The EMBO Journal vol.9 no.11 pp.3683-3689, 1990

A subset of hY RNAs is associated with erythrocyte Ro ribonucleoproteins

Charles A.O'Brien and John B.Harley

Arthritis and Immunology Program, Oklahoma Medical Research Foundation, Departments of Microbiology and Immunology, and Medicine, University of Oklahoma Health Sciences Center and Veterans Administration Medical Center, Oklahoma City, OK 73104, USA

Communicated by J.A.Steitz

The Ro autoantigen is a mammalian cellular ribonucleoprotein (RNP) of unknown function. We have demonstrated that hY1 and hY4 Ro RNAs are associated with erythrocyte Ro RNPs and represent a subset of the four hY RNAs found in HeLa cell and leukocyte Ro RNPs. We have cloned and sequenced hY4 RNA, the only hY RNA not sequenced previously, from a polymerase chain reaction amplified erythrocyte hY cDNA library. Sequencing of the erythrocyte hY RNAs in conjunction with Northern blot analysis confirms that the erythrocyte hY RNAs contain the same sequences as the respective HeLa cell RNAs of similar mobility. Ribonuclease inhibition activity has been found in erythrocytes and this activity inhibits the degradation of hY3 and hY5 in leukocyte lysates thereby favoring the possibility that the presence of hY1 and hY4 in erythrocytes is the result of differential expression of the hY RNAs in erythrocyte precursors.

Key words: hY4 RNA/erythrocytes/Ro protein/RNase inhibition

Introduction

Analysis of cellular components has been greatly aided by the use of antibodies produced in autoimmune disorders such as systemic lupus erythematosus and Sjögren's syndrome. The Ro ribonucleoprotein (RNP) was originally defined as a common target of the autoimmune response in these diseases (Clark *et al.*, 1969). Affinity purification, immunoprecipitation and immunoblottting with anti-Ro antibodies have all contributed to the structural analysis of this RNP. In human cells the Ro RNP is composed of the 60 kd Ro protein (Yamagata *et al.*, 1984; Deutscher *et al.*, 1988) which is bound to one of a number of small cytoplasmic RNAs (scRNAs) (Lerner *et al.*, 1981). Recent work has demonstrated that additional proteins of 52 and 54 kd may be a component of some human Ro RNPs (Rader *et al.*, 1989; Ben-Chetrit *et al.*, 1988).

The RNAs associated with the particle, designated hY1, (h for human and Y for cytoplasmic) hY2, hY3, hY4 and hY5 (hY2 being a truncated version of hY1) range in size from 83 to 112 bases, are products of RNA polymerase III, possess 5' triphosphate termini and contain no modified bases (Hendrick *et al.*, 1981). Three of the hY RNAs, hY1 and hY3 (Wolin and Steitz, 1983) and hY5 (Kato *et al.*, 1982) have been sequenced previously. Although the sizes of some of the Y RNAs appear to be roughly constant between several mammalian species, the number immunoprecipitated with the Ro protein varies, with four occurring in human and bovine cells, three in rabbit and rat cells and two in murine cells (Mamula *et al.*, 1989; Reddy *et al.*, 1983). The variation in the number of Y RNAs associated with the Ro RNP in different mammalian species suggests substantial evolutionary divergence.

Present in $1-5 \times 10^5$ copies/cell, the Ro particle is a relatively low abundance RNP and, while no function has as yet been ascribed to the particle, it has been postulated to be involved in specific mRNA translation or protein transport events (Wolin and Steitz, 1984). Ro RNAs have also been reported to be immunoprecipitated by antibodies to the La RNP (Hendrick *et al.*, 1981) which was recently shown to be an RNA polymerase III transcription termination factor (Gottlieb and Steitz, 1989). As nascent RNA polymerase III transcripts the Ro RNAs are expected to bind the La antigen via their 3'-terminal uridines (Francoeur and Matthews, 1982; Stefano, 1984). In addition, reassembly experiments indicate that some Ro RNP particles also contain the 50 kd La protein (Hendrick *et al.*, 1981), although sera with anti-La autoantibody precipitins may always also have anti-Ro autoantibody precipitins (Reichlin, 1986). The cellular location of the particle is controversial with reports of both cytoplasmic and nuclear localization (Clark *et al.*, 1969; Gaither *et al.*, 1987).

In this report we demonstrate that only a subset of the four hY RNAs, hY1 and hY4, are associated with the Ro RNP in erythrocytes. The hY1 and hY4 RNAs recovered from erythrocytes are smaller than their HeLa cell counterparts. Furthermore, factors in erythrocyte lysates are capable of protecting all four hY RNAs from degradation. We have also found that the Ro RNP is more concentrated in reticulocyte enriched erythrocytes but is also present in reticulocyte depleted erythrocytes. The Ro RNAs present in these erythrocytes are not immunoprecipitated by anti-La sera. In addition, we have sequenced the hY4 RNA and compared its primary and predicted secondary structure with those of the other hY RNAs.

