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Characterization of an Essential *Saccharomyces cerevisiae* Gene Related to RNA Processing: Cloning of *RNA1* and Generation of a New Allele with a Novel Phenotype

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The *RNA1* gene product is believed to be involved in RNA metabolism due to the phenotype of a single conditionally lethal, temperature-sensitive allele, *rna1-1*. We cloned the *RNA1* gene and determined that it produces a 1,400-nucleotide polyadenylated transcript. On a multicopy plasmid, the mutant *rna1-1* allele partially complements the *rna1-1* temperature-sensitive growth defect. This suggests that the temperature-sensitive nature of the *rna1-1* allele results from the synthesis of a product with lowered activity or stability at elevated temperatures or from a decrease in synthesis of the *rna1-1* product at the restrictive temperature. A chromosomal disruption of *RNA1* behaves as a recessive lethal mutation. Haploids bearing the distruption were isolated by sporulating a diploid heterozygous for the disrupted allele and the *rna1-1* allele and possessing an episomal copy of the *RNA1* gene. Analysis of the rescued haploids bearing the chromosomal disruption indicated that the recessive lethal phenotype of the *RNA1* disruption is not merely due to a block in spore germination. Unexpectedly, diploids heterozygous for the disruption and the *rna1-1* alleles become aneuploid for chromosome XIII at a frequency of 2 to 5%. It appears that the disrupted *RNA1* allele on a multicopy plasmid also promotes aneuploidy for chromosome XIII. Promotion of aneuploidy seems to be a phenotype of this particular allele of *RNA1*.

In eucaryotes, the maturation of an RNA may include 5′ and 3′ end processing, removal of intervening sequences, and modification of individual nucleosides (13). In *Saccharomyces cerevisiae*, *trans*-acting mutations have been isolated which interfere with this processing regimen. The temperature-sensitive, conditionally lethal recessive mutation *rna1-1* prevents growth at temperatures of 34°C and above (restrictive temperature). This mutation has a conditional pleiotropic effect, interfering not only with the production of rRNA, mRNA, and tRNA but apparently also with the transport of mRNA and rRNA from the nucleus to the cytoplasm (14, 15, 28).

At the restrictive temperature, *rna1-1* strains exhibit a rapid decrease in the rate of accumulation of newly synthesized RNA (15, 28). After 20 min at 36°C, an *rna1-1* strain accumulates ca. 60% of the total newly synthesized RNA that is accumulated in the permissive-temperature control (15). Approximately 60% of this is localized in the nucleus (15).

rRNA has been shown to be affected by *rna1-1* at the level of maturation of the nuclear 35S rRNA precursor (14). This normally short-lived precursor moderately accumulates and is not processed to the mature 25S, 5.8S, and 18S rRNAs (14). This processing block may not reflect the primary nature of the *rna1-1* defect since environmental factors or mutations which affect ribosomal protein synthesis also characteristically affect rRNA synthesis (5, 16, 17, 29, 35, 36). Therefore, a decline in the accumulation of translatable ribosomal protein mRNA conceivably could lead to such a phenotype.

The effect of the *rna1-1* mutation upon mRNA production is not clearly defined. At the restrictive temperature, the rate of polyadenylated RNA accumulation is 75% lower than that at the permissive temperature (28). The polyadenylated RNA which is synthesized appears to remain predominantly within the nucleus (28) and to be 10% larger than that produced at the permissive temperature (28). *rna1-1* does not appear to affect the removal of intervening sequences from precursor mRNAs (26). However, *rna1-1* strains incubated at the restrictive temperature produce two novel *GAL1* transcripts which are extended at the 3′ terminus (31). It is not known whether these novel transcripts are transient processing intermediates, stabilized in *rna1-1* strains, or merely reflect aberrant transcription termination (13).

The effect of *rna1-1* upon tRNA production has been more clearly defined. At the restrictive temperature, *rna1-1* strains accumulate tRNA species which correspond to intervening-sequence-bearing precursors. These accumulated precursors have the mature termini and most of the nucleoside modifications found in the mature tRNA (7, 18, 19, 23). The defect leading to this accumulation appears not to be at the level of the intervening-sequence-splicing activity, per se, since extracts from *rna1-1* strains contain tRNA-splicing activity in vitro (L. D. Schultz and A. K. Hopper, unpublished results).

The pleiotropic nature of the *rna1-1* defect upon the processing and possibly transport and transcription of multiple RNA species could be generated by interference with a process quite removed from events normally classified as RNA metabolism and transport. New mutations in the *RNA1* gene may not exhibit the pleiotropic nature of the *rna1-1* mutation and might more obviously reflect the primary function of the *RNA1* gene product. Since the *rna1-1* mutation affects the production of the three major classes of RNA, the elucidation of the function of the *RNA1* gene product could define an unknown common step in gene expression. We believe that information concerning the phenotype of new alleles of *RNA1* and the nature and cellular location of the gene product will provide strong

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clues as to the function of this product. Therefore, we cloned the *RNA1* gene and utilized this cloned sequence to examine the nature of the *RNA1* gene product and the *rna1-1* mutation. By in vitro manipulation of the cloned *RNA1* gene, we also created a new allele of *RNA1* which exhibits the novel phenotype of promotion of aneuploidy.

MATERIALS AND METHODS

Abbreviations. The following abbreviations were used: kb, kilobases; bp, base pairs; n, nucleotides.

General yeast genetic methods and media. Standard yeast genetic methods of mating, sporulation, and tetrad analysis were followed (22). Strains were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose supplemented with 0.4% adenine and 0.4% uracil) or defined dropout media with 2% glucose as the carbon source. Sporulation of diploids was carried out on plates containing 1% potassium acetate, 0.1% glucose, 0.25% yeast extract 0.01% adenine, 0.01% uracil, and 1.5% agar.

Concomitant loss of plasmid markers and mitotic stability of transformed strains were assayed by growing the strains to stationary phase in YEPD broth at 23°C and plating the culture for single colonies upon YEPD plates at a density of 100 to 300 colonies per plate. These plates were incubated at 23°C, and the colonies were replica plated to selective media or grown under selective conditions to determine the rate of loss of a particular trait.

Transformation and plasmid isolation. Yeast strains were transformed essentially as described by Hinnen et al. (10) except that 50 μg of sonicated denatured salmon sperm DNA per ml was added with the transforming DNA to the spheroplasts. Plasmids were isolated from yeast cells as described by Hirt (11) and used to transform *Escherichia coli* RR1 to ampicillin resistance by the calcium chloride procedure (20). Plasmid DNAs were prepared from bacteria by the alkaline lysis procedure (1) or by a modification of the Holmes and Quigley boiling procedure (12) as described in *Focus*, vol. 3, no. 2, published by Bethesda Research Laboratories.

