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Lithium toxicity in yeast is due to the inhibition of RNA processing enzymes

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Hal2p is an enzyme that converts pAp (adenosine 3′,5′ bisphosphate), a product of sulfate assimilation, into 5′ AMP and P_i. Overexpression of Hal2p confers lithium resistance in yeast, and its activity is inhibited by submillimolar amounts of Li^+ *in vitro*. Here we report that pAp accumulation in *HAL2* mutants inhibits the 5′→3′ exoribonucleases Xrn1p and Rat1p. Li^+ treatment of a wild-type yeast strain also inhibits the exonucleases, as a result of pAp accumulation due to inhibition of Hal2p; 5′ processing of the 5.8S rRNA and snoRNAs, degradation of pre-rRNA spacer fragments and mRNA turnover are inhibited. Lithium also inhibits the activity of RNase MRP by a mechanism which is not mediated by pAp. A mutation in the RNase MRP RNA confers Li^+ hypersensitivity and is synthetically lethal with mutations in either *HAL2* or *XRN1*. We propose that Li^+ toxicity in yeast is due to synthetic lethality evoked between Xrn1p and RNase MRP. Similar mechanisms may contribute to the effects of Li^+ on development and in human neurobiology.

Keywords: exonucleases/lithium/RNA processing/RNase MRP/sulfate assimilation

Introduction

The monovalent cation Li^+ has remarkable therapeutic effects in the treatment of various neural diseases and remains the most effective agent for the treatment of patients suffering from bipolar disorder (manic depressive psychosis) where it controls the occurrence of extreme mood swings (Rosenthal and Goodwin, 1982). However, due to its narrow therapeutic index and its widespread use, acute Li^+ intoxication occurs quite frequently, mostly because of overdosage (Grignon and Bruguerolle, 1996). In contrast to the mainly positive therapeutic effects of Li^+ as a drug are its profound effects on the development of various organisms which were first observed more than a century ago (Herbst, 1892). Li^+ inhibits the formation of the dorsal–ventral axis in *Xenopus laevis* embryos (Kao *et al.*, 1986) and induces vegetalization in sea urchin eggs (Livingston and Wilt, 1989). Moreover, Li^+ alters cell fate determination in the slime mold *Dictyostelium discoideum* (Peters *et al.*, 1989) and affects pattern formation in the unicellular ciliate *Tetrahymena thermophila* (Jerka-Dziadosz and Frankel, 1995).

Despite decades of clinical use, the molecular mechanisms underlying the therapeutic action of Li^+ have not been established definitively, nor are the molecular targets known which bring about the massive changes in the developmental program. Many of the proposed mechanisms for Li^+ action suggest the involvement of key components of signal transduction pathways as targets of Li^+ inhibition (Manji *et al.*, 1995). Currently, the most widely accepted model is the 'inositol depletion hypothesis' (Berridge *et al.*, 1989) which is based on the inhibition of inositol monophosphatases by Li^+, leading to depletion of cellular myo-inositol. Inositol depletion may result in insufficient regeneration of inositol 1,4,5-trisphosphate and thus block the response to exogenous signals which are mediated by inositol 1,4,5-trisphosphate-dependent signaling pathways (Berridge *et al.*, 1989). However, the use of a different inhibitor of inositol monophosphatases (the bisphosphonate L-690,330) failed to duplicate the developmental defects in *Xenopus* that are induced by Li^+ (Klein and Melton, 1996).

The *HAL2*/*MET22* gene was identified in two independent genetic screens; as a mutation leading to methionine auxotrophy (Masselot and de Robichon-Szulmajster, 1975) and as a gene which confers salt tolerance to yeast upon overexpression (Gläser *et al.*, 1993). Further characterization showed Hal2p to be a 3′(2′),5′ bisphosphate nucleotidase which is required for the turnover of a side product of sulfate assimilation, adenosine 3′,5′ bisphosphate (pAp) (Murguía *et al.*, 1995). Sulfate assimilation in *Saccharomyces cerevisiae* is initiated by the production of adenosine 5′-phosphosulfate (ApS) from ATP and sulfate. This is followed by phosphorylation of ApS forming 3′-phosphoadenosine, 5′-phosphosulfate (pApS). A subsequent pApS reductase reaction then yields sulfite and pAp. Sulfite is reduced further to sulfide which is incorporated into homocysteine which can be metabolized to methionine, one end product of sulfate assimilation (Thomas *et al.*, 1989). The methionine auxotrophy of *HAL2* mutants is not, however, simply due to the inhibition of sulfate assimilation since the auxotrophy cannot be relieved by inorganic sulfur sources such as sulfite or sulfide, even though the strains do posses all the enzymatic activities required for methionine synthesis (Thomas *et al.*, 1992; Murguía *et al.*, 1995, 1996; Peng and Verma, 1995). It was therefore proposed that the accumulation of pAp in strains lacking Hal2p activity is toxic to the cell (Murguía *et al.*, 1995, 1996), although the target of pAp inhibition was not identified. The role of methionine would then be to repress the sulfate assimilation pathway, thus reducing synthesis of pAp. The synthesis of enzymes required for sulfate assimilation (e.g. ATP sulfurylase that catalyzes

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the first step in sulfate assimilation) is strongly repressed by exogenous methionine in wild-type strains (Cherest *et al.*, 1971).

Overexpression of Hal2p suppresses Li^+ toxicity *in vivo* (Gläser *et al.*, 1993), and the activity of Hal2p was found to be strongly inhibited by submillimolar concentrations of Li^+ *in vitro* (Murguía *et al.*, 1995). Consistent with a role for pAp accumulation in Li^+ toxicity, methionine supplementation suppresses the cytotoxic effects of Li^+ in yeast (Gläser *et al.*, 1993; this study).

Enzymes with the same activity as Hal2p have been purified from a multitude of organisms, including mammals (Ramaswamy and Jakoby, 1987), and functional *HAL2* homologs have been isolated from *Escherichia coli* (Neuwald *et al.*, 1992), *Arabidopsis thaliana* (Quintero *et al.*, 1996) and rice (Peng and Verma, 1995). pApS is the sulfur donor for a host of sulfotransferase reactions (Falany, 1997, and references therein) and substantial generation of pAp is therefore to be expected in all organisms. Five sulfotransferases have been identified to date in humans (reviewed in Weinshilboum *et al.*, 1997).

RNase MRP is a ribonucleoprotein particle (RNP) with endoribonuclease activity, which cleaves the pre-rRNA at site A_3 in internal transcribed spacer 1 (ITS1) (Chu *et al.*, 1994; Lygerou *et al.*, 1994, 1996). RNase MRP also processes RNA complementary to the mitochondrial origin of replication *in vitro* (Chang and Clayton, 1987), although a function in mitochondrial DNA replication has not yet been demonstrated unambiguously *in vivo*. Since neither of these cleavages are essential for viability, RNase MRP, which is essential, must have additional substrates which have not yet been identified. We have performed a genetic screen for mutants which are synthetically lethal (sl) with *rrp2-1*, a temperature-sensitive (ts) mutation in the RNA component of RNase MRP (Dichtl and Tollervey, 1997). Synthetic lethality arises when the simultaneous inhibition of two interacting components in a biochemical pathway gives rise to a lethal phenotype that is not seen on inhibition of either single component. The screen identified a mutation in *HAL2* that is sl in combination with *rrp2-1*. Another sl interaction, between the exonuclease Xrn1p (*xrn1*-Δ) and RNase MRP (*rrp2-1*), is the basis of the sl interaction between *rrp2-1* and *hal2-1*. This arises because the accumulation of pAp in the *hal2-1* mutant inhibits Xrn1p activity. Furthermore, the sl interaction between Xrn1p and RNase MRP explains the toxicity of Li^+ to yeast cells. This suggests the testable hypothesis, that the inhibition of RNA processing enzymes may contribute to the diverse effects of Li^+ on neurobiology and development in higher eukaryotes.

Results

Synthetic lethality with RNase MRP identifies HAL2

We previously reported a genetic screen for mutants which are sl with a point mutation in the RNA component of RNase MRP RNA (*rrp2-1*) (Dichtl and Tollervey, 1997). Fifteen sl mutant strains were isolated, and one strain, SL158, displayed a tight cold-sensitive (cs) phenotype. We cloned a gene which complemented the cs phenotype and tested whether this also complemented the sl phenotype. To reduce background genetic effects of the mutagenesis, strain SL158 was first back-crossed twice to the parental strain CH1305, selecting for cold sensitivity. Eight plasmids which complemented the cs lethality of the resulting strain, YBD105, all contained overlapping regions of chromosome XV. Subcloning identified one gene, *HAL2/MET22*, that restored growth of strain YBD105 at 18°C (see Materials and methods).

Due to the sl mutation, growth of strain SL158 is dependent on the presence of a plasmid (pBD1) that carries a wild-type copy of the *RRP2* gene, as well as the *URA3* and *ADE3* marker genes. Since this strain contains a functional *URA3* gene, on pBD1, it is not viable on medium containing the toxic uracil analog 5-fluoroorotic acid (5-FOA). Complementation of the sl phenotype permits loss of the *RRP2* gene on pBD1 and therefore allows growth in the presence of 5-FOA. Figure 1B shows that strain SL158 was able to grow on medium containing 5-FOA when transformed with plasmids which carry the *LEU2* marker and either *HAL2* (pBD45) or *RRP2* (pBD4). *HAL2* therefore complemented both the cs and sl phenotypes, showing these to be due to the same mutation.

Overexpression of two protein components of RNase MRP, Snm1p and Pop3p, can suppress the *rrp2-1* allele (Schmitt and Clayton, 1994; Dichtl and Tollervey, 1997). Plasmids carrying *SNM1* (pBD11) or *POP3* (pBD10) allowed growth of SL158 on medium containing 5-FOA, showing that they suppressed the sl phenotype. However, neither these genes nor *RRP2* (pBD4) were able to complement the cs phenotype of SL158 (Figure 1C). Neither a plasmid carrying the *POP1* gene (pBD15), which encodes another protein component of RNase MRP (Lygerou *et al.*, 1994), nor the vector alone (pRS415) suppressed the cs or sl phenotypes.

Yeast strains mutant in *HAL2/MET22* have been reported to be auxotrophic for methionine (Masselot and de Robichon-Szulmajster, 1975). Figure 1D shows that SL158 was indeed non-viable on medium lacking methionine, and this phenotype was complemented only by the *HAL2* gene. A haploid yeast strain was constructed in which the *HAL2* gene is replaced by the *HIS3* marker (see Materials and methods). The *hal2::HIS3* mutant strain (*hal2*-Δ) was auxotrophic for methionine and was found to be cs-lethal (data not shown). Moreover, cold sensitivity, auxotrophy for methionine and exonuclease inhibition (see below) co-segregated in three tetrads analyzed from a diploid strain which was recovered from the second consecutive back-cross between SL158 and CH1305. We conclude that the cs mutation in strain SL158 lies in the *HAL2* gene and designate the mutant allele *hal2-1*. Furthermore, we conclude that *hal2-1* is sl with *rrp2-1*.