Results

Ro RNPs in erythrocytes contain a subset of hY RNAs

To compare the conservation of different RNPs in erythrocytes, several anti-RNP antibodies were used to immunoprecipitate RNPs from lysates of HeLa cells and erythrocytes. While HeLa cell Ro particles contain the usual complement of four hY RNAs (Figure 1, lane 3) only RNAs approximately comigrating with hY1 and hY4 were found in erythrocytes (Figure 1, lane 4). Both of the hY RNAs from erythrocytes consistently migrate slightly faster than their HeLa cell counterparts. Immunoprecipitation with anti-La, anti-Sm, anti-alanyl tRNA synthetase and anti-

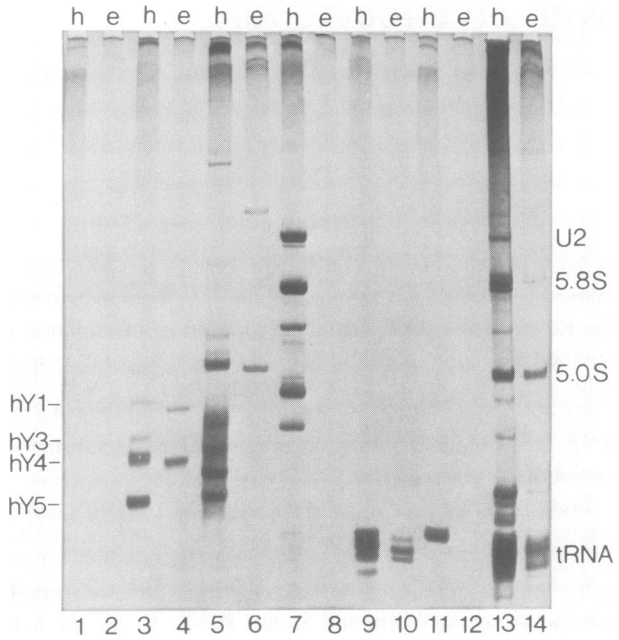

Fig. 1. Comparison of RNP RNAs immunoprecipitated from HeLa cells and erythrocytes. Antibodies with different autoimmune specificities were bound to Protein A−Sepharose and incubated with either HeLa cell or erythrocyte lysate. Approximately 100-fold more cells were used for the erythrocyte lysate than the HeLa cell lysate. After extraction and precipitation, the RNAs from the Hela cell and erythrocyte immunoprecipitates were fractionated on a denaturing polyacrylamide gel and silver stained. The RNAs from each cell type for a given RNP are run side by side with the HeLa cell (h) first followed by the erythrocyte (e). Lanes 1 and 2 are normal human serum controls for the two lysates. Lanes 3 and 4 compare Ro hY RNAs from HeLa cells and erythrocytes respectively. La/SSB RNAs are compared in lanes 5 and 6, Sm U RNAs in lanes 7 and 8, alanyl tRNAs in lanes 9 and 10, and threonyl tRNAs in lanes 11 and 12. Lanes 13 and 14 compare the total nucleic acid contained in the same amount of lysate from Hela cells and erythrocytes.

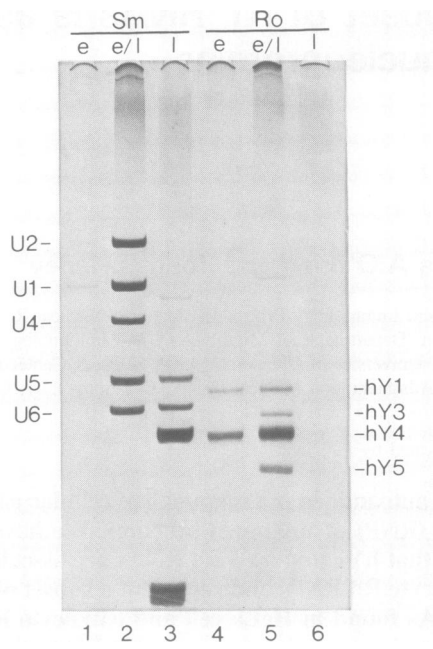

Fig. 2. Comparison of Sm and Ro RNAs in erythrocytes, leukocytes and erythrocyte/leukocyte mixtures. Lanes 1, 2 and 3 show Sm RNAs immunoprecipitated from erythrocytes (e), erythrocytes and leukocytes mixed together (e/l) and leukocytes alone (1), respectively. Lanes 4, 5 and 6 show Ro RNAs immunoprecipitated from cells in the same order as lanes 1, 2 and 3. The same number of cells was used in each immunoprecipitation including that cell type. For example, lanes 2 and 3 contain the same number of leukocytes from the same preparation.

threonyl tRNA synthetase using erythocytes did not, in general, preserve the pattern of RNA species immunoprecipitated from HeLa cells (Figure 1, lanes 5−12). With anti-La sera a previously unappreciated RNA species was immunoprecipitated, however, which is slightly larger than U2 RNA (Figure 1, lane 6). No RNAs comigrating with the hY RNAs were seen in the anti-La immunoprecipitate from erythrocytes.

Comparison of erythrocyte and HeLa cell RNAs led to attempts to immunoprecipitate RNPs from peripheral blood leukocytes. Only a small amount of apparently degraded RNA or no RNA at all was immunoprecipitated by anti-Ro from purified preparations of peripheral leukocytes. However, if the leukocytes were not fully depleted of erythrocytes some intact RNA was immunoprecipitated. This led to testing the hypothesis that mixing erythrocytes and leukocytes before lysis of the cells may lead to protection of the RNA from ribonuclease digestion.

A comparison of the Ro and Sm RNAs found in erythrocytes, leukocytes and mixtures of the two is shown in Figure 2. Antibodies binding the Sm particle immunoprecipitate intact U RNAs only from the mixture of the two cell types (lane 2). As expected, the erythrocytes alone contain little or no U RNAs (lane 1) and in the leukocyte lysate the U RNAs appear to be in various stages of degradation (lane 3). All four of the known hY RNAs are immunoprecipitated by anti-Ro antibodies from a mixture of erythrocytes and leukocytes (lane 5). Bands for each of the four intact hY RNAs are present while hY2, a truncated version of hY1, is completely absent. Only hY1 and hY4 are immunoprecipitated from erythrocytes alone and none of the hY RNAs were immunoprecipitable from the leukocyte lysate (lanes 4 and 6 respectively). These data establish the presence of hY1 and hY4 in erythrocytes and suggest that hY RNA containing Ro particles are present in mature polymorphonuclear leukocytes, the predominant cell type (>85%) in these leukocyte preparations.

