THE BRR GENES ENCODE NOVEL FACTORS INVOLVED IN RNA PROCESSING

by

SUZANNE M. NOBLE

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

Date **University Librarian**

Degree Conferred: . . . . . .

ACKNOWLEDGEMENTS

I am daunted by the task of thanking all of the people who have contributed to this thesis. Whatever else I have done in my time here, I feel that I have taken full advantage of the spirit of community at UCSF and am richer for the experience of having trained here. Many people in different labs have shared with me with their insights, expertise, reagents, and moral support during the course of this project. I would particularly like to thank past and present members of the Herskowitz lab, who have been consistently generous with their time and useful suggestions. Members of the Yamamoto, O'Farrell, Kirschner, Varmus, and Walter labs have also been tremendously helpful.

I would like to thank my advisor, Christine Guthrie, a formidable thinker and wise individual who continues to surprise me with her insights, and who has bowled me over with her exceptional courage, grace, and good humor during times of adversity. It is Christine's style to allow her students extraordinary freedom in pursuing science, and although this can seem a bit scary at first, it has promoted some of the most creative discoveries in our lab (and our field). For me personally, this approach has allowed me to achieve a level of independence and confidence in my own judgment which I would never have imagined previously.

One of Christine's most remarkable achievements has been to create a lab atmosphere which has remained close-knit and supportive, intellectually engaging, and chronically zany, despite an ever-changing cast of characters. Of course, each of these characters has left his imprint, even such dinosaurs as Roy Parker and Bruce Patterson, whom I never met while they were in the lab but whom I quote frequently, as does everyone else. Beth Shuster was my

rotation supervisor; between setting up her new job and buying a house, she managed to give me a good dose of classical genetics as well as a standard of meticulousness that I will never be able to meet. Evi Strauss was our chief composer of musicals and genetic mysteries; she was a charming and energetic force in the lab for many years, but I do harbor a grudge against her for inducing me to lip sync the "Motif" song in "West Wing Story." Sean Burgess was and is a true friend and a true believer in science as a fun and imaginative enterprise (despite initiating the "Bay of Bitterness" with Jim Umen). Cammie Lesser, tamer of postdocs, was my major rival for the presidency of the Brute Force Club; Cammie now uses her awesome strength to admit 12 patients a night. Shelly Jones was for many years our lab mom, looking out for us when we were down but also willing to cajole us into action when she thought we needed it; we have not been quite the same without her.

I have been fortunate to share many baymates over the years. Beate Schwer stands out as a real professional, accomplishing technical feats without ever imposing her frustrations on the rest of the lab (she did impose a few too many repetitions of "the penis song"). Dan Frank was my "still water" baymate #1, not talking much but having a lot to say if you could pry it out of him; he was also the instigator of the best practical jokes ever played on me, and I suppose I forgive him for leading me down the thorny path of *SLU1*. Leslie Stewart was "still water" #2 who, unlike Dan (collector of old sneakers and fungi), did a lot to beautify the bay; when she wasn't busy pursuing Prp28, Leslie contributed elaborate dryboard depictions of exotic tropical landscapes, to give us a taste of the outdoors in our windowless environment. Pratima Raghunathan has turned out to be my alter ego; it is unsettling to learn so late that one has an identical twin, but it is certainly a

pleasure to admire someone's opinions so unequivocally, and I hope that her remarkable perseverance and ingenuity are qualities that I will one day inherit.

Tanya Awabdy, Pratima's other twin, has brought her own light and energy to the lab; she is the clearest successor to the large (metaphorical) shoes of Shelly and Evi. Jim Umen (cohabitor of the Bay of Bitterness) will remain Chief Skeptic of the lab for years after he has left. Jim brought a much appreciated (as long as you weren't presenting) level of critical thinking to Group Meetings as well as a bit of debate for debate's sake; this and his terrific appetite for food and ideas make him a character never to be forgotten. Although not one to make to make a public spectacle, Yan Wang has added her own "little spark" to the lab; her mischievous good humor has lightened many a stressful day in the hot room. Jon Staley, king among victims, brought "kinetics versus thermodynamics" to the Guthrie lab; his questions at Group Meetings have changed lately, however, and I recently heard him ask, "What's the biological relevance of [whatever it was]?" Chris Siebel has taught me that speaking at a snail's pace bears no relationship (unless an inverse one) to one's thinking ability or the pace of one's experiments; Chris is a remarkable guy who can move mountains while appearing to be sitting around doing nothing or, more characteristically, talking on the phone. Anne de Bruyn Kops has introduced the lab to cell biology (and me in particular to immunofluorescence in yeast) as well as to her more recent genetic project, Tristan; she also takes our cultural education very seriously, and I am sure that it is for this reason that she is currently vacationing in France.