HAL2 mutant strains accumulate rRNA spacer fragments and pre-snoRNAs

Yeast strains carrying the *rrp2-1* ts allele show strong inhibition of pre-rRNA cleavage at site A_3 at 25°C and are non-viable at 37°C (Shuai and Warner, 1991; Lindahl *et al.*, 1992; Lygerou *et al.*, 1994). In an attempt to understand the molecular basis of the sl interaction between the *rrp2-1* and *hal2-1* alleles, pre-rRNA processing was analyzed in *hal2-1* and *hal2*-Δ strains.

Figure 2A shows the arrangement of the 35S pre-rRNA transcript which is processed to produce the 18S, 5.8S and 25S rRNAs. The early cleavages at sites A_0 and A_1

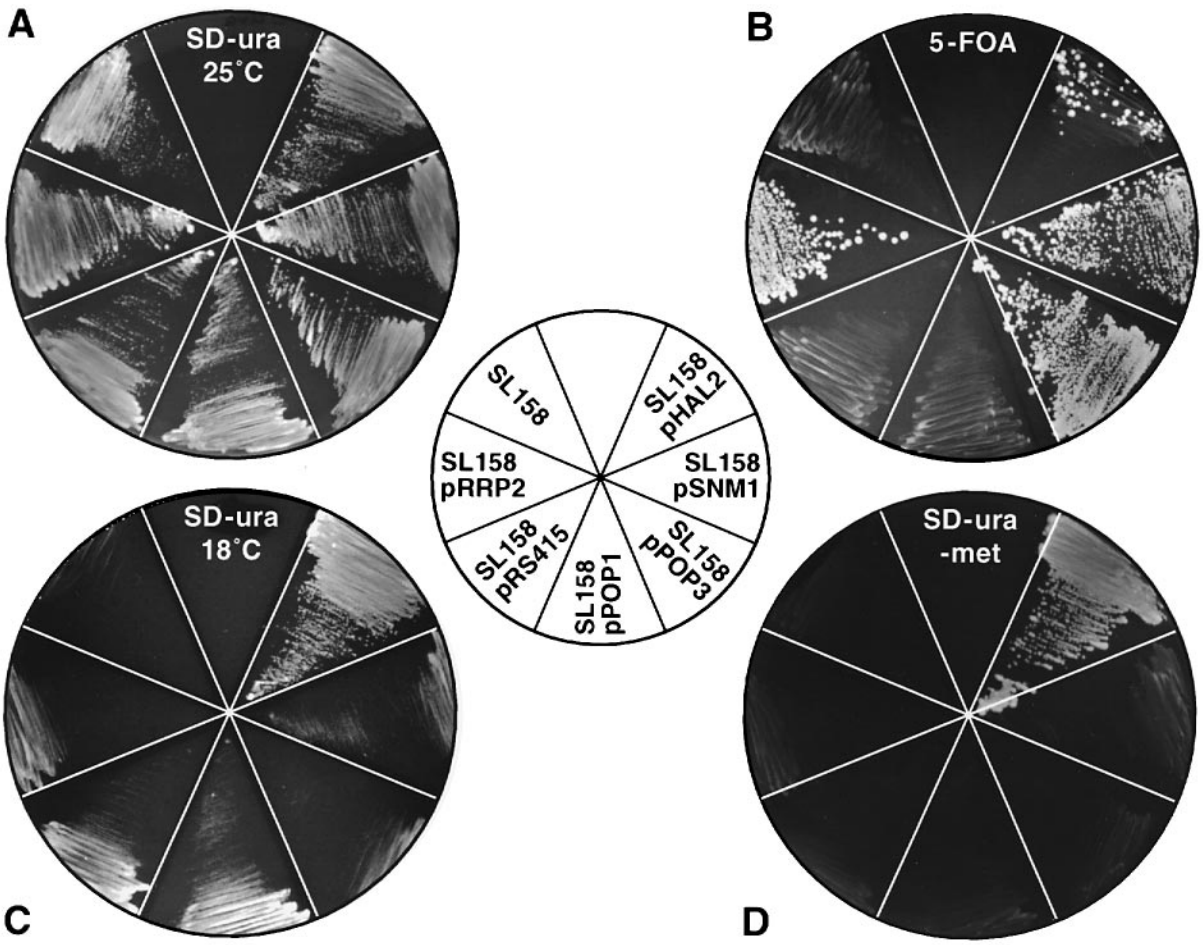

Fig. 1. *hal2-1* is synthetically lethal with *rrp2-1*. Strain SL158 carrying the *RRP2-URA3-ADE3* vector (pBD1) was co-transformed with plasmids *RRP2-LEU2-CEN* (pRRP2), empty vector (pRS415), *POP1-LEU2-CEN* (pPOP1), *POP3-LEU2–2*μ (pPOP3), *SNM1-LEU2–2*μ (pSNM1) and *HAL2-LEU2-CEN* (pHAL2). The resulting strains were streaked on SD –URA plates and incubated at 25 (**A**) or 18°C (**C**). Plates containing 5-FOA (**B**) and plates minus uracil and methionine (**D**) were incubated at 25°C.

release a 91 nucleotide fragment from the 5′ external transcribed spacer (5′ ETS). The *hal2-1* mutant strongly accumulated the A_0–A_1 fragment within 6 h after shift to 18°C (Figure 2B, probe a, lanes 3–6); accumulation of this fragment was non-conditional in the *hal2*-Δ strain (Figure 2B, lanes 7–10). Shorter fragments, which are likely to be degradation intermediates of the full-length fragment, also accumulated. Interestingly, we had observed a similar phenotype in strains carrying mutations in the 5′→3′ exonucleases Xrn1p and Rat1p (E.Petfalski, T.Dandekar, Y.Henry and D.Tollervey, submitted). The *xrn1*-Δ and the *rat1-1* single mutant strains showed mild accumulation of the A_0–A_1 fragment, but accumulation was much stronger in the *xrn1*-Δ, *rat1-1* double mutant (Figure 2B, lanes 11–16). This indicates that both exonucleases play roles in the degradation of this pre-rRNA region.

The pre-rRNA fragment which is released by the endonucleolytic cleavages at sites D and A_2 has been reported to require Xrn1p activity for its degradation (Stevens *et al.*, 1991). Consistent with this, we observed strong accumulation of this fragment in the *xrn1*-Δ and the *xrn1*-Δ, *rat1-1* strain, but not the *rat1-1* strain (Figure 2B, probe b). This fragment was slightly increased in the *hal2-1* strain at permissive temperature and accumulated strongly at 18°C (lanes 3–6). The *hal2*-Δ mutant also showed strong, non-conditional D–A_2 accumulation (lanes 7–10).

The next endonucleolytic cleavage event, further 3′ in ITS1, is performed by RNase MRP at site A_3 (Lygerou *et al.*, 1996), releasing the A_2–A_3 fragment. This fragment strongly accumulated in the *hal2-1* strain 6 h after shift to 18°C (Figure 2B, probe c, lanes 3–6), as well as in the *hal2*-Δ strain (lanes 7–10). Both exonuclease single mutant strains showed increased levels of A_2–A_3 (lanes 11–14) but the accumulation was much stronger in the double mutant strain, particularly after growth at the non-permissive temperature for *rat1-1* (lane 16).

Probe c also detected the accumulation of 5.8S which is 5′ extended to site A_2 (A_2–E) in the *hal2*-Δ mutant after shift to 18°C (Figure 2B, probe c lower, lanes 7–10). This RNA species is characteristic of mutants defective in components of RNase MRP (Lygerou *et al.*, 1994; Dichtl and Tollervey, 1997). This species did not accumulate in the *hal2-1* strain and the reason for its accumulation in the *hal2*-Δ strain is currently unclear.

A probe which is complementary to ITS1 3′ of cleavage site A_3 (Figure 2B, probe d) also detected the A_2–E fragment accumulated in the *hal2*-Δ mutant (lanes 7–10). In addition, probe d detected the RNA species labeled '*'

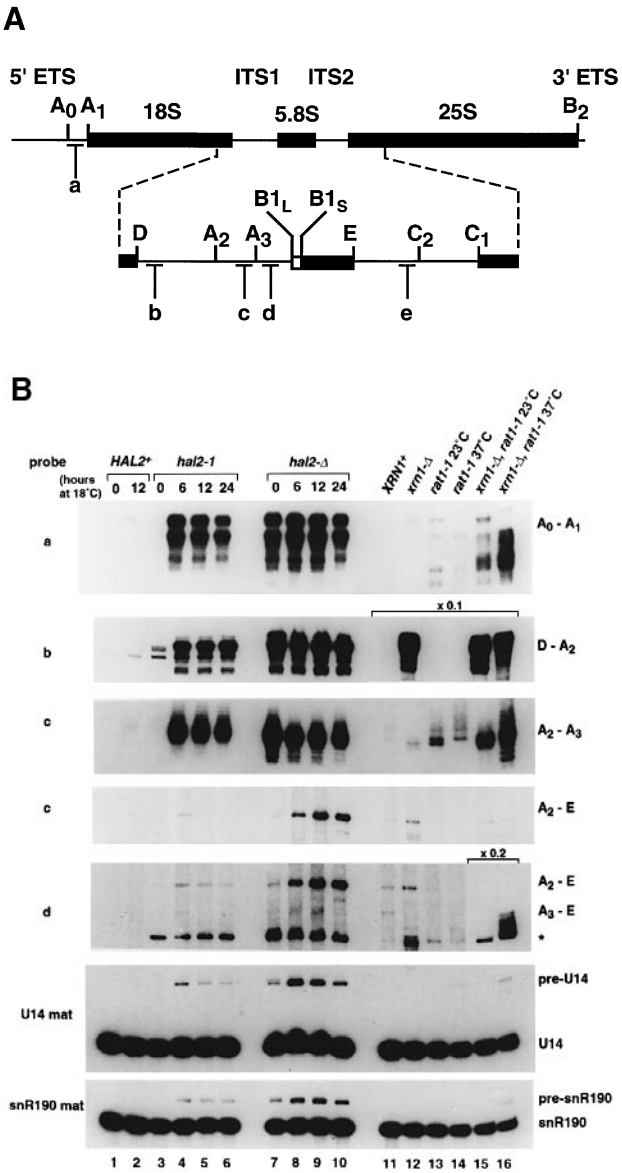

Fig. 2. *HAL2* mutants are inhibited in 5′→3′ exonuclease activity. (**A**) 35S pre-rRNA of *S.cerevisiae*. The major processing sites are indicated in upper case. Probes used for Northern hybridization and primer extension are labeled in lower case (a–e). (**B**) Northern analysis of total RNA extracted from a *hal2-1* strain (YBD105) carrying the empty vector pRS416 (*hal2-1*, lanes 3–6) or complemented by the *HAL2* gene (*HAL2*$^+$, lanes 1 and 2) and strain YBD128 carrying pRS416 (*hal2*-Δ, lanes 7–10) at 30°C, the permissive temperature (0 h lanes) and at intervals after transfer to 18°C, the non-permissive temperature (6–24 h lanes). RNA from strains mutant in the exonuclease Xrn1p (*xrn1*-Δ, D184) and an isogenic wild-type strain (*XRN1*$^+$, D185) was analyzed following growth at 30°C. Strains mutant in Rat1p (*rat1-1*, DAH18) or carrying both the *xrn1*-Δ and *rat1-1* mutations (966-c) were analyzed following growth at 25°C and after transfer to 37°C for 6 h. Probes used according to (A) are indicated on the left. U14mat and snR190mat are probes against the mature snoRNAs. The RNA species detected are indicated on the right. Differences in exposure time within one panel are indicated (×0.1 = 10 times shorter; ×0.2 = five times shorter).

in *xrn1*-Δ and *xrn1*-Δ, *rat1-1* strains (lanes 12 and 16). Previous analyses (Henry *et al.*, 1994) have shown this to represent 5.8S rRNA species which are 5′ extended to sites between A_3 and $B1_L$, due to inefficient exonuclease digestion from site A_3. These RNA species were also accumulated in the *hal2-1* and *hal2*-Δ mutant strains (lanes 3–10).

