Cell Cycle Arrest Promotes trans-Hammerhead Ribozyme Action in Yeast*

(Received for publication, February 29, 1996, and in revised form, May 7, 1996)

Gerardo Ferbeyre, J ohn Bratty, Hui Chen, and Robert Cedergren

From the Département de Biochimie, Université de Montréal, Montréal, Québec H3C 3J 7, Canada

A hammerhead ribozyme designed to cleave the yeast ADE1 mRNA has been expressed in yeast under the control of a galactose-inducible promoter. RNA prepared from the galactose-induced yeast cultures possesses an activity that cleaves ADE1 mRNA in vitro. However, in spite of high expression levels of the ribozyme, no cleavage activity could be demonstrated in vivo. On the other hand, when the yeast cells expressing hammerhead RNA were treated with the α-factor mating pheromone, the level of ADE1 mRNA was reduced by 50%. Similar reductions were observed when this strain was cultured in the presence of lithium acetate or in nitrogen-free medium. Moreover, control experiments in which disabled hammerhead genes were expressed showed no such reductions. Extension of the length of the flanking recognition arms of the ribozyme from a total of 10 to 16 or 24 nucleotides diminished the inhibitory effect of the ribozyme. These data suggest that ribozymes are able to cleave a trans-RNA target in yeast.

The selective repression of gene expression by antisense nucleic acids and ribozymes is a promising avenue for biomedical research (Van der Krol et al., 1988; Sarver et al., 1990; Bratty et al., 1993). In the case of ribozymes, the hammerhead domain has received preferential attention because it combines a simple target recognition mechanism with an RNA cleaving activity (Haseloff and Gerlach, 1988). Even though some success in the inhibition of mammalian gene expression has been reported using ribozymes (Seraver et al., 1990; Ojwang et al., 1992; Sullenger and Cech, 1993; Czubayko et al., 1994), our lack of knowledge concerning many aspects of RNA targeting in vivo has limited the application of this potentially powerful technology (Cotten and Birnstiel, 1989; L'Huillier et al., 1992; Lo et al., 1992). Improvements to ribozyme protocol such as co-localization of the ribozyme and the target (Sullenger and Cech, 1993), choosing a cleavage site in an exposed region of the RNA target (Lieber and Strauss, 1995), and varying the length of the flanking recognition arms (Crisell et al., 1993; Ellis and Rogers, 1993; Bertrand and Rossi, 1994; Bertrand et al., 1994) have been proposed to circumvent some of the shortcomings of in vivo ribozyme use.

This dilemma inspired us to consider the yeast Saccharomyces cerevisiae as a model organism even though, up until this time, yeast had been refractory to inhibition of gene expression with either antisense RNA or ribozymes (Atkins et al., 1994; Ferbeyre et al., 1995). In fact, ribozyme activity in yeast had been documented only in cases where a hammerhead gene was fused to the RNA target (cis-activity) and positioned close to the cleavage site (Egli and Braus, 1994; Ferbeyre et al., 1995).

The lack of trans-activity, in spite of the high expression levels of RNA from a galactose-inducible promoter (Ferbeyre et al., 1995), focused our thoughts on other phenomena likely to affect the in vivo efficacy of catalytic RNA. Considering that 1) a hammerhead ribozyme requires 1 min to cleave a small substrate devoid of extensive secondary structure in vitro, and the in vivo reaction velocity could easily be 10--100-fold slower; 2) cellular mRNAs possess extensive secondary structure and are in tight association with proteins (Bertrand et al., 1994); and 3) in vivo ribozymes must diffuse from their site of synthesis to their site of action in the cell, it is little wonder that ribozyme targeting of genes has not been successful in rapidly growing yeast cells. trans-Activity in mammalian cells may owe its feasibility to a markedly slower cell cycle and correspondingly slower RNA metabolism (Pardee, 1989).

The rationale for this study was to evaluate the hypothesis that yeast metabolism is simply too rapid to observe a ribozyme-based cleavage activity. In accord with this hypothesis, we show here that hammerhead ribozyme activity in trans is observed after treating the culture medium with α-factor or lithium acetate or starving the culture of nitrogen; these three treatments have in common the arrest of the yeast cell cycle in G₁. Furthermore, in light of the perceived problem of bringing catalyst and target together, our data surprisingly demonstrate that the shortest flanking recognition arm of the hammerhead ribozyme is associated with the highest inhibitory activity.