Ro RNPs are more concentrated in reticulocyte enriched erythrocytes

To localize the source of the Ro RNPs in the erythrocyte population, anti-Ro RNP immunoprecipitations from the same number of erythrocytes with either normal (1%) or enriched (5%) reticulocyte counts were performed. As can be seen in Figure 3A, more hY RNA was immunoprecipitated from reticulocyte enriched erythrocytes (lane 2) than from erythrocytes which were not enriched for reticulocytes (lane 1) although the hY RNA species found in the immunoprecipitates were the same. Although an internal standard for RNAs in erythrocytes is not possible, densitometry of the gel in Figure 3A showed a 1.8-fold increase in the enriched lane compared with the non-enriched. This comparison was repeated six times. In each experiment more hY1 and hY4 RNAs were immunoprecipitated in the reticulocyte enriched preparation. This result was obtained consistently using either density gradient enriched reticulocyte preparations or naturally elevated reticulocyte preparations from patients with various anemias. In addition, controlling for cell number with either cell counts or with packed cell volume demonstrated the same increase. In

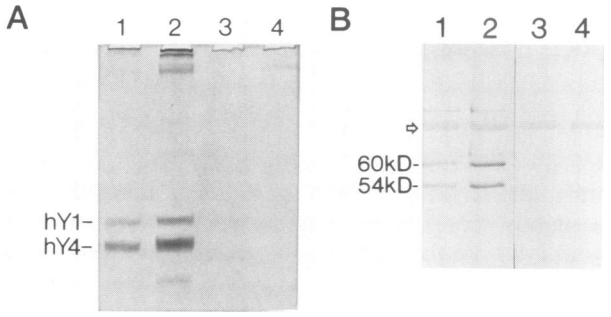

Fig. 3. Panel A. Comparison of hY RNAs from erythrocytes with normal and elevated reticulocyte counts. Erythrocytes with a normal (<1%) or elevated (5%) reticulocyte count were prepared with the same number of cells in each lysate. Lanes 1 and 2 show anti-Ro immunoprecipitated hY RNAs from erythrocytes with 1% and 5% reticulocytes, respectively. Lanes 3 and 4 show normal serum control immunoprecipitations from the same lysates as lanes 1 and 2, respectively. **Panel B.** Immunoblot comparison of Ro protein from erythrocytes with normal and elevated reticulocyte counts. Lanes 1 and 2 contain 5 μg of protein from the 1% reticulocyte or the 5% reticulocyte extract, respectively, transferred to nitrocellulose and probed with a human autoimmune serum containing antibodies to the 60 and 54 kd Ro proteins. Lanes 3 and 4 were loaded with identical samples to lanes 1 and 2, respectively, and probed with a normal human serum. The arrow indicates a band bound by the conjugate in both blots and used as an internal reference standard in densitometry analysis.

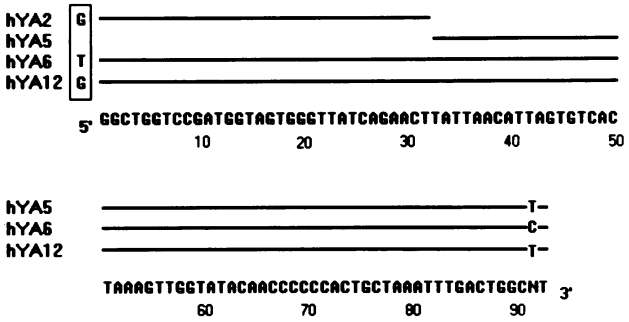

Fig. 4. Sequencing of the hY4 RNA. After selection by hybridization with the hY4 random primed probe, plasmids from four clones were purified and the inserts sequenced completely in both directions. The sequence between the homopolymer flanking regions was taken to be the RNA sequence. The sequence is shown at the bottom. The sequence from hYA6 contained two variations (discussed in the text). The nucleotide shown at the very 5'-end (enclosed by the box) of the lines representing sequence length indicates the variant base before the beginning of the dC homopolymer.

contrast, anti-Ro immunoprecipitations from erythrocytes depleted of reticulocytes by density gradient centrifugation did not show a change in the intensity or the number of hY RNA bands when compared with erythrocytes with a normal reticulocyte count (data not shown).

Comparison of the quantity of Ro protein in erythrocyte preparations with normal and elevated reticulocytes shows differences similar to those found with the immunoprecipitated hY RNA presented above. The Western blot presented in Figure 3B shows an increased amount of both the 60 kd and 54 kd Ro proteins in the enriched reticulocyte preparation (lane 2) compared with the normal (lane 1). Lanes 1 and 2 also contain a faint 90 kd band of unknown identity. Using the background band found in the normal serum control blots (arrow) as an internal standard, densitometry indicated a 3.5-fold increase in the 60 kd band and a 2.0-fold increase in the 54 kd in the 5% reticulocyte lane compared with the 1% lane. Again, depleting reticulocytes to <0.5% did not further reduce the level of the Ro proteins in Western blots compared with that found in erythrocyte preparations containing the normal 1% reticulocytes (not shown).

