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\textsubscript{i}. Overexpression of Hal2p confers lithium resistance in yeast, and its activity is inhibited by submillimolar amounts of Li\textsuperscript{+} \textit{in vitro}. Here we report that pAp accumulation in \textit{HAL2} mutants inhibits the 5'→3' exonucleases Xrn1p and Rat1p. Li\textsuperscript{+} 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\textsuperscript{+} hypersensitivity and is synthetically lethal with mutations in either \textit{HAL2} or \textit{XRNI}. We propose that Li\textsuperscript{+} toxicity in yeast is due to synthetic lethality evoked between Xrn1p and RNase MRP. Similar mechanisms may contribute to the effects of Li\textsuperscript{+} on development and in human neurobiology.

\textit{Keywords}: exonucleases/lithium/RNA processing/RNase MRP/sulfate assimilation

Introduction

The monovalent cation Li\textsuperscript{+} 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\textsuperscript{+} intoxication occurs quite frequently, mostly because of overdosage (Grignon and Bruguierolle, 1996). In contrast to the mainly positive therapeutic effects of Li\textsuperscript{+} as a drug is its profound effects on the development of various organisms which were first observed more than a century ago (Herbst, 1892). Li\textsuperscript{+} inhibits the formation of the dorsal–ventral axis in \textit{Xenopus laevis} embryos (Kao \textit{et al}., 1986) and induces vegetalization in sea urchin eggs (Livingston and Wilt, 1989). Moreover, Li\textsuperscript{+} alters cell fate determination in the slime mold \textit{Dictyostelium discoideum} (Peters \textit{et al}., 1989) and affects pattern formation in the unicellular ciliate \textit{Tetrahymena thermophila} (Jerka-Dziadosz and Frankel, 1995).

Despite decades of clinical use, the molecular mechanisms underlying the therapeutic action of Li\textsuperscript{+} 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\textsuperscript{+} action suggest the involvement of key components of signal transduction pathways as targets of Li\textsuperscript{+} inhibition (Manji \textit{et al}, 1995). Currently, the most widely accepted model is the ‘inositol depletion hypothesis’ (Berridge \textit{et al}, 1989) which is based on the inhibition of inositol monophosphatases by Li\textsuperscript{+}, 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 \textit{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 \textit{Xenopus} that are induced by Li\textsuperscript{+} (Klein and Melton, 1996).

The \textit{HAL2}/\textit{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 \textit{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) (Murguia \textit{et al}., 1995). Sulfate assimilation in \textit{Saccharomyces cerevisiae} is initiated by the production of adenosine 5'-phosphosulfate (ApS) from ATP and sulfate. This is followed by phosphorylation of ApS forming 3'5'-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 \textit{et al}., 1989). The methionine auxotrophy of \textit{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 sulfate, even though the strains do possess all the enzymatic activities required for methionine synthesis (Thomas \textit{et al}., 1992; Murguia \textit{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 (Murguia \textit{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...
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 (Murgia 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., 1992; 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 Weinsilboum et al., 1997).

RNase MRP is a ribonucleoprotein particle (RNP) with endoribonuclease activity, which cleaves the pre-rRNA at site A₃ in internal transcribed spacer 1 (ITS1) (Murgia 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., 1992; this study).

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 muta-

genesis, 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/MT22, 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, Smn1p 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/MT22 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 backcross 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 A₃ 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 A₀ and A₁,
release a 91 nucleotide fragment from the 5’ external transcribed spacer (5’ETS). The hal2-1 mutant strongly accumulated the A0–A1 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 A0–A1 fragment, but accumulation was much stronger in the xrn1-Δ, rat1-1 double mutant (Figure 2B, 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 A2 (A2–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 A3 (Figure 2B, probe d) also detected the A2–E fragment accumulated in the hal2-Δ mutant (lanes 7–10). In addition, probe d detected the RNA species labeled ‘*’...
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 exonuclease. Similarly, no accumulation of other pre-rRNA spacer fragments, the 5′ ETS region that lies 5′ to site A0 or the C1–C2 region of ITS2 was seen in either the HAL2 or exonuclease mutants (data not shown). Processing of the primary transcripts of tRNA^Thr^ and tRNA^Pro^ did not exhibit a phenotype characteristic of RNase P inhibition in the HAL2 or exonuclease mutant strains. However, the 5′-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 (Murguia 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 (Murguia 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.
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 (Murguia et al., 1995) and in vivo (Murguia 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 A₀–A₁ 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–A₂ (probe b) and A₂–A₃ (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 A₃ (A₃–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 A₃ (A₃–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 A₁, 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 A₃–E RNA is indeed due to inhibition of cleavage at site A₁, 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
Lithium toxicity in yeast

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.

C2. Figure 5 shows that the primer extension stop at site A3 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 A2 signal was dramatically increased when cells were grown in Li+ and no methionine (Figure 5, lanes 5–7), reflecting the accumulation of the A3–E RNA that was observed by Northern analysis (Figure 4B, lanes 9–12). On lithium treatment, cleavage at A3 by RNase MRP is reduced, but the subsequent processing of the residual A3-cleaved pre-rRNA is also strongly reduced due to the inhibition of the Xrn1p and Rat1p exonucleases. The primer extension stop at site A2 remains unaffected by lithium treatment (Figure 5, lanes 1–7).