Normal 5′ maturation of the tandemly transcribed snoRNAs snR190 and U14, as well as the intron-encoded snoRNA U24, also requires the activities of Rat1p and, to a lesser extent, Xrn1p (E.Petfalski, T.Dandekar, Y.Henry and D.Tollervey, submitted). In the exonuclease mutant strains, discrete 5′ extended snoRNA precursors accumulated mildly but clearly, especially in the *xrn1*-Δ, *rat1-1* double mutant at 37°C (lane 16 and data not shown). Figure 2B shows that significant levels of unprocessed precursor to U14 and snR190 accumulated at the non-permissive temperature in the *hal2-1* and *hal2*-Δ strains. We also observed accumulation of pre-U24 RNA in both *hal2* mutant strains (data not shown). The processing of the precursor to 5S rRNA was unaltered by mutations in either *HAL2* or in the exonucleases. Similarly, no accumulation of other pre-rRNA spacer fragments, the 5′ ETS region that lies 5′ to site A_0 or the C_1–C_2 region of ITS2 was seen in either the *HAL2* or exonuclease mutants (data not shown). Processing of the primary transcripts of $tRNA_3^{Leu}$ and $tRNA_{UGG}^{Pro}$ did not exhibit a phenotype characteristic of RNase P inhibition in the *HAL2* or exonuclease mutant strains. However, the 5′ and 3′ processed but unspliced tRNA precursors were observed to be one to two nucleotides longer than in the wild-type; this was also observed in the *rat1-1* strain (data not shown).

Both Xrn1p and Rat1p are required for the turnover of several excised spacer fragments of the pre-rRNA and for the normal 5′ maturation of snoRNAs and 5.8S rRNA (E.Petfalski, T.Dandekar, Y.Henry and D.Tollervey, submitted). The phenotypes of the *hal2-1* and *hal2*-Δ mutant strains closely resemble that of the exonuclease mutants. We conclude that strains mutated in *HAL2* are inhibited in the activities of both 5′→3′ exonucleases.

pAp is an inhibitor of the in vitro activities of the two 5′→3′ exonucleases, Xrn1p and Rat1p

Hal2p is a 3′(2′),5′ bisphosphate nucleotidase which converts pAp into 5′ AMP and P_i (Murguía *et al.*, 1995). Since *HAL2* mutant strains are inhibited in 5′→3′ exonuclease activity, we considered the possibility that this effect might be mediated by increased cellular levels of pAp, which are normally below 0.1 mM and can increase up to 3 mM upon inhibition of Hal2p (Murguía *et al.*, 1996). To test this hypothesis, the activities of highly purified Xrn1p and Rat1p were analyzed in the presence of variable concentrations of pAp *in vitro*. Both enzymes were inhibited by pAp with either poly(A) or an arbitrarily chosen RNA as the substrate (see Materials and methods) (Figure 3A). pAp at a concentration of 0.1 mM inhibited the activity of the two enzymes by 40–65% and 1 mM inhibited in the range 65–85%. The inhibition of Xrn1p by pAp was not affected by poly(A) concentration, suggesting that the inhibition is not competitive (data not shown). Supplementation of the reaction with Mg^{2+} did not restore activity (data not shown), indicating that inhibition of the exonucleases was not due to chelation of Mg^{2+} by pAp.

Figure 3B shows the results obtained with other nucleotides. 5′ AMP or 3′ AMP (Figure 3B), ADP and ATP (data not shown) inhibited <10% at concentrations from 0.1 to 1 mM. pApS inhibited the reactions less effectively

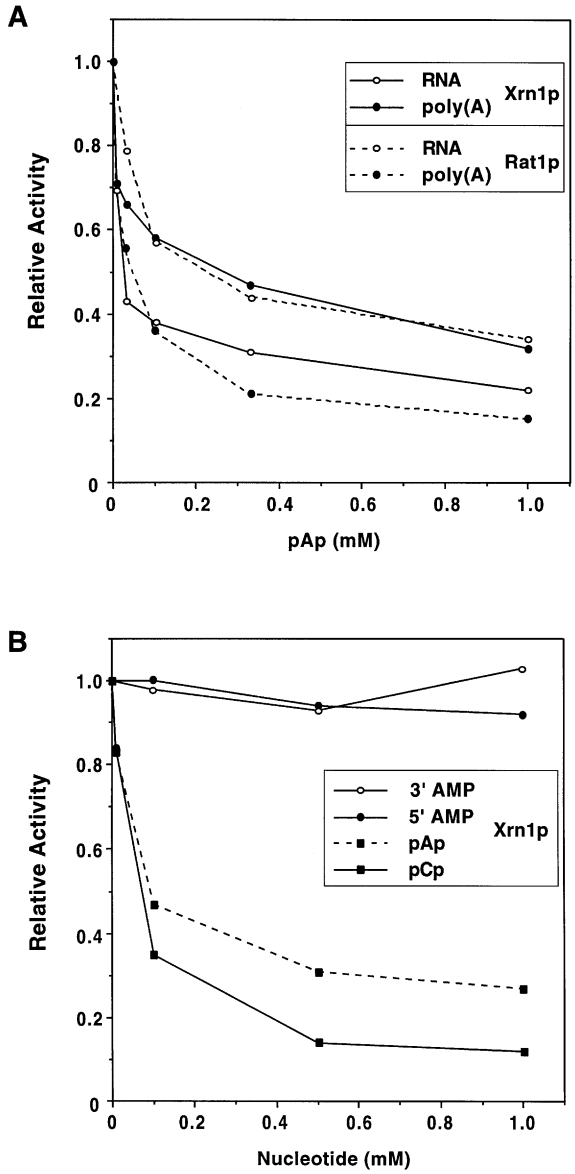

Fig. 3. pAp inhibits the exonuclease activities of Xrn1p and Rat1p *in vitro*. Exonuclease assays were performed using purified Xrn1p and Rat1p. (**A**) Relative exonuclease activity of Xrn1p (solid lines) and Rat1p (dashed lines) using poly(A) (●) or RNA (○) as substrates at different pAp concentrations. (**B**) Relative Xrn1p activity with poly(A) substrate upon increasing concentration of 5′ AMP (solid line, ●); 3′ AMP (solid line, ○); pAp (dashed line, ■); pCp (solid line, ■).

than pAp (data not shown). However, the supplier (Sigma) notes that pApS is both less stable and less pure than pAp, making the results obtained with pApS difficult to interpret. pCp inhibited slightly better than pAp (Figure 3B). pUp was as inhibitory as pAp (data not shown). These results show that the activities of Xrn1p and Rat1p were both strongly inhibited by nucleoside 3′,5′ bisphosphates. We conclude that the inhibition of exonuclease activity that we observe in *hal2* mutants *in vivo* is mediated by increased cellular pAp levels.

Lithium treatment of a wild-type yeast strain mimics the biochemical phenotype of hal2 mutants and inhibits RNase MRP activity

The enzymatic activity of Hal2p has been shown previously to be strongly inhibited by Li^+ *in vitro* (Murguía *et al.*, 1995) and *in vivo* (Murguía *et al.*, 1996). We therefore determined whether treatment with Li^+ salt *in vivo* results in inhibition of the 5′→3′ exonucleases in wild-type yeast strains. Since methionine and the overexpression of Hal2p have been reported to suppress the cytotoxic effects of Li^+ (Gläser *et al.*, 1993), we used synthetic minimal media which either contained or lacked methionine, and we also produced a wild-type yeast strain which carries an additional copy of the *HAL2* gene. The wild-type strain had a doubling time of 2.0 h, which increased to 10.5 h in medium containing 0.2 M LiCl and no methionine (Figure 4A). When the same strain was shifted to medium containing 0.2 M LiCl and methionine (20 mg/l), growth inhibition was strongly reduced and the strain grew with a doubling time of 2.8 h (Figure 4A). Overexpression of the *HAL2* gene also suppressed the Li^+-induced growth inhibition although to a slightly lesser extent (doubling time 3.4 h) (Figure 4A).

Northern analysis (Figure 4B, probe a) showed that the A_0–A_1 pre-rRNA spacer fragment accumulated very strongly in medium containing 0.2 M LiCl and no methionine (Figure 4B, lanes 9–12). This accumulation was suppressed completely in the presence of methionine (Figure 4B, lanes 4–8) and strongly reduced by overexpression of *HAL2* (Figure 4B, lanes 13–16), particularly after prolonged growth. The degree of accumulation of this fragment induced by Li^+ treatment was comparable with the accumulation of the same fragment in the *hal2-1* strain at 18°C (Figure 4B, lane 3). Similarly, we observed strong accumulation of the D–A_2 (probe b) and A_2–A_3 (probe c) fragments in medium containing 0.2 M LiCl and no methionine. These phenotypes were suppressed completely by the presence of methionine in the medium (lanes 5–8) and strongly reduced by an additional copy of the *HAL2* gene (lanes 13–16) in medium containing no methionine. Probes against the mature forms of the snoRNAs U14 and snR190 also detected Li^+-induced accumulation of pre-U14 and pre-snR190 RNAs in the absence of methionine (Figure 4B, lanes 9–12). Again, these RNA species were not detected in medium containing Li^+ and methionine (lanes 5–8) and were strongly reduced when *HAL2* was overexpressed (lanes 13–16). The same phenotype was observed for the intron-encoded snoRNA U24 (data not shown).

