U14 function in *Saccharomyces cerevisiae* can be provided by large deletion variants of yeast U14 and hybrid mouse–yeast U14 RNAs

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Communicated by J.Tooze

The functional equivalency of yeast and mouse U14 RNAs was examined in *Saccharomyces cerevisiae*. The test RNAs included mouse U14 and several yeast–mouse bi- and tri-partite hybrid RNAs, all transcribed from yeast U14 gene signals. The ability of the heterologous RNAs to provide essential U14 function was assessed in a test strain containing a single glucose-repressible wild-type U14 gene. Mouse U14 was not functional in yeast. However, wild-type growth was supported by hybrid RNAs that included universal sequence elements from either source, two yeast-specific segments and a 5',3' terminal stem domain. The universal sequences include box C, box D and a sequence complementary to 18S rRNA, all shown previously to be required for function of yeast U14. Deletion and substitution mapping defined the yeast-specific elements and showed that a major portion of neighboring non-conserved RNA is dispensable. The results are discussed with a view to defining a minimal consensus U14 molecule.

Key words: rRNA maturation/*Saccharomyces cerevisiae*/ snoRNA/sRNA/U14 RNA

Introduction

U14 RNA is one of nine small nucleolar RNAs (snoRNAs) linked to ribosome biogenesis in *Saccharomyces cerevisiae* (Tollervey and Guthrie, 1985; Tollervey, 1987; Hughes *et al.*, 1987; Zagorski *et al.*, 1988). U14 has been shown to be required for normal production of 18S rRNA. Repression of U14 synthesis results in impaired processing of 35S pre-rRNA, under-accumulation of 18S RNA precursors (20S) and an ensuing deficiency in mature 18S RNA (Li *et al.*, 1990). Similar patterns have also been observed with depletion of the U3 nucleolar RNA, and processing of 35S pre-rRNA is also defective in cells containing an inactive snR10 gene (Hughes and Ares, 1991; Tollervey, 1987).

U14 homologs also occur in a variety of metazoan organisms, including mouse, rat, hamster and *Xenopus laevis* (Maxwell and Martin, 1986; Liu and Maxwell, 1990). Direct sequence information is available for mouse U14 while the presence of the other variants was established by RNA hybridization assays (Trinh-Rohlik and Maxwell, 1988; E.S.Maxwell, personal communication). Particularly fascinating is the finding that the U14 DNA sequences of

mouse, rat, human and *Xenopus* occur within introns of the cognate heat shock gene *hsc70*, suggesting a link with *hsc70* protein expression (Liu and Maxwell, 1990).

The U14 RNA of *S.cerevisiae* is nearly 40 nucleotides larger than mouse U14 (Figure 1). As depicted, the RNAs contain three segments of strong sequence relatedness (regions 1–3) and a poorly conserved mid-portion of variable size. Discounting the latter region the two RNAs exhibit an overall sequence relatedness of 77%. Functional mapping of yeast U14 has shown that portions of each conserved region are required for U14 accumulation or activity (Jarmolowski *et al.*, 1990). The essential segments include sequences near the 5' and 3' ends, an internal 13-base element complementary to 18S rRNA (domain A) and elements of the non-conserved mid-region. The terminal segments are required for U14 accumulation while the 18S-related sequence and two portions of the non-conserved region are essential for function. The critical 5' and 3' elements include and overlap, respectively, with sequences known as box C and box D. Boxes A–D were first defined as sequence elements conserved among U3 species and boxes C and D are co-conserved among several snoRNAs including U3 (Wise and Weiner, 1980; Hughes *et al.*, 1987; Parker and Steitz, 1987; Jeppesen *et al.*, 1988; and summarized by Tyc and Steitz, 1989). The conserved domains required for yeast U14 activity are also present in the other metazoan U14 RNAs, along with segments in regions 2 and 3 that are not essential (Trinh-Rohlik and Maxwell, 1988).

The structural relatedness of the yeast and metazoan U14 RNAs suggests that they are probably functional homologs as well. The aim of the present study was to determine if mouse U14 could function in *S.cerevisiae* and, if so, to exploit this situation in order to develop a higher resolution functional map of yeast U14. Earlier deletion mapping of yeast U14 has also been extended. The results reveal that mouse U14 is not active in yeast whereas certain hybrid mouse–yeast RNAs are functional. Active variants included universal sequence elements from either source, two internal segments unique to yeast and a terminal stem domain. The results are discussed with a view to identifying a minimal consensus sequence for yeast U14 RNA.