Among the youngun's, Amy Kistler is the source of astonishing enthusiasm; her "Go, girl!!" admonishments helped me to make it through

the last few months. Cathy Collins has awakened in me an unexpected maternal instinct, as I realized in the middle of a long speech about mending her ripped bluejeans; she is very competent on her own, however, and I suspect that she will survive my departure better than I will. Kent Duncan can unleash such a flood of words that I had to establish a "Kent Free Zone" during my final months of thesis writing; he took it with pretty good humor, and I hope that he will be forgiving when I come groveling back to the lab for support once I return to medical school. Maki Inada has been Woman Without a Bench for some time now; her patience and positive attitude will serve her well in the coming years of graduate school. Guillaume Chanfreau is the newest member of the Guthrie lab; I am excited that such an eager and talented individual is willing to carry on the torch of *BRR5*, even if he does speak French with the wrong accent.

Heli Roiha, Lucita Esperas, and Carol Pudlow deserve to be mentioned apart, because it is they who keep the Guthrie lab running so smoothly. Heli is a master organizer and intrepid bargainer of great personal wit; she holds the lab together in ways that none of us will fully appreciate until we face the world without her. Tita is not only the toughest and most efficient lab cookie ever baked but also the sweetest-hearted; I will come back to visit her often--as long as she doesn't bite! Carol has become one of my favorite late night companions, despite her penchant for phone pranks and infomercials; I expect great things from Carol (once she finishes her resume!!).

I see that this section has turned into a series of thumbnail sketches instead of proper thank you's, but I think the reason is that what I have valued most in my labmates is not the advice, the protocols, the reagents, or even the chocolate that they have shared with me but rather their own nutty personalities. The cast of characters I have met in my time here would be

hard to assemble in a lifetime of looking, and I am grateful for their friendship.

I would like to thank Ira Herskowitz and Peter Walter for making my thesis meetings not only painless but pleasurable. Both Ira and Peter have been incredibly enthusiastic about my work, and each has provided an outlook which helped me to perceive my results in a different, more global, and more rigorous fashion.

Sue Adams and Jana Toutolmin have been very helpful in my encounters with the bureaucracy of graduate school. They are wonderful advocates--patient, flexible, and extremely adept at working the system.

Hiten Madhani has been my lab (and life) mate extraordinaire. His encouragement and sound judgment have influenced every part of this thesis, and I cannot imagine the last six years without him.

Finally, I would like to thank my mother, Joy Noble, who is my relentless #1 supporter and best friend. My mother is the most creative, energetic, original person I know, and I could not ask for a better role model as a human being. Although she herself has not always had an easy life, she has always wanted the best for me and has made countless personal sacrifices to provide me with opportunities that she never had. I am fortunate to be her daughter.

THE *BRR* GENES ENCODE NOVEL FACTORS INVOLVED IN RNA PROCESSING

Suzanne M. Noble

ABSTRACT

Despite intensive biochemical and genetic efforts, many factors involved in pre-mRNA splicing remain to be identified. To isolate such factors, I have screened a collection of cold-sensitive (cs) yeast strains for mutants with inefficient splicing and have identified nine genes, five of which are novel (designated *BRR1-BRR5* for *B*ad *R*esponse to *R*efrigeration). Four of the nine encode members of the RNA helicase family, suggesting that such proteins may be particularly mutable to the cs phenotype. These include the essential Brr2 protein, whose sequence defines a new subfamily of helicase-like factors.

Detailed characterization of Brr1 has revealed its role as a mediator of snRNP biogenesis. This possibility was suggested by several early observations, including: 1) diminished steady state levels of spliceosomal snRNAs in the *brr1-1* mutant; 2) partial suppression of the *brr1-1* cs growth defect by overexpression of a common snRNP protein, Smd1; and 3) physical association of wild type Brr1 with Sm snRNPs. Pulse-chase analysis of nascent snRNPs in wild type and *brr1* mutant cells has revealed the transcription and cap modification reactions to be unaffected by depletion of Brr1--as is the stability of mature snRNPs, whereas processing of snRNA 3' ends is slowed, implying a role for Brr1 at or before this step. Finally, a screen for overexpression suppressors of *brr1-1* identified three other yeast genes--

including a homolog of the human autoantigen La--with potential roles in snRNP biogenesis.

Brr5 was found to be an essential yeast homolog of the 100 kD subunit of the mammalian polyadenylation signal recognition factor, and the *brr5-1* mutant is defective in both splicing and 3' end formation. One explanation of these unexpected results is that Brr5 may couple the processes of pre-mRNA splicing and 3' end formation, a phenomenon which has been well-documented in mammalian cells, but which has not been described previously in yeast.

These data demonstrate the utility of cs mutants as a route to identifying novel components involved in pre-mRNA splicing. Further, the analysis of *BRR1* and *BRR5* highlight the interplay among cellular RNA processing events.