Enzymatic reactions. Restriction enzymes, bacterial alkaline phosphatase, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were purchased from Bethesda Research Laboratories or New England BioLabs, Inc. Restriction digestions were performed as per the instructions of the manufacturers. Bacterial alkaline phosphatase treatment of restricted DNA was as described by Ullrich et al. (34). Overhanging ends of restriction sites were converted to blunt ends with 20 U of the Klenow fragment of DNA polymerase I in a 10-μl reaction mix in nick translation buffer with 8 nmol each of dATP, dCTP, dGTP, and dTTP. Ligations were performed 14°C overnight in the buffer prescribed by the manufacturer. The concentrations of vector and insert sequences were determined according to the equations of Dugaiczyk et al. (6) and the recommendations of Bethesda Research Laboratories (*Focus*, vol. 2, no. 2 and 3). Probes were radiolabeled by using a Nick Translation Reagent Kit (Bethesda Research Laboratories) with [^{32}P]dCTP (New England Nuclear Corp.). DNA and unincorporated nucleotides were separated by the spun-column procedure (20).

Preparation of yeast genomic DNA. High-molecular-weight genomic DNA was prepared by the rapid yeast DNA isolation procedure (32) with the following modifications. The genomic DNA was precipitated by addition of ammonium acetate to 2.5 M and 2 volumes of isopropanol followed by centrifugation at 12,000 × *g* for 15 s. DNA pellets were vacuum dried, suspended in 10 ml of 10 mM Tris base–1 mM EDTA (pH 8.0), and extracted with distilled phenol saturated with 10 mM Tris base–1 mM EDTA (pH 8.0).

Gel electrophoresis of DNA, transfer to nitrocellulose, and hybridization. Agarose gel electrophoresis was carried out in 0.7% agarose gels in 100 mM Tris base–99 mM boric acid–2.5 mM EDTA (pH 8.6). The size standards used were *Hin*dIII-digested lambda DNA or *Rsa*I-digested YEp24 DNA. DNA in gels was acid depurinated and denatured (20) and transferred to nitrocellulose (Schleicher & Schuell, Inc.) with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (33). Blots were rinsed in boiling deionized water after being baked (30) and then placed upon the inside wall of screw cap jars, and prehybridization buffer (33) was added. The jars were rotated in a 40°C warm room for 9 to 15 h. Hybridization to nick-translated DNA probes was performed similarly with hybridization buffer (33). Filters were exposed to XAR-5 X-ray film (Eastman Kodak Co.) with intensifying screens (Du Pont Co.) at −70°C.

Preparation of RNA and Northern analysis. Total cellular RNA was prepared by breaking mid-log-phase yeast cells with glass beads in the presence of buffer-saturated phenol as described by Hopper et al. (14). RNA preparations were stored in 10 mM Tris base–1 mM EDTA (pH 8.0) at −70°C. RNAs were electrophoresed in 1% agarose-formaldehyde gels as described by Maniatis et al. (20), transferred to nitrocellulose in 20× SSC as described by Thomas (33), and boiled in deionized water after being baked (30). Prehybridization, hybridization, and exposure to film were as described for DNA blots. Size standards were as described above for Southern analysis.

Colony hybridization. Colony hybridization was carried out with nitrocellulose by the protocol of Schleicher & Schuell, Inc. Positively hybridizing colonies were those which hybridized to the probe in two consecutive rounds of hybridization.

Gene disruption. Disruption of a genomic copy of the *RNA1* gene was performed essentially as described by Rothstein (27). A 3-kb *Bgl*II fragment from YEp13 (3) containing the *LEU2* gene was a gift from Tim Torchia. This fragment was ligated into the sole *Bam*HI site of YEpRNA1(7.7) to construct YEpRNA1(7.7)::LEU2. A 10-μg portion of a 5.4-kb *Sst*I fragment which contained the disrupted *RNA1* gene was used to transform the leucine auxotrophic strain 2b×3b to leucine prototrophy. γδ mutagenesis was performed essentially as described by Guyer (8, 9).

Construction of YCpRNA1(7.7). Plasmid YCpRNA1(7.7) was constructed from YEpRNA1(7.7) and YCL1. YCL1 (a gift from Stephen Johnston) was constructed from pYE (CEN3)41 (4). A 13.4-kb *Eco*RI fragment from YEpRNA1 (7.7) and a 4-kb *Eco*RI fragment from YCL1 were ligated and transformed into *E. coli*. This yielded plasmid YCpRNA1(7.7), which contains the pBR322 sequences and yeast *URA3* and *RNA1* genes from YEpRNA1(7.7) and the *ars1* and *CEN3* sequences from YCL1. The mitotic and meiotic loss rates of YCpRNA1(7.7) were 10 and 62%, respectively.

RESULTS

Cloning and subcloning of *RNA1*. The *RNA1* gene was cloned from a pool of yeast genomic sequences carried in the vector YEp24 (provided by D. Botstein). YEp24 is a derivative of pBR322 which contains the yeast *URA3* gene and the 2μm origin of replication (2).

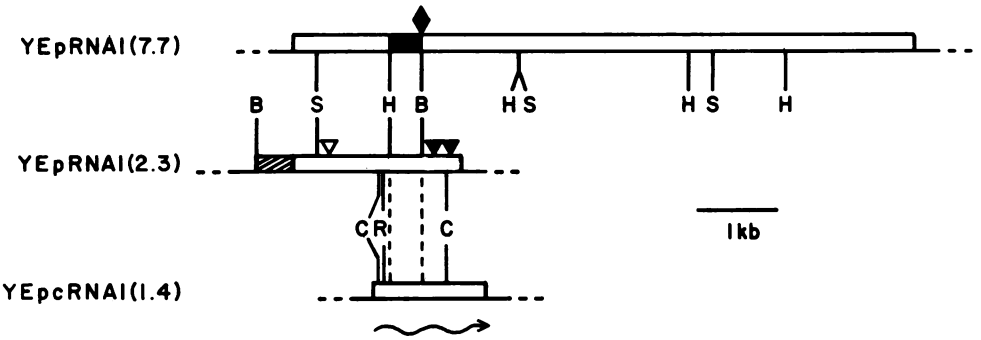

FIG. 1. Restriction maps of recombinant plasmids which complement the *rna1-1* mutation. The open rectangles represent genomic DNA [YEpRNA1(7.7) and YEpRNA1(2.3)] or cDNA insert sequences [YEpcRNA1(1.4)]. The thin lines represent the plasmid vector DNA. Diagonal lines represent a duplication of pBR322 sequences. Open and solid triangles are sites of γδ insertions which do not and do prevent complementation, respectively. The solid diamond is the site of insertion of a *LEU2*-bearing DNA fragment which prevents complementation of *rna1-1*. The darkened area of YEpRNA1(7.7) represents the *RNA1*-specific fragment in the probe pBRRNA1(0.45). The arrow represents the *RNA1* transcript and the direction of transcription. Restriction sites: B, *Bam*HI; S, *Sst*I; R, *Eco*RV; C, *Hin*cII; H, *Hin*dIII.