Cloning and sequencing of the hY4 RNA

The results of sequencing four polymerase chain reaction (PCR) amplified cDNA clones which hybridized to the random primed hY4 cDNA probe are shown in Figure 4. Two clones contain possible full length (93 bases) hY4 sequences and two contain partial sequences. The length of the proposed hY4 sequence is consistent with the migration of hY4 RNA on denaturing gels, between 90 and 95 bases. Since pppGGC has been shown to be the 5'-end of hY4 (Wolin and Steitz, 1984), variation at the 5'-terminal base between the three clones with 5'-end sequence is possibly due to the incorporation of additional bases by the reverse transcriptase as occurs during hairpin loop formation. As another possibility, an artifact may be generated during the tailing procedure due to contamination with unincorporated deoxynucleotide triphosphates (dNTPs) from the cDNA synthesis. If the unincorporated dNTPs are not entirely removed after cDNA synthesis, the terminal transferase used in the homopolymer tailing reaction will incorporate them with the dGTP which can result in a partial dG tail with various other bases included. However, if there is a sufficiently long stretch of dG, the PCR primer with the dC homopolymer can bind and after amplification, the only non-dG bases would be found downstream from the homopolymer. In addition, there is an internal variation in clone hYA6, with a C residue at position 92 while hYA5 and hYA12 contain a T residue at this position. Either sequence is also in complete agreement with all previous fingerprinting data (Wolin and Steitz, 1984).

During the screening, a clone containing a partial cDNA of hY1 was detected by cross-hybridization to the hY4 probe. This clone, containing 82 bases of the 3'-end of the 112 base hY1 RNA, was used to rescreen the library for additional hY1 clones. A clone containing 110 bases of the published sequence for hY1 was obtained. This sequence terminated with one rather than the three uridylate residues reported to be the 3'-end of hY1 (Wolin and Steitz, 1983) and was otherwise consistent with that previously published. In addition, this clone also contained an additional three bases at the 5'-end, between the hY1 sequence and the dC homopolymer.

Erythrocyte hY RNAs are homologous to their HeLa cell counterparts

In order to confirm the identity of the cDNA clones and to determine if the hY RNAs from HeLa cells and erythrocytes correspond not only in size but also in sequence, Northern blots were performed with random primed probes for hY1 and hY4. The probes were prepared from erythrocyte hY PCR clones for hY1 and hY4. The hY1 probe consisted of a 70 bp *Hin*dIII−*Sau*3A fragment of the partial hY1 clone. The probe for hY4 consisted of a 111 bp *Hin*dIII−*Sma*I fragment of the complete hY4 clone hYA12 (Figure 4).

hY RNAs from HeLa cells and erythrocytes were fractionated and stained with ethidium bromide (Figure 5, panel A) and then blotted onto nylon and probed consecutively with the hY1 and hY4 probes (Figure 5, panels B and C). When hybridized with the hY1 probe, two bands

corresponding to hY1 and hY2 can be seen in the HeLa cell Y RNAs (panel B, lane 2) and a single band migrating between these two is present in the erythrocyte Y RNAs (panel B, lane 3). Lane 1 in each panel contains total nucleic acid (TNA) from HeLa cells. When the same blot was hybridized with the hY4 probe (panel C) a band of ~90 bases is present (lanes 2 and 3). The same slightly faster migration of the erythrocyte hY RNA is reflected in these blots as was previously appreciated in Figure 1. The sizes of hY3 and hY5 are 100–101 bases and 83–84 bases respectively (Wolin and Steitz, 1983), and are easily distinguished from hY1 and hY4. These data establish that the slightly more rapidly migrating hY RNAs found in erythrocytes are the homologs of the hY1 and hY4 found in HeLa cells.

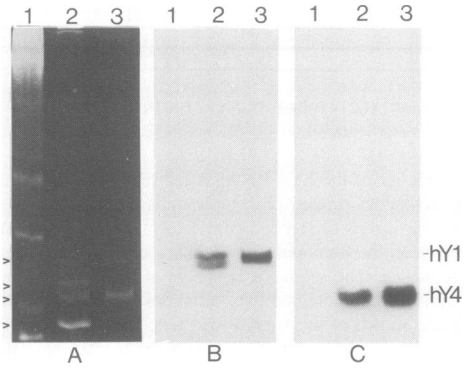

Fig. 5. Northern blot confirmation of hYPCR clones and comparison of Y RNAs from Hela cells and erythrocytes. **Panel A** shows an ethidium bromide stain of an immunoprecipitation using anti-Ro sera with HeLa cells and erythrocytes. Lane 1 contains total nucleic acid (TNA) from HeLa cells. Lanes 2 and 3 contain hY RNAs immunoprecipitated from Hela cells and erythrocytes, respectively. The arrows to the left of lane 1 indicate the positions of the four hY RNAs visible in lane 2. **Panels B** and **C** show Northern blots of the same gel transferred to nylon membrane and probed with the hY1 and an hY4 PCR clone, respectively.

Primary and secondary structure comparisons

Figure 6A shows a comparison of the sequences of the four hY RNAs. The complete sequence of hY1 is shown at the top and only those bases differing from this sequence are shown for the remaining RNAs. As indicated by the blank regions, there is considerable homology between all of the RNAs at the 5′ and near the 3′-ends. These are the regions predicted to be base-paired and form the stem region to which the Ro protein apparently binds (Wolin and Steitz, 1984).

