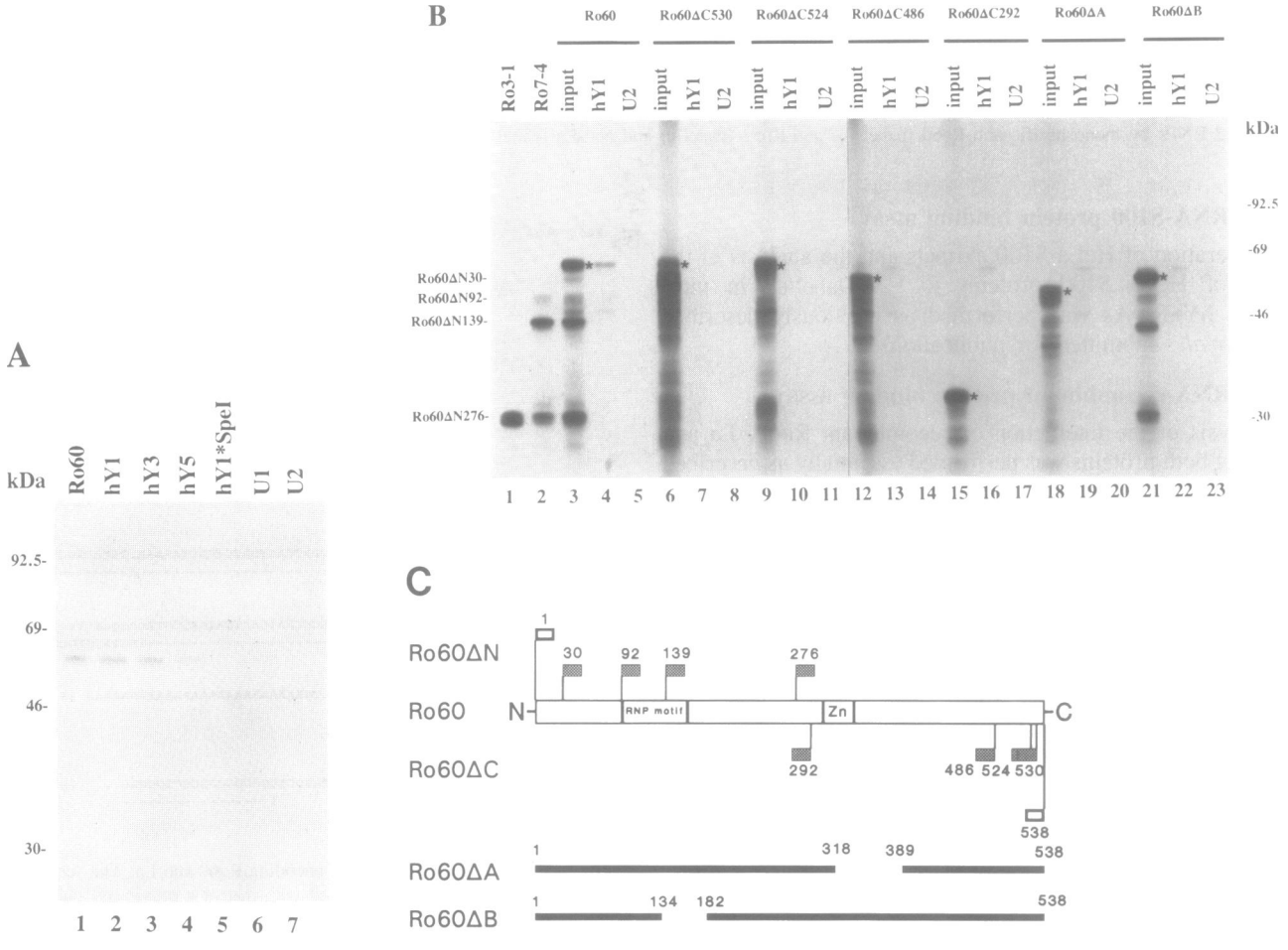

Figure 4. Analysis of the interaction of recombinant Ro60 with hY RNA. Recombinant Ro60 was translated in wheat germ extract in the presence of [^{35}S]-Methionine, incubated with biotinylated RNA and precipitated with streptavidin-agarose. Precipitated proteins were analysed by SDS-PAGE followed by autoradiography. **(A)** Wild type Ro60 (lane 1 contains 25% of the input) was incubated with biotinylated hY1 RNA (lane 2), hY3 RNA (lane 3), hY5 RNA (lane 4), hY1*SpeI RNA (lane 5), U1 RNA (lane 6) and U2 RNA (lane 7). **(B)** RNA-binding of Ro60 deletion mutants. In lanes 1, 2 and 3 translates obtained by translation using mRNAs derived from the truncated Ro60 cDNA clones Ro3-1 and Ro7-4 and from the complete Ro60 cDNA clone, respectively, are shown. Ro3-1 and Ro7-4 translation products mark the position of N-terminal deletion mutants (indicated on the left), produced by translation initiation at internal AUG codons of the wild type mRNA. The binding of Ro60 and of a number of Ro60 deletion mutants to hY1 and U2 RNA was analysed. Each set of three lanes contain 25% of input protein, protein bound by hY1 RNA and protein bound by U2 RNA from left to right. 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 46 kDa co-occurring with RNA-bound Ro60 is due to Ro60 degradation after the RNA binding assay. **(C)** Structure of Ro60 deletion mutants and summary of RNA-binding analyses. The Ro60 protein (538 amino acids) is drawn schematically. The positions of the RNP motif and the putative zinc finger are indicated. The first and last amino acid of N- and C-terminal deletion mutants, respectively, are indicated by flags and 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.

to the presumed binding site, that is the 3'-oligouridine stretch, only but is also able to interact with other parts of hY3 and hY5 (Figure 2B, panel d, lanes 1–6). For hY1 and hY5 RNA these results were confirmed using hY1*SpeI and hY5*AluI (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, but not hY5c 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, lanes 7–11.

The interaction of Ro60, Ro52 and La with the hY5 RNA point-mutants was analysed using the immunoprecipitation assay described above. The results, shown in Figure 2B, indicate that all mutations in the presumed Ro-protein binding site 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 an approximately 5-fold lower efficiency. These results confirm that the highly conserved stem structure of hY RNAs including the bulged C-residue is an important determinant for Ro-protein binding. Since neither of the hY5 point-mutants contained nucleotide changes within the presumed La binding site, La interacted efficiently with all of these mutants (Fig. 2B, panel d, lanes 7–11).

Interaction of recombinant Ro60 and La with hY RNAs

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.* (20) for Ro60 and Chambers *et al.* (22) 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