Strain EE1b (relevant genotype: *rna1-1 ura3-52*) was transformed with the pool, and transformants were selected by the ability to grow on medium lacking uracil. Among these transformants, putative *RNA1* clones were identified by the ability to complement the *rna1-1* temperature-sensitive growth defect. Plasmid YEpRNA1(7.7) contains a 7.7-kb genomic insert (Fig. 1). Transformants containing YEpRNA1(7.7) showed simultaneous and consistent loss of both the Ura^+ and temperature resistance phenotypes.

The genetic identity of a clone may be determined by integrating the plasmid into the genome by homologous recombination and then mapping the site of integration by genetic techniques. Orr-Weaver et al. (24) have shown that transformation of yeast cells with plasmids which have been linearized within a region homologous to genomic DNA greatly enhances the frequency of integration at the homologous site. Plasmid YEpRNA1(7.7) was cleaved at a unique *Bam*HI site within the insert and used to transform strain EE1b. Transformants were isolated (EE1b:1 and EE1b:2) which exhibited stable maintenance of both the *URA3* and temperature-resistant markers characteristic of integrants.

Integrants EE1b:1 and EE1b:2 were mated to strain M25-3D (relevant genotype: *ura1 RNA1*). Spore clones of 20 asci resulting from the mating EE1b:1 × M25-3D all segregated 4:0 for temperature resistance to sensitivity. These results are consistent with a map distance of less than 2.5 centimorgans between the *rna1-1* locus and the sequence conferring temperature resistance. Eight tetrads from the mating EE1b:2 × M25-3D yielded one ascus with a single temperature-sensitive spore clone, consistent with a distance of 6.3 centimorgans between the integration site and the *rna1-1* locus. These data indicate that the integration is at or near the *rna1-1* locus. The single temperature-sensitive spore clone probably resulted from an intrachromosomal recombination between the endogenous *rna1-1* and integrated *RNA1* sequences. Complementation of the *rna1-1* growth defect and the genomic integration site confirm the genetic identity of at least one gene carried by YEpRNA1(7.7) as *RNA1*.

The gene was subcloned by ligating a *Sau*3A partial digest of YEpRNA1(7.7) into the *Bam*HI site of the vector YEp24. The ligation products were transformed into strain EE1b. The smallest complementing plasmid obtained, YEpRNA1(2.3), contained a 1.8-kb yeast insert and a small duplication of pBR322 sequences (Fig. 1).

Localization of the *RNA1* coding region. For localization of the *RNA1* coding region within the cloned sequences, deletions and disruptions were constructed. Two deletions were individually constructed by using the two *Bam*HI sites of YEpRNA1(2.3) (Fig. 1). This plasmid was digested with *Bam*HI, and the 2.2-kb *Bam*HI fragment was ligated into the *Bam*HI site of YEp24, thereby deleting sequences to the right of the internal *Bam*HI site. Secondly, *Bam*HI-digested YEpRNA1(2.3) was ligated under dilute conditions to yield a plasmid which contained only the insert sequences to the right of the internal *Bam*HI site. Neither of these deleted plasmids could complement the *rna1-1* temperature-sensitive growth defect. A third deletion was created by removing all insert sequences to the left of the unique *Sst*I site in YEpRNA1(2.3). The plasmid was digested with *Sst*I, and the 3′ overhanging ends were converted to blunt ends. This DNA was then digested with *Pvu*II, which produces a single cut within the pBR322 sequences, and blunt-end ligated to produce a plasmid deleted for all sequences between the *Sst*I and *Pvu*II sites. This plasmid was capable of complementing the temperature-sensitive phenotype of *rna1-1*.

A second method often utilized to localize coding sequences is inactivation of the gene after insertion of a transposable element into the coding region. We utilized the γδ transposable element to construct transposable element insertions into the insert sequences of YEpRNA1(2.3). γδ is a 5.7-kb transposable element carried upon the F′ episome of *E. coli* (8). We have observed that plasmids containing γδ elements may exhibit deletions and rearrangements of sequences internal and external to the γδ element (unpublished results). Therefore, in this study, only isolates which appeared to be the products of simple insertions were further examined. These γδ-containing plasmids were transformed into yeast strain EE1b to test their abilities to complement the *rna1-1* temperature-sensitive phenotype. Of the γδ insertions, three fell within the insert sequences; the two to the right of the *Bam*HI site inactivated the *RNA1* gene, and the one to the left of the *Sst*I site did not (Fig. 1).

Finally, an approximately 3-kb *Bgl*II fragment carrying the yeast *LEU2* gene was ligated into the *Bam*HI site of YEpRNA1(7.7). The resulting plasmid was not able to complement the *rna1-1* temperature-sensitive growth defect. The data from the deletions and γδ and *LEU2* insertions indicated that sequences immediately flanking and including the *Bam*HI site of the insert DNA of YEpRNA1(2.3) are

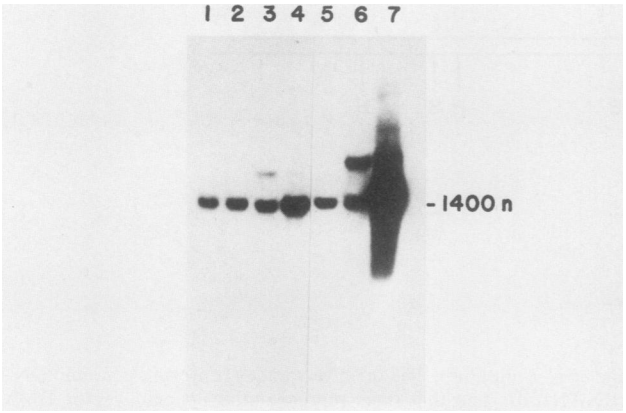

FIG. 2. Northern analysis of total cellular RNA with the probe pBRRNA1(0.45). Lanes: 1, A364a (*RNA1*); 2, 1c5 (*rna1-1 ura3-52*); 3, 1c5 transformed with YEp24; 4, 1c5 transformed with YEpRNA1 (7.7); 5, AKH1008 (*rna1-1 trp1*); 6, AKH1008 transformed with pMAC561; 7, AKH1008 transformed with YEpcRNA1(1.4). The bands greater than 1,400 n in length are vector-specific transcripts which hybridize to the pBR322 portion of the probe, pBRRNA1(0.45).