**EXPERIMENTAL PROCEDURES**

**Growth Conditions and Cell Cycle Arrest—**The yeasts strain SC252 (ade1 leu2-112 ura3-52) was grown either on 1% yeast extract, 2% peptone, and 2% glucose or on synthetic complete (SC) medium supplemented with 30 mg/liter leucine or 20 mg/liter uracil when required (Rose et al., 1990). For G₁ arrest, cells were initially grown overnight in 5 ml of SC galactose medium supplemented with leucine. They were then used to inoculate an SC galactose medium to an initial density of A₆₀₀ = 0.05. When the cell density attained A₆₀₀ = 0.4, the culture was divided in two, and α-factor (Bachem Biosciences Inc., Philadelphia) was added to one-half to give a final concentration of 3 μM. The other half was centrifuged, and the cell pellet was immediately frozen with liquid nitrogen for posterior RNA purification. N-[α-Tosyl]-l-arginine methyl ester (Sigma) was added together with α-factor up to a final concentration of 5 mM. Treatment with the pheromone was for 3 h. Then, cells were harvested by centrifugation, and after washing, RNA was extracted as described below. Cell division arrest induced by α-factor was evaluated by determining the number of budding forms in the medium (Moore, 1983). Treatment with lithium acetate (Sigma) was performed in the same way as α-factor treatment except that the final

---

*This work was supported by the Medical Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† On leave from the Centro de Ingenierı́a Genética y Biotecnologı́a, Havana, Cuba.

‡ Fellow of the Evolutionary Biology Program of the Canadian Institute for Advanced Research. To whom correspondence should be addressed. Tel.: 514-343-6320; Fax: 514-343-2177.

1 The abbreviations used are: SC, synthetic complete; PCR, polymerase chain reaction.
concentration of LiAc was 50 mM. Nitrogen starvation was accomplished by collecting the cells after they had grown to \( A_{600} = 0.4 \). The cells were then washed and subjected to 6 h of further growth on SC medium lacking ammonium sulfate.

Construction of Ribozyme Expression Plasmids—Plasmids used in this study are summarized in Table I and Fig. 1C. Plasmids pVTDAE, YEpGAL, YEpGALhh (where “hh” is hammerhead), and YEpGALhhx (where “hhx” is disabled hammerhead) have previously been described (Ferbeyre et al., 1995). The sequences of all ribozymes used in this study and their complementary target sites in the ADE1 mRNA are illustrated in Fig. 1A. All constructions were performed according to standard protocols (Sambrook et al., 1989). The expression vector YEpGAL (Ferbeyre et al., 1995) was modified to simplify cloning: the single BamHI site was substituted for the part of the pUC19 polylinker between the EcoRI and BamHI sites. The resulting plasmid was named pKB.T. Ribozyme cassettes flanked with BamHI and KpnI overhangs were obtained by annealing the appropriate top and bottom cloned between the KpnI and BamHI sites of pKB.T to generate plasmid pKBThh16 with flanking recognition arms of 16 nucleotides. Inactive ribozyme controls were prepared by replacing G\(^5\) with C during the chemical synthesis of the ribozyme genes and were cloned like their wild-type counterparts.

RNA Purification and Northern Blotting—RNA was purified according to Rose et al. (1990) and separated on a 1% agarose-formaldehyde gel (Sambrook et al., 1989). RNA was then transferred to Hybond-N nylon membranes (Amersham Corp.). Hybridization was performed by incubation of the membranes with \(^{32}P\)-labeled probes at 65°C in 7% SDS, 0.25 \( \times \) Na\(_2\)HPO\(_4\) (pH 7.4), and 1% bovine serum albumin. The membranes were washed twice at 65°C in 2\( \times \) SSC (10% SDS, 0.25 M Na\(_2\)HPO\(_4\) (pH 7.4), and 1% SDS) for 20 min and once in 0.2 \( \times \) SSC. Probes were prepared by PCR amplification of the 1.1-kilobase pair ADE1 gene and the 1.1-kilobase pair fragment from exon 2 of the yeast actin gene (nucleotides 1204–2109, GenBank\textsuperscript{TM}); both were labeled using the T7 Quickprime kit (Pharmacia Biotech Inc.). Autoradiograms of Northern blots were quantified using an LKB densitometer.

