INTRODUCTION

Eucaryotic gene expression depends on a large number of events that follow polymerase II transcription, but which occur before mRNA translation. The mRNA maturation steps, including 5'-end capping, 3'-end polyadenylation and mRNA splicing, are nuclear events. Many factors involved in mRNA maturation are localized in the nucleus, such as the snRNPs involved in splicing (Ruby and Abelson, 1991) and the hnRNPs probably involved in pre-mRNA packaging, splicing and also in mRNA export (Bennett et al., 1992; Pinõl-Roma and Dreyfuss, 1992; Amberg et al., 1993). The 3'-end cleavage and polyadenylation (Wahle and Keller, 1992) occur in the nucleus, and after mRNA export other events such as mRNA deadenylation, where a poly(A)-nuclease (PAN) activated by a poly(A)-binding protein (PAB) is implicated, take place in the cytoplasm (Sachs and Deardoff, 1992). The PAB has been localized in the cytoplasm, but a shorter PAB product has also been identified in the nucleus (Sachs et al., 1986). Other factors, for example RNA1p, are localized in the cytoplasm although they are also involved in nuclear events such as pre-tRNA and pre-rRNA processing, mRNA production and RNA export to the cytosol (Hopper et al., 1978; Minvielle-Sebastia et al., 1991). Each gene is essential for cell viability. The lethality of the double temperature-sensitive mutant *rna14-rna15* at the permissive temperature, as well as the cross-correction of *rna14* mutants by multicopy plasmids bearing the *RNA15* gene and vice versa, suggest a possible interaction between the *RNA14* and *RNA15* proteins (*RNA14p* and *RNA15p*). *RNA15p* possesses a RNA-binding domain in its N-terminal sequence (Minvielle-Sebastia et al., 1991).

Until now, despite biochemical and genetic experiments, it has not been possible to link the function of *RNA14p* and *RNA15p* with those of other proteins that are involved in mRNA stability but not yet localized, such as UPF1 (Leeds et al., 1991), or with nuclearly located proteins involved in polyadenylation, such as the poly(A)-polymerase (PAP) (Wahle, 1991), or with cytoplasmically located proteins which control the poly(A)-tail length such as PAN, or with the mRNA translation initiation apparatus such as PAB (Sachs and Deardoff, 1992). In order to understand at which stage of mRNA metabolism *RNA14p* and *RNA15p* could be involved, we have localized them in yeast cells, using corresponding antibodies.

MATERIALS AND METHODS

Strains and plasmids

All the yeast strains used in these experiments are presented in Table 1. These strains were maintained on YPD (1% yeast extract, 2% peptone, 2% glucose) or on a selective YNB medium supplemented with the appropriate nutritional ingredients to maintain plasmids.
Strains harboring plasmids under the control of the GAL 10-CYC1 promoter were grown under induction conditions (0.7% yeast nitrogen base DIFCO, 2% galactose, 0.1% Casamino acids, 20 μg/ml tryptophan and adenine). The E. coli strain BMH71-18 (Table 1) was maintained on LB medium containing ampicillin to select for plasmid maintenance. Table 2 describes the plasmids used in this study; all the nucleotide sequence positions are numbered according to the sequence previously published (Minvielle-Sebastia et al., 1991).

**Immune sera and affinity purification of antibodies**

pU14 and pU15C-term (Table 2) were overexpressed in the BMH71-18 E. coli strain, after induction with 1 mM of isopropylthigalactoside (IPTG) as described by Fiehzy et al. (1986). After induction, cells were harvested and lysed directly in loading buffer. Typically, for resuspension, 100 μl of loading buffer was added for every one unit of optical density at 600 nm. Samples were boiled before being loaded onto protein gels (Laemmli, 1970). Coomassie Blue staining was performed to visualise the proteins on the gel. The gel slices that contained the overproduced protein; were used to immunize New Zealand rabbits. The sera IS14 (directed against the pU14 product) and IS15 (directed against pU15C-term product) thus obtained were used directly in immunoblot analysis or were purified. The purification was done in two steps: first, IgGs were purified on a Protein A-Sepharose CL-4B column (Pharmacia Corporation), the specific antibodies were then purified from whole IgGs by immunoadsorption to the wild-type RNA14 protein bound to nitrocellulose filters for E14, or to the mutated rna15-1 protein for E15. The proteins used on the nitrocellulose strips were produced by IPTG induction of E. coli containing plasmids pU14 or pU15s, respectively (see Table 2). The pU15s plasmid was able to produce some amount of full length protein at 37°C without death of the host bacteria, probably because the sensitive temperature protein is not functional under these temperature conditions. The antibodies were eluted from nitrocellulose filters in 0.2 M glycine hydrochloride (pH 2.2), neutralized with Tris-