Results

Genetic strategy

The ability of U14 variants to function in *S.cerevisiae* was assessed in test strain YS153 containing a single inducible U14 gene located in the chromosome (Jarmolowski *et al.*, 1990). The inducible allele consists of a yeast U14 sequence fused to the galactose-inducible, glucose-repressible *GAL1* promoter (Johnston and Davis, 1984). Test RNAs were expressed from single-copy *CEN* plasmids using normal yeast U14 transcription signals and functionality was evaluated by screening growth phenotype after shifting transformants from galactose to glucose (Jarmolowski *et al.*,

Fig. 1. Relatedness of yeast and mouse U14 RNAs. The yeast and mouse RNAs are aligned to show regions of sequence relatedness. The segments highlighted correspond to regions of RNA homology (elements 1–3), sequences within these conserved regions that are complementary to the cognate 18S rRNAs (domains A and B) and boxes C and D, sequences common to several snoRNAs.

1990). Expression levels of the test RNAs was examined by Northern analysis of total RNA. The product of the chromosomal *GAL1::SNR128* allele has five extra nucleotides at the 5' end, making it readily distinguishable from the plasmid encoded test RNAs (Li *et al.*, 1990).

Two types of U14 variants were analyzed. One class consisted of heterologous RNAs with mouse sequences—either mouse U14 alone or mouse–yeast hybrids. The second class consisted of yeast U14 variants deleted or substituted in the non-conserved middle region. The purpose in analyzing the latter set was to define functional elements specific for yeast inferred in our earlier mapping analysis (Jarmolowski *et al.*, 1990). The test genes were constructed by (i) assembly of natural and synthetic DNA fragments at existing or newly created restriction sites and (ii) base substitution or deletion mutagenesis of natural U14 DNA.

Can mouse U14 RNA support yeast growth?

To test the functional equivalency of the yeast and mouse RNAs, plasmids expressing mouse U14 were constructed by replacing the coding sequence in a yeast U14 plasmid (pJZ45) with synthetic mouse DNA. Two variant expression vectors were obtained as a consequence of using two different methods for blunting the DNA ends to be joined. The resulting constructs were predicted to yield RNA starting at position –2 or +2 relative to the normal 5' end of mouse U14. The vectors are designated pM(ouse)5(–2) and pM5(+2), respectively.

Neither of the two mouse constructs was able to support growth of the test strain in glucose at 25, 32 and 37°C. Expression was examined by Northern analysis using a probe specific for the 5' end of mouse U14 (Figure 2). Internal control RNAs included yeast U14 produced from the *GAL1* chromosomal allele and (later) a hybrid yeast–mouse U14 RNA (M-Y-Y) which accumulates to about wild-type level (see below). These and other results showed that the mouse U14 variants accumulated at levels similar to that of wild-type yeast U14 and were of the expected size. Taken together, the expression and activity results argue that the

Fig. 2. Expression of mouse U14 in yeast. Mouse U14 DNA was introduced into the test strain YS153 on a single copy *CEN* plasmid and transcribed from yeast U14 gene signals (Jarmolowski *et al.*, 1990). Strain YS153 features a *GAL1* chromosomal U14 allele (Li *et al.*, 1990). RNA was isolated from transformants grown in selective medium containing galactose and detected by Northern analysis. By lane the test DNAs and media were: (1) pM-Y-Y in glucose; (2 and 3) two independent pM5(–2) transformants; (4 and 5) two independent pM5(+2) transformants. Constructs M5(–2) and M5(+2) correspond to recombinant mouse U14 DNAs with predicted transcriptional start sites at positions –2 or +2 relative to the 5'-terminus of mouse U14 RNA. (A) Pattern obtained with a 5' labeled probe specific for nt 3–39 of mouse U14 RNA. (B) Reprobe of blot with an oligonucleotide complementary to nt 41–71 of yeast U14 RNA. The U14 RNA derived from the chromosomal allele (*GAL*-U14) has five additional bases at the 5' end. Y and M designate segments of yeast and mouse RNA.