The predicted secondary structures of the four hY RNAs are shown in Figure 6B. While hY1 consists of two stem–loops and a stacked region in which the 5′ and 3′-ends are brought together, the hY3, hY4 and hY5 structures consist of a single stem in which the ends are base-paired. Each of the structures contain a pyrimidine-rich interior or

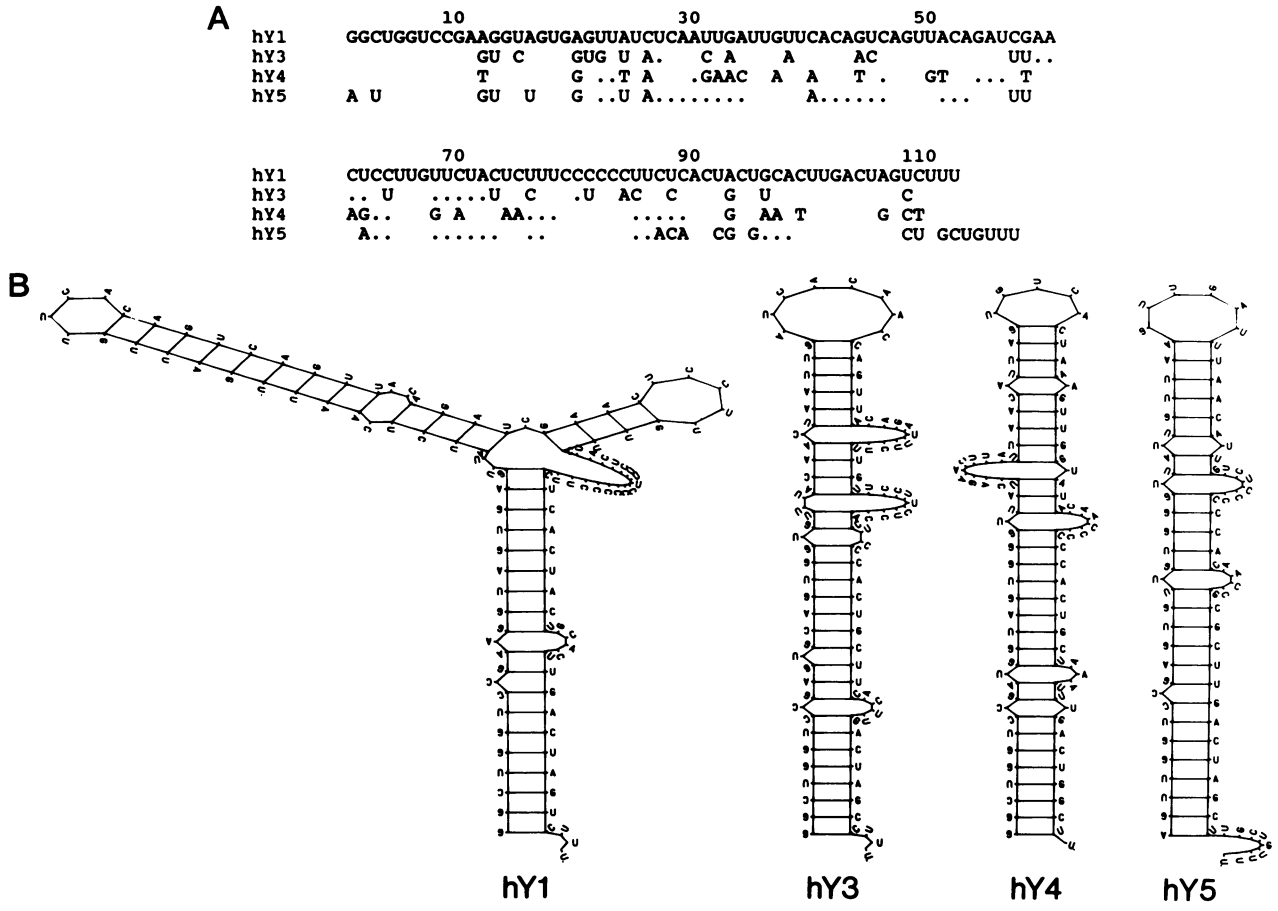

Fig. 6. (A) Sequence comparison of the four hY RNAs. The complete sequence of hY1 is shown at the top. Only the bases differing from this sequence are shown for the other RNAs. The periods indicate regions where gaps were introduced to aid in the alignment of the sequences. **(B)** Proposed secondary structures for the hY RNAs. Proposed secondary structures for hY1, hY3, hY4 and hY5 were generated as described in Materials and methods.

bifurcation loop 22−24 nucleotides from the bottom of the stem region. In addition, all of the proposed structures have a predicted bulge or interior loop consisting of a single cytidine exactly nine bases from the 5′-end of the RNA. The calculated free energies of formation for the proposed structures are −23.5, −26.2, −24.8 and −23.9 kcal/mol for hY1−hY5, respectively.

Discussion

A subset of hY RNAs is found in erythrocytes

The observation that hY1 and hY4 alone are found on Ro RNPs in erythrocytes provides the first example of a human cell containing Ro RNPs in which only a subset of the four hY RNAs are present. As might be expected, each of the anti-RNP specificities tested showed a decrease in the amount of RNA immunoprecipitated from erythrocytes compared with HeLa cells. The lack of any U RNAs in erythrocytes (Figure 1) is a reflection of the lack of a nucleus in these cells since U RNAs are predominantly of nuclear origin. The other cytoplasmic RNPs assayed were maintained in various ratios between the HeLa cells and erythrocytes. However, only the Ro and perhaps the La RNAs demonstrate a definite selection of a subset in erythrocytes.