### Table 1. Yeast and E. coli strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotypes and comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. pombe h-shizosaccharomyces pombe, Urs Leupold, Institute of General Microbiology, University of Bern, Switzerland.</td>
<td></td>
</tr>
<tr>
<td>W303</td>
<td>MATa/MATa, ura3-1/ura3-1 tp1-1/tpl-1 ade2-1/ade2-1 1-leu2-3,112/1-leu2-3,112 his3-11,15/his3-11,15 can1-100/can1-100, Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>W303-1B</td>
<td>MATαura3-1 tp1-1 ade2-1 leu2-3,112 his3-11,15 can1-100, R. Rothstein, Columbia University, New York.</td>
</tr>
<tr>
<td>LM21</td>
<td>MATa, RNA14::TRP1 ade2-1 ura3-1 his3-11,15 tp1-1 leu2-3,112 can1-100pLM28.</td>
</tr>
<tr>
<td>LM31</td>
<td>MATa, RNA15::TRP1 ade2-1 ura3-1 his3-11,15 tp1-1 leu2-3,112 can1-100pLM13.</td>
</tr>
<tr>
<td>LM53</td>
<td>MATa, RNA15::TRP1 ade2-1 ura3-1 his3-11,15 tp1-1 leu2-3,112 can1-100pYeF1H-RNA15, W303-1B strain disrupted for the RNA15 gene and transformed with pYeF1H-RNA15 (see Table 2).</td>
</tr>
<tr>
<td>LM54</td>
<td>MATa, RNA15::TRP1 ade2-1 ura3-1 his3-11,15 tp1-1 leu2-3,112 can1-100pYeF1H-RNA15, W303-1B strain disrupted for the RNA15 gene and transformed with pYeF2H-RNA15 (see Table 2).</td>
</tr>
<tr>
<td>LM33</td>
<td>MATα RNA15::TRP1 ade2-1 ura3-1 his3-11,15 tp1-1 leu2-3,112 can1-100pYeF1H-RNA15, W303-1B strain disrupted for the RNA15 gene and transformed with pYeF1H-RNA15.</td>
</tr>
<tr>
<td>LM35</td>
<td>MATα RNA15::TRP1 ade2-1 ura3-1 his3-11,15 tp1-1 leu2-3,112 can1-100p YeF2H-RNA15.</td>
</tr>
</tbody>
</table>

BMH71-18 supE thi (lac–proA) B' [proAB+ lacI] lacZAM15 E. coli strain kindly provided by H. Bujard (University of Heidelberg).

### Table 2. Plasmid constructions

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Constructions</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUHE21-2</td>
<td>Contains the phage T7-promoter A combined with two lac operators, which is tightly repressible and also permits a high level of transcription on induction. Kindly provided by H. Bujard (University of Heidelberg).</td>
</tr>
<tr>
<td>pU15</td>
<td>Full length RNA15 open reading frame (ORF) generated by PCR with a 5'-end oligonucleotide No 890 and the 3'-end oligonucleotide No 865, including a HinDIII site downstream of the stop codon. The PCR product was then cloned into the BamHI and the HindIII sites of the pUHE21-2 vector.</td>
</tr>
<tr>
<td>pU15C-term</td>
<td>A deleted RNA15 ORF generated by PCR with the 3'-end oligonucleotide No 865 and a 5'-end oligonucleotide including a HinDIII site upstream of the ORF (removing the endogenous ATG) and a 3'-end oligonucleotide No 865 which added a HindIII site downstream of the stop codon.</td>
</tr>
<tr>
<td>pU15Δ</td>
<td>Using the 5'-end oligonucleotide No 890 and the 3'-end No 865, the rna15-1 ORF bearing the thermosensitive mutation was PCR-amplified from the genomic DNA of the rna15-1 mutant strain DNA and cloned in pUHE21-2.</td>
</tr>
<tr>
<td>pU14</td>
<td>Full length RNA14 ORF generated by PCR with a 5'-end oligonucleotide introducing a BamHI site just upstream of the ORF (removing the endogenous ATG) and a 3'-end oligonucleotide adding a HindIII site downstream of the stop codon. The PCR product was then cloned into the BamHI and the HindIII sites of the pUHE21-2 vector.</td>
</tr>
<tr>
<td>pLM27</td>
<td>A HindIII genomic fragment containing the full RNA14 gene was cloned in the HindIII cloning site of YEp351, a LEU2 yeast 2 μm-based plasmid (Hill et al., 1986).</td>
</tr>
<tr>
<td>pLM28</td>
<td>A HindIII genomic fragment containing the full RNA14 gene was cloned in the HindIII cloning site of YEp352, a URA3 yeast 2 μm-based plasmid (Hill et al., 1986).</td>
</tr>
<tr>
<td>pLM13</td>
<td>The 5′ end genomic fragment containing the RNA15 gene was cloned in YEp352, a URA3 yeast 2 μm-based plasmid (Hill et al., 1986).</td>
</tr>
<tr>
<td>pYeF1H-RNA15</td>
<td>pYeF1H, a His3 yeast plasmid with a GAL10-CYC1 promoter which allows conditional expression. The complementary oligonucleotide primers (FLU-1 and FLU-2) were designed to yield an in-frame insertion of the amino acids ‘YPYDVPDYA’ at the N-terminal end of the RNA15 ORF cloned into pYeF1H*.</td>
</tr>
<tr>
<td>pYeF2H-RNA15</td>
<td>pYeF2H Ha His3 yeast plasmid with a GAL10-CYC1 promoter which allows conditional expression. The complementary oligonucleotide primers (FLU-1 and FLU-2) were designed to yield an in-frame insertion of the amino acids ‘YPYDVPDYA’ at the C-terminal end of the RNA15 ORF cloned into pYeF2H*.</td>
</tr>
</tbody>
</table>