TABLE OF CONTENTS

LIST OF TABLES

Chapter 1

Appendix

LIST OF FIGURES

Chapter 1

Chapter 2

Appendix

PROLOGUE

This work was initiated in order to identify novel genes and steps in the process of pre-mRNA splicing. The removal of introns from mRNA precursors is an essential event in eukaryotic gene expression, as well as a frequent target of regulation. The chemistry of splicing is rather simple, occurring as two transesterification reactions which, in principle, require no exogenous energy source. In contrast, the splicing machinery is complex, consisting of five small nuclear RNAs (U1, U2, U4, U5 and U6 snRNAs) and a set of 50-100 proteins, in addition to the pre-mRNA substrate (reviewed by Moore et al., 1992; Rymond and Rosbash, 1992; Madhani and Guthrie, 1994). *In vitro* splicing requires ATP.

At the time this work was undertaken, the conservation of splicing in the yeast *S. cerevisiae* had only recently been demonstrated, with the twin discoveries of intron-containing yeast genes (Gallwitz and Sures, 1980; Ng and Abelson, 1980) and the full set of cognate yeast snRNAs (reviewed by Guthrie and Patterson, 1988). Despite the relative infancy of the field, progress in yeast had been rapid, quickly outstripping the mammalian field in the identification of protein splicing factors. A set of temperature-sensitive (ts) yeast mutants, now known as *prp2-prp11*, had been identified in a screen for components which participate in RNA synthesis (Hartwell, 1970); these were subsequently shown to have primary defects in RNA splicing, leading to feedback inhibition of ribosomal RNA synthesis as a secondary consequence (Rosbash et al., 1981). A second screen was performed, this time assessing 1000 ts yeast mutants directly for *in vivo* splicing activity; the splicing candidates *PRP17-PRP27* emerged (Vijayraghavan et al., 1989). A similar screen was performed on a small scale and led to the identification of the cold-sensitive (cs) mutant, *prp28-1* (Strauss and Guthrie, 1991). Finally, in the

only screen (at that time) directed to identify factors with a particular splicing function, *prp16-1* was found as a mutant which could suppress a mutation in the branchpoint sequence, one of three conserved sequences within the intron substrate for splicing (Couto et al., 1987).

In addition to genetics, biochemical and sequence analysis of the *PRP* gene products had led to some understanding of their functions (reviewed by Rymond and Rosbash, 1992). Most of them were shown to be required for the first step of splicing, whereas three were found to act at the second step (Prp16, Prp17, Prp18); one gene product, Prp22, was shown to participate in the release of the mRNA product from the spliceosome. Several splicing proteins were found to associate physically with snRNAs in small nuclear ribonucleoprotein particles (snRNPs); these included Prp3, Prp4, Prp6, and Prp8. Because of the ability of a mutant *prp16-1* to suppress normally deleterious changes in the conserved branchpoint helix, Prp16 was proposed to regulate the fidelity of splicing, ensuring that only those substrates with correct branchpoint sequences undergo the second chemical step of splicing (Couto et al 1987; Burgess et al., 1990). Finally, several of the Prp proteins were found to bear sequence homology to a family of RNA helicase and helicase-related proteins; these included Prp2, Prp5, Prp16, Prp22, and Prp28.

From *in vitro* work performed both in mammalian cells and in yeast, it was known that the formation of a spliceosome involves many events prior to the chemical reactions of splicing. This complex assembly process requires ATP and is thought to maintain the accuracy of splicing as well as to correctly position the substrate relative to the catalytic splicing apparatus. The various precursors to the spliceosome can be fractionated either by native gel electrophoresis or by glycerol gradient sedimentation. The identification of the earliest appearing complex to contain splicing intermediates suggested the

identity of the active spliceosome. It was found that the form corresponding to the catalytically active intermediate contained all of the snRNAs except for U4, which is normally extensively base-paired to U6 snRNA (Cheng and Abelson, 1987; Konarska et al., 1987; Lamond et al., 1988). These and other observations led to the hypothesis that the highly conserved U6 snRNA is the catalytic component of the spliceosome and that its usual association with U4 sheathes this enzyme until the correct time for splicing (Guthrie and Patterson, 1988).

Although many splicing factors had already been identified in yeast, it was clear that many remained to be discovered (as none of the previous screens had been saturating). Furthermore, there existed biochemical evidence for important events in splicing for which no protein mediator had been identified, such as the U4-U6 helix destabilizing factor. In planning a method to identify these additional splicing components, I decided to use a variation of the approach which had been most fruitful in identifying large numbers of splicing factors. Screening collections of ts mutant strains of yeast for those with defects in splicing had identified all but two of the known *PRP* genes. With good secondary screens to suggest a possible function for newly-identified protein products, this approach seemed to offer the highest chance of success. The main drawbacks were: 1) the ts approach had already been used twice, and the existence of ts hotspots would likely reduce the proportion of novel genes to result from a subsequent screen; and 2) studies from the Pringle lab suggested that many essential genes will not mutate detectably to temperature-sensitivity (Diehl and Pringle, 1991; Harris and Pringle, 1991). My eventual choice to screen cs mutants seemed likely to overcome these limitations, both in principle and because of the previous success of a cs screen for cell cycle defective mutants in yeast by Moir and

Botstein (1982). In addition, I hoped that cs mutants would provide a useful biochemical tool to identify "frozen complexes" which might correspond to previously undetected splicing intermediates.