The 5.8S rRNA 5′ extended to site A_3 (A_3–E) and the shorter '*' RNA species (Figure 4B, probe d) accumulated in medium containing Li^+ and no methionine. Its accumulation was strongly suppressed by methionine (lanes 5–8) or pHAL2 (lanes 13–16).

Probes c and d detect 5.8S rRNA which is 5′ extended to site A_2 (A_2–E). The accumulation of this RNA species was also induced by Li^+ (Figure 4B, lanes 9–12), strongly indicating that cleavage of pre-rRNA at site A_3, and therefore the activity of RNase MRP, was inhibited. Strikingly, however, neither methionine (lanes 5–8) nor the overexpression of *HAL2* (lanes 13–16) could suppress its accumulation. This species also did not accumulate in the *hal2-1* mutant (lanes 1–3).

To show that the accumulation of the A_2–E RNA is indeed due to inhibition of cleavage at site A_3, we analyzed RNA from the Li^+-treated wild-type strain by primer extension, using oligonucleotide e (see Figure 2A), which hybridizes to a region of the pre-rRNA 3′ of cleavage site

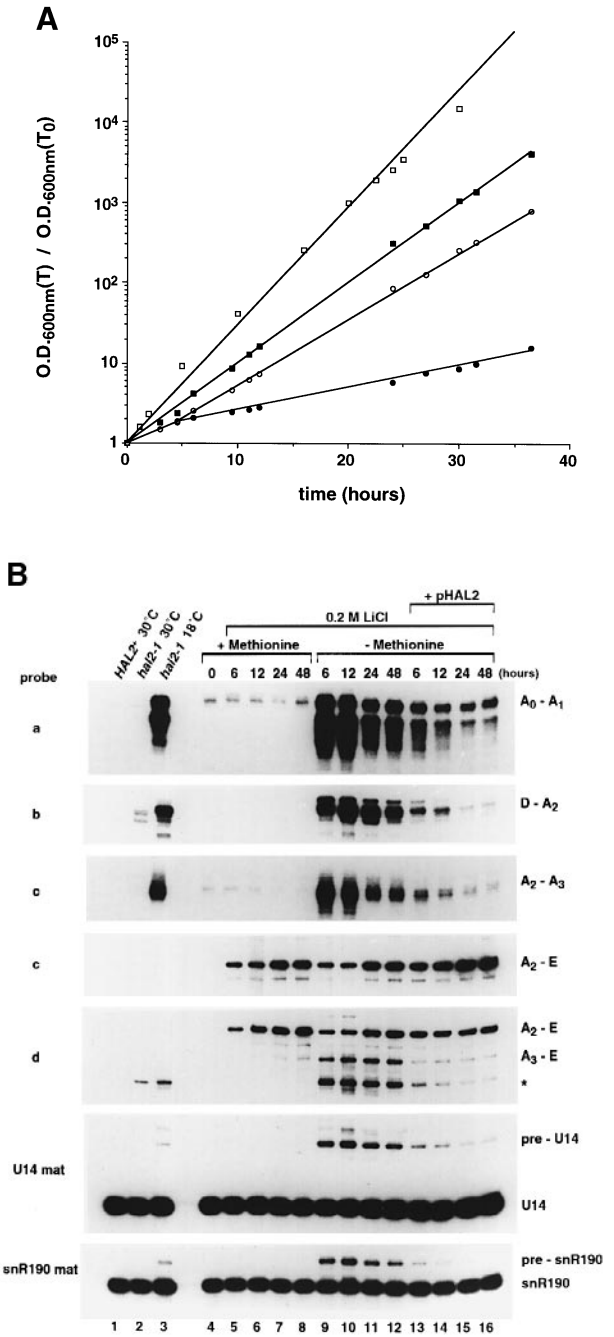

Fig. 4. Lithium induces inhibition of the 5′→3′ exonucleases and also inhibits RNase MRP activity. (**A**) Growth curves of wild-type strain BWG1-7A carrying an empty vector (pRS416) before (□) and after shift to medium containing 0.2 M LiCl without methionine (●) or 0.2 M LiCl with methionine (■). The same strain carrying an additional copy of *HAL2* (pHAL2) was shifted to medium containing 0.2 M LiCl without methionine (○). (**B**) Northern hybridization analysis on total RNA extracted from strains grown as described in (A). As reference, RNA from the *hal2-1* mutant strain (see Figure 2B) was analyzed in parallel. Probes used (see Figure 2B) are indicated on the left; RNA species detected are indicated on the right.

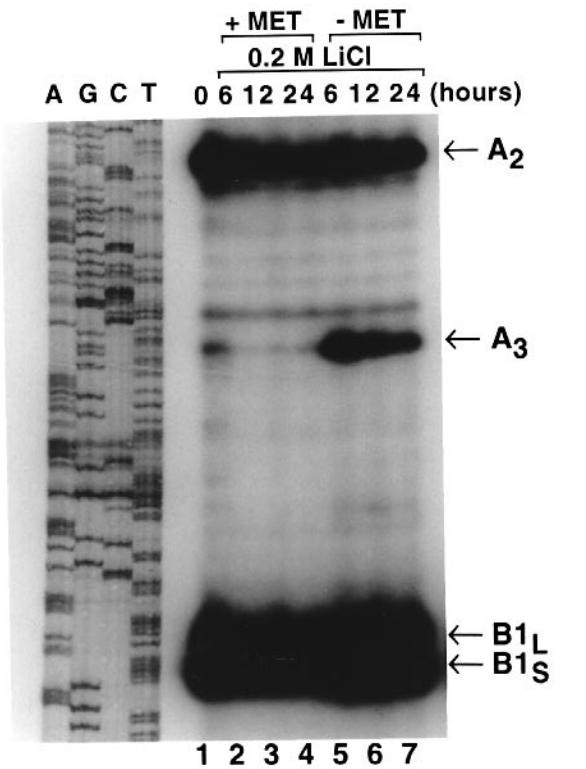

Fig. 5. Lithium inhibits pre-rRNA cleavage at site A_3. Primer extension analysis through site A_3, employing oligonucleotide e (see Figure 2A) on total RNA derived from a wild-type strain (BWG1-7A) growing in medium lacking LiCl (0 h lane) and after transfer to medium containing 0.2 M LiCl (6–24 h lanes) with and without methionine (± MET). RNA samples are identical to Figure 4B. The processing sites revealed as major primer extension stops are indicated on the right (see Figure 2A). DNA sequencing reactions on a wild-type rDNA plasmid using the same primer are indicated. The autoradiogram is overexposed in order to visualize the wild-type level of the weak primer extension stop at site A_3 (lane 1).

C_2. Figure 5 shows that the primer extension stop at site A_3 was strongly reduced when cells were grown in medium containing Li^+ and methionine (Figure 5, lanes 2–4), as compared with wild-type levels (Figure 5, lane 1). In contrast, the A_3 signal was dramatically increased when cells were grown in Li^+ and no methionine (Figure 5, lanes 5–7), reflecting the accumulation of the A_3–E RNA that was observed by Northern analysis (Figure 4B, lanes 9–12). On lithium treatment, cleavage at A_3 by RNase MRP is reduced, but the subsequent processing of the residual A_3-cleaved pre-rRNA is also strongly reduced due to the inhibition of the Xrn1p and Rat1p exonucleases. The primer extension stop at site A_2 remains unaffected by lithium treatment (Figure 5, lanes 1–7).

A_3 cleavage is required for the formation of the major, short form of 5.8S rRNA, $5.8S_S$ (Henry *et al.*, 1994). Consistent with this, we observed under-accumulation of the major, short form of 5.8S rRNA ($5.8S_S$) after 48 h of lithium treatment (in the presence of methionine) due to inhibition of A_3 cleavage (data not shown).

As was found for mutants defective in Hal2p or the 5′→3′ exonucleases, the processing of the precursor to 5S rRNA was unaltered by Li^+ treatment and no accumulation of other pre-rRNA spacer fragments, the 5′ ETS region that lies 5′ to site A_0 or the C_1–C_2 region of ITS2 was seen (data not shown). Processing of the primary transcripts of $tRNA_3^{Leu}$ and $tRNA_{UGG}^{Pro}$ did not exhibit a phenotype characteristic of RNase P inhibition upon Li^+ treatment; however, the 5′ and 3′ processed but unspliced tRNA precursors were 1–2 nucleotides longer than in untreated cells, a phenomenon which was also observed in the *hal2-1* and *rat1-1* strains (data not shown).

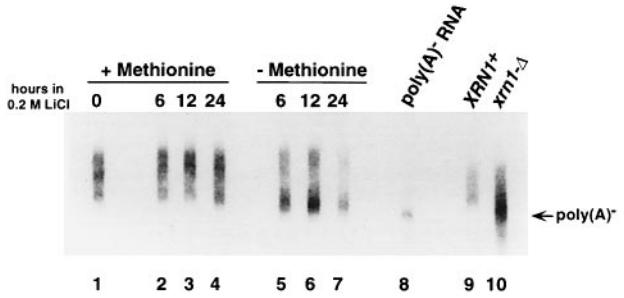

Fig. 6. Lithium treatment results in accumulation of deadenylated *MFA2* mRNA. Northern analysis of total RNA derived from a wild-type strain (BWG1-7A) growing in medium lacking LiCl (0 h lane) and after transfer to medium containing 0.2 M LiCl (6–24 h lanes) with and without methionine (± Methionine). RNA was also analyzed from the *xrn1*-Δ (D184) and the otherwise isogenic *XRN1*$^+$ strain (D185); the same RNA preparations were used as in Figures 2B and 4B. As a size marker, deadenylated RNA from strain BWG1-7A was separated on the same gel [poly(A)$^-$ RNA]. The filter was probed with an oligonucleotide against *MFA2* mRNA. Migration of deadenylated *MFA2* mRNA is indicated.

Li^+ therefore induced a biochemical phenotype in a wild-type yeast strain which resembled that seen in the *HAL2* mutant strains. We conclude that the inhibition of Hal2p by Li^+ leads to increased cellular pAp levels which then inhibit the 5′→3′ exonucleases, Xrn1p and Rat1p. Consistent with this proposal, methionine suppressed the inhibition of the exonucleases, presumably by repressing sulfate assimilation (Cherest *et al.*, 1971), thus reducing production of pAp. Overexpression of *HAL2* also suppressed the exonuclease inhibition, probably by reducing the cellular pAp concentration due to increased enzyme levels (Murguía *et al.*, 1995). In addition, Li^+ inhibits the activity of RNase MRP in cleavage of the pre-rRNA at site A_3. This inhibition does not appear to be mediated by pAp, since neither methionine supplementation nor *HAL2* overexpression could rescue the Li^+-induced phenotype.