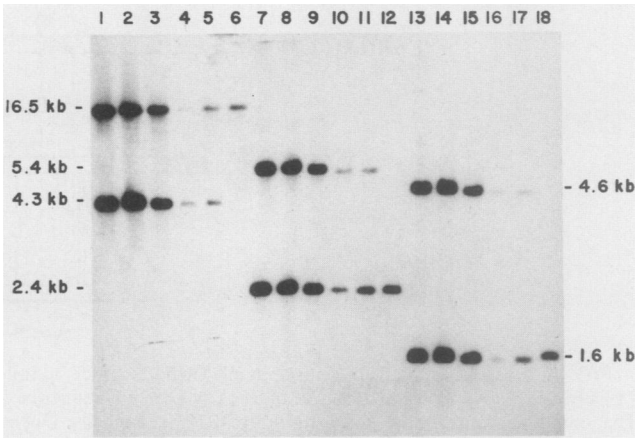

FIG. 3. Southern analysis of *Eco*RI-digested (lanes 1 through 6), *Sst*I-digested (lanes 7 through 12), and *Hin*dIII-digested (lanes 13 through 18) genomic DNA with pBRRNA1(0.45) as the probe. Lanes 1, 7, and 13 are genomic DNA from spore 2a, which is a Leu$^+$ spore from 2b×3bRNA1::LEU2A. Lanes 2, 8, and 14 are genomic DNA from 8d, which is a Leu$^+$ spore from 2b×3bRNA1::LEU2B. Lanes 3, 9, and 15 are genomic DNA from 3a, which is a Leu$^+$ spore from 2b×3bRNA1::LEU2A. Lanes 4, 10, and 16 are genomic DNA from 2b×3bRNA1::LEU2B; lanes 5, 11, and 17 are genomic DNA from 2b×3bRNA1::LEU2A; and lanes 6, 12, and 18 are genomic DNA from the undisrupted parental strain 2b×3b.

required, whereas sequences to the left of the *Sst*I site are not required for complementation.

Map of the *RNA1* transcription unit. Based upon the deletion and insertion mapping data, it appeared likely that the 450-bp *Hin*dIII-*Bam*HI fragment (Fig. 1, darkened area) would contain sequences homologous to the *RNA1* transcript. This fragment was cloned into pBR322, and the recombinant plasmid pBRRNA1(0.45) or the gel-purified fragment itself was used in all subsequent hybridizations.

To map more finely the *RNA1* coding region on the *RNA1* clones, an *rna1-1*-complementing cDNA clone was isolated from the yeast cDNA plasmid pool of McKnight and McConaughy (21). The vector used to construct this pool, pMAC561, contains the yeast gene *TRP1*. This plasmid pool was constructed such that a cDNA sequence is juxtaposed at its 5′ end and transcribed from the *ADH1* promoter (21). Yeast cDNA pool transformants of *E. coli* RR1 were screened by colony hybridization to the 450-bp *Hin*dIII-*Bam*HI restriction fragment of YEpRNA1(2.3). Plasmid DNA from positively hybridizing colonies was used to transform the *rna1-1* strain AKH1008 (relevant genotype: *trp1 rna1-1*). Resulting tryptophan prototrophs which were able to grow at 34°C showed simultaneous loss of tryptophan prototrophy and temperature resistance after nonselective growth. Transformation of *E. coli* RR1 with DNA isolated from temperature-resistant yeast transformants yielded bacterial clones carrying plasmid YEpcRNA1(1.4). The map of the 1,400-bp cDNA insert of YEpcRNA1(1.4) is shown in Fig. 1.

Isolation of a complementing cDNA clone from a cDNA pool constructed from polyadenylated RNA indicates that *RNA1* produces a polyadenylated RNA. This was confirmed by Northern analysis. Both polyadenylated RNA (data not shown) and total RNA (Fig. 2, lane 1) contain a 1,400-bp species homologous to pBRRNA1(0.45). The fact that the only RNA detected was the same size as the complementing cDNA insert of YEpcRNA1(1.4) implicated this insert as a cDNA copy of the mature *RNA1* message.

Restriction analysis of the cDNA plasmid YEpcRNA1(1.4) confirmed the identity of the cDNA sequences with a region of YEpRNA1(7.7). Due to the method of construction of the yeast cDNA plasmid pool (21), the direction of transcription of the *RNA1* message could be deduced as proceeding from left to right on the map in Fig. 1. Alignment of the restriction maps of the cDNA and genomic clones indicates that the coding region of *RNA1* begins ca. 220 bp 5′ to the *Hin*dIII site within the *RNA1* gene and ends 800 bp 3′ to the *Bam*HI site internal to the gene.

***RNA1* is a single-copy gene.** To determine the *RNA1* gene copy per haploid genome, we performed Southern analysis, utilizing pBRRNA1(0.45) as the probe. Digestions of genomic DNA from strain 2b×3b with *Eco*RI, *Sst*I, and *Hin*dIII each produced a single band of homology (Fig. 3, lanes 6, 12, and 18). Similar results were obtained with a number of other restriction endonucleases (data not shown). If multiple copies of *RNA1* exist, the reiterated sequences must include at least 16.5 kb of the region surrounding *RNA1*. Therefore, within the limits of this analysis, it appears that *RNA1* exists in a single copy per haploid genome.

Cloning of the *rna1-1* allele. We cloned the mutant *rna1-1* allele in the vector YEp24. Genomic DNA from strain 2b×3b (relevant genotype: *rna1-1/rna1-1 ura3-52/ura3-52 leu2-112,-3/leu2-112,-3*) was digested with *Bgl*II and ligated into the *Bam*HI site of YEp24. Since *Bgl*II does not cut within the *RNA1* coding region, this pool should contain intact copies of *rna1-1*. *E. coli* transformants containing *rna1-1* sequences were identified by colony hybridization with the 450-bp *Hin*dIII-*Bam*HI *RNA1*-specific fragment as the probe. Four thousand transformants were screened, and three positively hybridizing colonies were identified. The restriction map of the insert of all three plasmids is colinear with the YEpRNA1(7.7) restriction map for the section spanning the *RNA1* coding region (data not shown).

