A temperature sensitive mutant of *Saccharomyces cerevisiae* defective in pre-rRNA processing

Ke Shuai⁺ and Jonathan R. Warner^{*}

Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York, NY 10461, USA

Received April 1, 1991; Revised and Accepted August 22, 1991

ABSTRACT

A recessive temperature sensitive mutant has been isolated that is defective in ribosomal RNA processing. By Northern analysis, this mutant was found to accumulate three novel rRNA species: 23S', 18S' and 7S', each of which contains sequences from the spacer region between 25S and 18S rRNA. 35S pre-rRNA accumulates, while the level of the 20S and 27S rRNA processing intermediates is depressed. Pulse-chase analysis demonstrates that the processing of 35S pre-rRNA is slowed. The defect in the mutant appears to be at the first processing step, which generates 20S and 27S rRNA. 7S' RNA is a form of 5.8S RNA whose 5' end is extended by 149 nucleotides to a position just 5 nucleotides downstream of the normal cleavage site that produces 20S and 27S rRNA. 7S' RNA can assemble into 60S ribosomal subunits, but such subunits are relatively ineffective in joining polyribosomes. A single lesion is responsible for the pre-rRNA processing defect and the temperature sensitivity. The affected gene is designated *RRP2*.

INTRODUCTION

In *Saccharomyces cerevisiae*, the processing of the primary 35S rRNA transcript involves accurate recognition and processing of the mature 5' and 3' termini, removal of transcribed spacer sequences, and modification of individual nucleosides. Ribosomal proteins and small nuclear RNAs are involved in this process (1). The sequence of rRNA processing steps has been proposed (2,3) (Fig. 1). However, the timing of the various cleavage events has not been precisely determined. No enzyme involved in yeast pre-rRNA processing has been identified.

In bacteria, studies on prokaryotic pre-rRNA processing have been greatly facilitated by isolation and characterization of mutants involved in various steps of rRNA maturation (4). In yeast a temperature sensitive mutant has been found that blocks specifically a single step in the formation of rRNA: the processing of 27S rRNA to 25S rRNA (5). New 60S ribosomal subunits are not assembled in this mutant. The unprocessed 27S rRNA and the newly synthesized 60S subunit proteins are quickly

degraded, most with half-lives of less than 7.5 min (6). The mutant gene is designated *rrp1* (*rrp* for ribosomal rRNA processing); the *RRP1* gene has been cloned (7). The *RRP1* mRNA is in lower abundance than are the mRNAs for ribosomal proteins, suggesting that *RRP1* gene product probably does not code for a ribosomal protein.

Other mutants involved in pre-rRNA processing have also been reported. Mitlin and Cannon (8) have described a temperature sensitive mutant which has a slowed conversion of 20S rRNA to 18S rRNA. Particles containing 20S rRNA are found to accumulate in the nucleus and are only slowly transported to the cytoplasm. The processing of 27S pre-rRNA to 25S rRNA is normal in this mutant. Further characterization of this mutant has not been reported.

In order to study the mechanism of pre-rRNA processing, we have screened a temperature sensitive bank for mutants involved in rRNA processing. The isolation and characterization of a temperature sensitive mutant in a new gene, *RRP2*, is reported in this paper.

MATERIALS AND METHODS

Yeast strains

Yeast strain W303 (*MATa*, *leu2-3,112*, *trp1-1*, *ura3-1*, *can1-100*, *ade2-1*, *his3-11,15*) was obtained from R. Rothstein, Columbia University; J1003.1D (*MATa*, *ade2-1*, *ura3-1*, *trp1*, *leu2-3,112*, *can1-100*) and J1003.1E (*MATa*, *his3-111*, *ade2-1*, *ura3-1*, *leu2-3,112*, *can1-100*) were derivatives of W303 in which the intact *HIS3* and *TRP1* genes, respectively, had been introduced by transformation. Except where indicated, yeast strains were grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose). The growth of yeast cells in liquid culture was measured by light scattering.

A bank of 517 temperature sensitive mutants was generated using ethyl-methane sulfonate mutagenesis to approximately 45% killing. Permissive conditions were 23°C, non-permissive 37°C, both in YPD. The subject of this paper is mutant strain ts517. This was backcrossed to the parental strain and the mutant spore used for most of the biochemical experiments is termed KS1D.