Fig. 3. Structures of experimental mouse and yeast–mouse hybrid U14 genes. The alignment of the yeast and mouse U14 coding sequences is shown at the top and the structures of four test U14 genes are shown below, including mouse, M-Y-Y, M-Y-M and Y-Y-M. The sites of recombination are indicated with arrows. Growth properties and RNA content are summarized at the right.

inability of mouse U14 to function is due to a structural deficiency rather than a problem at the expression level.

The possibility that U14 function might be achieved by increasing the intracellular concentration of mouse RNA was tested. To this end the U14 fragments of pM5(–2) and pM5(+2) were cloned into the *Clal* site of the multicopy vector, YEP24, to yield YEP24-M5(–2) and YEP24-M5(+2). As before, no growth was observed at any of the temperatures screened (25, 32 and 37°C). Interestingly, no over-production of mouse U14 was evident on Northern analysis, suggesting a gene dosage compensation effect (data not shown). However, wild-type yeast U14 was over-expressed 5- to 10-fold from an otherwise matching YEP24 vector, pJZ37. The basis for the different expression effects observed with the mouse and yeast U14 sequences is not known and provides a subject for future study.

Hybrid yeast – mouse RNAs are functional

With a view to defining common and unique functional domains, hybrid U14 molecules were made that featured both mouse and yeast sequences. Figure 3 shows the structures and properties of the test RNAs. The RNAs analyzed included three mouse–yeast hybrids featuring different combinations of the individual coding sequences. The coding regions were divided into three segments corresponding to: (i) the highly conserved 5' portion (yeast nt 1–42) which includes conserved regions 1 and 2 and exhibits 76% homology, (ii) the poorly conserved interior region (nt 43–101) and (iii) the 3' conserved region (nt 102–126) with 82% homology. Engineering involved the use of a natural restriction site (*BclI*) for joining the 5' mouse elements to the yeast expression signals, an engineered *SacII* restriction site at the border of the 5'-conserved and unique middle sequences (Jarmolowski *et al.*, 1990) and oligonucleotide-directed mutagenesis to convert the conserved 3' segment from yeast to mouse DNA.

Fig. 4. Expression of hybrid M-Y-Y RNA in yeast. Expression was examined by Northern hybridization analysis using RNA prepared from transformants grown in selective medium containing galactose or glucose. Lanes: (1) the parent strain GRF167 in glucose; (2+3 and 4+5) independent pM-Y-Y transformants in galactose and glucose respectively. (A) Probing with oligonucleotide complementary to bases 89–115 in yeast U14 RNA. (B) Reprobe of the blot in panel A with an oligonucleotide specific for nucleotides 3–39 of mouse U14. The structure of the hybrid gene is shown in Figure 3.

Function was observed for two of the three hybrid RNAs, all of which included the yeast mid-region (Figure 3). One of the functional variants was bipartite (M-Y-Y), the other tripartite (M-Y-M). The M-Y-Y species was active at all three temperatures tested and Northern analysis showed that RNA accumulated at the wild-type level (Figure 4). Its identity was established by hybridization with a probe specific for the 5' portion of mouse U14. The 5' end was

mapped by primer extension analysis and shown to have the same terminus as natural yeast U14. The M-Y-Y results show that the 5' portions of the mouse and yeast RNAs are interchangeable and, thus, functionally equivalent.

The highly conserved 3' sequences are not interchangeable, however, as the hybrid Y-Y-M did not support growth. Northern assays revealed that this variant did not accumulate (Figure 5). Good yields and function were obtained for a tripartite hybrid that combined the two end segments of mouse U14 with the yeast mid-segment (M-Y-M), indicating that the conserved terminal sequences of yeast and mouse U14 are, indeed, functionally equivalent (Figure 5). This result also suggests that the inability of mouse U14 to function may be due to the absence of essential sequences in the yeast-specific region. Taken with the positive result for the M-Y-Y hybrid, the mixed results obtained for the Y-Y-M and M-Y-M species suggests the possibility that the 5' and 3' terminal segments interact and that productive interaction does not occur for the 5'-yeast and 3'-mouse segments. Functional interaction does occur in the mouse-yeast and mouse-mouse terminal variants, but not for the 5', 3' yeast-mouse hybrid.