*These plasmids will be described in more detail by Cullin and Minvielle-Sebastia (unpublished data).
HCl (pH 8.9), then concentrated using Centricron filtration tubes (Amicon Corporation). They were stored at −20°C after addition of 20 µg/ml BSA and 0.02% sodium azide.

**Preparation of crude extracts for western blot (immunoblot)**

Crude yeast protein extracts were prepared as described by Sachs and Davis (1989). HeLa cells and Drosophila larvae were simply boiled in loading buffer. 50 µg of each extract and, for Drosophila, an equivalent of 50 larvae, were loaded and run on a 10% SDS-polyacrylamide gel and electrotransferred to nitrocellulose filters in a Bio-Rad transblot apparatus (Towbin et al., 1979). These blots were processed for immunological detection of antigens. The blots were first blocked with 5% nonfat milk powder in PBS, and immune-sera were used at a dilution of 1/2000 for IS14, 1/1000 for IS15 and 1/100 for E15 and E14. The protein bands were detected using anti-rabbit antibodies coupled to horseradish peroxidase in a 1/2000 dilution (Amersham), then revealed with the ECL-KIT system (Amersham) and exposed to Amersham hyperfilm for two or ten minutes. The emitted signal decreased completely after one hour and some blots were used a second time for another detection. The monoclonal antibodies mAb12CA5 that recognize the FLU epitope sequence (YPYDVPDYA), were purified from ascitic fluid (kindly provided by C. Conessa; C.E.A. Saclay), used at a dilution of 1/50000, revealed C. Conessa; C.E.A. Saclay), used at a dilution of 1/50000, revealed

**Synthesis of RNA14p and RNA15p proteins in vitro and immunoprecipitation**

To examine the affinities of the antibodies for the RNA14 and the RNA15 proteins, we synthesized 35S-labelled RNA14p or RNA15p in vitro with a rabbit reticulocyte lysate system from the Promega Corporation. A plasmid that contains the 1911 nucleotides of the RNA14 ORF or 891 nucleotides of RNA15 ORF, cloned into the pGEM3 vector, was used to perform the in vitro transcription (Promega Corporation). For immunoprecipitation, sera were mixed with Protein A-Sepharose-6MB (from Sigma) in 150 mM NaCl, 10 mM Tris-HCl pH 8.0 and 0.1% Nonidet-P40 (Ipp150) (Scherly et al., 1989). The 35S-labelled proteins were added at 4°C for 90 minutes. The Protein A-Sepharose/antibodies/35S-labelled protein complex was pelleted, washed and directly boiled in loading buffer for analysis by 10% SDS-polyacrylamide gel electrophoresis. Standard molecular mass markers loaded on the gels were 14C-labelled and ranged from 14.3 to 200 kDa. After electrophoresis the gels were treated with amplifier (Amersham), then dried for 30 minutes at 80°C and subjected to autoradiography.