In the major mRNA decay pathway in yeast, deadenylation is followed by decapping; this then exposes the body of the mRNA to 5′→3′ degradation by Xrn1p (Muhlrad *et al.*, 1994). *MFA2* encodes a well-studied mRNA which follows this decay pathway (Muhlrad *et al.*, 1994). Figure 6 shows the effects of Li^+ treatment on the size distribution of the *MFA2* mRNA; the heterogeneity represents the distribution of poly(A) tail lengths. Growth in medium containing 0.2 M LiCl and no methionine led to the accumulation of shortened forms of *MFA2* mRNA (Figure 6, lanes 5–7). These migrated faster than the shortest mRNA species detected in the wild-type control (Figure 6, lane 1), indicating that they had poly(A) tails shorter than the length at which decapping and Xrn1p-mediated degradation are normally triggered. The size of these mRNA species was, however, slightly longer than poly(A)$^-$ RNA (Figure 6, lane 8), consistent with reports that the final 10–12 adenine residues of the poly(A) tail are removed more slowly than the initial, fast deadenylation. The effects of Li^+ on the *MFA2* mRNA were suppressed in medium supplemented with methionine (Figure 6, lanes 2–4). As expected (Muhlrad *et al.*, 1994), the *xrn1*-Δ strain strongly accumulated the short *MFA2* mRNA (Figure 6, lane 10). This indicates that Li^+ can strongly alter mRNA degradation rates via the pAp-mediated inhibition of Xrn1p.

The exonuclease Rat1p was identified as a ts mutant allele which accumulates poly(A)$^+$ RNA in the nucleus at non-permissive temperature (Amberg *et al.*, 1992). Although the function of the exonuclease in mRNA export is unclear, we tested whether Li^+ inhibition of Rat1p activity also results in nuclear poly(A)$^+$ accumulation. *In situ* hybridization experiments were performed using yeast cells from the wild-type strain BWG1-7A after growth in medium containing 0.2 M LiCl and no methionine for 6 h and the *hal2*-Δ mutant strain after growth for 6 h at the non-permissive temperature (18°C). Using fluorescein isothiocyanate (FITC)-labeled oligo(dT) as a probe, no accumulation of poly(A)$^+$ RNA in the nucleus was detected in either the Li^+-treated wild-type strain or the *hal2*-Δ mutant at 18°C (data not shown). In contrast, a yeast strain mutated in the nuclear pore protein Nup133p (Doye *et al.*, 1994) gave clear poly(A)$^+$ accumulation at the restrictive temperature (data not shown). We conclude that Li^+ did not inhibit nuclear mRNA export.

rrp2-1 strains are lithium hypersensitive, and the combination of the *xrn1-Δ* and *rrp2-1* alleles results in synthetic lethality

The pAp-independent inhibition of RNase MRP activity by Li^+ prompted us to test whether mutants in the RNA component of RNase MRP were hypersensitive to Li^+. Figure 7A shows that growth of the *rrp2-1* mutant strain (YBD39) was strongly inhibited compared with the isogenic *RRP2* wild-type strain (YBD40) on plates containing increasing concentrations of LiCl (50, 100 and 200 mM LiCl, in the presence of 20 mg/l methionine). In contrast, the *hal2*-Δ strain (YBD128) was not inhibited in growth relative to the isogenic *HAL2* sister strain (YBD127) on these media. In a related experiment (Figure 7B), we tested the growth of the *rrp2-1* strain used for sl screening, YBD1, on 5-FOA (which selects against the *RRP2-URA3-ADE3* plasmid; pBD1) and 5-FOA supplemented with 0.2 M LiCl; both media also contained methionine. Strain YBD1 was viable on medium containing 5-FOA alone but unable to grow in the presence of 5-FOA + LiCl. This shows that the *rrp2-1* strain requires the *RRP2* gene on the plasmid in order to grow in the presence of LiCl. The wild-type control strain BWG1-7A showed only slightly reduced growth on 5-FOA + 0.2 M LiCl compared with 5-FOA alone, indicating that the inability of strain YBD1 to grow on 5-FOA + 0.2 M LiCl was not simply due to a reduced growth rate. Since methionine was present in these plates, the exonuclease defects should be suppressed (see Figure 4B). These results show that the *rrp2-1* mutant strain is hypersensitive to Li^+ and that this is not mediated by the Li^+ inhibition of Hal2p, consistent with the direct inhibition of RNase MRP activity by Li^+.

Our finding that *HAL2* mutants are inhibited in 5′→3′ exonuclease activity, together with the observation that *hal2-1* is sl with *rrp2-1*, prompted us to test for an sl interaction between *rrp2-1* and *xrn1*-Δ. We therefore disrupted the non-essential *XRN1* gene in the strain used for screening for synthetic lethality with *rrp2-1* (YBD1). Figure 7C shows that the resulting strain, YBD125, did not grow on 5-FOA, indicating that viability of the strain depended on the wild-type *RRP2* gene, which was present

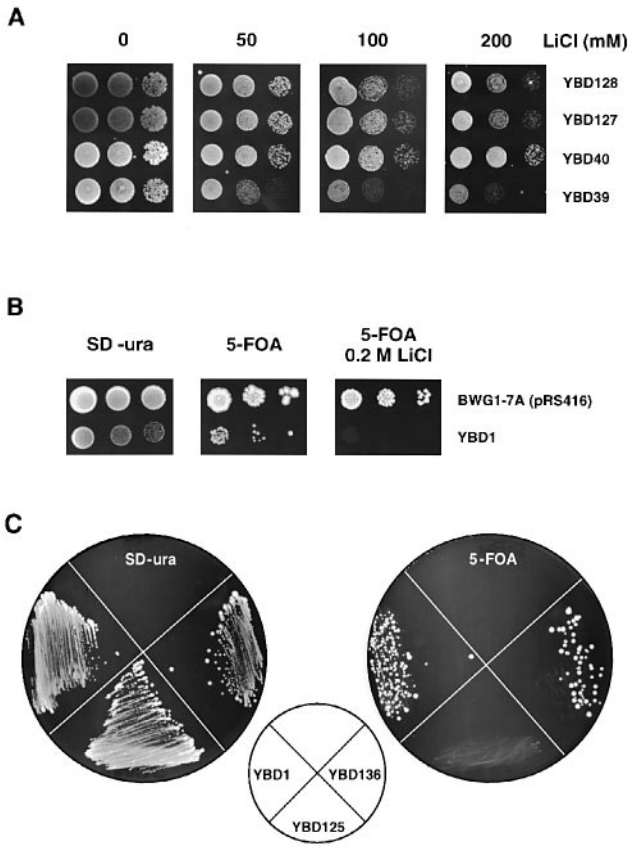

Fig. 7. *rrp2-1* strains are lithium hypersensitive and the combination of the *xrn1*-Δ and *rrp2-1* alleles results in synthetic lethality. (**A**) The *hal2*-Δ (YBD128) and the *rrp2-1* (YBD39) strains as well as the otherwise isogenic wild-type strains (YBD127 and YBD40) were grown in YPD and diluted to $OD_{600\ nm}$ of 0.4. Aliquots of 5 μl from 10-fold serial dilutions were spotted on plates lacking lithium, or containing 50, 100 and 200 mM LiCl. All plates contained methionine and were incubated at 25°C. The growth shows that the *rrp2-1* strain is hypersensitive to LiCl whereas the *hal2-1* strain is not. (**B**) Yeast strains YBD1 (*rrp2-1*) carrying pBD1 (*RRP2-URA3-ADE3-CEN*) and the wild-type strain BWG1-7A (carrying the empty vector pRS416) were grown to saturation in SD –URA medium and 5 μl of 10-fold serial dilutions were spotted on SD –URA plates, 5-FOA plates and 5-FOA plates containing 0.2 M LiCl. All plates contained methionine and were incubated at 25°C. The *rrp2-1* strain is unable to lose the *RRP2-URA3-ADE3-CEN* plasmid in the presence of 0.2 M LiCl, as shown by its failure to grow on medium containing 5-FOA which selects against the *URA3* marker on the plasmid. (**C**) Yeast strains YBD1 (*rrp2-1*), carrying vector pBD1 (*RRP2-URA3-ADE3-CEN*), YBD125 (*rrp2-1* and *xrn1::LEU2*), carrying vector pBD1 (*RRP2-URA3-ADE3-CEN*) and YBD136 (*hal2-1* and *xrn1::LEU2*), carrying vector pBD38 (*HAL2-URA3*), were streaked on SD –URA plates and on plates containing 5-FOA; incubation was at 25°C. Growth of the *xrn1*-Δ, *rrp2-1* strain (YBD125) is dependent on the presence of the *RRP2-URA3* plasmid (pBD1), as shown by its failure to grow on medium containing 5-FOA, demonstrating that the *xrn1*-Δ and *rrp2-1* mutations are synthetic lethal. The *hal2*-Δ and *xrn1*-Δ mutations in YBD136 are not synthetic lethal.

on the *URA3*, *ADE3* plasmid (pBD1). We conclude that the combination of the *xrn1*-Δ and the *rrp2-1* alleles results in synthetic lethality.

We also disrupted the *XRN1* gene in a *hal2-1* mutant strain which carries the *HAL2* gene on a *URA3* plasmid (pBD38). Viability of the resulting strain (YBD136) did not depend on the presence of a wild-type copy of the *HAL2* gene, as shown by its ability to grow on 5-FOA (Figure 7C). The *hal2-1* and *xrn1*-Δ alleles are, therefore,