* To whom correspondence should be addressed

⁺ Present address: The Rockefeller University, Laboratory of Molecular Cell Biology, 1230 York Avenue, New York, NY 10021, USA

Yeast RNA preparation

An eight ml culture logarithmically growing at 23°C at $0.6-2.0 \times 10^7$ cells/ml was shifted to 37°C for 90 min and then poured onto sterile crushed ice. Total RNA was extracted essentially as described (9).

For preparation of small RNA a 200 ml culture at $0.7-2.0 \times 10^7$ cells/ml was centrifuged at 7,000 rpm for 5 min and washed with sterile water. Cells were resuspended in 3 ml of 20 mM Tris-HCl (pH 7.4) and 5 mM EDTA (pH 7.4) and were transferred to a 15 ml Sarstedt polypropylene tube to which 3.0 ml of phenol equilibrated with the same buffer was added. The tube was capped and rotated end over end on a roller drum for 30 min at 37°C. The aqueous fraction was extracted twice with phenol/CHCl₃. RNA was precipitated by addition of 5M NaCl to 0.1 M and 2.5 volume of 95% ethanol, and chilling at -20°C for at least 3 hrs.

Pulse-chase Analysis

A 3 ml culture at $0.75-2.0 \times 10^7$ cells/ml in synthetic medium without methionine at 23°C was shifted to 37°C for 1 hr and was pulse-labeled with 60 μ Ci/ml [methyl-³H] methionine for 2.5 min. 500 μ g/ml of cold methionine was immediately added. Samples of 1 ml were withdrawn at the indicated times and were transferred to eppendorf tubes with crushed ice. Total RNA was prepared and analyzed on a 1.5% agarose gel in formaldehyde (10). The agarose gel was soaked with EN³HANCE [NEN-DuPont] for 3 hrs and washed in cold sterile water for 1 hr while the gel was shaking slowly. The gel was then dried and visualized by fluorography at -70°C.

Figure 1. rRNA processing pathway in *Saccharomyces cerevisiae*. The scheme shows the steps involved in the processing of the 35S primary transcript to produce mature 18S, 5.8S and 25S rRNA. The 18S and 25S RNAs are shown with interruptions to save space. A1, A2, B1, B2, C1, C2, D, and E represent cut sites of proposed processing enzymes. (Adapted from Veldman et al., 1981)

Screening of the temperature sensitive mutant bank

The temperature sensitive bank of 517 mutants was screened by Northern analysis. Mutant cells growing at 23°C were shifted to 37°C for 90 min. RNA was extracted from each mutant and analyzed by Northern hybridization using probes from rDNA regions (Fig. 2). Potential mutants involved in pre-rRNA processing were identified by comparing the phenotype of each mutant on the Northern blot with that of a wild type strain.

Miscellaneous techniques

Northern blot analysis was performed essentially as described by Thomas (10). Oligonucleotides were synthesized on an Applied Biosystems Model 380A DNA Synthesizer. Oligonucleotide probes were 5'-end labeled with [γ^{32} P]ATP (Amersham, 3000 μ Ci/mmol) and T4 polynucleotide kinase. Polyribosome preparation was performed as described (11). Slot blot analysis was performed as described (12). Primer extension analysis was carried out as described (13).

RESULTS

Identification of a Recessive Mutant That Accumulates Unusual rRNA Processing Products

To screen the bank of ts mutants, RNA was prepared from each mutant after it had been at the restrictive temperature for 90 minutes. Each RNA sample was subjected to Northern analysis, and hybridized to a number of probes representing different regions of the ribosomal RNA transcript, as well as from several protein encoding genes (Fig. 2). The mutant ts 517 was identified by its novel phenotype shown on the Northern blot when probed with the oligonucleotide probe JW-134 (Fig. 3B). In addition to a significant accumulation of 35S rRNA and unusually low levels of 27S and 20S rRNA precursors, there were three distinctive bands, termed 23S', 18S', and 7S' (Fig. 3B). On an overexposed film, one can identify several additional minor bands. For the most part the level of mRNAs is unaffected by the mutation, but in certain cases, e.g. *ENO1* (Fig 3E), there is a reproducible deficiency of mRNA for unknown reasons. The splicing process seems to be normal in this mutant as indicated by probing with the *ACT1*, *RPS10*, and *RPL32* genes (Fig. 3D, 3E, 3F). The small amount of unspliced mRNA from *RPL32* is the usual consequence of an imbalance of rRNA and ribosomal protein production (14,15).