**Subcellular fractionation and detection**

Cellular separation of cytoplasm and nuclei was performed by a combination of the methods described by Fisher et al. (1982) and Ide and Saunders (1981). Spheroplasts were lysed in 30 ml of buffer A (18% Ficoll DL-400, 0.5 mM CaCl2, 20 mM KHPO4, pH 6.5, and 1 mM PMSF) and disrupted with a Dounce homogenizer. After a first centrifugation (2500 g) to remove whole cells and large debris, nuclei were pelleted at 30,000 g. Supernatant was considered to be the cytoplasmic fraction. Supernatant and crude nuclear pellets were DAPI-stained and inspected by fluorescence microscopy. The nuclear pellet was resuspended in 1 ml of buffer B (buffer A, supplemented with 0.1% Triton X-100) and incubated for 15 minutes on ice, to remove the proteins and debris trapped on the outer membrane of the nuclei. Nuclei were then pelleted (5000 g, 10 minutes) and the supernatant discarded. The pellet served as the source of nuclear fraction. 50 µg of each fraction were boiled in Laemmli buffer, loaded and migrated on a 10% SDS-polyacrylamide gel and then transferred to nitrocellulose. The immunodetection was performed at a dilution of 1/2000 for IS14 and 1/1000 for IS15.

**Indirect immunofluorescence**

Cells were harvested by centrifugation and prefixed for 1 hour in 3.7% formaldehyde in 0.1 M KHPO4, pH 6.5, then washed in the same buffer, and resuspended in S buffer (1.2 M sorbitol, 0.12 M KHPO4 and 0.033 M citric acid, pH 5.9). They were converted into spheroplasts by addition of 25 µg/ml of Zymolyase (100,000 units) for 20 minutes to one hour to thoroughly remove cell walls. The spheroplasts were centrifuged, the pellet was washed twice with buffer S and resuspended in the same buffer. About 5x10^6 spheroplasts were applied to polylysine-coated slides, fixed with 3.7% formaldehyde and passed through −20°C acetone for 5 minutes (Kilmartin and Adams, 1984). These spheroplasts were then stained for immunofluorescence by addition of a 1/2 dilution of the E15 antibodies or a 1/2000 dilution of IS14. The secondary antibody was goat anti-rabbit IgG, conjugated with FITC (Jackson Corporation). Nuclear and mitochondrial DNAs were stained by DAPI to localize these structures. We used a serum raised against the S. cerevisiae RNA polymerase A (a kind gift of A. Sentenac from CEA Saclay) as a nuclear control immunodetection marker. The slides were mounted in glycerol and observed with a Reichert microscope using Nomarsky Interference and FITC excitation wavelengths.

**RESULTS**

**Identification in S. cerevisiae extracts of RNA14p and RNA15p**

To characterize the RNA14 and RNA15 gene products in yeast, we prepared antisera to the corresponding proteins. We inserted the previously cloned RNA14 and RNA15 sequences in the E. coli expression vector pUHE21-2, as described in Materials and Methods. The RNA14 expression plasmid (pU14) was able to produce a 73 kDa protein in E. coli, which was used to immunize rabbits and to produce the serum IS14. The full length RNA15 sequence cloned in the pUHE21-2 plasmid (pU15) induced death of E. coli at the start of IPTG induction. We therefore decided to delete the RNA15 sequence starting from its N terminus, until we obtained a product capable of being overexpressed in E. coli. Only the pU15C-term plasmid (described Table 2) allowed normal growth of E. coli transformants. After IPTG induction, an abundant 17 kDa protein accumulated in these transformants, and this was used to immunize rabbits and to produce the serum IS15.