Each clone was transformed into the yeast strain EE1b. Ura$^+$ transformants were selected and tested for the ability to grow at both 34 and 37°C. All transformants were capable of growing at 34 but not 37°C (Table 1). *RNA1* strains are capable of growth at 34 and 37°C, whereas *rna1-1* strains are normally incapable of growth at these temperatures (Table 1). The plasmid-borne nature of the Ura$^+$ and partially

TABLE 1. Temperature sensitivity of various combinations of *RNA1* alleles

Strain	*RNA1* allele	Plasmid	Plasmid copy no.	Temperature dependence of growth on YEPD at:		
				23°C	34°C	37°C
A364a	*RNA1*			+	+	+
EE1b	*rna1-1*			+	−	−
EE1b	*rna1-1*	YEpRNA1(7.7)	5–30	+	+	+
EE1b	*rna1-1*	YEpRNA1(2.3)	5–30	+	+	+
AKH1008	*rna1-1*	YEpcRNA1(1.4)	5–30	+	+	+
EE1b	*rna1-1*	YEprna1-1(4.1)a	5–30	+	+	−
EE1b	*rna1-1*	YEprna1-1(4.1)b	5–30	+	+	−
EE1b	*rna1-1*	YEprna1-1(12.6)	5–30	+	+	−
2b×3b	*rna1-1/rna1-1*			+	−	−
2b×3b	*rna1-1/rna1-1*	YEpRNA1(7.7)	5–30	+	+	+
2b×3b	*rna1-1/rna1-1*	YEpRNA1(2.3)	5–30	+	+	+
2b×3b	*rna1-1/rna1-1*	YCpRNA1(7.7)	1	+	+	+
2b×3b	*rna1-1/rna1-1*	YCpRNA1(2.3)	1	+	+	+
2b×3b	*rna1-1/rna1-1*	YEpRNA1(7.7)::LEU2	5–30	+	40% +	−
2b×3bRNA1::LEU2A	*rna1-1/RNA1::LEU2*			+	5% +	−
2b×3bRNA1::LEU2B	*rna1-1/RNA1::LEU2*			+	5% +	−

temperature resistance phenotypes was confirmed by observing the concomitant loss of both markers during growth under nonselective conditions.

The copy number of YEp24 plasmids has been reported to be 5 to 30 copies per cell (32). Increasing the copy number of the *rna1-1* gene into this range is apparently sufficient to partially overcome the *rna1-1* thermosensitive growth defect.

Disruption of the *RNA1* gene at its chromosomal locus. *RNA1* is considered to be an essential gene; however, this assumption is based upon the phenotype of a single mutation, *rna1-1*. To test this hypothesis, we created a genomic disruption of the *RNA1* coding sequences. YEpRNA1(7.7)::LEU2, which contains an insertion of a *LEU2*-bearing *Bgl*II restriction fragment into the unique *Bam*HI site of YEpRNA1(7.7), was utilized. Two lines of evidence support the interpretation that this fragment is inserted into the *RNA1* coding region: (i) transcript mapping and (ii) the inability of YEpRNA1(7.7)::LEU2 to complement the *rna1-1* mutation.

A gene replacement of *rna1-1* with the 5.4-kb *Sst*I fragment of YEpRNA1(7.7)::LEU2 was performed essentially as described by Rothstein (27; see Materials and Methods). A diploid recipient, 2b×3b (relevant genotype: *rna1-1/rna1-1 leu2-112,-3/leu2-112,-3 ura3-52/ura3-52*), was utilized since it was anticipated that the disruption would be lethal in the haploid. A substantial number of leucine prototrophic transformants was obtained. Two transformants which stably maintained the Leu$^+$ phenotype were chosen for further study, 2b×3bRNA1::LEU2A and 2b×3bRNA1::LEU2B.

The leucine prototrophy of the transformants was presumed to have arisen by a gene replacement of the *rna1-1* coding region with a *LEU2*-disrupted *RNA1* gene. Southern analysis was used to confirm this interpretation. A unique disruption of the *RNA1* gene should affect the size of *RNA1*-specific restriction fragments in a predictable manner. Since the *LEU2*-containing restriction fragment used to construct the disruption is ca. 3 kb in length and does not contain *Hin*dIII or *Sst*I sites, all *Hin*dIII and *Sst*I genomic fragments homologous to the *RNA1*-specific probe pBRRNA1(0.45) should increase in size by ca. 3 kb.

Digestion of 2b×3b genomic DNA with *Hin*dIII and with *Sst*I produced bands of 1.6 and 2.4 kb, respectively, that were homologous to the probe (Fig. 3, lanes 18 and 12). *Hin*dIII digestion of genomic DNA from the two independently isolated disrupted diploid strains (2b×3bRNA1::LEU2A and 2b×3bRNA1::LEU2B) produced 1.6- and 4.6-kb bands homologous to the probe (Fig. 3, lanes 16 and 17). An *Sst*I digest of the disupted strains produced two bands of homology to the probe, a 2.4- and a 5.4-kb band (Fig. 3, lanes 10 and 11). The smaller band of homology in both digests corresponds in size to the intact *rna1-1* locus, whereas larger bands are of the sizes predicted for a 3-kb insertion at this locus.

The previous analysis confirms that the *LEU2* gene is inserted at the *RNA1* locus; however, it does not establish whether the transformation produces a unique replacement. Tandem repeats of the transforming fragment could occur if the transforming sequence became circular before integration. Southern analysis of *Eco*RI-digested genomic DNA was used to determine that the transformants contained a unique replacement of the *rna1-1* coding region with the *LEU2*-disrupted *RNA1* sequences. *Eco*RI digestion of 2b×3b genomic DNA produced a single large band (about 16.5 kb) homologous to the probe pBRRNA1(0.45) (Fig. 3, lane 6). The DNA fragment used to disrupt *RNA1* contained a single *Eco*RI site. Therefore, a unique heterozygous disruption produces two *Eco*RI fragments hybridizable to the probe. One band arises from the disrupted *RNA1* locus, and the other arises from the undisrupted *rna1-1* locus. Tandem repeats would produce three bands of homology. *Eco*RI digestion of the genomic DNA from the disrupted strains yielded two bands of homology (16.5 and 4.3 kb) (Fig. 3, lanes 4 and 5). It appears then that this transformation produced a unique heterozygous disruption of *RNA1*.