The inability of the anti-La sera to immunoprecipitate any of the hY RNAs from the erythrocyte lysate indicates that in these cells, the Ro RNAs are not a subset of the La RNAs. This inability may result from insufficient 3′-terminal uridylate residues on the erythrocyte Ro RNAs since none of the clones from the erythrocyte hY cDNA library contain more than two uridines and the La protein requires at least three for binding. The slightly faster migration of erythrocyte hY1 and hY4 compared with their HeLa cell counterparts may be a reflection of this difference. It is possible that partial exonuclease digestion is responsible for these slightly truncated versions of the hY RNAs in erythrocytes. However, the presence of the ribonuclease inhibitor demonstrated in Figure 2 favors the possibility that alternative transcription or processing is responsible for the differences. In fact, it has been demonstrated that inadequate levels of La protein result in RNA polymerase III transcripts with fewer 3′-terminal uridylate residues (Gottlieb and Steitz, 1989).

Comparison of the Sm and Ro RNP RNAs immunoprecipitated from erythrocytes, leukocytes and mixtures of the two demonstrates that intact RNAs are obtained from peripheral blood leukocytes only when they are first mixed with erythrocytes before the cells are lysed. The lack of intact RNA band from leukocytes alone is presumably due to excessive ribonuclease activity in the polymorphonuclear leukocytes.

Preservation of the leukocyte RNAs by combination with erythrocytes before lysis of the cells, Figure 2, is consistent with the presence of a previously reported ribonuclease inhibitor found in erythrocytes and reticulocytes (Kraft and Shortman, 1970; Goto and Mizuno, 1971). These results show that leukocytes contain all four hY RNAs and that hY1 and hY4 from both leukocytes and erythrocytes have the same mobility under these conditions. In addition, it is apparent that factors in the erythrocyte are able to support the stability of the hY3 and hY5 Ro RNPs *in vitro*, indicating that the presence of hY1 and hY4 alone in erythrocytes may not be due to increased stability of these particular Ro RNPs, but rather, may reflect differential expression of hY RNA in erythrocyte precursors.

Due to the requirement for many more erythrocytes than HeLa cells to obtain RNA bands of comparable intensity, we postulated that the Ro RNPs might reside in reticulocytes. Reticulocytes in normal individuals constitute ~1% of the total RBCs and ~100-fold more cells were required for erythrocyte immunoprecipitations than those with HeLa cells. Our results indicate that reticulocyte enriched populations of erythrocytes contain slightly more Ro RNP than erythrocyte populations with normal reticulocyte counts. However, depletion of reticulocytes to below normal levels (not shown) did not significantly affect the amount of hY RNA seen in immunoprecipitations, indicating that Ro RNPs are also present in erythrocytes beyond the reticulocyte stage.

Cellular location of Ro RNPs

Localization of Ro RNPs in the nucleus as well as the cytoplasm has been reported (Clark *et al.*, 1969; Gaither *et al.*, 1987). The La RNP, often described in association with the Ro RNP, has been shown to shuttle between the nucleus and the cytoplasm (Bachmann *et al.*, 1989). Therefore, it would not seem unreasonble that Ro RNPs may reside in both places also. However, the demonstration of the particle in human erythrocytes, which contain no nucleus, would seem to support convincingly the idea that at least some of the Ro RNPs are found in the cytoplasm and presumably have a cytoplasmic function.

Although our data indicate that both reticulocytes and mature erythrocytes contain Ro RNPs, the two cells have some significant differences. The reticulocyte has a lifetime of 24−48 h and during that time synthesizes the remaining 20% of red cell hemoglobin (Williams *et al.*, 1983). Mitochondria, a small number of ribosomes, and remnants of the Golgi apparatus are still present but are lost as the reticulocyte matures (Simpson and Kling, 1968). If the Ro RNPs are not involved in one of the functions of mature erythrocytes, they may be present in the reticulocyte to take part in some aspect of the last burst of protein synthesis and then diminish slowly as the erythrocyte ages. Comparisons of the level of hY RNA in mature erythrocytes of different ages have not been performed.

Sequencing of the hY4 RNA

Because erythrocytes contain a subset of the hY RNAs, these RNAs, hY1 and hY4, were analyzed to determine if they shared any common features with each other or with hY3 and hY5. However, since the sequence of hY4 was previously unknown, we sequenced erythrocyte hY4 from a series of cloned PCR amplified cDNAs. The strategy employed yielded consistent results with the exception of a possible added base at the 5′-end and a single base difference near the 3′-end of one of the clones as presented above. A possible explanation for the variation near the 3′-end is that an error was introduced during the PCR amplification. However, considering the short length of the amplified sequence and the fidelity of the *Taq* polymerase, this seems unlikely. In addition, since erythrocytes from three different individuals were used to prepare the RNA, it is also possible that the variation represents allelic variation in the hY4 gene. Of the four clones, two complete and two partial, these were the only sequence variations. The Northern blot analysis verifies the identity of the hY4 and hY1 clones. In addition, the blots demonstrate that the hY

RNAs found in erythrocytes are homologous to their HeLa cell counterparts. This observation is complemented by the exact sequence agreement of the two erythrocyte partial hY1 sequences with the known hY1 sequence, one of them lacking only two uridylate residues from the 3'-end.

hY RNA structural comparisons

Comparison of the hY RNA sequences with one another reveals a high degree of similarity, especially at the 5' and 3'-ends which are the regions predicted to base-pair and form the stem region involved in binding to the Ro protein (Wolin and Steitz, 1984). However, no striking level of homology is observed internally suggesting that each hY RNA subsumes a different function in cellular metabolism which none the less must share important elements of similarity between the hY RNA species. The predicted secondary structures for the two hY RNAs found in erythrocytes do not share any obvious similarities that are not observed in all four of the hY RNAs.