Fig. 5. Patterns of chimeric yeast-mouse RNAs. RNAs were detected by Northern hybridization analysis as in Figure 4. The blot was hybridized with probes for yeast U14 and snR190. The U14 probe was specific for nucleotides 41–71; snR190 was detected with an oligonucleotide complementary to nucleotides 158–190. Lanes: (1) parent cells GRF167 in glucose; (2) test strain YS153 in galactose; (3) a pY-Y-M transformant in galactose; (4) a pM-Y-M transformant in glucose; and (5) a pY*-Y-M transformant in glucose. Y* is the 5' yeast segment with A3 changed to G. The structures of the hybrid RNAs are depicted in Figure 3.

Fig. 6. Proposed structure of a common 5',3' terminal stem of U14 RNA. Examination of the folding patterns predicted for natural yeast and mouse RNAs and the hybrids active in yeast revealed a common terminal stem, consisting of 4 bp for yeast and 5 bp for the mouse species. The stem sequences for all species are the same except at the third position (box), where the base pair is preserved in yeast, mouse and two active variants (M-Y-Y and M-Y-M), but not in the non-functional Y-Y-M hybrid RNA. Activity was restored to a non-functional Y-Y-M hybrid by correcting the hypothetical mismatch in the stem by changing A3 to G, to yield Y*-Y-M.

Activity requires pairing of the 5' and 3' ends

Examination of predicted secondary folding patterns for the wild-type and hybrid RNAs suggests that the imperfection in the non-effective hybrid might be the absence of a terminal stem domain common to both yeast and mouse U14 RNAs and to the active hybrids as well (Figure 6; Zuker and Stiegler, 1981). Stems of 4 and 5 bp are predicted for the functional variants, whereas a critical centrally located base pair is absent in the inactive Y-Y-M hybrid; the absence of this base pair would be expected to preclude stem formation from occurring. Stem formation can be maintained in the case of the M-Y-Y hybrid where a G:U base pair replaces a yeast A:U pair. The Y-Y-M variant has unpaired A and C bases at this position.

The essential-stem hypothesis was tested by creating a point mutation in the 5' portion of the inactive Y-Y-M hybrid to permit formation of the putative stem (A3→G). The new hybrid Y*-Y-M was active and accumulated to about one-fifth of the wild-type level (Figure 5). This result provides evidence that the terminal segments do, indeed, interact as predicted and that the stem is important for U14 stability. The occurrence of the terminal stem and its role in U14 stabilization is supported by additional mutational results for yeast U14 to be described elsewhere (A.Jarmolowski, J.Struck, M.Huang and M.Fournier, in preparation). We return to this issue below and discuss the hybrid RNA findings in context with the other mutational results available.

Mapping the essential spacer region

Earlier deletion mapping of yeast U14 showed that two portions of the non-conserved region are required for function and the requirement for yeast-specific segments is verified by the present results with the heterologous RNAs (Jarmolowski *et al.*, 1990). The previous deletions implicated 12 and 25 base segments delimited by A43–A54 and U59–G83. Additional deletions and multibase substitutions were made in the non-conserved region to obtain a higher resolution map. Sets of Bal31 deletions were generated from restriction sites at A54 (*MluI*) and G83 (*BstEII*) and the resulting variants were screened for U14 content and function. The *MluI* site was introduced by a single base substitution (no effect) whereas the *BstEII* site is natural (Jarmolowski *et al.*, 1990). The results obtained for these deletion mutants are summarized in Table I.

The 24 mutants listed contained deletions ranging from one to 46 nucleotides. Mutant RNA was produced in every

Table I. Bal31 deletion variants of the yeast U14 gene

Clone ^a	Deletion	No. of nt	Growth			RNA level ^{b,c}
			RT ^b	32°C	37°C	
M2	U49–U61	13	–	–	–	+
M3	U60–G85	26	–	–	–	+
M5	U60–U63	4	+	+	+	+
M10	U49–A62	14	–	–	–	+
M11	A45–A56	12	–	–	–	+
M14	C46–C72	27	–	–	–	+
M16	U27–C59	33	–	–	–	+
M17	C45–U71	27	–	–	–	+
M18	U60–C90	31	–	–	–	+
M20	U49–U69	21	–	–	–	+
M21	G48–A66	19	–	–	–	+
M23	A56–U101	46	–	–	–	+
M25	U55–U61	7	+	+	+	+
M26	U60–A62	3	+	+	+	+
M28	U61–A70	10	–	–	–	+
M30	U61	1	+	+	+	+
M33	U46–U61	13	–	–	–	+
M36	U61–C84	24	–	–	–	+
M38	G48–U71	24	–	–	–	+
M39	G48–U75	28	–	–	–	+
M40	C37–U61	25	–	–	–	+
BS4	C91–U109	19	+	+	+	+
BS7	C91–U101	11	+	+	+	+
BS20	C63–G85	23	–	–	–	+
BS39	C91–A102	12	+	+	+	+

^a Clone designations M and BS refer to deletions initiated at *Mlu*I and *Bst*EII sites respectively.