In Vitro Cleavage and RNA Ligation-dependent PCR—A sample of 5 \( \mu \)g of total RNA from each yeast strain was incubated under cleavage conditions in 1 \( \mu \)mol polymerase buffer (New England Biolabs Inc.) at 37°C for 30 min. To assay cleavage, the RNA ligase-dependent PCR method was used. First, the RNA was phosphorylated by adding ATP (1 \( \mu \)mol) and 10 units of polynucleotide kinase to the cleavage reaction, and incubation was continued for another 30 min at 37°C. Following this step, the RNA was ligated to 100 ng of the 14-mer oligoribonucleotide 5'-ACGGUCAGACGG in 50 mM HEPES (pH 7.5), 10 mM MgCl\(_2\), 1 mM ATP, 20 mM dithiothreitol, 1 \( \mu \)g of RNase-free bovine serum albumin, 10% dimethyl sulfoxide, and 6 units of T4 RNA ligase (New England Biolabs Inc.) in a final volume of 20 \( \mu \)l. The reaction was incubated overnight at 15°C and stopped by heating, and the products were recovered by ethanol precipitation. Ligated RNA was reverse-transcribed in Taq polymerase buffer (Pharmacia Biotech Inc.) supplemented with 2.5 mM MgCl\(_2\), and 1 \( \mu \)g each dNTP in a volume of 20 \( \mu \)l using 50 pmol of the primer 5'-GAGCTGGCTAGCAGGAGAC, which is complementary to a region 328 nucleotides downstream from the start codon. Moloney reverse transcriptase was added after a 45-min annealing step, and the reaction was carried out for 1 h at 37°C. Amplification of cDNA by PCR was then performed using the reverse transcription primer and 50 pmol of the primer 5'-ACGGUCAGACGG, whose sequence corresponded to that of the 14-mer oligoribonucleotide used in the ligation step plus the first 5 nucleotides of the presumed cleavage product. The reaction volume was adjusted to 100 \( \mu \)l with Taq DNA polymerase buffer, and 1 unit of Taq polymerase was added per reaction. PCR was carried out for 30 cycles of 30 s each at 94, 57, and 72°C (Ferbeyre et al., 1995).

**RESULTS**

Ribozyme Activity in Vitro—The hammerhead ribozyme used in these experiments targeted the ADE1 gene of the yeast S. cerevisiae in the region of the initiation codon (Fig. 1A). In a previous report, we showed that this ribozyme in trans was unable to produce the ade1 phenotype or reduce the concentration of the ADE1 mRNA in vivo (Ferbeyre et al., 1995). The lack of ribozyme activity could not be due to poor expression or degradation of the ribozyme RNA in vivo because ribozymes were shown to be present in high concentrations in the cell.

To ascertain whether cellular ribozymes were modified by the cellular machinery or remained active in the cell, total cellular RNA was extracted from cells, and ribozyme activity was determined. RNA was purified from the strain Sc252 grown on galactose and harboring one of the following: 1) plasmid YEpGAL hh (see Table I and Fig. 1C), which expresses the hammerhead ribozyme against the ADE1 gene under the control of the GAL1 promoter; 2) plasmid YEpGAL hhx, which expresses a disabled ribozyme from a GAL1 promoter; or 3) plasmid pVTDAE, which expresses the ADE1 gene under the control of the ADH promoter. The RNA containing the ADE1 mRNA target was incubated under cleavage conditions with the two ribozyme preparations, and cleavage was assayed by the RNA ligation-dependent PCR technique (Ferbeyre et al., 1995), which was designed to amplify selectively the 3′-cleavage product of the ADE1 mRNA.

Band of amplified DNA in Fig. 2 clearly demonstrate that the hammerhead ribozyme expressed by the yeast cells is catalytically active. Cleavage was observed when the wild-type hammerhead extract and ADE1 transcripts were incubated together (Fig. 2, lane 5). On the other hand, no bands were observed when the ADE1 mRNA sample alone (lane 1), either of the two ribozymes in the absence of the ADE1 mRNA (lanes 2 and 3), or the ADE1 mRNA and the disabled hammerhead ribozyme (lane 4) were incubated under cleavage conditions. Catalytically active RNA preparations were also obtained from a strain containing both plasmids pVTDAE and YEpGAL hh (data not shown).

Ribozyme Activity in \( \alpha \)-Factor-treated Cells—Normal progression of yeast cells through the cell cycle is assured by the cyclin-dependent protein kinases (Nasmuth, 1993). However, during mating, these kinases are inhibited by \( \alpha \)-factor, and as a result, cells are arrested in late G\(_1\) phase, and resume growth as diploid cells (Cross, 1988; Cross and Tinkelenberg, 1990; Peter and Herskowitz, 1994). Artificial cell cycle arrest for up to 6 h can be induced by the treatment of a heterothallic yeast strain with synthetic or purified \( \alpha \)-factor. Cell division resumes eventually due to pheromone degradation or adaptation of the signal transduction pathway to the presence of the
pheromone (Ciejek and Thorner, 1979; Moore, 1984; Stefan and Blumer, 1994).