**Yeast immunoblot analysis and immunoprecipitation**

In order to identify the RNA14p and RNA15p proteins, crude yeast extracts were analyzed by SDS-PAGE and blotted. The IS14 and IS15 antibodies were used to probe this blot. To evaluate the specificity of the detected products, we purified the IgGs from the IS14 and IS15 sera, then immunopurified the antibodies on nitrocellulose strips containing the RNA14p or the RNA15p, to obtain E14 and E15. These were used in parallel with IS14 and IS15 to probe crude yeast extract. IS14 recognized a polypeptide with an apparent molecular mass of 73 kDa and another one with a variable apparent molecular mass, depending on the IS14 serum used. The serum used for the detection presented in the Fig. 1a reveals a second product with a molecular mass of 30 kDa. A single 73 kDa product appeared when the E14 immunopurified antibodies were used to probe a blot of crude yeast extract. We conclude that the polyclonal sera are able to recognize some other products in S. cerevisiae, but none could be identified as secondary RNA14 products. IS15
revealed three major bands with apparent molecular masses of 51 kDa, 42 kDa and 28 kDa (see Fig. 1b). When the E15 immunopurified antibodies were used in the same manner (Fig. 1b), a 28 kDa protein could still be identified, but the 42 kDa species was more prominent compared to the initial IS15 detection. The 51 kDa product is completely lost, confirming that it was an artefact. The proteins identified by the immune-sera and purified antibodies showed no cross-reactivity with either of the preimmune-sera (data not shown). The anti-RNA14p and anti-RNA15p affinities were tested by an immunoprecipitation experiment (Fig. 2). In vitro translations of RNA14 and RNA15 (described in Materials and Methods) were performed in rabbit reticulocyte extracts and the 35S-labelled proteins were immunoprecipitated with IS14, IS15 and the preimmune-sera and loaded on a protein gel. When the immunoprecipitation was performed with the 35S-labelled RNA14p and IS14 antibodies, a 73 kDa protein was detected (Fig. 2, lane 1). A minor product with a molecular mass of 50 kDa appeared in this experiment and was probably due to a partial degradation of the 73 kDa 35S-labelled protein. No labelled products were seen when using the preimmune-serum (Fig. 2, lane 3). IS15 antibodies were used to immunoprecipitate the 35S-labelled RNA14p and, as expected, no product was detected (Fig. 2, lane 8). In the case of the RNA15 in vitro translation, the 35S-labelled RNA15p 42 kDa product was only immunoprecipitated by the IS15 antibodies (Fig. 2, lane 5) and not with the corresponding preimmune-serum (Fig. 2, lane 7) or with the IS14 antibody (Fig. 2, lane 4). A black spot appeared in lane 4; this is a non-specific radioactive spot as it is not focussed or centered on the track, and does not correspond to the expected 42 kDa product of the in vitro translation. In the light of these results, we conclude that the protein of 73 kDa apparent molecular mass corresponds to the RNA14 gene product, in agreement with the size of the 75.3 kDa product predicted from the DNA sequence (Minvielle-Sebastia et al., 1991). The IS14 antibodies recognize the RNA14p with good specificity. Since the in vitro-synthesized product shows the same apparent molecular mass as the 42 kDa band revealed in in vivo extracts, we have to conclude that the 42 kDa protein corresponds to the RNA15 gene product in spite of the size of 32.8 kDa predicted by the RNA15 sequence. Other RNA-binding proteins have been previously described to have aberrant migration (Query et al., 1989). The 28 kDa band could be a truncated RNA15 protein or a different protein with a common epitope.

Epitope-tagged RNA15 protein reveals a single 42 kDa product

In order to determine whether the 28 kDa product could result from a post-translational modification of the 42 kDa RNA15p, we decided to construct epitope-tagged RNA15 proteins. We have fused the FLU epitope to either the N terminus or the C terminus of the RNA15 ORF using the yeast expression vectors pYeF1H and pYeF2H (whose expression is controlled by the GAL10-CYC1 promoter). These constructions generated pYeF1H-RNA15 and pYeF2H-RNA15 (see Table 2). A haploid strain W303-1B disrupted for the RNA15 gene and transformed by pYeF1H-RNA15 (N-terminal tagged) (LM33) and the cor-
We have shown that, unlike the 42 kDa RNA15p, the 28 kDa product detected by the IS15 antibodies was not overexpressed in the LM33 strain. In addition, this product was not detectable when RNA15p was tagged either in the C-terminal or N-terminal position. Thus it is very unlikely that the 28 kDa protein is an RNA15p maturation product and the hypothesis of a different cross-reacting protein is favoured. Nevertheless we cannot rule out the possibility that the 28 kDa protein could be derived from RNA15p by a double cleavage at both its C terminus and its N terminus. However, if this is the case the product is strictly regulated, possibly because of a stoichiometric involvement in a structure for which all excess of an individual component is rapidly degraded.

RNA14p and RNA15p cellular localization

Subcellular fractionation of yeast cells

Having established that our antibodies were able specifically to recognize RNA14p or RNA15p, we used them to determine the cellular localization of these proteins. The subcellular localization of the RNA14p and RNA15p was performed by fractionation of yeast cells. Subcellular fractions enriched for nuclei (i.e. fractions containing nuclei, as assayed by 4′,6-diamidino-2-phenylindole, hydrochloride (DAPI)-staining and observation under the light microscope) were isolated as described in Materials and Methods, where the counterpart of the nuclei fraction is considered to be the cytosolic fraction. Fig. 4 shows the results obtained after detection with IS14 (top) and with IS15 (bottom) in a total crude extract (lane T), in the cytosolic fraction (lane C) and in the nuclear fraction (lane N). RNA14p is found in the nuclear fraction and in the cytosol. The RNA15p 42 kDa polypeptide appears to be exclusively found in the nuclear fraction and the 28 kDa exclusively in the cytosolic fraction. An anti-RNA polymerase B185 subunit serum (a kind gift of A. Sentenac from CEA, Saclay, France) used as a control confirmed the presence of the polymerase B in the nuclei-enriched fraction and its absence in the cytosolic fraction (data not shown).