In Chapter 1, I describe a screen of 340 cs strains for those with defects in pre-mRNA processing. Twelve mutants identified nine complementation groups with association between the splicing and cs defects. Five of these corresponded to novel genes (named *BRR1-BRR5* for Bad Reponse to Refrigeration), validating the approach of using an alternative conditional phenotype to identify novel splicing factors. Included in this chapter is the characterization of the essential gene, *BRR2*, which is interesting in light of its homology to RNA helicase-like proteins, along with three of the four *PRP* genes recovered in this screen. Further analysis of the sequence of Brr2 demonstrated that it belongs to a new subfamily of helicase-related proteins that exhibits extensive sequence conservation in regions outside the helicase domain and which includes the yeast antiviral protein, Ski2.

Chapter 2 represents a detailed analysis of the *BRR1* gene product. Because the *brr1-1* mutant exhibited a decrease in the levels of the spliceosomal snRNAs specifically and because its cs growth defect is partially suppressed by overexpression of a common snRNP protein, I hypothesized that Brr1 might normally participate in the biogenesis of snRNPs. Biochemical experiments demonstrated that Brr1 associates physically with the spliceosomal snRNAs. Finally, comparison of the stability of mature snRNPs in *brr1* mutant versus wild type cells, as well as the development of a transcriptional pulse-chase system for assessing snRNP biogenesis in vivo, provided direct evidence for a role for Brr1 in the manufacture of spliceosomal snRNPs.

Chapter 3 describes the initial characterization of *BRR5*. The *brr5-1* mutant exhibits a defect in the first step of splicing. Sequence analysis of the Brr5 protein revealed an unexpected similarity to a subunit of the mammalian factor responsible for recognizing the conserved AAUAAA cleavage-polyadenylation signal (cleavage and polyadenylation specificity factor, CPSF). The detection of 3' processing defects in *brr5-1* suggests a possible coupling between splicing and polyadenylation in yeast, as has been well-documented in mammalian cells. Brr5 and its mammalian homolog may mediate crosstalk between the two RNA processing systems.

Finally, in the Appendix, I return to the topic of snRNP biogenesis. A screen for overexpression suppressors of *brr1-1* was intended to identify other participants in the process of snRNP biogenesis. Three suppressors (in addition to *BRR1* itself) were recovered from the screen: one is the yeast homolog of the human autoantigen, La; a second is the likely yeast homolog of a mammalian nuclear helicase-like protein; and the third is homologous to a subunit of the mammalian basal transcription factor, TFIIH.

In summary, the search for novel factors and steps in the pre-mRNA splicing processes via cs mutants proved successful on both counts. I was able to identify five genes which were previously undescribed; at least one of these (*BRR2*) is likely to be directly involved in splicing, whereas two others (*BRR1* and *BRR5*) are involved in snRNP biogenesis and 3' end processing, respectively. The isolation of *BRR1* and *BRR5*, though unexpected, underscores our growing awareness of the intimate relation between the various processes which govern RNA metabolism.

CHAPTER 1

Identification of Novel Components of the Yeast pre-mRNA Splicing Machinery by Means of Cold-Sensitive Mutations

ABSTRACT

Pre-mRNA splicing occurs in the spliceosome, a large and dynamic ribonucleoprotein that contains five small nuclear RNAs and an estimated 50 to 100 protein factors. Genetic approaches in the yeast *Saccharomyces cerevisiae* have led to the identification of 38 genes required for efficient RNA splicing. The majority of these have been found by screening collections of (high) temperature-sensitive (ts) mutants for those that fail to splice endogenous pre-mRNAs. Despite the success of this approach, the presence of ts hotspots reduces the proportion of novel genes recovered from successive screens; moreover, studies of mutagenesis in yeast suggest that many essential genes rarely mutate to the ts phenotype. With a goal of revealing novel genes, we screened a collection of 340 cold-sensitive (cs) mutants for diminished splicing of several pre-mRNAs. Twelve mutants in nine complementation groups exhibited association between the cs growth and RNA splicing defects. Four of these affected known genes *(PRP8, PRP16, PRP22, PRP28)*, three of which encode RNA helicase homologs. Five genes are novel *(BRR1, BRR2, BRR3, BRR4, BRR5; B*ad *R*esponse to *R*efrigeration); mutations in these genes inhibited splicing prior to the first chemical step of the reaction. Nucleotide sequence and gene disruption analysis of *BRR2* revealed it to encode an essential member of a new class of RNA helicase-like proteins which includes the product of the yeast antiviral gene *SKI2*. These data validate the use of cs mutants in genetic screens and raise the possibility that RNA helicase family members are particularly prone to mutation to cold-sensitivity.