To test whether cell cycle arrest might improve the performance of the hammerhead ribozyme in vivo, yeast cells expressing high levels of both the ribozyme and the target ADE1 gene were treated with α-factor (see “Experimental Procedures”). The ribozyme was expressed from the galactose-inducible promoter. High expression of the ADE1 gene effectuated by the strong constitutive ADH1 promoter was necessary to facilitate Northern analysis due to the low expression level of the chromosomal gene. α-Factor and a protease inhibitor to reduce pheromone degradation were added to the yeast culture in the early log phase of growth, and an aliquot of the culture provided an RNA sample before and 3 h after addition of the pheromone. The results presented in Fig. 3B demonstrate that during G1 arrest, the hammerhead ribozyme effectively reduces levels of the ADE1 mRNA in a concentration-dependent manner. As this initial study could not differentiate between reduced mRNA levels due to catalytic activity or to other inhibitory mechanisms, the experiment was repeated using a disabled ribozyme control (Ferbeyre et al., 1995) in cultures grown either on dextrose, where no ribozyme expression is anticipated, or on galactose, where ribozyme production is induced.

It can be observed in Fig. 4 that the active hammerhead ribozyme, but not the mutated version, significantly reduced the level of the mRNA target in galactose medium (cf. lanes 11 and 12). These results have been reproduced several times, and densitometric analysis of the autoradiograms from Northern hybridizations indicated a ribozyme-dependent reduction in ADE1 mRNA concentration of 30–50% when corrected for the level of actin mRNA. The inconsistency of this value could result from the variable efficiency of cell cycle arrest obtained...
in each experiment as evidenced by the percentage of budding forms in each culture. Furthermore, Fig. 4 shows that treatment with α-factor does not modify ADE1 gene expression in glucose cultures, although an ~15% reduction in ADE1 mRNA levels is observed in galactose cultures even in the absence of the active ribozyme. The fact that little or no observable cleavage occurs in the presence of the active ribozyme, in the absence of cell cycle arrest, indicates that the reductions in ADE1 mRNA concentrations are not due to in vitro cleavage during the extraction and purification of the RNA, as has been recently suggested (Beck and Nassal, 1995).

Ribozyme Activity in Cells Treated with Lithium Acetate or Grown on Nitrogen-free Medium—At this point, the observed hammerhead effect in vivo could be due to the cell cycle arrest as we initially hypothesized or to any of a number of pheromone-dependent phenomena. To distinguish more clearly between possible mechanisms, we used two other growth conditions that induce cell arrest in G_1: treatment with lithium acetate (Smith et al., 1995) and nitrogen starvation (Pringle and Hartwell, 1981). The effect of adding lithium acetate to yeast cultures expressing the hammerhead ribozyme is shown in Fig. 5. A significant hammerhead-dependent reduction in ADE1 mRNA levels is observed in lane 6. Quantitative analysis of the probed filters corrected for the quantity of actin mRNA indicated that the wild-type ribozyme reduced the concentration of the target by 63% compared with a 47% reduction for the disabled ribozyme.

Nutrient limitation, particularly that of nitrogen, decreases cyclin activity and thus modulates progression through the G_1 phase in yeast (Wittenberg et al., 1990). Nitrogen starvation induces an arrest in the G_1 phase much like α-factor and also triggers a signal transduction pathway that shares components with the pheromone pathway (Herskowitz, 1995). Therefore, cells were grown to early log phase and then transferred to nitrogen-free medium. As expected, nitrogen deprivation stimulated a reduction in the ADE1 mRNA level in cells expressing a wild-type hammerhead ribozyme (Fig. 5, lane 12). Corrected quantitative analysis of the filters showed that the ribozyme reduced the concentration of the target by 75%, the disabled ribozyme reduced the level by only 29%.

Effect of the Length of the Hammerhead Ribozyme Flanking Recognition Arms on Ribozyme Activity—The length of the flanking recognition arms can be a major factor in the efficiency of the hammerhead ribozyme in vivo (Crisel et al., 1993; Ellis and Rogers, 1993; Bertrand and Rossi, 1994; Bertrand et al., 1994). Since cell cycle arrest promoted hammerhead activity in yeast, we thought that this would be an excellent opportunity to explore the effect of the flanking region.