Figure 2. Probes for rRNA used to screen the temperature sensitive bank. The exact positions of these probes are indicated in parentheses.

The mutant strain was crossed with the wild type strain J1003.1D and the diploid was found to grow at 37°C, indicating that the temperature sensitivity of *ts* 517 is recessive. RNA from the diploid strain also showed a wild type phenotype (Fig. 4), suggesting its phenotype on the Northern blot is also recessive.

This diploid was sporulated and its tetrads analyzed. Eleven sets of tetrads were obtained; all showed 2:2 segregation for temperature sensitivity. RNA from five sets of those tetrads were extracted and analyzed on a Northern blot. It was found that temperature sensitivity and the appearance of 23S', 18S' and 7S' RNAs cosegregated 2:2 (Fig. 4) suggesting that the two phenotypes of *ts* 517 are due to a single mutation or to mutations in closely linked genes. Subsequent experiments were carried out with the strain KS1D derived from spore D, except where indicated.

Spontaneous revertants that grew at the restrictive temperature were obtained at a frequency of about 10^{-6} . Twenty *ts* revertants were analyzed by Northern blots; all showed a wild type phenotype (Fig. 5). The reversion is at or near the site of the mutation since dissection of 18 tetrads from a cross of the revertant with wild type revealed no *ts* spores.

All together the results demonstrate that a single mutation causes both temperature sensitivity for growth and the appearance of 23S', 18S', and 7S'. We tentatively term the gene mutated as *RRP2* (7). The *rrp2* allele complements *rrp1* (data not shown), showing that they are different genes.

Temperature Sensitivity of the *rrp2* mutant

The growth of the *rrp2* mutant strain on YPD plates at 37°C was examined. Mutant cell cultures growing at 23°C were shifted to 37°C for 2 hours and cells were streaked onto a pre-warmed

YPD plate which was then incubated at 37°C. Growth of mutant cells was then examined under a microscope over a period of time. The mutant cells formed micro-colonies of only 4 to 30 cells.

In liquid medium at 23°C the doubling time of the *rrp2* mutant strain is 225 min, while that of the wild type strain is 145 minutes (Fig. 6A). The slower growth is presumably due to a residual effect of the mutation, since some 7S', 18S' and 23S' RNA is present in cells growing at 23°C (Figure 7).

The growth curve was determined after cultures were shifted from 23°C to 37°C (Fig. 6B). The mutant cells grew progressively more slowly at 37°C, showing no significant growth after four to five generations. Microscopic examination revealed no enrichment of cells at a particular phase of the cell cycle.

Figure 3. Northern analysis of *ts* 517. 5 μ g of RNA extracted from mutant cells growing at 37°C was analyzed on the Northern blot and probed with A) JW-144; B) JW-134; C) JW-164; D) *ACT1*; E) *ENO1* and *RPS10*; F) *RPL3* and *RPL32*; G) the locations of 23S', 18S' and 7S'. The blots have been rearranged in the vertical dimension for clarity. The actual sizes of the mRNAs (and their unspliced precursors) are approximately: *ACT1*: 1500, (1800); *ENO1*: 1357; *RPS10*: 799, (1151); *RPL3*: 1320; *RPL32*: 535, (765).

Figure 4. Tetrad analysis of mutant *rrp2*. The diploid *ts*517 $\times$ J1003.1D was sporulated, its tetrads dissected and RNA from four spores A, B, C, D was extracted and analyzed on the Northern blot as described under *Methods*. RNA from the diploid strain (*a/a*) was also analyzed on the same blot. The Northern blot was probed with JW-134.

Figure 5. Reversion analysis of the mutant allele of *ts*517. 5 μ g of RNA from log-phase cultures of *ts*517 (*ts*), a revertant (*rev*), and wild type (*WT*) cells was analyzed on a Northern blot as described for Figure 3. The Northern blot was probed with JW-134.