INTRODUCTION

The removal of intervening sequences from mRNA precursors is a fundamental event of eukaryotic gene expression. Splicing occurs via two successive transesterification reactions. The first step involves nucleophilic attack by the 2' hydroxyl of a conserved intronic adenosine on a phosphate group at the 5' splice site, thereby generating a free 5' exon and an intron lariat-3' exon intermediate. In the second step, the 3' hydroxyl of the free 5' exon attacks the phosphate at the 3' splice site, yielding the ligated exons as well as a precisely excised lariat intron (Ruskin et al., 1984; Konarska et al., 1985; Moore et al., 1993). Despite this simple chemistry and the fact that some Group II organellar introns are able to complete the reaction *in vitro* without the aid of proteins, nuclear splicing requires a complex machinery of five small nuclear RNAs (snRNAs) and an estimated 50-100 proteins (reviewed by Moore et al., 1993; Rymond and Rosbash, 1992). Work in recent years suggests that the active site of the spliceosome is built as a network of RNA-RNA interactions that involve the U2, U5, and U6 snRNAs as well as the intron-containing substrate (reviewed by Madhani and Guthrie, 1994). Several of these interactions require disruption of RNA secondary structures and the formation of mutually-exclusive alternative structures. It has been proposed (see Guthrie, 1991) that the roles of the protein elements are to assure that spliceosome assembly is ordered and precise, to correctly orient and rearrange the trans-acting snRNAs within the spliceosome, and, in some cases, to mediate alternative splicing choices. Determination of such functions requires the isolation of the protein factors.

A number of proteins required for splicing have been identified. In vertebrate cells, biochemical stategies have been used to isolate and clone the

genes for all eight of the core (Sm) snRNP proteins, as well as a number of snRNP-specific and trans-acting proteins (reviewed by Moore et al., 1993; see also Bennett and Reed, 1993; Brosi et al., 1993; Patton et al., 1993; Krämer et al., 1994; Champoin-Arnaud and Reed, 1994; Chiara et al., 1994; Krämer et al., 1995); however, this approach is made challenging by the low abundance of many splicing proteins. In yeast, tractable genetics and genome sequencing efforts have permitted the identification of about forty splicing proteins in a "gene-first" manner. Several genes have been identified in screens for suppressors or enhancers of conditional lethal mutations in known components of the splicing apparatus (or the spliced substrate). These include the cold-sensitive (cs) mutant, *prp16-1*, discovered by its ability to suppress an intron mutation in a reporter gene (Couto et al., 1987); *SPP2*, which was isolated as a high copy suppressor of *prp2-1* (Last et al., 1987); *SLU1*, *SLU2*, and *SLU7*, first identified as mutants which are synthetically lethal with a mutant U5 snRNA (Frank et al., 1992); and *MUD1* and *MUD2*, found in a similar screen with mutant U1 snRNA (Liao et al., 1993; Abovich et al., 1994). Recently, homologs of several of the mammalian Sm proteins as well as the U1 70K snRNP-specific protein have been identified by a combination of genome sequencing and genetic analysis (Smith and Barrell, 1991; Kao and Siliciano, 1992; Rymond, 1993; Séraphin, 1995; Cooper et al., 1995; Roy et al., 1995). Virtually all other published mRNA splicing components have been discovered by screening collections of ts lethal mutants for those that fail to efficiently splice endogenous pre-mRNAs following a shift to the nonpermissive temperature (Hartwell et. al., 1970, these were first screened for failure to synthesize total RNA and subsequently shown to affect RNA splicing; Rosbash et al., 1981; Vijayraghavan et. al., 1989; Blanton et al., 1992; Lockhart and Rymond, 1994; B. Rymond, personal communication; J.

Woolford, personal communication). A total of 28 genes have been identified by this method, which in aggregate has involved screening more than 2000 ts mutants.

Conditional-lethal splicing mutants are useful for many reasons, including ease of cloning, the ability to propagate cells at permissive temperatures, and, most importantly, the ability to selectively inactivate components *in vivo* and *in vitro*. Although the majority of known splicing genes in *S. cerevisiae* have been identified in screens of collections of ts mutants (described above), there exist serious obstacles to identifying the remainder by the same means. First, studies of *S. cerevisiae Chromosome* I demonstrate that only a minority of essential genes mutate to temperature-sensitivity, at least when the target of the mutagen is the intact cell (Harris and Pringle, 1991; Diehl and Pringle, 1991). In these studies of diploid yeast strains monosomic for *Chromosome* I, 53 ts alleles were mapped to only six genes on *Chromosome* I, whereas no ts alleles were obtained for at least 14 other essential genes (as defined by gene disruption studies; Harris and Pringle, 1991; Diehl and Pringle, 1991). If true in general, these data suggest that many splicing proteins probably cannot be detected in screens that rely upon ts mutants. Second, although screens for ts splicing mutants have not been saturating, the existence of mutational hotspots makes the detection of additional splicing factors a case of diminishing returns (Hartwell et. al., 1970; Vijayraghavan et. al., 1989). For instance, mutations in the *PRP2* and *PRP3* genes compose 24% and 16%, respectively, of the mutants isolated in the aforementioned screens (Hartwell et al., 1970; Vijayraghavan et al., 1989; J. Woolford, personal communication; B. Rymond, personal communication).