Yeast cells transformed with plasmid constructions expressing hammerhead ribozymes with flanking recognition arms of 10, 16, and 24 nucleotides were grown in SC galactose medium, and RNA samples were collected before and 3 h after the addition of α-factor. A disabled hammerhead ribozyme with flanking recognition arms of 24 nucleotides was used to control the antisense effect. The results from Northern blots hybridized to both the ADE1 probe and the actin probe were quantified and are summarized in Fig. 6. Only the ribozyme with 10 nucleotides complementary to the target proved to be active. Northern blot analysis showed similar and high expression levels for all ribozymes, and RNA ligation-dependent PCR demonstrated that all ribozymes were active in vitro (data not shown).

**DISCUSSION**

This report is the first demonstrating a trans-hammerhead ribozyme activity in yeast. Previous attempts in assembling an RNA-based gene inhibition model in yeast met with the disappointing observation that neither antisense RNAs nor ribozymes were functional in trans (Atkins et al., 1994; Law and Devenish, 1988; Ferbeyre et al., 1995) in spite of the fact that both had been used successfully, albeit with variable results, in the more complex mammalian cells (Van der Krol et al., 1988; Rossi, 1992; Bratty et al., 1993; Marschall et al., 1994). In a previous paper, we reported that the hammerhead ribozyme was catalytically active in yeast as long as the ribozyme was close and in cis to the cleavage site (Ferbeyre et al., 1995). Similar results were reported independently by Egli and Braus (1994), where a cis-acting hammerhead ribozyme was able to simulate the cleavage step in 3' terminal processing of primary transcripts.
We have shown here that although a hammerhead ribozyme is not active in vivo against its target under normal growth conditions, these same ribozymes display good activity against the target when cells are arrested in the G₁ phase. Significantly, RNA catalytic activity was demonstrated using three different methods of arresting the cell cycle: 1) the α-factor pheromone, which arrests the cell at the stage called “start” in late G₁ phase; 2) lithium acetate, which slows the progression of the cell through G₂ by inhibiting inositol 1-phosphatase (Smith et al., 1995); and finally, 3) growing the cells in nitrogen-free medium, which also slows the progression through G₂ (Pringle and Hartwell, 1981). In these cases, the ribozyme induced a 50–75% reduction in the concentration of the Ade₁ mRNA target during incubation of the yeast cultures for 3–6 h under the given conditions. A relatively high disabled hammerhead reduction in mRNA levels was also observed, but if this can be attributed to the antisense effect, then cell cycle arrest should stimulate this activity in yeast as well. These results are in good agreement with the hypothesis presented above that the kinetics of cell growth and RNA metabolism in yeast limits the effectiveness of ribozymes under normal conditions. We believe that it is for these reasons that antisense RNAs and ribozymes perform better in mammalian cells, where passing through the G₁ phase takes ~12 h.

The importance of the flanking recognition arms in the hammerhead ribozyme activity in vivo has been amply documented (Crisell et al., 1993; Ellis and Rogers, 1993; Bertrand et al., 1994). However, the prior failure of trans-acting ribozymes in yeast was not due to the length of the flanking arms since ribozymes with arms of 10, 16, and 24 nucleotides were similarly inactive under normal conditions. In addition, we had previously tried an antisense RNA covering the entire length of the Ade₁ gene and found it to be nonfunctional (data not shown).

In this study, the shortest flanking recognition arm performs better than longer ones, in contrast to the more usual observation that increasing the length increases the activity (Crisell et al., 1993). Possibly, this behavior is related to differences in RNA annealing activities of nuclear proteins in yeast and mammalian cells that can speed RNA-RNA interactions in vivo (Pontius and Berg, 1990) and increase the hammerhead ribozyme activity in vitro (Tsuchihashi et al., 1993; Bertrand and Rossi, 1994). Indeed, the activity of these proteins depends both on their concentration (Tsuchihashi et al., 1993) and on the length of the complementary base pair interaction between the ribozyme and its target (Bertrand and Rossi, 1994). It is possible that in yeast, the nature and the concentration of nuclear proteins do not allow hybridization between long complementary sequences.