^b RT, room temperature.

^c + indicates detectable level of RNA, varying from ~30 to 200% of wild-type RNA levels.

case, at levels similar to wild-type and of the expected size (data not shown). The largest functional variant is BS4, which lacks 19 nt between C89 and U107. This segment includes 15 nt of yeast sequence and the first three nucleotides in conserved region 3. Because no small deletions were obtained upstream of G83 (*Bst*EII) this region was probed with multibase substitutions. The substituting fragments were designed as follows: A→C; C→A, G→T; and T→G. Two substitution mutants were developed, one for T73–C80 and the other for A64–A68 (Figure 7). The T73–C80 mutant is cold-sensitive (cs), but showed wild-type growth at 37°C; no growth was observed at 25 or 32°C. The A64–A68 substitution is lethal, lacking function at all three temperatures tested. The key mutagenesis data are summarized in Figure 7.

Taken together, the data identify two essential yeast specific segments, Y1 and Y2. Presently these segments correspond to G45–G52 (Y1) and C61–U72 (Y2). The essentiality of element Y1 is consistent with a previously discovered cs point mutation (A48→G; Jarmolowski *et al.*, 1990). The importance of Y2 is supported by the lethality of a five base substitution at A64–A68. The 5' border of Y1 (G45) was defined by the functional deletion A43–A44 and the 3' border (G52) by the functional U53–U59 deletion and non-functional U47–U59 deletion. The 5' border of Y2 is presently placed at C61 by functional deletions U53–U59 and U58–A60 and the lethal U59–A68 deletion. The 3' boundary of Y2 is defined as U72 based on the findings that

the substitution U73–C80 and the deletions G84–G90 and C89–U107 are non-lethal, while the deletion C61–G83 is lethal. A current functional map of yeast U14 is shown in Figure 8, based on all mutagenic mapping results available.

Discussion

The results from the hybrid RNA analyses show that U14 function can be provided in yeast by variants containing mouse U14 sequences. Wild-type growth was obtained for hybrids featuring either the 5' conserved region of mouse U14 (M-Y-Y) or both 5' and 3' conserved regions from mouse (M-Y-M). No activity was observed for mouse U14 itself, indicating that yeast-specific nucleotides are required for function in yeast. The 5' segments encoding universal regions 1 and 2 are completely interchangeable, functioning with both homologous and heterologous 3' segments. The 3' fragments can also be exchanged, but activity required an apparently compensating base change in the yeast 5' segment. Taken together, the results indicate that U14 RNA activity in yeast requires both phylogenetically conserved sequence elements and sequences specific to yeast.

Also required apparently is the formation of a terminal stem domain. RNAs unable to form the presumed stem do not accumulate, arguing that this domain is at least necessary for accumulation. Similar losses of U14 have also been observed for specific point mutations in the box C and box D sequences (G.Huang and M.Fournier, in preparation). These elements and the proposed terminal stem might serve as signals for U14 binding proteins. Alternatively, the stem could provide stability against exonuclease attack. Lack of productive protein binding could disrupt U14 maturation or incorporation of U14 into a functional RNP complex. Either type of defect could lead to rapid turnover of U14. This view is supported by studies of snoRNA patterns in yeast depleted of fibrillarin, an abundant nucleolar protein associated with several snoRNA species (Tollervey *et al.*, 1991). Repression of fibrillarin synthesis was shown to cause decreases in U14 and snR190 content, both of which are normally precipitated with antibodies against fibrillarin. Interestingly, levels of other fibrillarin-associated snoRNAs were unaffected.