A possible solution to this quandary is to select for mutants based on a different conditional phenotype, such as cold-sensitivity. Early studies of

bacteriophage mutants indicated that cs and ts mutants tend to fall in different subsets of genes (Scotti 1968; Cox and Strack, 1971; Jarvik and Botstein, 1975). Moreover, Guthrie et al. (1969a; 1969b) and Tai et al. (1969) demonstrated that, while extensive searches for ts mutants affecting prokaryotic ribosomal assembly had proven fruitless, cs mutants are remarkably easy to obtain. In yeast, comparative data exist from genetic analysis of the cell division cycle (cdc). Moir et. al. (1982) generated and screened a collection of 350 cs mutants for those which arrest with morphologies characteristic of a cdc defect. Whereas their rationale in choosing cs mutants had been to use them for pseudoreversion analysis, the screen proved to be successful in another way, yielding at least six novel complementation groups that had not been identified in a previous screen of 1500 ts yeast strains (Hartwell et. al., 1973). Only one complementation group from the cs screen had been previously identified (Moir et al., 1982), consistent with the idea that, for *cdc* mutations, ts and cs alleles occur in largely nonoverlapping sets of genes. We reasoned that a strategy employing a collection of cs yeast strains might be equally useful when applied to studies of mRNA splicing. In support of this idea, Strauss and Guthrie (1991) identified a novel splicing factor, *PRP28* , in a pilot screen of only 18 cs mutants.

We examined the cs collection produced by Moir et al. (1982) for strains with defects in the splicing of endogenous pre-mRNAs. Genetic analysis of the initial candidates revealed nine complementation groups in which the cs phenotype was associated with the RNA splicing defect. To identify which of these genes were novel, complementation analysis was performed with the previously identified *prp* strains. Four of the complementation groups were found to correspond to previously-identified genes (*PRP8, PRP16, PRP22,*

PRP28), whereas five had not been described previously (*BRR1*, *BRR2*, *BRR3*, *BRR4*, *BRR5*; *B*ad *R*esponse to *R*efrigeration). Nucleotide sequence and gene disruption analysis of *BRR2* demonstrate it to encode an essential member of a novel family of RNA helicase-like proteins which includes the product of the yeast antiviral gene *SKI2*. As *PRP16*, *PRP22*, and *PRP28* also encode RNA helicase homologs, it is possible that genes for helicase-like proteins are preferentially mutable to cold-sensitivity. Like the previous findings of Moir et al. (1982), our results demonstrate that screening mutants of a different conditional phenotype can be an efficient method for identifying novel genes involved in an essential cellular process.

MATERIALS AND METHODS

Yeast and molecular biology methods

Cultivation and genetic manipulation of *S. cerevisiae* were performed by standard methods (Guthrie and Fink 1991). Molecular biological methods are described in Sambrook et al. (1989).

Temperature shift of cs collection and Northern analysis of *CRY1* mRNA

Of the 350 cs strains described by Moir et al. (1982; Table 1), 340 were recovered from storage at -80°C. Single colonies from each of these as well as the wild-type parental strains were inoculated into 5 ml of YEPD and grown for 2 days at 30°C until the cultures were saturated. Adenine prototrophs (DBY4157-4482) were then inoculated into 25 ml of fresh YEPD to an optical density at 600 nm (O.D. 600) of about 0.15; adenine auxotrophs (DBY4483-4497)--which grow somewhat more slowly--to 0.2. The cells were allowed to recover for 3 hours at 30°C, a period sufficient for most cells to double once. The cultures were then moved to a shaking water bath at 16°C, where they were incubated for 10 hours. Subsequently, the cells were pelleted by centrifugation, frozen quickly using liquid nitrogen, and total cellular RNA was prepared.

For Northern analysis, 20 ug of RNA from each mutant as well as the wild-type parent and a positive control mutant (*prp18-1*^{ts}, shifted to 37°C for 4 hours) was electrophoresed overnight at 30 V on 1.5% agarose/6% formaldehyde gels. The resolved material was then electroblotted onto a Hybond-N (Amersham) nylon membrane for 3 hours at 40 volts. The blots were crosslinked with ultraviolet light using a Stratalinker (Stratagene) on the automatic setting. Hybridization was performed overnight at room

temperature using standard methods and ^{32}P 5'end-labeled oligonucleotides complementary to *CRY1* second exon sequences and to U5 snRNA (see below for oligonucleotide sequences). Autoradiograms were scanned visually for candidates which displayed differences from the wild-type control in the ratio of *CRY1* mRNA to the U5 snRNA internal standard.