Figure 6. Growth of wild type (J1003.1E) and *rrp2* mutant (KS1D) strains. At the indicated times, turbidity was measured in a Klett colorimeter, using a red filter. A) Log-phase cultures of KS1D and J1003.1E growing at 23°C in YPD medium. The doubling time of J1003.1E is 145 min. The doubling time of KS1D is 225 min. B) Similar cultures growing at 23°C at a concentration of 1×10^7 /ml were shifted to a 37°C water bath. ■—■: J1003.1E at 23°C and 37°C. □—□: KS1D at 23°C and 37°C. For measuring growth at 37°C, dilution was made when the cell density reached 2.5×10^7 in order to maintain log-phase growth.

Figure 7. Mutant processing products at the permissive temperature. Northern analysis as described in Figure 3 was carried out on RNA isolated from *rrp2* mutant cells (KS1D) and wild type cells (WT) growing at permissive and at non-permissive temperatures. The blot was probed with oligonucleotide JW 134, complementary to sequences just upstream of 5.8S RNA. (See Figure 2)

Pulse-chase Analysis of the *rrp2* mutant

The processing of newly synthesized pre-rRNA can be followed by pulse-chase analysis after labelling with the methyl group of [C^3H_3]methionine (16; Fig. 8). After a 2.5 minute pulse at the beginning of the chase, 35S, 27S and 20S pre-rRNA could be detected in the wild type strain and about one third of 20S rRNA had already been processed to 18S rRNA. In the mutant strain, however, the major rRNA detected at the beginning of the chase was 35S rRNA. Only a small fraction of 35S rRNA had been processed to 27S and 20S rRNA; no mature RNA could be detected. After a chase of 3 min, the wild type strain showed a 27S/25S ratio of about 1:1 and 20S rRNA was completely processed to 18S rRNA, while in the *rrp2* mutant strain little of the 27S rRNA had been processed to 25S rRNA, and the ratio of 20S/18S rRNA was about 1:1. After a chase of 10 min, the pre-rRNA processing was completed and the 25S/18S ratio is similar in both wild type and mutant strains. A similar experiment carried out at the permissive temperature revealed that the mutant had only a slightly slower rate of processing than the wild type. (data not shown). The pulse-chase analyses suggest that, in the mutant at the restrictive temperature, 35S rRNA is processed relatively slowly, but yields 25S and 18S RNA in approximately the amounts and ratios of the wild type. No 23S' RNA is apparent. The 23S' RNA detected in the Northern analysis (Fig. 2) may be rare but stable aberrant molecules. 7S' RNA would not be detected in this experiment because it has no methyl groups.

Identification of the distinctive RNA species of the *rrp2* mutant

By comparison with the migration of the known rRNA precursor species, the 23S', 18S' and 7S' RNA species are estimated to be about 3000 nt, 1600 nt and 310 nt in length, respectively (Fig. 3). All three RNA molecules could be detected by oligonucleotide probes JW-134 and JW-164. The 23S' and 18S' species but not 7S' could be detected by oligonucleotide probes JW-144 or JW-166 (Fig. 3A, 3B, 3C). The 23S' RNA could also be detected by oligonucleotide JW-127. None of these three species could be detected by oligonucleotide probes JW-145 or

Figure 8. Pulse-chase analysis. Three ml log-phase cultures of wild type and KS1D strains growing at 23°C were shifted to 37°C for 1 hr and labelled with 60 μ Ci/ml [C^3H_3]methionine for 2.5 min. 500 μ g/ml of non-radioactive methionine was then added. 1 ml samples were withdrawn at indicated times. RNA was prepared and 5×10^4 cpm was analyzed on a 1.5% agarose gel. The gel was treated with EN 3 HANCE and visualized by fluorography.

JW-128 (data not shown). These RNA species are therefore identified as the molecules shown in Figure 3G.

The appearance of 7S' RNA on the Northern blot is especially interesting for two reasons: 1) It is relatively abundant as indicated by its signal on the Northern blot. 2) While the 7S rRNA that is the precursor of 5.8S RNA includes spacer sequence at its 3' end (3; Fig. 1), the 7S' RNA found in the *rrp2* mutant strain contains 5.8S RNA with spacer sequence at its 5' end. To determine the exact 5' terminus of 7S' RNA, primer extension analysis was carried out on small RNA prepared from the *rrp2* mutant strain, using oligonucleotide JW-134 as a specific primer (Fig. 9). The single primer extension product is 131 bp in length. The 5' terminus of 7S' RNA is therefore at a site 5 nucleotides downstream of the normal cleavage site (A2 in Fig. 1) that generates the 20S and 27S intermediates (17,3). Alternatively, the 5' end of 7S' RNA may be the normal A2 cleavage site, with 5 nucleotides usually being removed by an exonuclease.