Northern analysis of the disrupted strains. The *LEU2*-bearing fragment was inserted ca. 670 bp 3′ to the beginning of the *RNA1* coding region (as measured by comparison with the cDNA clone). This could cause the production of a truncated *RNA1* transcript. Alternatively, transcription could proceed through the interrupting sequences, producing a hybrid transcript of the 5′ portion of *RNA1* and the inserted sequences.

We prepared total cellular RNA from the undisrupted, diploid parent strain, 2b×3b; and from two disrupted diploid strains, 2b×3bRNA1::LEU2A and 2b×3bRNA1::LEU2B. The RNA was analyzed by Northern analysis with pBRRNA1(0.45) as the probe. All strains produced a 1,400-n

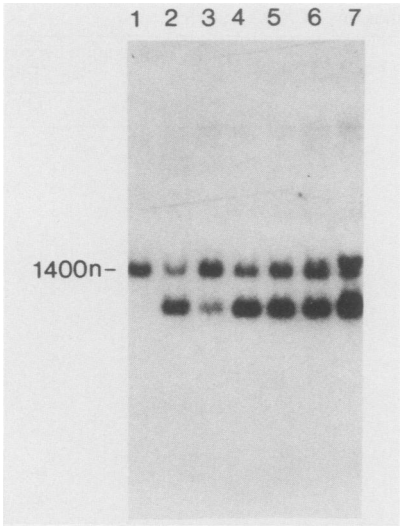

FIG. 4. Northern analysis of total RNA from undisrupted and disrupted strains. Lanes containing RNA from: 1, the undisrupted parental strain 2b×3b; 2, 2b×3bRNA1::LEU2B; 3, 2b×3bRNA1::LEU2A; 4, 2c, which is a Leu$^+$ spore from 2b×3bRNA1::LEU2A; 5, 3a, which is a Leu$^+$ spore from 2b×3bRNA1::LEU2A; 6, 2a, which is a Leu$^+$ spore from 2b×3bRNA1::LEU2A; 7, 8d, which is a Leu$^+$ spore from 2b×3bRNA1::LEU2B.

band homologous to the probe corresponding to the message from the undisrupted *rna1-1* gene (Fig. 4, lanes 1 through 3). The disrupted strains, in addition, produced a homologous band estimated as 700 to 800 n (Fig. 4, lanes 2 and 3). The size of the shorter transcript agrees well with the interpretation that it arises due to a truncated *RNA1* transcription unit generated by the insertion. Interestingly, the abundance of the truncated transcript and the *rna1-1* message are comparable. Therefore, truncation of ca. one-half of the transcript does not greatly affect its stability.

***RNA1* disruption is recessive lethal.** Disrupted strains 2b×3bRNA1::LEU2A and 2b×3bRNA1::LEU2B were analyzed by tetrad analysis to determine the phenotype of a disruption of *RNA1* in a haploid background. If the disruption is recessive lethal, then the meiotic products should segregate 2:2 for viability, and the *LEU2* gene should be tightly linked to the lethal phenotype.

Of the 18 tetrads examined from the strain 2b×3bRNA1::LEU2A, 17 segregated 2:2 for viability to lethality and produced no Leu$^+$ survivors. One tetrad segregated 2:2 for viability to lethality, and contained one Leu$^+$ spore. Disruption strain 2b×3bRNA1::LEU2B yielded the following tetrad types: 2:2 for viability to lethality, no Leu$^+$ spores; 3:1 for viability to lethality, one Leu$^+$ spore; and 4:0 for viability to lethality, two Leu$^+$ spores. These tetrad types appeared in the ratio of 17:1:1. The exceptional Leu$^+$ survivors are described below.

The previous analysis does not distinguish between recessive lethality occurring at spore germination or mitotic growth. To determine whether *RNA1* is essential for mitotic growth, we isolated, from a diploid disruption strain, spores which contain an *RNA1*- and *URA3*-bearing plasmid. These spores were then examined for the capacity to grow after mitotic loss of the plasmid sequences. Disruption diploid 2b×3bRNA1::LEU2B was transformed with the *CEN3*- and *RNA1*-bearing plasmid YCpRNA1(7.7) (see Materials and Methods). Sporulation of diploid transformants produced tetrads in which all four spores were Ura$^+$ and temperature resistant and exhibited 2:2 segregation for leucine prototrophy. During nonselective propagation, spore clones auxotrophic for leucine (undisrupted *rna1-1*) showed simultaneous loss of the Ura$^+$ and temperature resistance phenotypes at a frequency of 10%. In spore clones prototrophic for leucine (disrupted *RNA1*), no loss of the plasmid-borne phenotypes was detected (600 mitotic progeny examined). In a control experiment with the undisrupted parent strain, 2b×3b, all four spores in a tetrad exhibited mitotic loss of the plasmid of 10%.

The rescue of the recessive lethal phenotype of the *LEU2* disruption of the *RNA1* locus by an *RNA1*-bearing plasmid was a further confirmation that the lethal phenotype was a result of the insertion at the *RNA1* locus. The fact that rescued haploid disruptions which had lost the plasmid were not obtained implies that loss of the plasmid was a lethal event, suggesting that *RNA1* is a gene required for mitotic growth.

Temperature-resistant isolates of the disrupted strains. During characterization of the disrupted diploid strains, it was noted that partially temperature-resistant colonies, capable of growth at 34 but not 37°C, arose at a frequency of 2 to 5% during nonselective propagation (YEPD, 23°C) (Table 1). *RNA1* strains grow at temperatures between 34 and 37°C, whereas *rna1-1* strains are incapable of growth at or above 34°C (Table 1). The partially thermoresistant isolates did not grow at 37°C, indicating that they did not result from a reversion of the *rna1-1* locus to *RNA1*. Tetrad analysis of these partially thermoresistant isolates from the diploid disruption strain 2b×3bRNA1::LEU2A produced Leu$^+$ and Leu$^-$ spores, some of which were also partially thermoresistant. Tetrad analysis of the original temperature-sensitive disruption strains also produced a minority of tetrads with Leu$^+$ survivors, some of which exhibited to a lesser degree the partial thermoresistance phenotype (growth at 30 but not 34°C).