Oligonucleotides used in this study

Name/Sequence

CRY1 exon 2/GTA TCG TTG AAA GAA GCG

U5-7wtsmnr/AAG TTC CAA AAA ATA TGG CAA GC

U3 exon 2/CCA AGT TGG ATT CAG TGG CTC

RP51A exon 2/CGC TTG ACG GTC TTG GTT C

U1-19K/CAA TGA CTT CAA TGA ACA ATT AT

RP51A intron/GTA TGA CTT TAT TGC GCA TGT CGA CTC

Primer extension analysis of transcripts from the *SNR17A, SNR17B,* and *RP51A* genes

For the initial rescreening of the 98 candidates identified by Northern analysis of the *CRY1* message, an abbreviated primer extension protocol was used to monitor the splicing of the U3 pre-snoRNA (Lesser and Guthrie, 1993). ^{32}P-labeled oligonucleotide U3 exon2 was used to detect the U3 precursor and mature species; oligonucleotide U5wtsmnr was used to detect U5 snRNA, which served as the internal control for RNA loading. In subsequent primer extension of U3, we employed our standard protocol (Patterson and Guthrie 1991).

To monitor the splicing of pre-*RP51A*, we employed our standard primer extension protocol and primers RP51A exon2 (for visualization of pre-*RP51A*, mature *RP51A*, and lariat-exon2) or RP51A intron (for visualization of pre-*RP51A* and lariat-exon2/lariat intron); in these primer extensions, primer U5-7wtsmnr was used to visualize the internal control, U5 snRNA.

Complementation analysis among the cs mutants

Cs mutant candidates were crossed to wild-type strains of similar S288C genetic background (either DBY640 or DBY473 for the first cross and YGS1 in subsequent matings; Table 1). The resulting diploids, heterozygous for the cs mutation, were sporulated and the resulting ascospores dissected. **a** and α spores from each mutant were mated by cross-stamping patches of spore progeny on YEPD. After incubation at 30°C for one day, the plates were replica-plated to prechilled YEPD plates and incubated at 17°C for two days. Outcrossed descendants of strains DBY4234, 4236, and 4489 formed a single complementation group, as did descendants of strains DBY4172 and 4423. The DBY4172 and 4423 progeny were crossed to each other and dissected. In nineteen four-spore tetrads, the cs phenotype segregated in a pattern of 4:0 (cs^- to cs^+) indicating that the two mutants are allelic. Descendants of strains DBY4234 and 4489 were subsequently shown to be linked to a previously-identified *prp* mutation (see below), indicating that they are also allelic to each other.

Linkage analysis of cs growth and splicing defects

The progeny of five tetrads from each cross (described in the section above) were assessed for growth at 16°C and also for splicing defects. The latter was accomplished by performing a temperature shift similar to the one

described above, except this time the cultures were grown in tubes placed in a roller drum in a 16°C incubator for 12 hours. Total cellular RNA was prepared and analyzed for splicing of pre-U3 snRNA as described above.

Cloning of genomic complementing plasmids

Complementing clones for DBY4483 (*BRR1*), DBY4340 (*BRR2*), DBY4217 (*BRR3*), DBY4475 (*BRR4*), DBY4490 (*PRP8*), and DBY4172 (*PRP22*) were obtained in the following manner: cs splicing mutants which had been outcrossed one to four times were transformed with DNA from the Rose library; this library consists of wild-type yeast genomic DNA carried in the *URA3*-marked, centromeric vector, YCp50 (Rose et al., 1987). Transformation reactions were plated directly on pre-chilled plates lacking uracil and incubated at 17°C for four to six days. Calculated transformation efficiencies indicate that more than twenty genome equivalents of transformants underwent the selection for each mutant. For each mutant, approximately twenty candidate transformants were colony purified on plates lacking uracil which were incubated at 17°C. To determine whether the cold-resistant phenotype results from the introduced plasmid, single colonies were then streaked to plates containing 5-fluorootic acid (FOA), which selects against the *URA3*-marked library plasmid. Colonies from the FOA plates were assessed for growth at 17°C; those which reverted to cold-sensitive growth in the absence of the plasmid were processed further. Plasmids recovered from strains passing the 5-FOA test were retransformed into the original cs mutant strains and scored once again for their ability to confer growth at 17°C. For a given cs mutant, those plasmids which passed this retransformation test were compared by digestion with multiple restriction enzymes for the presence of common restriction fragments. In all cases but one, multiple complementing

plasmids contained overlapping inserts. For *BRR1*, two classes of plasmids were obtained: multiple isolates of plasmids which conferred wild-type growth at 17°C and which contained overlapping inserts, and a single plasmid which partially suppressed the cold-sensitive growth defect of *brr1-1* and which contained DNA of distinct sequence from the fully complementing ones. In analysis described elsewhere (Noble and Guthrie, in preparation), the fully complementing plasmid was found to encode the authentic *BRR1* gene.

BRR5 could not be cloned by direct selection at the nonpermissive temperature because of a relatively high reversion frequency. Thus, *brr5-1* transformants were plated first on plates lacking uracil and incubated at the permissive temperature of 30°C, followed by replica-plating to prechilled plates at 17°C. Plates were monitored daily, and large flattened patches (corresponding to primary transformants) which grew at 17°C were chosen as candidate *BRR5* transformants (as opposed to papillations, which were likely to be revertants). The remainder of the cloning strategy was the same as for the other mutants. A list of the complementing plasmids appears in Table 2.