Polyribosome Analysis

Does 7S' RNA assemble into ribosomal subunits? In order to identify the complexes with which 7S' RNA may be associated and to study the effects of the *rrp2* mutation on ribosome assembly, ribosomes and polyribosomes from the wild type and

Figure 9. Primer extension analysis of 7S' RNA. 2 μ g of total yeast small RNA was analyzed by primer extension using 20 ng of 5'- 32 P labeled JW-134 as a primer. A portion of the primer extension product was loaded adjacent to a dideoxy sequencing ladder. The 5' end of the primer extension product deduced from the dideoxy sequencing ladder is indicated. (Note that this is the anti-sense sequence.)

Figure 10. Location of 7S' RNA. Samples of 0.1 ml from the 40S, 60S, 80S and polysome fractions of sucrose gradients of extracts of wild type and KS1D strains were applied to a slot blot. The blot was probed with oligonucleotides JW-134 to reveal 7S' RNA and JW-71 to reveal 5S RNA.

mutant strains growing at 23° were prepared as described under *Methods*. Fractions containing 40S, 60S, 80S and polyribosomes were applied to a slot blot (12) that was probed with JW-71 (an oligonucleotide complementary to a sequence within 5S rRNA) to detect 5S RNA, as well as with oligonucleotide JW-134 that is specific for 7S' RNA (Fig. 10). The 7S' RNA is found in 60S, 80S, and polyribosome fractions. However, the 7S'/5S rRNA ratio decreases in the order of 60S, 80S and polyribosome fractions. These results suggest that 7S' RNA can assemble into 60S subunits, but that such 60S subunits enter polyribosomes very inefficiently.

DISCUSSION

We have identified a temperature sensitive mutation, in a gene termed *RRP2*, that leads to defective pre-rRNA processing. Pre-mRNA processing is not affected. A different mutant allele of *RRP2*, confirmed by lack of complementation, has been isolated by L. Lindahl, R.H. Archer and J.M. Zengel (pers. comm.). The defect in RNA processing is evident in two ways. The processing of 35S precursor to the mature 25S and 18S rRNAs is slowed substantially, and several aberrant RNA species accumulate. Chief among these is a molecule we have termed 7S' RNA that consists of the entire 5.8S RNA with an additional 149 nucleotides at its 5' end. This molecule can be incorporated into a 60S subunit. Such subunits are inefficient at entering polyribosomes.

Although the processing pattern shown in Figure 1 has been generally accepted for a number of years, recent evidence suggests that in higher organisms there is a primary cleavage site near the 5' end of the transcript. (18) U3 RNA has been implicated in this cleavage step, (19) and some evidence suggests it may have a role at the A2 site as well. (20) The precise role of the snRNAs in the processing of yeast ribosomal precursor RNA has not yet been established, but their involvement in some steps seems beyond doubt. (21,22,23)

The mutation in *RRP2* leads to an accumulation of 35S rRNA and the appearance of 23S', 18S' and 7S' RNA species, suggesting that the mutant has a defect in the processing step that generates 20S and 27S rRNA. Instead of being cleaved at A2, the normal site, 35S rRNA is cleaved at site E to generate the 23S' RNA species. Subsequent cleavage at or near site A2 would yield a 7S' species that might no longer be susceptible to cleavage at site B1. The defect either could be in a processing enzyme responsible for the first cleavage in wild type strains (Fig. 1), or could be due to a defective factor that leads the pre-rRNA to form an altered conformation in the processing complex. It is unlikely to be in U3 RNA because (a) the mutant is recessive, (Fig. 4) and (b) there are two genes for U3 RNA, either of which will support growth. (21)

While we have demonstrated genetically that the temperature sensitivity and the aberrant processing of rRNA are caused by the same mutation, the biochemical basis of the temperature sensitivity remains unclear. Apparently normal 25S and 18S RNA continue to be synthesized, albeit at a somewhat reduced rate. The 7S' RNA is clearly observable even at the permissive temperature. (Figure 7) It accumulates to no more than 5–10% of the 5.8S RNA even after six hours at 37°C, when growth has declined substantially (data not shown). The mutant cell grows more slowly than wild-type at 23°C, but at same rate as wild type at 18°C. Does even a low amount of 7S' RNA affect the rate or the accuracy of protein synthesis sufficiently to cause this

phenotype? Or is the appearance of 7S' RNA simply a symptom of a more profound biochemical effect of the mutation that we have not yet detected?