These results establish the presence of a subset of Ro RNPs in a terminally differentiated cell. One may suppose that hY1 and hY4 Ro RNPs are found in erythrocytes because they are more stable than hY3 and hY5 Ro RNPs and are only remnants of prior cellular activity. That hY1 and hY4 are more stable is inconsistent with the observed protection of leukocyte hY3 and hY5 by factors in erythrocytes, Figure 2. In addition, it may be that hY3 and hY5 are relatively more nuclear specific hY RNAs while hY1 and hY4 are more cytoplasmic, although the studies of Kato *et al.* (1982), indicate that in HeLa cells the amount of nuclear hY5 is one-half to one-third that of the cytoplasm. One intriguing possibility is that only hY1 and hY4 Ro RNPs, as opposed to the entire set, are involved in the expression, either transcriptionally or translationally, of certain proteins found in the erythrocyte. If these results then indicate that the pre-erythrocyte expresses a subset of the Ro RNPs, and retains them for a specific purpose, then the number of potential functions that need to be considered for these hY RNA containing particles is greatly reduced relative to nucleated and dividing cell types.

Materials and methods

Immunoprecipitation

Immunoprecipitations using HeLa cells were performed according to the method of Forman *et al.* (1985). Immunoprecipitations using blood cells were modified in the following manner. For leukocyte immunoprecipitations, white cells were prepared from whole blood by washing EDTA anticoagulated blood successively in TBS and removing erythrocytes until the volume of the white cells was at least twice that of the red cells. These cells were used to prepare lysates according to Forman protocol. For assays using erythrocytes, TBS (10 mM Tris−HCl pH 7.4, 150 mM NaCl) diluted heparinized whole blood was passed over nylon wool. The flow-through cells were washed three times with TBS, removing any residual leukocytes from the surface of the erythrocytes. Observation of >10 000 cells in a smear revealed no leukocytes. These cells were used as above except that a 100-fold higher concentration of cells was used to prepare erythrocyte lysates than for HeLa cells or leukocytes.

Density fractionation of red blood cells

Erythrocyte preparations slightly enriched for reticulocytes were prepared by using the density gradient system of Vettore *et al.* (1980). After washing the erythrocytes as described above, 0.5 ml were gently mixed with 10 ml of a gradient mixture consisting of 35% (v/v) colloidal silica particles (Percoll™, Pharmacia Fine Chemicals AB, Uppsala, Sweden) 15% (v/v) of 60% meglumine iothalamate (Mallinckrodt, St Louis, MO) and 40 mM NaCl. This mixture was centrifuged at 35 000 *g*, 4°C, for 10 min. Fractions enriched or depleted of reticulocytes (top or bottom of the red cell band, respectively) were recovered by side puncture and washed twice in TBS and used as described for immunoprecipitations or Western blotting. A 5-fold increase in reticulocyte count was consistently obtained with this method.

Western blots

Anionic extracts of erythrocyte lysates were prepared by incubating the same lysates used in the immunoprecipitations with one tenth volume DE 52 (Whatman) for 1 h at 4°C. After washing four times in TBS, the DE 52 was eluted with 1 M NaCl and the protein concentration of the extract was measured (Smith *et al.*, 1985). The extracts were fractionated on an SDS−10% polyacrylamide gel (Laemmli, 1970) and immunoblotted by standard techniques (Towbin *et al.*, 1979).

Purification of specific hY RNAs

Specific hY RNAs were prepared by scaling up the immunoprecipitation reaction and fractionating the precipitated RNA on preparative denaturing 10% polyacrylamide−10 M urea gels. After visualization of the RNA bands with ethidium bromide, specific bands were excised with a clean scalpel and the RNA electroeluted (Schleicher and Schuell Elutrap, Keene, NH).

cDNA probes

Randomly primed cDNA probes were generated by incubation of gel purified hY RNA or cloned DNA with random hexamers and cold and hot dNTPs in 50 mM Tris−HCl pH 8.0, 8 mM $MgCl_2$, 2 mM DTT and 10 U of AMV reverse transcriptase (Promega), Shank *et al.* (1978) or the Klenow fragment of DNA polymerase I (Boehringer), Feinberg and Vogelstein (1983). The reaction was incubated for 1 h at 42°C and the unincorporated deoxynucleotides separated on a G-50 spun column.

Northern blots

RNAs fractionated on denaturing polyacrylamide gels were transferred to Hybond-N (Amersham) membranes electrophoretically and then hybridized with randomly primed probe under standard conditions (Maniatis *et al.*, 1982). After washing to high stringency (0.2 × SSPE, 65°C), the blots were autoradiographed overnight at 25°C.

hY4 sequencing

An erythrocyte hY RNA library was made by polyadenylating immunoprecipitated erythrocyte hY RNA (Sippel, 1973), followed by first strand cDNA synthesis (Gubler and Hoffman, 1983). Following spermine precipitation, terminal transferase was used to tail the first strand of cDNA with dGTP (Loh *et al.*, 1989). The tailed cDNA was used in a PCR with the anchored homopolymer primers LinT (CGCGCATGCCTGCAGAA-GCTTTTTTTTTTTTTTTTT) and LinC (GGCGAGCTCGAATTCG-GTACCCCCCCCCCCCCC). The PCR product was fractionated on a 4% Nusieve (FMC BioProducts) agarose gel and the appropriate size band excised. The product was reamplified and digested with *Hin*dIII and *Eco*RI (Bethesda Research Labs) and the products fractionated on a 5% polyacrylamide gel. The area between the 118 bp and 234 bp DNA size markers was excised and the DNA electroeluted. The restricted DNA was ligated into pUC18 restricted with the same enzymes and these recombinant plasmids were used to transform competent DH5α *E.coli*. Colony hybridizations were performed as described by Maniatis *et al.* (1982) with the hY4 or hY1 randomly primed probes described above. Plasmid DNA mini-preps were prepared from positive colonies and the inserts sequenced completely in both directions (Sanger *et al.*, 1977; Tabor and Richardson, 1987).