The hybrid RNA results provide valuable information about the structural requirements for U14 activity. Each active variant, all containing the yeast spacer, differs substantially in sequence from yeast U14. The variant sequences have the following features: M-Y-Y has seven substitutions, four deletions and one insertion; M-Y-M has ten substitutions, four deletions and three insertions; and Y*-Y-M has four substitutions, no deletions and two insertions; recall that this last variant differs from the inactive Y-Y-M by a single base change. In combination with other mutagenic results these data have been especially useful in predicting folding patterns for U14 (in preparation).

The deletion mapping results reveal that at least 34 bases in the 57 base mid-region unique to yeast U14 are not essential and might therefore be eliminated from a minimum functional structure (Figure 8). It is important to point out, however, that this is a summary of many individual mutagenesis results. Simultaneous deletion of all these and other dispensible nucleotides would probably be lethal.

Previous mapping results showed that the signal for correct 3'-end formation of U14 is most probably located in the coding sequence (Li *et al.*, 1990). This view is based on

Fig. 7. Mutational analysis of the yeast-specific mid-region. The importance of the non-conserved mid-portion nucleotides (boxed) was evaluated by deletions and directed base substitutions. The effects on function are indicated below the spacer sequence. Asterisks identify mutants described earlier (Jarmolowski *et al.*, 1990); open boxes, non-lethal mutations; shaded boxes, lethal mutations. Two yeast-specific segments deduced to be essential are indicated as Y1 and Y2.

Fig. 8. Functional map of yeast U14. Five sequence elements required for U14 accumulation or function are identified. Also highlighted is the terminal stem region determined to be essential for accumulation of hybrid RNAs. Closed circles, essential nucleotides; open circles, non-essential nucleotides; boxes at termini identify stem elements. The map of non-essential segments is a hypothetical summary for all deletion and substitution variants—derived from the present study and from that of Jarmolowski *et al.* (1990) and unpublished deletions defined by H.V.Li. The structures are drawn to scale, but the circles and boxes do not correspond to individual nucleotides. From the 5' end the essential segments include box C, the 18S RNA-related domain A (18S-A), the yeast-specific sequence Y1 (G45–G52), the yeast spacer Y2 (C61–U72), and box D. The 18S-related domain B (18S-B) is not essential (Jarmolowski *et al.*, 1990).

the knowledge that RNAs of expected size are produced from recombinant genes lacking DNA that normally occurs upstream of the coding sequence and have only two bases of yeast DNA at the 3' junction (i.e. the *GAL1* promoter fusions). The present study revealed that U14 variants with deletions of up to 46 nt accumulate and are of the expected size. Taken together the present and earlier mutagenesis data suggest that the signal for 3' end formation is located at one or both ends of U14 (Jarmolowski *et al.*, 1990). One attractive hypothesis is that the 3' end—and perhaps the 5' end too, is generated by processing and the terminal stem may serve as a signal for nuclease cleavage (Liu and Maxwell, 1990).

The results of the present study establish that the structural homologs of U14 are also related functionally. While the findings underscore the strong functional conservation of the

U14 molecules, the results also demonstrate that at least two non-universal elements are required for U14 activity in yeast. The requirement for the yeast-specific segments could reflect differences in synthesis of the two snoRNAs or the corresponding RNP structures. Differences could occur in the mechanisms by which a common U14 function is mediated or, alternatively, additional unique functions could be carried out by one or both RNAs. Uncovering the roles of the conserved and divergent U14 elements offers an interesting challenge for the future.

Materials and methods

Strains and culture conditions

The yeast strain used in this study was YS153 (Ura⁻, Trp⁻, His^{+/+}, Gal^d, *HIS::GAL1::SNR128*; Jarmolowski *et al.*, 1990). Synthesis of U14 in both strains is under control of the *GAL1* promoter (Johnston and Davis, 1984;

Li *et al.*, 1990). The parent of YS153 was GRF167 (*MAT α* , *his3 Δ* , *ura3–167 Gal⁺*) obtained from G.Fink.

Cells were maintained in rich medium containing galactose (YPG: 1% yeast extract, 2% peptone, 2% galactose) or in minimal medium (SD or SG: 0.67% yeast nitrogen base with 2% dextrose or galactose) supplemented with histidine (20 μ g/ml) and uracil (20 μ g/ml). Cells were cultured at 32°C except where indicated. All other yeast manipulations were carried out as described by Sherman *et al.* (1985).