Alternatively, it could be that the longer ribozymes with 16- and 24-nucleotide flanking recognition arms have a greater potential to engage in alternate, inactive conformations than the smaller arm. However, two facts argue against this hypothesis. First, all ribozymes showed the same activity in vitro. Second, each ribozyme is embedded in a much larger RNA fragment derived from the flanking non-complementary sequences of the vector, so the few additional nucleotides added to the ribozyme in going from 10 to 24 nucleotides in the flanking recognition arms are unlikely to make a big structural change. A more provocative hypothesis to explain these activities relates to catalytic turnover. A longer base pairing region may slow or even eliminate turnover by sequestering the product in a ribozyme-product complex. The shorter flanking recognition arms would allow a faster dissociation of the ribozyme from the product, enabling it to bind a new substrate. However, multiple turnover kinetics would also imply that only a small fraction (a compartmentalization fraction?) of the ribozyme molecules would be actively engaged in catalysis.

Our rationale for this study derived from the consideration of yeast as an excellent model system, and our results could now open new avenues for the study of the hammerhead ribozyme in vivo. Of particular note is that both the genetic and molecular tools available in yeast can now be applied to improve the activity of the hammerhead ribozyme. In addition, our observations with cell cycle modulators may give new clues to the pursuit of more efficient ribozyme-based gene inhibitions in higher eukaryotes. However, the key to our findings was the realization that yeast is a microorganism that evolved fast processes for subsisting in slow growing organisms.

REFERENCES

Atkins, D., Arndt, G. M. & Izant, J. G. (1994) Biud. Chem. Hoppe-Seyler 375, 721–729.

Bertrand, E. & Rossi, J. J. (1994) EMBO J. 13, 2904–2912.

Bratt, J., Kaufman, R. & Grange, T. (1994) Nucleic Acids Res. 22, 293–300.

Crisell, P., Thompson, S. & James, W. (1993) Nucleic Acids Res. 21, 3513–3522.

Cross, F. R. (1988) Mol. Cell. Biol. 8, 4765–4768.

Cross, F. R. & Tinkelenberg, A. H. (1990) Cell 65, 875–883.

Crisell, P., Thompson, S. & James, W. (1993) Nucleic Acids Res. 21, 3513–3522.

L'Huillier, P. L., Davis, S. R. & Bellamy, A. R. (1992) Biochem. Int. 21, 673–679.

Luther, W. W. (1988) Biochem. Int. 21, 673–679.

L'Huillier, P. L., Davis, S. R. & Bellamy, A. R. (1992) EMBO J. 11, 4411–4418.

Lieber, A. & Strauss, M. (1995) Mol. Cell. Biol. 15, 540–551.
Lo, K., Biasolo, M. A., Dehni, G. & Haseltine, W. A. (1992) Virology 190, 176–183
Marschall, P., Thomson, J. B. & Eckstein, F. (1994) Cell. Mol. Neurobiol. 14, 523–538
Moore, S. (1983) J. Biol. Chem. 258, 1004–1010
Nasmyth, K. (1993) Curr. Opin. Cell Biol. 5, 166–170
Ojwang, J. O., Hampel, A., Looney, D. J., Rappaport, J. & Wong-Staal, F. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10802–10806
Pardee, A. B. (1989) Science 246, 603–608
Peter, M. & Herskowitz, I. (1994) Science 265, 1228–1231
Pontius, B. W. & Berg, P. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8403–8407
Pringle, J. R. & Hartwell, L. H. (1981) in The Molecular Biology of the Yeast Saccharomyces cerevisiae LifeCycle and Inheritance (Strathern, J. N., Jones, E. W. & Broach, J. R., eds) pp. 97–142, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Rossi, J. J. (1992) Curr. Opin. Biotechnol. 3, 3–7
Russo, P. & Sherman, F. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8348–8352
Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Sarver, N., Cantin, E. M., Chang, P. S., Zaia, J. A., Ladne, P. A., Stephens, D. A. & Rossi, J. J. (1990) Science 247, 1222–1225
Smith, B. E., O’Day, D. H. & Proteau, G. A. (1995) Biochem. Biophys. Res. Commun. 206, 401–407
Stefan, C. J. & Blumer, K. J. (1994) Mol. Cell. Biol. 14, 3339–3349
Sullenger, B. A. & Cech, T. R. (1993) Science 262, 1566–1569
Tsuchihashi, Z., Khosla, M. & Herschlag, D. (1993) Science 262, 99–102
Van der Krol, A., Mol, J. N. M. & Stuitje, A. R. (1988) BioTechniques 6, 958–976
Wittenberg, C., Sugimoto, K. & Reed, S. I. (1990) Cell 62, 225–237