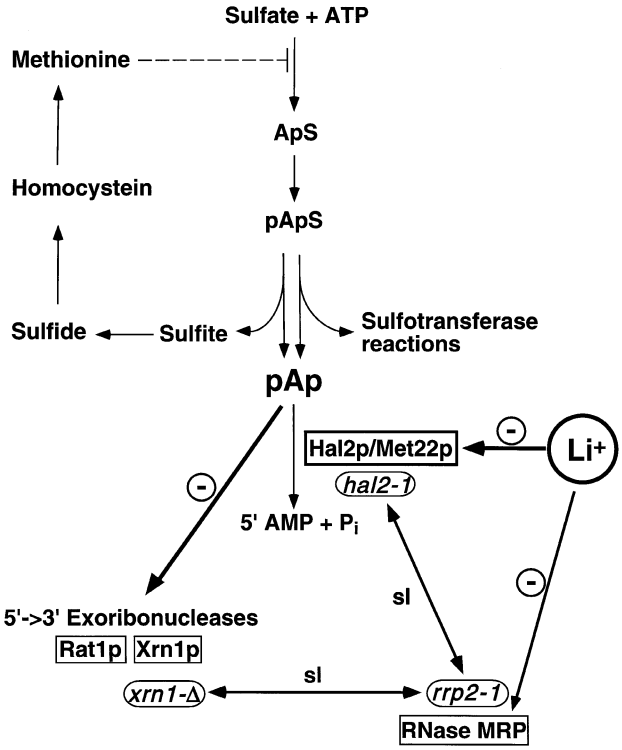

Fig. 8. Schematic representation of the inhibitory effects of lithium on RNA processing enzymes. Sulfate is activated by formation of pApS, which is then reduced to sulfite by pApS reductase, releasing pAp as a side product. Methionine, one end product of sulfate assimilation, represses the sulfate assimilation pathway (indicated by dashed line) and thus pAp production. In addition, sulfotransferases use pApS as sulfur donor, contributing to pAp production. A mutation in Hal2p (*hal2-1*) leads to accumulation of pAp, resulting in the inhibition of the exonucleases Rat1p and Xrn1p (indicated by the minus sign). A deletion of *XRN1* (*xrn1*-Δ) is synthetically lethal (sl) with a mutation in RNase MRP RNA (*rrp2-1*). We, therefore, conclude that the sl interaction originally observed between *rrp2-1* and *hal2-1* is a consequence of Xrn1p inhibition. The lethality of Li^+ treatment is due to synergistic toxicity between Hal2p and RNase MRP. Li^+ inhibition of Hal2p leads to the accumulation of pAp, which inhibits the exonucleases Xrn1p and Rat1p. Li^+ also inhibits the activity of RNase MRP, by a mechanism which is independent of pAp.

not sl with each other, consistent with our conclusion that the *hal2-1* mutation acts epistatically in the inhibition of Xrn1p. We conclude that the initially identified sl interaction between the *rrp2-1* and the *hal2-1* alleles is caused by indirect (pAp-mediated) inhibition of Xrn1p, which then results in synthetic lethality with *rrp2-1*. Moreover, following treatment with Li^+, the pAp-mediated inhibition of Xrn1p (due to Li^+ inhibition of Hal2p) and pAp-independent Li^+ inhibition of RNase MRP activity results in 'synergistic toxicity', which forms the basis of the inhibition of growth by Li^+ (Figure 8).

Discussion

We have undertaken an sl screen with a point mutation in the RNA component of RNase MRP *(rrp2-1)* in order to identify new genes which functionally and/or physically interact with RNase MRP. One of the sl strains, SL158, displayed a tight cs-lethal phenotype. The *HAL2* gene was cloned by complementation of this cs-lethality and was found also to complement the sl phenotype, making it

very probable that both are due to the same mutation. Strains carrying a complete deletion of the *HAL2* gene were found also to be cs-lethal and are methionine auxotrophs. Strain SL158 is a methionine auxotroph and this phenotype was complemented by *HAL2*. Genes which act as multicopy suppressors of the *rrp2-1* mutation, *SNM1* and *POP3*, suppressed the sl phenotype of SL158, but did not suppress the cs phenotype or methionine auxotrophy. We conclude that strain SL158 carries a mutation in *HAL2* which leads to synthetic lethality with a point mutation in the RNase MRP RNA; the mutant allele was designated *hal2-1*. None of the 14 other sl strains identified in the genetic screen are methionine auxotrophs, indicating that only SL158 has a mutation in *HAL2*.

Following transfer of the *hal2-1* strain to the non-permissive temperature, we observed strong accumulation of several pre-rRNA spacer fragments, from the A_0–A_1, D–A_2 and A_2–A_3 regions. 5′ Extended forms of several snoRNAs tested, U14, snR190 and U24, also appeared. Non-conditional accumulation of these RNA species was observed in the *hal2*-Δ strain. A requirement for the 5′→3′ exonuclease, Xrn1p, in the degradation of the D–A_2 pre-rRNA spacer fragment had been reported previously (Stevens *et al.*, 1991). In addition, both the Rat1p and Xrn1p exonuclease activities are required for normal degradation of the A_0–A_1 and A_2–A_3 pre-rRNA fragments and for the normal 5′ maturation of the snoRNAs U14, snR190 and U24 (E.Petfalski, T.Dandekar, Y.Henry and D.Tollervey, submitted). The mature 5′ end of the major, short form of 5.8S rRNA ($5.8S_S$) is also generated by an exonuclease activity that requires Xrn1p and Rat1p, following cleavage of the pre-rRNA at site A_3 by RNase MRP (Henry *et al.*, 1994). We observed the accumulation of 5′ extended forms of $5.8S_S$ rRNA in the *HAL2* mutant strains. Both mutant strains accumulated mainly the RNA labeled '*' in Figure 2B, which has its 5′ end in the region between the A_3 and $B1_L$ processing sites and is characteristic of the inhibition of Xrn1p and Rat1p (Henry *et al.*, 1994), together with low levels of the species which are 5′ extended to site A_3. Taken together, we conclude that the activities of both Xrn1p and Rat1p are strongly inhibited in the *hal2* mutant strains.

Hal2p exhibits a 3′(2′),5′ bisphosphate nucleotidase activity which converts pAp to 5′ AMP, and inhibition of Hal2p results in strong accumulation of pAp (Murguía *et al.*, 1995, 1996). This suggested that the 5′→3′ exonucleases might be inhibited directly by pAp in *hal2* mutants. Exonuclease assays using purified Xrn1p and Rat1p showed that pAp strongly inhibited the activity of both enzymes *in vitro*. About 70% inhibition was observed with 1 mM pAp, while little inhibition was seen at 0.1 mM or below; the normal intracellular pAp concentration in yeast cells is <0.1 mM (Murguía *et al.*, 1996). pApS also inhibited, although to a lesser extent; and other nucleoside 3′,5′ bisphosphates tested, pUp and pCp, are also inhibitory. No *in vitro* inhibition of the exonucleases was seen with other nucleotides tested (5′ AMP, 3′ AMP, ATP or ADP) at a concentration of 1 mM. We conclude that the inhibition of Xrn1p and Rat1p activities in the *hal2* mutants is due to increased cellular levels of pAp.

The observations that Hal2p overexpression confers salt tolerance on yeast and also suppresses Li^+ toxicity (Gläser *et al.*, 1993, and data presented here), together with the sensitivity of Hal2p activity to Li^+ inhibition *in vitro*, support the view that Hal2p is the most sensitive target of Li^+ inhibition *in vivo*. The *in vitro* enzymatic activity of Hal2p is non-competitively inhibited by submillimolar amounts of Li^+ (50% inhibition at 0.1 mM Li^+) (Murguía *et al.*, 1995). Consistent with this, the intracellular concentration of pAp increases from <0.1 mM to 2–3 mM upon Li^+ treatment of wild-type cells (Murguía *et al.*, 1996). We observed strong inhibition of the Xrn1p and Rat1p activities at 1 mM pAp *in vitro*. Li^+ treatment of wild-type yeast strains also resulted in strong inhibition of all the RNA processing reactions known to require the activities of Xrn1p and Rat1p. We conclude that Li^+ inhibition of Hal2p results in increased cellular levels of pAp which in turn inhibit the exonucleases. Inhibition of Xrn1p is not, in itself, the basis of Li^+ toxicity, since Xrn1p is not required for viability. Rat1p is essential, but of the biochemical defects so far observed in *rat1* mutant strains the only one that would be expected to cause lethality is the accumulation of nuclear poly(A)$^+$ RNA (Amberg *et al.*, 1992). We did not detect nuclear poly(A)$^+$ RNA accumulation in Li^+-treated wild-type cells or in the *hal2*-Δ mutant strain at non-permissive temperature, indicating that this is also not the sole basis of Li^+ toxicity.

The *in vivo* activity of RNase MRP, i.e. cleavage of pre-rRNA at site A_3, was inhibited by Li^+. This is independent of pAp since neither methionine supplementation nor overexpression of Hal2p suppressed the biochemical phenotype. Since *rrp2-1* mutant strains, which have a single nucleotide substitution in the MRP RNA (Chu *et al.*, 1994), are Li^+ hypersensitive even in the presence of methionine, it is very likely that RNase MRP is inhibited directly by Li^+. However, the inhibition of RNase MRP by Li^+ does not directly result in lethality, since Li^+ toxicity in cells can be relieved by overexpression of Hal2p.

These observations, together with the isolation of *hal2-1* as a mutation which is sl with a point mutation in RNase MRP, suggested that the toxicity of Li^+ is due to the simultaneous inhibition of Hal2p and RNase MRP. Since there is no obvious direct connection between the activities of RNase MRP and Hal2p, we speculated that the real basis of the lethality was an sl interaction between RNase MRP and the 5′→3′ exonucleases; increased pAp levels in the sl strain, SL158, might inhibit Xrn1p resulting in lethality in combination with the *rrp2-1* allele. Consistent with this model, strain SL158 showed substantial inhibition of the exonucleases even at 24°C, the temperature at which the sl screen was performed (data not shown). The combination of the *xrn1*-Δ and the *rrp2-1* alleles was therefore tested and, indeed, resulted in synthetic lethality. This previously unsuspected genetic interaction between Xrn1p and RNase MRP underlies the toxicity of Li^+. Xrn1p activity is blocked indirectly by pAp accumulation, due to Li^+ inhibition of Hal2p, while RNase MRP is inhibited directly, evoking an sl interaction (see Figure 8). This model is strongly supported by the Li^+ hypersensitivity of the *rrp2-1* strains. It remains possible that pAp inhibition of Rat1p also contributes to lethality. The conclusion is that there is not a single target of lithium toxicity in yeast; lethality arises via the simultaneous inhibition of two components, where inhibition of neither

Table I. Yeast strains

Strain	Genotype	Reference/Source
YBD1	*MATα, ade2, ade3, leu2, his3, trp1, ura3, rrp2-1,* [pRRP2-URA3-ADE3]	Dichtl and Tollervey (1997)
YBD39	*MATα, ade2, ade3, his3, leu2, trp1, ura3, rrp2-1*	this work
YBD40	*MATα, ade2, ade3, his3, leu2, trp1, ura3*	this work
YBD105	*MAT**a**, ade2, ade3, leu2, lys2, ura3, hal2-1*	this work
YBD125	*MATα, ade2, ade3, leu2, his3, trp1, ura3, rrp2-1, xrn1::LEU2* [pRRP2-URA3-ADE3]	this work
YBD127	*MAT**a**, ade2-1, his3-Δ200, leu2-3,112, trp1-1, ura3-1, can1-100*	this work
YBD128	*MAT**a**, ade2-1, his3-Δ200, leu2-3,112, trp1-1, ura3-1, can1-100, hal2::HIS3*	this work
YBD136	*MAT**a**, ade2, ade3, leu2, lys2, ura3, hal2-1, xrn1::LEU2* [pHAL2-URA3]	this work
SL158	*MATα, ade2, ade3, leu2, his3, trp1, ura3, rrp2-1, hal2-1* [pRRP2-URA3-ADE3]	this work
CH1305	*MAT**a**, ade2, ade3, leu2, lys2, ura3*	C.Holm (Harvard, USA)
BWG1-7A	*MAT**a**, ade1-100, his4-519, leu2-3,112, ura3-52*	L.Guarente (MIT, USA)
966-1c	*MAT**a**, ura3-52, xrn1::URA3, rat1-1*	Henry *et al*. (1994)
D184	*MAT**a**, ade2-1, ura3-52, his3-11, trp1-1, xrn1::URA3*	S.Kearsey (Oxford, UK)
D185	*MAT**a**, ade2-1, ura3-52, his3-11, trp1-1*	S.Kearsey (Oxford, UK)
DAH18	*MATα, leu2-Δ1, ura3-52, his3-Δ200, rat1-1*	Henry *et al*. (1994)/ C.Cole (Dartmouth, USA)

individual component is lethal. We refer to this phenomenon as synergistic toxicity.