We conclude that the 73 kDa RNA14p is located both in the nuclear and in the cytosolic compartments. The 42 kDa RNA15p is detected only in the nuclear fraction and the 28 kDa product is found only in the cytosolic fraction. With the aim of confirming the localization of these proteins we decided to perform immunofluorescence experiments.
Detection of RNA14p and RNA15p in yeast cells by immunofluorescence

We prepared spheroplasts of diploid strains of *S. cerevisiae*, both wild type and transformed by plasmids carrying the genes *RNA14* and *RNA15* (see Materials and Methods). These cells were decorated with the antiserum against RNA14p, IS14 (Fig. 5a,c and g). Immunodetection was also effected with semi-purified immunoglobulin G (E15) prepared from the IS15 serum (Fig. 5e), since the crude serum against this protein did not give a response under the conditions used. Fig. 5a shows the detection of RNA14p with IS14 in the wild-type strain W303. A light, diffuse staining of the nucleus is apparent but

![Images of W303, LM54, LM35 yeast cells with immunofluorescence.]
this is significantly less intense than the fluorescence associated with certain cytoplasmic structures. The Nomarski visualisation of the cells shown in Fig. 5b confirms that the cells are in a good cytological state with clearly visible nuclei. In the wild-type strain W303 it was never possible to detect the RNA15 protein, presumably due to the very low level of expression of the gene. In order to detect RNA15p in situ, we decided to overexpress this protein, initially under the control of its own promoter. However, western blot analysis (result not shown) showed that the strain W303 transformed with the 2µ based plasmid (pLM13) carrying the RNA15 gene under the control of its own promoter is not able to overexpress the RNA15 protein. The same result was obtained with the plasmid pLM27 which contains the RNA14 gene. It is only when the cell is co-transformed with both plasmids pLM13 and pLM27 (to yield the strain LM54) that it becomes possible to increase the amount of the proteins. The results of this overexpression are shown in Fig. 5. In Fig. 5c the strain LM54 was treated with the serum IS14. In this case it is apparent that the nuclear staining of RNA14p is significantly increased, and the cytoplasmic decoration remains. The stained spots in the cytoplasm appear to correspond to mitochondria which were localised by a corresponding DAPI stain (Fig. 5d). When this same strain is analysed with antisera E15, a clear nuclear staining is apparent (Fig. 5e). The corresponding staining by DAPI (Fig. 5f) confirms this nuclear localization. The unequal intensity of the nuclear staining was a result of a corresponding variation in the expression of the gene on a 2µ plasmid from one cell to another (Futcher and Cox, 1984). These results show clearly that the RNA14 and RNA15 proteins are both located in the nucleus and the former is also present in the cytoplasm. These observations confirm the results previously obtained from the western blot analysis of the cellular fractions shown in Fig. 4. The fact that RNA14p only seems to increase in quantity in the nucleus when both genes are overexpressed (Fig. 5c) led us to investigate whether this was due to a displacement of RNA14p from the cytoplasm to the nucleus when the quantity of RNA15p is itself increased in the nucleus, or whether it was simply the result of the overexpression of RNA14p itself. In an initial experiment, we placed the RNA14 gene under the control of an inducible GAL promoter using a system equivalent to the one used for the overexpression of RNA15p. This overexpression in the wild-type strain proved to be toxic. In an attempt to answer this question in a different way, we studied the behaviour of RNA14p in cells that overexpressed only RNA15p. It should be pointed out that a cell transformed by a multicopy plasmid containing the RNA15 gene under the control of its own promoter (pLM13) cannot overexpress RNA15p. Accordingly, we used the diploid strain LM35 which permits the overexpression of the RNA15 gene when it is under the control of a GAL promoter, and compared the results with those obtained with the strains W303 and LM54. The immunodetection of RNA14p in LM35 (see Fig. 5g) and comparison with the corresponding DAPI staining (Fig. 5h) again showed a faint and diffuse staining of the nucleus and spot-staining of cytoplasmic structures. Thus it is apparent that the strong staining of the nucleus by IS14 in the strain LM54 (see Fig. 5c) is due to the overexpression of the RNA14 gene. This result is in agreement with those obtained by western blotting when the RNA14 protein was detected in the strains LM33 and W303-1B (Fig. 3). In particular, we noticed that the quantity of RNA14p remained the same as that observed in the wild-type strain when RNA15p was overexpressed using a GAL promoter. The overexpression of RNA15p in the strain LM35 was investigated by immunofluorescence (result not shown) by using the antibody E15; the nuclear staining was equivalent to that obtained with LM54 (Fig. 5e). Finally it should be noted that the detection of epitope-tagged RNA15p by the monoclonal antibody mAb12CA5 was not possible in this strain for unknown reasons.