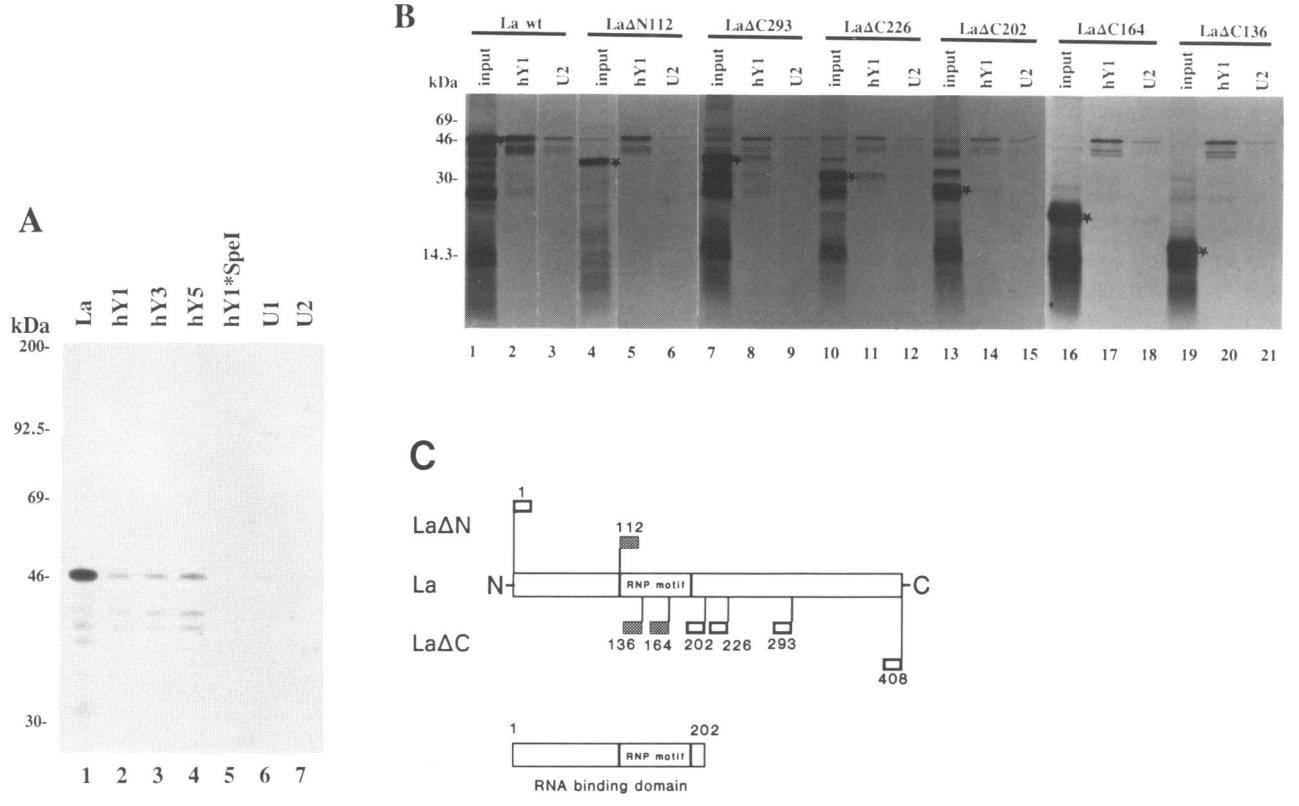

Figure 5. Analysis of the interaction of recombinant La with hY RNA. Recombinant La was translated in wheat germ extract in the presence of [^{35}S]-Methionine, incubated with biotinylated RNA and precipitated with streptavidin-agarose. Precipitated proteins were analysed by SDS-PAGE followed by autoradiography. **(A)** Wild type La (lane 1 contains 25% of the input) was incubated with biotinylated hY1 RNA (lane 2), hY3 RNA (lane 3), hY5 RNA (lane 4), hY1*SpeI RNA (lane 5), U1 RNA (lane 6) and U2 RNA (lane 7). Polypeptides migrating faster than La represent degradation products of La. **(B)** RNA-binding of La deletion mutants. The binding of La and of a number of La deletion mutants to hY1 and U2 RNA was analysed. Each set of three lanes contains 10% of input protein, protein bound by hY1 RNA and protein bound by U2 RNA. The positions of N- and C-terminal deletion mutants are marked by asterisks. Wild type La was added as an internal positive control to the deletion mutant analyses. **(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 motif is indicated. The first and last amino acid of N- and C-terminal deletion mutants, respectively, are indicated by flags and 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.

Ro60 cDNA clone is distinct from the Ro60 cDNAs published before (20, 21): 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 a wheat germ extract in the presence of ^{35}S-Methionine resulted in radiolabelled proteins of the expected molecular weights (Ro60: 60 kDa; La: 47 kDa). Both the size of the obtained proteins and immunoprecipitation experiments (not shown) confirm that our cDNAs encode genuine Ro60 and La, respectively.

To study the interaction of recombinant proteins with the hY RNAs we employed the assay described by Scherly *et al.* (27). In short, biotinylated RNA is incubated with radiolabelled protein translated *in vitro* and protein-RNA complexes are precipitated with streptavidin-agarose. Finally, precipitated protein is analysed by SDS-PAGE. The results in Figure 4A show that Ro60 specifically interacts with hY1, hY3 and hY5 RNA but 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. Also partially purified recombinant Ro60 produced in a bacterial expression system was found to associate specifically with hY RNAs.

We next wanted to analyse which parts of Ro60 are involved in RNA binding. As has been documented by Deutscher *et al.* (20) and Ben-Chetrit *et al.* (21) at the primary sequence level two (potentially nucleic acid binding) motifs can be discerned. One of these, the RNP motif (28), 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 (28, 29). 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 (30).

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 could be 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 show that all deletions eliminated the capacity of Ro60 to bind specifically to hY1 RNA (Figure 4B). 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. Similar RNA binding data were obtained with partially purified recombinant La expressed in bacteria.

hY1 and U2 RNA, as a control, 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; 22, 23). Several La deletion mutants were produced *in vitro* and the effects of these deletions on RNA-binding of La were analysed (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. The result of the N-terminal deletion mutant LaΔN112 further indicates that the presence of the RNP motif alone is not sufficient for RNA binding of La (see also Moreau *et al.* (31) and Kenan *et al.* (32)). We conclude that the minimal RNA-binding region of La consists of the RNP motif and an adjacent part of the N-terminus of the protein (Figure 5C).