Xrn1p is the major exonucleolytic activity which is required for the turnover of mRNAs (Muhlrad *et al*., 1994). We have shown that this function of Xrn1p is also inhibited by Li^+-induced pAp accumulation, resulting in the accumulation of deadenylated mRNA. Mutations in *XRN1* can result in pleiotropic effects and, consequently, the gene has been identified independently in a number of different genetic screens as *SEP1*, *DST2*, *KEM1*, *RAR5* and *XRN1* (reviewed in Kearsey and Kipling, 1991). Whether all of these phenotypes can be attributed directly to alterations in mRNA turnover or other RNA processing defects is not yet established. RNase MRP cuts site A_3 in the pre-rRNA but this function is non-essential for cell viability (Henry *et al.*, 1994), whereas all known components of the RNase MRP RNP are essential. We therefore concluded that RNase MRP has additional, as yet unidentified, substrates (Dichtl and Tollervey, 1997). The biochemical basis of the sl interaction between Xrn1p and RNase MRP is also unclear. Two obvious possibilities are that they collaborate in the synthesis of some (as yet unidentified) essential RNA species, or that they collaborate in mRNA turnover. No mRNA turnover pathway in yeast has been shown to involve an endonucleolytic cleavage, but several examples are known from higher eukaryotes. In *E.coli*, the endonucleases RNase E and RNase III participate in both mRNA turnover and pre-rRNA processing.

The pAp-mediated inhibition of Xrn1p and Rat1p and direct inhibition of RNase MRP might also contribute to the effects of Li^+ in neurobiology and in development. Gene expression is known to be affected by therapeutic concentrations of Li^+ (Manji *et al.*, 1995, and references therein). Although the concentrations of Li^+ used in this study are two orders of magnitude higher than blood plasma levels in patients treated with Li^+ (~1–1.5 mM), it is known that yeast has very efficient mechanisms to reduce intracellular Li^+ (Rodríguez-Navarro and Asensio, 1977). A central role in Li^+ and Na^+ efflux in yeast is played by the *ENA1–4* gene cluster, and deletion of these genes reduces the toxic concentration of Li^+ to <10 mM (Quintero *et al.*, 1996). Moreover, mutations in a number of other transport systems increase sensitivity to Li^+, indicating that these also contribute to normal Li^+ efflux. The Li^+ concentration at which Hal2p activity is inhibited *in vitro* is well below the therapeutic dose. *HAL2* homologs have been identified in *E.coli (cysQ)*, *A.thaliana (SAL1)* and rice (*RHL*), and enzymes with the activity of Hal2p have been characterized in mammals (Ramaswamy and Jakoby, 1987). A multitude of sulfotransferase reactions which use pApS as the donor exist in higher eukaryotes, resulting in the production of pAp. It seems probable that Li^+ will also inhibit Hal2p activity when applied as a drug in humans, or when injected into developing *Xenopus* embryos, leading to pAp accumulation. The 5′→3′ exonucleases are highly conserved in evolution since the mouse *XRN1* homolog can complement a yeast *xrn1* mutant strain (Bashkirov *et al.*, 1997). By analogy to yeast, the accumulation of pAp could inhibit Xrn1p, leading to mRNA stabilization and consequent changes in the pattern of gene expression. These observations raise the possibility that alterations in RNA metabolism may contribute to the effects of Li^+ in development and in human neurobiology.

Materials and methods

Strains, media and microbiological techniques

Growth and manipulations of *E.coli* (Maniatis *et al.*, 1982) and *S.cerevisiae* (Sherman, 1991) were performed by standard techniques. Yeast transformations were carried out using a lithium acetate method (Gietz *et al.*, 1992). Plasmid recovery from yeast into *E.coli* was performed as described in Robzyk and Kassir (1992). The yeast strains used in this study are listed in Table I.

All media used in this study contained methionine, unless explicitly stated otherwise. For analysis of the effects of Li^+ on growth, yeast strains were grown at 30°C in SD minimal medium to mid-exponential phase. Cells were harvested by centrifugation and resuspended in medium containing 0.2 M LiCl with and without methionine (20 mg/l), and exponential growth was maintained. Growth on 5-FOA was tested on synthetic medium agar plates containing 0.1% 5-FOA (w/v), uracil (20 mg/l) and nutrients.

Cloning of HAL2

Mutagenesis and isolation of yeast strains which are sl with *rrp2-1* has been described (Dichtl and Tollervey, 1997). Strain SL158 was backcrossed twice to the parental strain CH1305, selecting for cold sensitivity, and one cs strain (YBD105) of the resulting progeny was transformed with a yeast genomic library in pUN100 (Bergès *et al.*, 1994). Library plasmids were recovered in *E.coli* from eight independent non-cs yeast transformants. All plasmids contained an overlapping fragment of yeast

chromosome XV. One plasmid (pBD36) was used to produce deletion mutants. A 2.3 kb *Sac*I–*Spe*I fragment (containing the *HAL2/MET22* gene) released from pBD36 was shown to complement the cs phenotype of strain YBD105 and the sl phenotype of SL158 when transformed into those strains as plasmid pBD38 or pBD45, respectively.

Plasmids

The 2.3 kb *Sac*I–*Spe*I fragment of pBD36 (see above) was cloned into pRS316 (*CEN-URA3*, Sikorski and Hieter, 1989) to give plasmid pBD38 and into pRS415 (*CEN-LEU2*, Stratagene) to give plasmid pBD45 (pHAL2).

Plasmids pBD1 (*RRP2-URA3-ADE3-CEN*), pBD4 (pRRP2, *RRP2-LEU2-CEN*), pBD10 (pPOP3, *POP3-LEU2-2*μ), pBD11 (pSNM1, *SNM1-LEU2-2*μ) and pBD15 (pPOP1, *POP1-LEU2-CEN*) have been described (Dichtl and Tollervey, 1997).

Gene disruptions

To construct a *HAL2* null allele, we replaced the complete *HAL2* open reading frame in the diploid yeast strain BMA38 using a one-step PCR method (Baudin *et al.*, 1993). The *HIS3* marker gene was PCR amplified using oligonucleotide HAL2-pro, ATATGTACTCATATATTTATGTCTA-TCAATAAAGTAAAATATATGCTCGTTCAGAATGACACG and oligonucleotide HAL2-term, TTAGTAAGTAAGAAGTTTAAAGACAAC-TCAGAAGACATCAGCACTCTTGGCCTCCTCTAG. Sporulation and tetrad dissection of one heterozygous *HAL2/hal2::HIS3* transformant resulted in four viable strains per tetrad. Correct integration was verified by Southern hybridization. Strains carrying the *hal2::HIS3* allele (*hal2*-Δ) were auxotrophic for methionine and cs.

XRN1 gene disruptions in strains YBD1 and YBD105 were performed using a 5.8 kb *xrn1::LEU2* disruption construct recovered from plasmid LBW503 (kindly provided by Lydia Jane, Oxford, UK). Correct integration was verified by Southern hybridization.

RNA analysis

RNA extraction (Tollervey and Mattaj, 1987), Northern hybridization (Tollervey, 1987) and primer extension (Beltrame and Tollervey, 1992) were performed as described. Probes for Northern hybridization and primer extension, indicated in Figure 2A, were as follows: oligonucleotide b, GCTCTTTGCTCTTGCC; oligonucleotide c, ATGAAAACTCCACA-GTG; oligonucleotide d, CCAGTTACGAAATTCTTG; and oligonucleotide e, GGCCAGCAATTTCAAGT; probe a is a riboprobe overlapping the A_0–A_1 fragment (Venema *et al.*, 1995). Oligonucleotides against the mature snoRNAs U14 and snR190 were as follows: U14mat, TCACTCAGACATCCTAGG; and snR190mat, CGTCATGGTCGAAT-CGG. The probe against *MFA2* mRNA was the oligonucleotide GGCGG-GATCCCAGAAGAGGCCCTTGATTAT (Michaelis and Herskowitz, 1988).

In situ hybridization was performed as described (Amberg *et al.*, 1992), using FITC-labeled oligo(dT).

In vitro exonuclease assays

Xrn1p was purified according to the procedure of Johnson and Kolodner (1991) and Rat1p was purified as described (Stevens and Poole, 1995). Nucleotides were from Sigma. pApS is 60–80% pure and described as unstable at room temperature. The exonuclease assays were carried out as described for determination of the reaction specificity of Rat1p (Stevens and Poole, 1995). The reaction mixtures (50 μl) contained 4 nmol (as nucleotide) of [^{3}H]poly(A) (8.5×10^4 c.p.m.), 33 mM Tris–HCl, pH 8.0, 2 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 30 μg of acetylated albumin, and an amount of Xrn1p or Rat1p to give 15–30% hydrolysis of the poly(A). The mixtures were incubated at 37°C for 10 min. Activity was determined by measurement of acid-soluble label released.

[^{3}H]RNA was prepared using the T7 Ribo-Max high level RNA production system with the luciferase control DNA (Promega) as the template and [^{3}H]UTP (ICN). The triphosphate-ended RNA was then hydrolyzed with tobacco acid pyrophosphatase (Epicentre) to give a 5′ phosphate terminus on the RNA (Stevens and Poole, 1995). For the [^{3}H]RNA assay, 2 nmol (as nucleotides), 3×10^4 c.p.m., were incubated in reaction mixtures as described above.

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References

Amberg,D.C., Goldstein,A.L. and Cole,C.N. (1992) Isolation and characterization of *RAT1*: an essential gene of *Saccharomyces cerevisiae* required for the efficient nucleocytoplasmic trafficking of mRNA. *Genes Dev.*, **6**, 1173–1189.

Bashkirov,V.I., Scherthan,H., Solinger,J.A., Buerstedde,J.-M. and Heyer,W.-D. (1997) A mouse cytoplasmic exoribonuclease (mXRN1p) with preference for G4 tetraplex substrates. *J. Cell Biol.*, **136**, 761–773.