Detection of RNA14- and RNA15-related proteins in other organisms

A databank search revealed that a suppressor gene of the forked locus in D. melanogaster [su(f)] locus, has a significant homology with the S. cerevisiae RNA14 protein and that the [su(f)]-encoded protein has a predicted molecular mass of 84 kDa (Mitchelson et al., 1993). With this information in mind and in order to determine whether proteins homologous to RNA14p and RNA15p are present in other eucaryotic organisms, we electrophoresed crude proteins extracts from S. cerevisiae, D. melanogaster, HeLa cells and D. melanogaster, for immunodetection by IS14 and IS15. The immunodetection results are shown in Fig. 6. The detection by IS14 (top) shows a 73 kDa band for S. cerevisiae, S. pombe and also D. melanogaster extracts, but no signal appears in HeLa cell extracts. The detection by IS15 (bottom) does not reveal anything for the D. melanogaster extract but gives two bands for S. cerevisiae, S. pombe and HeLa cells: a common band of about 42 kDa (similar to RNA15p) and a supplementary band of 28 kDa for S. cerevisiae and S. pombe and of 35 kDa for HeLa cells.

The identical pattern obtained for S. cerevisiae and S. pombe when the immunobLOTS were probed with IS14 or with IS15, suggests a strong evolutionary conservation of these proteins, even though for many cytosolic proteins the percentage of sequence identity between S. cerevisiae and S. pombe is not better than found between each of these fungi and human genes (Moreno et al., 1991). The maintenance of a 28 kDa band in S. pombe suggests that its recognition by IS15 in S. cerevisiae Fig. 6. Recognition of foreign proteins by IS14 and IS15 antibodies; 50 µg of crude extract from S. cerevisiae (a), S. pombe (b), HeLa cells (c) and an equivalent of 50 D. melanogaster larvae (d), were separated on SDS-PAGE, blotted and immunodetected with IS14 at dilutions of 1/2000 (top) and IS15 at 1/1000 (bottom).
is not fortuitous, but corresponds to an epitope strongly conserved during evolution.

Concerning higher eucaryotes, it is possible that the 73 kDa band seen in D. melanogaster extracts and the 42 kDa band found in HeLa cells extracts are homologous to RNA14p and to RNA15p, respectively. Nevertheless only a true molecular study, by microsequencing of these proteins followed by cloning of the corresponding cDNAs, would confirm this possibility.

**DISCUSSION**

The cellular localization of the RNA14p shows clearly that the protein is partly nuclear and partly cytosolic, being associated with continuous filaments and with small dots, correlated with the position of mitochondrial DNA revealed by DAPI-staining. At this level of resolution, it is unfortunately impossible to discern whether this corresponds to the rough endoplasmic reticulum surrounding the mitochondria or to an internal location inside mitochondria. This RNA14p localization is correlated with the immunolocalization in the cellular fraction by immunoblot (Fig. 4), where 50 μg of nuclear fraction and the same quantity of cytosolic fraction were loaded on the gel. The nuclear fraction analysed represents 0.46% of the totality of the nuclear extract, whereas the cytosolic fraction analysed represents 0.04% of the totality of the cytoplasmic extract. In this experiment, the cytoplasm/nucleus ratio is thus at least 10 times smaller than in the cell. This explains the stronger intensity of the signal obtained for the nuclear fraction in comparison with that obtained with the immunofluorescence in the wild-type strain. We have tested the respiratory abilities of our two different *rna14* ts alleles by growth on different substrates such as lactate and ethanol without any evidence for an impairment in mitochondrial functions (data not shown). Moreover, two lines of evidence suggest that one of the RNA14p functions occurs at the level of the polyribosomes, which would favour an ER localization. The first is a pulse-chase experiment, where labelled mRNAs are synthesized at the permissive temperature, chased for five minutes still at the permissive temperature (where they should be mainly engaged in polysomes), and then transferred for ten minutes to the non-permissive temperature. In this experiment the level of poly(A)*-radiolabelled mRNAs found in an *rna14* or an *rna15* mutant is about four times less than in the wild-type control, showing a decrease in cytoplasmic mRNA stability (Bloch et al., 1978). The second line of evidence comes from the suppressor studies performed by A. Petitjean in our laboratory. They show that the overexpression in multicopy plasmids of either of two wild-type ribosomal proteins, SSM1p and SSM2p associated with the large ribosomal subunit, partially suppress the ts phenotype of *rna14-1* and *rna14-3* but not of *rna15-1*. This also suggests a function of RNA14p in polysomes (Petitjean et al., unpublished observations).