Secondary structure modeling

Predicted RNA secondary structures were produced on a VAX computer using the Genetic Computer Group (Devereux *et al.*, 1984) FOLD program of Zuker and Stiegler (1981) and energies defined by Freier *et al.* (1986).

Acknowledgements

We would like to thank Drs J.Gimble, B.Frank and M.Gilmore for valuable discussions, Dr I.Targoff for suggesting the cell mixing experiment and K.Rayno and M.J.Easley for blood samples. This work was supported by the NIH (AI24717, AR39577 and AI21568), March of Dimes Birth Defects Foundation (1−1109), and Veterans Administration.

References

Bachmann,M., Pfeifer,K., Schröder,H.C. and Müller,W.E.G. (1989) *Mol. Cell. Biochem.*, **85**, 103−114.

Ben-Chetrit,E., Chan,E.K.L., Sullivan,K.F. and Tan,E.M. (1988) *J. Exp. Med.*, **167**, 1560−1571.

Clark,G., Reichlin,M. and Tomasi,T.B. (1969) *J. Immunol.*, **102**, 117–122.
Deutscher,S.L., Harley,J.B. and Keene,J.D. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 9479–9483.
Devereux,J., Haeberli,P. and Smithies,O. (1984) *Nucleic Acids Res.*, **12**, 387–395.
Feinberg,A.P. and Vogelstein,B. (1983) *Anal. Biochem.*, **132**, 6–13.
Forman,M.S., Nakamura,M., Mimori,T., Gelpi,C. and Hardin,J. (1985) *Arthritis Rheum.*, **28**, 1356–1361.
Francoeur,A.M. and Mathews,M.B. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 6772–6776.
Freier,S.M., Kierzek,R., Jaeger,J.A., Sugimoto,N., Caruthers,M.H., Neilson,T. and Turner,D.H. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9373–9377.
Gaither,K.K., Fox,O.F., Yamagata,H., Mamula,M.J., Reichlin,M. and Harley,J.B. (1987) *J. Clin. Invest.*, **79**, 841–846.
Goto,S. and Mizuno,D. (1971) *Arch. Biochem. Biophys.*, **145**, 71–77.
Gottlieb,E. and Steitz,J.A. (1989) *EMBO J.*, **8**, 841–850.
Gubler,U. and Hoffman,B.J. (1983) *Gene*, **25**, 263–269.
Hendrick,J., Wolin,S.L., Rinke,J., Lerner,M.R. and Steitz,J.A. (1981) *Mol. Cell. Biol.*, **1**, 1138–1149.
Kato,N., Hoshino,H. and Harada,F. (1982) *Biochem. Biophys. Res. Commun.*, **108**, 363–370.
Kraft,N. and Shortman,K. (1970) *Biochim. Biophys. Acta*, **217**, 164–175.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Lerner,M.R., Hardin,J.A. and Steitz,J.A. (1981) *Science*, **211**, 400–402.
Loh,E.Y., Elliott,J.F., Cwirla,S., Lanier,L.L. and Davis,M.M . (1989) *Science*, **243**, 217–220.
Mamula,M.J., O'Brien,C.A., Harley,J.B. and Hardin,J.A. (1989) *Clin. Immunol. Immunopath.*, **52**, 435–446.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 383–389.
Rader,M.D., O'Brien,C., Liu,Y., Harley,J.B. and Reichlin,M. (1989) *J. Clin. Invest.*, **83**, 1293–1298.
Reddy,R., Tan,E.M., Henning,D., Nohga,K. and Busch,H. (1983) *J. Biol. Chem.*, **258**, 1383–1386.
Reichlin,M. (1986) *J. Clin. Immunol.*, **6**, 339–348.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Shank,P.R., Cohen,J.G., Varmus,H.E., Yamamoto,K.R. and Ringold,G.M. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 2112–2116.
Simpson,C.F. and Kling,J.M. (1968) *J. Cell Biol.*, **36**, 103–109.
Sippel,A.E. (1973) *Eur. J. Biochem.*, **37**, 31–40.
Smith,P.K., Krohn,R.I., Hermanson,G.T., Mallia,A.K., Gartner,F.H., Provenzano,M.D., Fujimoto,E.K., Goeke,N.M., Olson,B.J. and Klenk,D.C. (1985) *Anal. Biochem.*, **150**, 76–85.
Stefano,J.E. (1984) *Cell*, **36**, 145–154.
Tabor,S. and Richardson,C.C. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4767–4771.
Towbin,H., Staehlin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354.
Vettore,L., De Matteis,M.C. and Zampini,P. (1980) *Am. J. Hematol.*, **8**, 291–297.
Williams,W.J., Beutler,E., Erslev,A.J. and Lichtman,M.A. (1983) *Hematology.* 3rd edn, McGraw-Hill, New York.
Wolin,S.L. and Steitz,J.A. (1983) *Cell*, **32**, 735–744.
Wolin,S.L. and Steitz,J.A. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1996–2000.
Yamagata,H., Harley,J.B. and Reichlin,M. (1984) *J. Clin. Invest.*, **74**, 625–633.
Zuker,M. and Stiegler,P. (1981) *Nucleic Acids Res.*, **9**, 133–148.

Received on May 14, 1990; revised on July 19, 1990