DISCUSSION

RNA-protein interactions occurring within Ro RNP particles have been analysed. 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 analyse the association of individual proteins with RNA and mutants thereof. 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

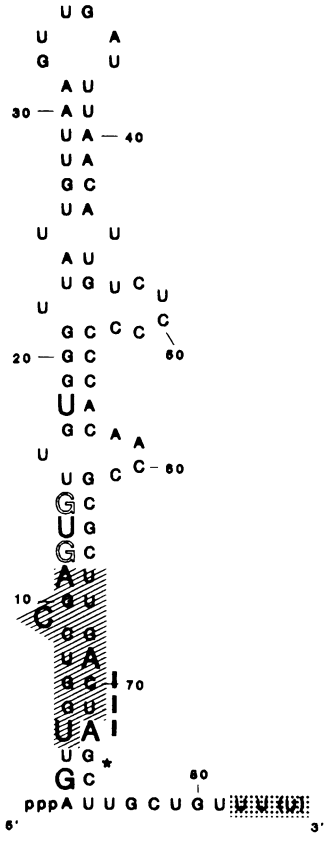

Figure 6. Summary of Ro60 binding to hY5 mutants. In the predicted secondary structure of hY5 the most highly conserved regions are shaded. The 3′-end of hY5*AluI and of the oligonucleotide/RNaseH truncated mutants are marked by an asterisk and by a dashed line, respectively. Mutated nucleotides in the hY5 mutants (see Fig. 2A) are drawn as large capitals: bold nucleotides were mutated in non-Ro60-binding RNAs; open nucleotides were mutated in mutants still binding Ro60. The residues at position 2 (G), 13 (U), 18 (U) and 72 (A) were mutated in mutants containing also mutations within the conserved region thought to be responsible for Ro60-binding.

interaction of these proteins and mutants thereof with biotinylated hY RNAs was analysed by precipitation with streptavidin-linked agarose beads (27). It should be pointed out that most hY RNA molecules used for these analyses contained some additional nucleotides at their extremities, which, however do not influence the predicted secondary structures nor eliminate Ro protein binding.

The results of both approaches strongly suggest that Ro60 and La directly bind to *in vitro* transcribed hY RNAs. The possibility that a protein present in the wheat germ extract, which is used for expression of the recombinant proteins, mediated the interaction of Ro 60 and La with hY RNAs is unlikely because of the finding that both proteins obtained via three different systems, a HeLa cell extract, recombinant proteins expressed in a wheat germ extract as well as partially purified recombinant proteins expressed in bacteria (unpublished observations), all specifically interact with hY RNAs.

The RNA binding site for Ro60 and La

Previous RNase protection experiments (5) 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.* (20), 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. The importance of bulge structures for the interactions of proteins with RNA has been found in other systems as well, e.g. the bacteriophage R17 coat protein (33) and the HIV-1 Tat protein (34). 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 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 (Slobbe *et al.*, submitted). 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 (1, 2, 9). The most plausible explanation for this discrepancy is that hY1 might be cleaved to hY2 in cell(extract)s after its association with Ro60 and that the cleaved RNA remains to be bound by the protein.

Previous reports have shown that the La protein binds to a short stretch of uridylate residues at the 3′ terminus of La-associated RNAs (10, 11). However, our data with *in vitro* transcribed RNAs containing an oligouridylate stretch near the 3′ terminus show that this sequence motif should not necessarily be located at the extreme 3′-end of the RNA molecule for La to bind. 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 are present in hY3 and hY5, but also in hY1) or whether completely divergent sequences are bound. Interestingly, Wolin and Steitz (2) have previously shown that a 3′-truncated version of hY3, designated hY3**, is still included in anti-La precipitates. Moreover, La does also interact with U1 RNA both *in vitro* (Fig 5A) and *in vivo* (35).

The predicted secondary structure of hY5 RNA and a summary of our data concerning the Ro60 and La binding sites of hY RNA are shown in Figure 6.

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 (28, 36, 37). In several cases, e.g. poly(A) binding protein (38), U1−70K (37), U1-A (27) and U2-B″ (28), this RNP motif with only a few flanking amino acids has been shown to be sufficient for specific RNA binding. We have shown that for hY RNA binding much larger parts of the RNP motif flanking sequences of Ro60 as well as La 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, although not having a direct role in the interaction with RNA, might be required for the RNP motif to fold properly.

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 motif 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 (10, 11, 27, 28, 37, 38).

Several reports have provided evidence for the heterogeneity of Ro RNPs (for review see ref. 4). 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 (19) may be predicted based upon sequence homology), can not or only partially explain differences in protein composition of the individual Ro RNPs. Interestingly, a third conserved sequence motif has been identified within the hY RNAs (4). 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. The identification of an hY5 RNP specific epitope (39) 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 will prove to 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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