Nucleic acid manipulations

Procedures for preparing and analyzing yeast RNA, DNA sequencing and site-directed mutagenesis have been described (Jarmolowski *et al.*, 1990). Directed mutagenesis was carried out in the phagemid pJZ45 (Jarmolowski *et al.*, 1990). This vector contains (i) a 1.3 kb *Clal* yeast DNA fragment that includes both the *SNR190* and *SNR128* genes (Zagorski *et al.*, 1988), (ii) the yeast *TRP-ARS* for replication and selection and *CEN3* for maintenance at single copy [from pYe(CEN3)30 (Fitzgerald-Hayes *et al.*, 1982)] and (iii) *Amp^R*, *ori* and *fl* from pJ150 for propagation in *E. coli* and single-stranded template isolation. Primer extended DNA synthesis was according to Ausubel *et al.* (1987). Yeast transformation was by the lithium acetate procedure of Ito *et al.* (1983).

Plasmid construction

Two plasmids expressing mouse U14 were constructed from pJZ45. Assembly involved replacing a *BclI*–*AccI* DNA fragment containing the yeast U14 coding sequence with synthetic mouse DNA (Zagorski *et al.*, 1988). In this strategy pJZ45 was first digested with *BclI* which cleaves at position –2 relative to the 5' end of U14 RNA. The termini were converted to blunt ends either by trimming away the ACGT overhang with S1 nuclease or by filling in with the Klenow enzyme repair activity. The resultant plasmid was then digested with *AccI* at a site located four nts downstream of the U14 coding sequence. Finally, mouse U14 DNA assembled from synthetic oligonucleotides was inserted into the vector. The nature of the two fusions between the mouse and yeast DNA predicts that mouse U14 transcripts will start at position –2 (Klenow) or +2 (S1 nuclease) relative to the normal 5' end of mouse U14. The plasmids were designated pM5(–2) and pM5(+2). The two constructions were verified by DNA sequencing and two point mutations found in the mouse sequence were corrected by site-directed mutagenesis. The multicopy vectors expressing mouse U14 were constructed by cloning the 1.3 kb *Clal* fragment from pM5(–2) or pM5(+2) into the *Clal* site of YEP24.

The hybrid yeast–mouse U14 gene M-Y-Y was developed by replacing the *BclI*–*SacII* fragment (nt –2 to 42) in pJZ45 with the corresponding mouse DNA. A *SacII* site was introduced into yeast U14 DNA by a single base change (Jarmolowski *et al.*, 1990). Substitution of mouse DNA was achieved by incorporation of two pre-annealed oligonucleotides synthesized *in vitro* as described by Theriault *et al.* (1990). The Y-Y-M hybrid DNA was constructed by oligonucleotide-directed mutagenesis designed to change the A102–A126 region of yeast U14 to the corresponding mouse U14 sequence. The tripartite hybrid M-Y-M was generated by cloning the *MboI*–*BstEII* SNR fragment (nt –2 to 81) from the M-Y-Y vector into the *BclI* and *BstEII* sites of the Y-Y-M plasmid. The relevant regions of all constructs were verified by DNA sequencing.

Bal31 deletions

Linearized DNA was digested with an experimentally determined amount of *Bal31* nuclease ('slow form'; ICN) at 30°C in 250 μ l of 20 mM Tris–HCl, pH 8.0, 600 mM NaCl, 12.5 mM MgCl₂, 12.5 mM CaCl₂ and 1 mM EDTA. 50 μ l aliquots of the reaction mixture were sampled at different times, extracted with an equal volume of phenol, re-extracted with phenol–chloroform and precipitated with ethanol. For deletion mapping, plasmid pJZ45 was digested with *MluI* (C55) or *BstEII* (G83) and treated with *Bal31* nuclease; development of novel restriction sites has been described previously (Jarmolowski *et al.*, 1990). A collection of bidirectional deletions was constituted by combining plasmid digests. DNAs were treated with T4 DNA polymerase to create blunt ends, digested with *BglII* which cleaves ~70 nt upstream of *SNR128* and then ligated. The deletion variants were screened by DNA sequencing.

Acknowledgements

We are grateful to E.Stuart Maxwell for providing synthetic mouse U14 DNA fragments. We also thank him and our laboratory colleagues for helpful advice and stimulating discussions and John Zagorski for help with preliminary experiments. This work was supported by a National Institutes of Health grant (GM19351) to M.J.F.

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Received on September 26, 1991; revised on October 29, 1991