A3 cleavage is required for the formation of the major, short form of 5.8S rRNA, 5.8Ss (Henry et al., 1994). Consistent with this, we observed under-accumulation of the major, short form of 5.8S rRNA (5.8Ss) after 48 h of lithium treatment (in the presence of methionine) due to inhibition of A3 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 A0 or the C1–C2 region of ITS2 was seen (data not shown). Processing of the primary transcripts of tRNA3Leu and tRNAProUGG 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).
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 (Murguia et al., 1995). In addition, Li⁺ inhibits the activity of RNase MRP in cleavage of the pre-rRNA at site A₃. 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.

rpp2-1 strains are lithium hypersensitive, and the combination of the xrn1-Δ and rpp2-1 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 rpp2-1 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 rpp2-1 strain used for sl screening, YBD1, on 5-FOA (which selects against the RRP2-UR3-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 rpp2-1 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 rpp2-1 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 rpp2-1, prompted us to test for an sl interaction between rpp2-1 and xrn1-Δ. We therefore disrupted the non-essential XRN1 gene in the strain used for screening for synthetic lethality with rpp2-1 (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.
YBD136 are not synthetic lethal. The HAL2 gene, as shown by its ability to grow on 5-FOA (Figure 7C). 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, 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 LiCl, the pAp-mediated inhibition of Xrn1p (due to LiCl inhibition of Hal2p) and pAp-independent LiCl inhibition of RNase MRP activity results in ‘synergistic toxicity’, which forms the basis of the inhibition of growth by LiCl (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 A0–A1, D–A2 and A2–A3 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–A2 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 A0–A1 and A2–A3 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 (5.8Ss) is also generated by an exonuclease activity that requires Xrn1p and Rat1p, following cleavage of the pre-rRNA at site A3 by RNase MRP (Henry et al., 1994). We observed the accumulation of 5′-extended forms of 5.8Ss RNA 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 A3 and B1L 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 A3. 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 (Murguia 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 (Murguia 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+) (Murguia 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 (Murguia 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.

In vivo activity of RNase MRP, i.e. cleavage of pre-rRNA at site A3, 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 MRN 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 Hal2p, we speculated that the real basis of the lethality was an sl interaction between RNase MRP and Hal2p, we speculated that the real basis of the lethality was an sl interaction between RNase MRP and Hal2p, we speculated that the real basis of the lethality was an sl interaction between RNase MRP and Hal2p, we speculated that the real basis of the lethality was an sl interaction between RNase MRP and Hal2p, we speculated that the real basis of the lethality was an sl interaction between RNase MRP and Hal2p, we speculated that the real basis of the lethality was an sl interaction between RNase MRP and Hal2p, we speculated that the real basis of the lethality was an sl interaction between RNase MRP and Hal2p. 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
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 A3 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 Ratlp and direct inhibition of RNase MRP might also contribute to the effects of Li\(^+\) in neurobiology and in development.

Materials and methods

**Strains, media and microbiological techniques**

Growth and manipulations of E.coli (Maniatis et al., 1982) and Scerevisiae (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 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.

**Cloning of HAL2**

Mutagenesis and isolation of yeast strains which are sI with rrp2-1 have been described (Dichtl and Tollervey, 1997). Strain SL158 was back-crossed 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 SacI–SpeI 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 SacI–SpeI 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–LEU2-2μ), pBD11 (pSNM1, SNM1–LEU2-2μ) 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, ATATGGAATATATGTGCTGAAATGCAGGACG and oligonucleotide HAL2-term, TTAAGAATGAGGTTACTAAGAACAATCTCAAAAGAAGACATCACTGTTCCGGGTTTCCGAGGCACTGACCGC 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 Xln1::LEU2 disruption construct recovered from plasmid LBWS03 (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, GCCCTTTGCTCCTGGCC; oligonucleotide c, ATGAGAAACCTACCAAGAGTG; oligonucleotide d, CCAGTTACGAAATTCTTG; and oligonucleotide e, GGCAGCAAGATTTCAAGT; probe a is a riboprobe overlapping the A0–A1 fragment (Venema et al., 1995). Oligonucleotides against the mature snoRNAs U14 and snR190 were as follows: U14mat, GTG; oligonucleotide c, ATGAAAACTCCACAGGAGC and snR190mat, CGTCA TGGTCGAATCTCTCT. GATCCCAGAAGAGGCCCTTTATATGACTCATATATTATGCTA (Michaelis and Herskowitz, 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 according to the reaction specificity of Rat1p (Stevens and Poole, 1995). The reaction mixtures (50 μl) contained 4 mmol (as nucleotides) of [3H]poly(A) (8.5×10^6 c.p.m.), 33 mM Tris-HCl, pH 8.0, 2 mM MgCl2, 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.

[3H]RNA was prepared using the T7 Ribo-Max high level RNA production system with the luciferase control DNA (Promega) as the template and [3H]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 [3H]RNA assay, 2 nmol (as nucleotides), 3×10^6 c.p.m., were incubated in reaction mixtures as described above.

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References

7194