The viability of these Leu$^+$ isolates and the partial thermoresistance might be due to a gross physical change at the disrupted *RNA1* locus, a duplication of the intact *rna1-1* locus, or a second-site suppressor which alleviates the lethal nature of the disruption. We performed Southern analysis upon genomic DNA from three Leu$^+$ meiotic products of disrupted diploids and compared the hybridization patterns with those of the parental disruption strains and an undisrupted strain. 2a and 3a are meiotic products from a partially thermoresistant diploid isolate of 2b×3bRNA1::LEU2A. 8d is a Leu$^+$ meiotic product which was isolated from a tetrad with four viable spores obtained during the original genetic characterization of 2b×3bRNA1::LEU2B. Genomic DNAs from 2a, 3a, 8d, diploid disruption strains 2b×3bRNA1::LEU2A and 2b×3bRNA1::LEU2B, and undisrupted strain 2b×3b were digested with *Eco*RI, *Sst*I, and *Hin*dIII, respectively, and analyzed by Southern analysis with pBRRNA1(0.45) as the probe (Fig. 3). For each endonuclease used, the genomic DNAs from 2a, 3a, 8d, 2b×3bRNA1::LEU2A, and 2b×3bRNA1::LEU2B produced two bands homologous to the probe. The bands from genomic DNA of the meiotic products were the same size as those produced from disrupted diploid strains (Fig. 3). 2a, 3a, and 8d produced bands corresponding to a disrupted *RNA1* locus and an intact *rna1-1* allele. Similarly, Northern analysis of total RNA isolated from 2a, 3a, 8d, and 2c (which is a sister spore of 2a) identified two transcripts hybridizable to pBRRNA1(0.45) which appear identical in size to the two transcripts produced by the parental, diploid disruption strains 2b×3bRNA1::LEU2A and 2b×3bRNA1::LEU2B (Fig. 4).

This established that 2a, 3a, and 8d, which were expected to be haploid, each contained two *RNA1* alleles, *RNA1::LEU2* and probably *rna1-1*.

Southern and Northern analyses indicated that these Leu$^+$ meiotic products were the result of an amplification of the region surrounding and including the *rna1-1* allele. This amplification may have resulted from an increase in ploidy for the chromosome harboring the *RNA1* locus (chromosome XIII) or an amplification of a specific region of chromosome XIII, including the *rna1-1* allele. An increase in the copy number of the *rna1-1* allele could account for both the partial thermoresistance and the suppression of Leu$^+$-associated lethality phenotypes. We did not attempt to estimate the copy numbers of the *rna1-1* allele and the *RNA1::LEU2* allele based upon the relative intensities of bands in the Southern analysis of genomic DNA since the efficiency of DNA transfer to nitrocellulose varied with fragment size. To determine whether multiple copies of chromosome XIII exist, we genetically examined the partially thermoresistant spore 2c (relevant genotype: Leu$^+$ Lys$^+$ *ura3-52 RNA1::LEU2*; a meiotic product from a partially thermoresistant isolate of the diploid strain 2b×3bRNA1::LEU2A). 2c was mated to X4037-14C (relevant genotype: *lys7*; Lys$^-$ at 30°C, obtained from the Berkeley Stock Center). *lys7* is located ca. 120 centimorgans from *RNA1* upon chromosome XIII. If 2c is aneuploid for chromosome XIII (1 n + 1 or greater), then we should observe a preponderance of Lys$^+$ progeny. In 29 tetrads from this cross, we observed 42 Lys$^+$ and 5 Lys$^-$ spores, indicating that 2c is aneuploid for chromosome XIII. As a control, X4037-14C was mated to NA3b (relevant genotype: Lys$^+$). NA3b is one of the parental strains to the diploid 2b×3b. In this cross, ca. 50% of the spores were Lys$^+$, and 50% were Lys$^-$, demonstrating that aberrant segregation is not merely a characteristic of this *lys7* marker. 2c was also mated to (NA3b×EE1b:1)-6d (relevant genotype: *rna1-1–URA3–RNA1 leu2-112, -3*). This test strain is a derivative of EE1b:1 and, therefore, carries a *URA3* marker integrated at the *RNA1* locus. If 2c is aneuploid for chromosome XIII, we should observe a large number of Leu$^+$/Ura$^+$ progeny, whereas if 2c contains a single chromosome XIII, then the *LEU2* and *URA3* markers should behave as alleles and segregate into sister spores. Sixteen tetrads from this cross yielded 36 testable spores, 15 of which were Leu$^-$/Ura$^+$, and 7 of which were Leu$^+$/Ura$^+$. This result confirms that 2c is aneuploid for chromosome XIII.

The frequently arising partial thermoresistance in the disrupted strains, presumably due to aneuploidy for chromosome XIII, may be due to a selection for at least two functional *rna1-1* genes per diploid cell. Alternatively, it may be the result of a truncated *RNA1* gene product encoded by the disrupted *RNA1* allele. To help distinguish between these hypotheses, we examined the effect of plasmid YEpRNA1(7.7)::LEU2 on the temperature-sensitive phenotype of diploid strain 2b×3b (relevant genotype: *rna1-1/rna1-1 ura3-52/ura3-52 leu2-112,3/leu2-112,-3*). This strain bearing plasmid YEpRNA1(7.7)::LEU2 was grown to the stationary phase and plated for single colonies under nonselective conditions. Replica plating was performed to test for the presence of plasmid-borne markers and partial thermoresistance. Seventy-five percent of the colonies exhibited the plasmid-borne *URA3* and *LEU2* markers, and 46% of the plasmid-bearing colonies were also partially thermoresistant (460 colonies examined) (Table 1). Colonies which did not contain the plasmid markers were never observed to be partially thermoresistant. The control experiment with 2b×3b in the absence of a plasmid produced only temperature-sensitive isolates. Similarly, the control consisting of 2b×3b carrying plasmid YEpRNA1(7.7) produced only completely temperature-resistant colonies.