The screening of more than 500 ts mutants, each with probes for several regions of the transcribed spacer regions of the rRNA, led to only a single clear mutant in rRNA processing, and perhaps to a few that were marginal. In contrast many were defective in mRNA processing. This suggests either that aberrant processing intermediates are degraded extremely rapidly, or that there are very few genes involved in rRNA processing, or that such genes are duplicated. Clearly, alternative methods will be required to identify more mutations in this process.

ACKNOWLEDGMENTS

We would like to thank Ian Willis for advice, Lasse Lindahl for communicating results, Lenore Neigeborn, Qida Ju, Diane Baronas-Lowell and Lefa Alksne for generating the temperature sensitive mutant bank for this study and Pei Ying Yuan for technical assistance. This work has been supported by grants #GM25532 and CA13330 from the National Institutes of Health and by American Cancer Society grant # MV-323T.

REFERENCES

1. Warner, J. R. (1989) *Micro. Rev.* **53**, 256–271.
2. Udem, S. A. and Warner, J.R. (1973) *J. Biol. Chem.* **48**, 1412–1416.
3. Veldman, G. M., Klootwijk, J., van Heerikhuizen, H. and Planta, R.J. (1981) *Nucleic Acids Res.* **9**, 4847–4862.
4. Srivastava, A.K. and Schlessinger, D. (1990) *Annu. Rev. Microbiol.* **44**, 105–129.
5. Andrew, C., Hopper, A.K. and Hall, B.D. (1976) *Mol. Gen. Genet.* **144**, 29–35.
6. Gorenstein, C. and Warner, J.R. (1977) *Mol. Gen. Genet.* **157**, 327–332.
7. Fabian, G. R. and Hopper, A.K. (1987) *J. Bacteriol.* **169**, 1571–1578.
8. Mitlin, J. A. and Cannon, M. (1984) *Biochem. J.* **220**, 461–467.
9. Elion, E. A. and Warner, J.R. (1984) *Cell* **39**, 663–673.
10. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5201–5205.
11. Warner, J. R., Mitra, G., Schwindinger, W. F., Studeny, M. and Fried, H.M. (1985) *Mol. Cell. Biol.* **5**, 1512–1521.
12. de Benedetti, A. and Baglioni, C. (1986) *J. Biol. Chem.* **261**, 15800–15804.
13. Pikielny, C. W. and Rosbash, M. (1985) *Cell* **41**, 119–126.
14. Dabeva, M.D., Post-Beittenmiller, M.A. and Warner, J.R. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5854–5857.
15. Wittekind, M., Kolb, J.M., Dodd, J., Yamagishi, M., Memet, S., Buhler, J.-M. and Nomura, M. (1990) *Molec. Cell. Biol.* **10**, 2049–2059.
16. Warner, J.R. (1991) In Guthrie, C. and Fink, G. (eds.), *Methods of Enzymology*. Academic Press, Vol. 194, pp 423–428.
17. Veldman, G.M., Brand, R.C., Klootwijk, J. and Planta, R.J. (1980) *Nucl. Acids Res.* **8**, 2907–2920.
18. Miller, K.G., and Sollner-Webb, B., (1981) *Cell* **27**, 165–174.
19. Kass, S., Tyc, K., Steitz, J.A., and Sollner-Webb, B. (1990) *Cell* **60**, 897–908.
20. Savino, R. and Gerbi, S.A. (1990) *EMBO J.* **9**, 2299–2308.
21. Hughes, J.M., Konings, D.A., and Cesareni, G. (1987) *EMBO J.* **6**, 2145–2155.
22. Tollervey, D. (1987) *EMBO J.* **6**, 4169–4175.
23. Li, H.V., Zagorski, J. and Fournier, M.J. (1990) *Mol. Cell Biol.* **10**, 1145–1152.