Baudin,A., Ozier-Kalogeropoulos,O., Denouel,A., Lacroute,F. and Cullin,C. (1993) A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Res.*, **21**, 3329–3330.

Beltrame,M. and Tollervey,D. (1992) Identification and functional analysis of two U3 binding sites on yeast pre-ribosomal RNA. *EMBO J.*, **11**, 1531–1542.

Bergès,T., Petfalski,E., Tollervey,D. and Hurt,E.C. (1994) Synthetic lethality with fibrillarin identifies Nop77p, a nucleolar protein required for pre-rRNA processing and modification. *EMBO J.*, **13**, 3136–3148.

Berridge,M.J., Downes,C.P. and Hanley,M.R. (1989) Neural and developmental actions of lithium: a unifying hypothesis. *Cell*, **59**, 411–419.

Chang,D.D. and Clayton,D.A. (1987) A novel endoribonuclease cleaves at a priming site of mouse mitochondrial DNA replication. *EMBO J.*, **6**, 409–417.

Cherest,H., Surdin-Kerjan,Y. and de Robichon-Szulmajster,H. (1971) Methionine-mediated repression in *Saccharomyces cerevisiae*: a pleiotropic regulatory system involving methionine transfer ribonucleic acid and the product of gene *ETH2*. *J. Bacteriol.*, **106**, 758–772.

Chu,S., Archer,R.H., Zengel,J.M. and Lindahl,L. (1994) The RNA of RNase MRP is required for normal processing of ribosomal RNA. *Proc. Natl Acad. Sci. USA*, **91**, 659–663.

Dichtl,B. and Tollervey,D. (1997) Pop3p is essential for the activity of the RNase MRP and RNase P ribonucleoproteins *in vivo*. *EMBO J.*, **16**, 417–429.

Doye,V., Wepf,R. and Hurt,E.C. (1994) A novel nuclear pore protein Nup133p with distinct role in poly(A)$^+$ RNA transport and nuclear pore distribution. *EMBO J.*, **13**, 6062–6075.

Falany,C.N. (1997) Sulfation and sulfotransferases; introduction: changing view of sulfation and the cytosolic sulfotransferases. *FASEB J.*, **11**, 1–2.

Gietz,D., St Jean,A., Woods,R.A. and Schiestl,R.H. (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.*, **20**, 1425.

Gläser,H.-U., Thomas,D., Gaxiola,R., Montrichard,F., Surdin-Kerjan,Y. and Serrano,R. (1993) Salt tolerance and methionine biosynthesis in *Saccharomyces cerevisiae* involve a putative phosphatase gene. *EMBO J.*, **12**, 3105–3110.

Grignon,S. and Bruguerolle,B. (1996) Cerebellar lithium toxicity: a review of recent literature and tentative pathophysiology. *Therapíe*, **51**, 101–106.

Henry,Y., Wood,H., Morrissey,J.P., Petfalski,E., Kearsey,S. and Tollervey,D. (1994) The 5′ end of yeast 5.8S rRNA is generated by exonucleases from an upstream cleavage site. *EMBO J.*, **13**, 2452–2463.

Herbst,C. (1892) Experimentelle Untersuchungen über den Einfluss der veränderten chemischen Zusammensetzung des umgebenden Mediums auf die Entwicklung der Tiere. I. Versuche and Seeigeleiern. *Z. Wiss. Zool.*, **55**, 446–518.

Jerka-Dziadosz,M. and Frankel,J. (1995) The effects of lithium chloride on pattern formation in *Tetrahymena thermophila*. *Dev. Biol.*, **171**, 497–506.

Johnson,A.W. and Kolodner,R.D. (1991) Strand Exchange Protein 1 from *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **266**, 14046–14054.

Kao,K.R., Masui,Y. and Elinson,R.P. (1986) Lithium-induced respecification of pattern in *Xenopus laevis* embryos. *Nature*, **322**, 371–373.

Kearsey,S. and Kipling,D. (1991) Recombination and RNA processing: a common strand? *Trends Cell Biol.*, **1**, 110–112.

Klein,P.S. and Melton,D.A. (1996) A molecular mechanism for the effect of lithium on development. *Proc. Natl Acad. Sci. USA*, **93**, 8455–8459.
Lindahl,L., Archer,R.H. and Zengal,J.M. (1992) A new rRNA processing mutant of *Saccharomyces cerevisiae*. *Nucleic Acids Res.*, **20**, 295–301.
Livingston,B.T. and Wilt,F.H. (1989) Lithium evokes expression of vegetal-specific molecules in the animal blastomeres of sea urchin embryos. *Proc. Natl Acad. Sci. USA*, **86**, 3669–3673.
Lygerou,Z., Mitchell,P., Petfalski,E., Séraphin,B. and Tollervey,D. (1994) The *POP1* gene encodes a protein component common to the RNase MRP and RNase P ribonucleoproteins. *Genes Dev.*, **8**, 1423–1433.
Lygerou,Z., Allmang,C., Tollervey,D. and Séraphin,B. (1996) Accurate processing of a eukaryotic precursor ribosomal RNA by ribonuclease MRP *in vitro*. *Science*, **272**, 268–272.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Handbook*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Manji,H.K., Potter,W.Z. and Lenox,R.H. (1995) Signal transduction pathways, molecular targets for lithium's actions. *Arch. Gen. Psychiatr.*, **52**, 531–543.
Masselot,M. and de Robichon-Szulmajster,H. (1975) Methionine biosynthesis in *Saccharomyces cerevisiae*. I. Genetical analysis of auxotrophic mutants. *Mol. Gen. Genet.*, **139**, 121–132.
Michaelis,S. and Herskowitz,I. (1988) The **a**-factor pheromone of *Saccharomyces cerevisiae* is essential for mating. *Mol. Cell. Biol.*, **8**, 1309–1318.
Muhlrad,D., Decker,C.J. and Parker,R. (1994) Deadenylation of the unstable mRNA encoded by the yeast *MFA2* gene leads to decapping followed by 5′→3′ digestion of the transcript. *Genes Dev.*, **8**, 855–866.
Murguía,J.R., Bellés,J.M. and Serrano,R. (1995) A salt-sensitive 3′(2′),5′-bisphosphate nucleotidase involved in sulfate activation. *Science*, **267**, 232–234.
Murguía,J.R., Bellés,J.M. and Serrano,R. (1996) The yeast *HAL2* nucleotidase is an *in vivo* target of salt toxicity. *J. Biol. Chem.*, **271**, 29029–29033.
Neuwald,A.F., Krishnan,B.R., Brikun,I., Kulakauskas,S., Suziedelis,K., Tomcsanyi,T., Leyh,T.S. and Berg,D.E. (1992) *cysQ*, a gene needed for cysteine synthesis in *Escherichia coli* K-12 during aerobic growth. *J. Bacteriol.*, **174**, 415–425.
Peng,Z. and Verma,D.P.S. (1995) A rice *HAL2*-like gene encodes a Ca^{2+}-sensitive 3′(2′),5′-diphosphonucleoside 3′(2′)-phosphohydrolase and complements yeast *met22* and *Escherichia coli cysQ* mutations. *J. Biol. Chem.*, **270**, 29105–29110.
Peters,D.J.M., Van Lookeren Campagne,M.M., Van Haastert,P.J.M., Spek,W. and Schaap,P. (1989) Lithium ions induce prestalk-associated gene expression and inhibit prespore gene expression in *Dictyostelium discoideum*. *J. Cell Sci.*, **93**, 205–210.
Quintero,F.J., Garciadeblás,B. and Rodríguez-Navarro,A. (1996) The *SAL1* gene of *Arabidopsis*, encoding an enzyme with 3′(2′),5′-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase activities, increases salt tolerance in yeast. *Plant Cell*, **8**, 529–537.
Ramaswamy,S.G. and Jakoby,W.B. (1987) (2′)3′,5′-Bisphosphate nucleotidase. *J. Biol. Chem.*, **262**, 10044–10047.
Robzyk,K. and Kassir,Y. (1992) A simple and highly efficient procedure for rescuing autonomous plasmids from yeast. *Nucleic Acids Res.*, **20**, 3790.
Rodríguez-Navarro,A. and Asensio,J. (1977) An efflux mechanism determines the low net entry of lithium in yeasts. *FEBS Lett.*, **75**, 169–172.
Rosenthal,N.E. and Goodwin,F.K. (1982) The role of the lithium ion in medicine. *Annu. Rev. Med.*, **33**, 555–568.
Schmitt,M.E. and Clayton,D.A. (1994) Characterization of a unique protein component of yeast RNase MRP: an RNA-binding protein with a zinc-cluster domain. *Genes Dev.*, **8**, 2617–2628.
Sherman,F. (1991) Getting started with yeast. *Methods Enzymol.*, **194**, 3–21.
Shuai,K. and Warner,J.W. (1991) A temperature sensitive mutant of *S.cerevisiae* defective in pre-rRNA processing. *Nucleic Acids Res.*, **19**, 5059–5064.
Stevens,A. and Poole,T.L. (1995) 5′-Exonuclease-2 of *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **270**, 16063–16096.
Stevens,A., Hsu,C.L., Isham,K.R. and Larimer,F.W. (1991) Fragments of the internal transcribed spacer 1 of pre-rRNA accumulate in *Saccharomyces cerevisiae* lacking 5′→3′ exoribonuclease 1. *J. Bacteriol.*, **173**, 7024–7028.
Thomas,D., Cherest,H. and Surdin-Kerjan,Y. (1989) Elements involved in *S*-adenosylmethionine-mediated regulation of the *Saccharomyces cerevisiae MET25* gene. *Mol. Cell. Biol.*, **9**, 3292–3298.
Thomas,D., Barbey,R., Henry,D. and Surdin-Kerjan,Y. (1992) Physiological analysis of mutants of *Saccharomyces cerevisiae* impaired in sulfate assimilation. *J. Gen. Microbiol.*, **138**, 2021–2028.
Tollervey,D. (1987) A yeast small nuclear RNA is required for normal processing of pre-ribosomal RNA. *EMBO J.*, **6**, 4169–4175.
Tollervey,D. and Mattaj,I.W. (1987) Fungal small nuclear ribonucleoproteins share properties with plant and vertebrate U-snRNPs. *EMBO J.*, **6**, 469–476.
Venema,J., Henry,Y. and Tollervey,D. (1995) Two distinct recognition signals define the site of endonucleolytic cleavage at the 5′ end of yeast 18S rRNA. *EMBO J.*, **14**, 4883–4892.
Weinshilboum,R.M., Otterness,D.M., Aksoy,I.A., Wood,T.C., Her,C. and Raftogianis,R.B. (1997) Sulfation and sulfotransferases 1; sulfotransferase molecular biology: cDNAs and genes. *FASEB J.*, **11**, 3–14.

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