Concerning RNA15p, its nuclear location is clear and there is no evidence for its presence in the cytosol. This raises a problem, since the pulse-chase experiment described above also suggests a polysomal function for RNA15p. There are two possible ways to reconcile these data: first, the 28 kDa protein recognized in the cytosol by the E15 antibodies may be the result of a double processing of RNA15p (see the epitope-tagged results). Second, RNA14p may be modified by an activity directly or indirectly dependent on RNA15p to be functional in polysomes. It is also possible, although unlikely, that the full length RNA15p is present in the cytosol, but at too low an abundance to be revealed under our experimental conditions.

RNA14p and RNA15p were expected to be located in the cytoplasm for the reasons evoked above. Nevertheless, RNA14p has also been found in the nucleus and RNA15p is only detectable in the nucleus. The nuclear localization of both the RNA14 and RNA15 proteins, without being a proof, strongly suggests a nuclear function for these two proteins. It is not possible at the present time to attribute any precise nuclear role to these proteins. Nevertheless it is worth remembering that besides the involvement of the two proteins in mRNA stability and poly(A)-tail shortening, a moderate but significant transcriptional effect has been observed (Minvielle-Sebastia et al., 1991), perhaps implying a role in transcription termination. Moreover the RNA15p sequence shows an RNA-binding motif, with poly-U and poly-dT specificity and a putative polymerase module, which could implicate this protein in the re-addition of poly(A) residues to the poly(A)-tail (L. Minvielle-Sebastia et al., unpublished observations).

The use of strains that overexpress RNA15p in order to permit the detection of this protein, normally present in small amounts, allowed us to localize RNA15p and also led to interesting perspectives concerning the study of the regulation of RNA14p and RNA15p as well as their possible interaction. The overexpression of one of the genes on a 2μ plasmid is capable of compensating for the temperature-sensitive mutation of the other. Nevertheless, the same overexpression in a wild-type strain does not result in a detectable increase in the amounts of the proteins encoded by either of these genes. In order to observe an increase in RNA14p and RNA15p in the nucleus it is necessary to overexpress both genes on multicopy plasmids in the wild-type strain. Therefore it seems that, individually, each protein can only be overexpressed when the other is deficient, or when the quantities of the two wild-type proteins are maintained at parity. This argument is supported by considerations of the lethal effect of the double mutant *rna14-rna15* at the permissive temperature. Until now we have been unable to co-precipitate both proteins with any of the available antibodies; this could be explained if the interaction is weak. These assays will be repeated after protein cross-linking.

A more detailed study concerning the interaction between RNA14p and RNA15p, and of possible interactions with other proteins which function with them, is at present being undertaken in our laboratory.

Our present approaches for trying to understand the function of RNA14p and the RNA15p reside mainly in an extension of the search for suppressors, in a search for synthetic lethals of the *rna14* and *rna15* mutants, and in the construction of a collection of new ts alleles by plasmid-shuffling (Mann et al., 1987), with a view to finding new phenotypes. We hope also to extend our knowledge of the conservation of the function during evolution of these proteins by cloning mammalian and plant homologs.

We thank Dr Michel Bornens for hospitality in his laboratory where IgG purification was performed and for the invaluable help of Nicole...
REFERENCES


Bordes. We thank Braun Spencer and his collaborators for assisting with the epifluorescence microscopy and Alexis Harington for looking over the English. We are grateful to Denise Menay for synthetic oligonucleotides. The work reported here was supported by ARC grant no. 6892 and in part by the LNC foundation. NOTE: The Saccharomyces cerevisiae mutant strains: cor1-1 and cor2-1, studied by Bloch et al. (1978), have been renamed rna14 and rna15 by Minvielle-Sebastia et al. (1991).


(Received 21 September 1993 - Accepted 22 December 1993)