DISCUSSION

We have isolated a 7.7-kb genomic clone which complements the *rna1-1* temperature-sensitive growth defect. We confirmed the genetic identity of the cloned sequences by integrating the plasmid into the yeast genome by homologous recombination and genetically mapping the integration site. These data indicate that the cloned sequences are homologous to sequences at or near the *rna1-1* locus. Northern analysis and analysis of the cDNA clone indicated that the *RNA1* gene encodes a 1,400-n polyadenylated transcript. Although not stringently proven, the presence of a polyadenylated *RNA1* transcript suggests that the functional *RNA1* product is a protein moiety. This interpretation is supported by preliminary data from a hybrid selection translation experiment which indicate that *RNA1* produces a protein of ca. 50,000 daltons (R. W. Dunst and A. K. Hopper, unpublished data). The restriction maps of the genomic clones and the cDNA clone are colinear for at least five restriction sites dispersed throughout the cDNA insert. Colinearity of restriction maps is a strong indication that *RNA1* does not contain any intervening sequence greater than 25 bp. It is interesting that the 3′ end of the cDNA is ca. 300 bp longer than the genomic subclone, YEpRNA1(2.3). This subclone complements the *rna1-1* growth defect when present in low copy number upon a CEN plasmid (Table 1). We do not know yet whether the subclone is missing translated sequences. If it is, then we have defined a portion of the protein not necessary for complementation.

The *rna1-1* allele was cloned to determine the nature of the mutant defect. We are interested in the relative leakiness of the *rna1-1* mutation since it is pertinent to the analysis of second-site suppression of the *rna1-1* mutation by *SRN1-1* (25) and *srn1-2* (S. L. Nolan, R. W. Dunst, N. S. Atkinson, and A. K. Hopper, manuscript in preparation) and the suppression of the *rna1-1* temperature-sensitive phenotype due to gene amplification of the *rna1-1* allele itself. The observation that multiple copies of the *rna1-1* gene promote growth at 34 but not 37°C (mimicing the degree of suppression by *SRN1-1* and *srn1-2*) indicates that *rna1-1* produces a product of lowered activity or stability at elevated temperatures or that transcription from the *rna1-1* allele is reduced at the restrictive temperature. Therefore, second-site suppressors of *rna1-1* could act by increasing the abundance of the *rna1-1* gene product.

RNA1 is assumed to be a gene essential for mitotic growth. To examine this hypothesis, we created a new allele of *RNA1* by disrupting the *RNA1* coding region upon one chromosome in a diploid with a DNA fragment bearing the *LEU2* gene. This disruption behaves as a recessive lethal. To verify that this lethality was not restricted to spore germination, we transformed the diploid heterozygous disruption strain with a CEN plasmid carrying the *RNA1* gene. This plasmid was capable of rescuing the recessive lethal phenotype of the Leu$^+$ meiotic progeny of the diploid disruption strains. Spores which contained an undisrupted *rna1-1* allele exhibit normal mitotic loss of the plasmid, whereas spores containing the *RNA1::LEU2* allele which had lost the plasmid were never obtained. This indicates that loss of the plasmid is a lethal event, suggesting that an intact *RNA1* or *rna1-1* allele is essential for mitotic growth.

Tetrads containing Leu$^+$ meiotic progeny were produced

from the disrupted diploids at a frequency slightly in excess of 5%. Similarly, the disrupted diploid became partially temperature resistant at a frequency of 2 to 5%, and these temperature-resistant isolates produced Leu$^+$ spores (temperature sensitive or partially thermoresistant). We have demonstrated that the Leu$^+$ spores contain at least one intact copy of the *rna1-1* allele in addition to the disrupted *RNA1* allele. A test cross of the partially thermoresistant Leu$^+$ spore, 2c, to a strain carrying a second marker for chromosome XIII (*lys7*) indicates that 2c contains multiple copies of chromosome XIII. Data from the tetrad analysis of the cross 2c × (NA3b×EE1b:1)-6d also suggest that 2c is aneuploid for chromosome XIII. We are currently examining the disruption strains to determine whether the *RNA1::LEU2* allele promotes aneuploidy for a number of chromosomes. Preliminary data suggest that the promotion of aneuploidy is not restricted to chromosome XIII (data not shown). The degree of temperature resistance is analogous to that produced by *rna1-1* upon a multicopy plasmid. Some of the partially thermoresistant aneuploids produce temperature-sensitive segregants during mitotic propagation, presumably due to a decrease in the ploidy of chromosome XIII. The resulting Leu$^+$ segregants must remain at least 1 n + 1 to be viable.

We have demonstrated that the *RNA1::LEU2* allele upon a multicopy plasmid promotes the appearance of partial thermoresistance in a diploid strain. Since such strains exhibit similar characteristics to the partially thermoresistant diploids obtained from the genomic disruption, we have inferred that they too arise due to aneuploidy for chromosome XIII. The strain 2b×3b carrying plasmid YEpRNA1(7.7)::LEU2 becomes partially thermoresistant at a frequency of 46%, whereas, in the genomic disruption strain, partial thermoresistance occurs at a frequency of 2 to 5%. Apparently, increasing the copy number of the disruption from 1 to between 5 and 30 has enhanced the promotion of aneuploidy, presumably due to a higher concentration of the aberrant product. If this interpretation is correct, then this experiment demonstrates that aneuploidy is not the result of selection for at least two copies of *rna1-1* per diploid genome. Rather, it suggests that the disrupted *RNA1* allele itself produces a product with altered activity. It is interesting that the heterozygous diploid disruption strains produce a truncated transcript homologous to an *RNA1*-specific probe. This transcript corresponds in length to a transcript read from the *RNA1* promoter and terminating at or immediately within the inserted sequences. Intensities upon Northern analyses of the truncated transcript and the *rna1-1* transcript are comparable, indicating that the truncated transcript is fairly stable. Translation of this transcript might produce a product with an aberrant activity. Such a product might occupy *RNA1*-bindng sites but be incapable of performing the *RNA1* function, adversely affecting cell growth. Increasing the copy number of *rna1-1* would then be presumed to increase the level of functional protein product, outcompeting the aberrant product and providing a selective advantage for aneuploidy. Alternatively, the protein product produced from the *RNA1::LEU2* allele may itself catalyze aneuploidy. A model in which *RNA1* is involved in the maintenance of nuclear structure accounts for both the RNA metabolism defect of *rna1-1* and the promotion of aneuploidy phenotype of *RNA1::LEU2* since the perturbation of nuclear structure could reasonably be expected to interfere with multiple nuclear processes.

We are currently constructing a variety of truncations of the *RNA1* gene to determine whether the unusual phenotype of the *RNA1::LEU2* allele is actually a property of the *RNA1* sequences or of the disrupting *LEU2*-bearing sequences. Preliminary results with a gene fusion of the *RNA1* gene to the bacterial β-galactosidase gene suggest that this phenotype is a property of the *RNA1* sequences.

We are continuing to construct new alleles of *RNA1* since our *RNA1* disruption suggests that new mutations in *RNA1* have the potential for unexpected phenotypes. We are also continuing to characterize the gene products of the existing *RNA1* alleles.

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