Determination of allelism with previously-identified splicing factors

At least one cs mutant from each of the nine complementation groups for which there was association between the splicing and cs growth phenotypes was crossed to cs alleles of *PRP16* (*prp16-101*; strain YS111) and *PRP28* (*prp28-1*; strain YGS5). As failure to complement was observed between the outcrossed progeny of DBY4406 and *prp16-101*, as well as between progeny of DBY4234, DBY3236, DBY4489 and *prp28-1*, further crosses were made for linkage analysis. A DBY4406 descendant was crossed to a temperature-sensitive strain containing the *prp16-2* mutation, YEJS35 (Table 1). In six of six tetrads, 2:2 segregation of the cs^-:ts^- phenotypes was observed,

indicating that the two mutations are allelic. Likewise, descendants of DBY4234 (YSN113) and DBY4489 (YSN150), both members of the same complementation group, were crossed to a cs strain containing the *prp28-1* mutation (YGS5; Table 1). Of twelve and nine four-spore tetrads, respectively, 4:0 segregation of the cs^-:cs^+ phenotypes was observed for each of the tetrads, again indicating allelism of the mutations.

For the remaining 22 previously identified pre-mRNA processing mutants for which conditional lethal alleles existed, as well as several unpublished mutants (kindly provided by J. Woolford), only ts alleles were available (Table 1). Therefore, complementation analysis of these strains was performed by transformation of the strains with the wild-type clones for the remaining candidates from this screen. Transformants were streaked on YEPD plates, and growth at 37°C was compared between transformants with candidate complementing clones versus a vector control. As the clone for DBY4172/4423 complemented *prp22*, and that for DBY4490 complemented *prp8*, their inserts were examined further. Restriction fragment analysis of both clones as well as PCR analysis of the *PRP22* clone confirmed the identity of the complementing DNAs. Segregation analysis of the spore progeny was not performed, however, leaving open the possibility that the single copy plasmids fully suppress the cs defects but are not allelic to the mutant genes.

Molecular analysis of *BRR2*

In order to better delineate the *BRR2* gene within the 12 kilobase (kb) insert of pSN21, we prepared a library of size-fractionated fragments of pSN21 which had been digested only partially with *Sau*IIIA. Fragments were isolated in the following size classes: 1-3.5 kb, 3-5 kb, and greater than 5 kb; these were

ligated to *Bam*HI-digested pSE360, a centromere, ARS-containing vector (pSE360 is identical to pUN50; Elledge and Davis, 1988).

Complementing clones were obtained by transforming the three minilibraries in parallel into YSN405 (a *brr2-1* strain which has been outcrossed four times; Table 1) and plating directly to prechilled plates lacking uracil. Approximately 10^6 transformants were plated for each library; subsequent steps in the screen were identical to those described above for cloning of the original complementing plasmids.

Plasmids which were able to confer wild-type growth at 17°C to YSN405 cells upon retransformation were compared for the size of their inserts. The smallest complementing plasmid had a 7.1 kb insert (pSN108) and was selected for further analysis. Sequencing reactions were performed with primers homologous to regions of the vector which flank the insert. Using this sequence information, a search of the database was performed, and an exact match was located on *Chromosome* V (Swiss Protein accession number P32639).

Linkage of the cloned *BRR2* gene to *brr2-1*

To determine whether the minimal complementing plasmid corresponded to the wild-type version of *brr2-1* (rather than a suppressor), we created a plasmid which could be used to target the *URA3* selectable marker to the chromosomal locus of the cloned gene. A 877 base pair (bp)*Sal*I-*Sal*I fragment from pSN108 which includes the C-terminus of the cloned gene was subcloned into the integrating vector, pRS306 (*URA3*; Sikorski and Hieter, 1989). This construct was linearized with *Bst*XI, which cuts within the 877 bp insert, and transformed into the wild-type strain, YGS1, which is auxotrophic for uracil. Uracil prototrophs were screened using a PCR method for

integration to the correct locus (i.e. to chromosomal sequences homologous to the cloned gene). One of the correct integrants was crossed to a *ura3, brr2-1* strain and the meiotic progeny scored for segregation of the cs and uracil auxotrophic phenotypes.

Disruption of the *BRR2* gene

A disrupted allele of *BRR2* was created by replacing a 2493 bp *Bgl*II-*Bgl*II fragment corresponding to the middle of the Brr2 ORF with the *LEU2* gene to yield pSN211. The *Bgl*II-*Bgl*II fragment encodes almost the entire helicase homology region of Brr2. pSN211 was digested with *Sal*I to liberate the *LEU2* gene flanked by 1660 and 3143 bp of *BRR2* flanking sequences. This restriction fragment was gel-purified and transformed into the wild-type diploid strain, JO226, in a one-step gene replacement procedure. Integration at the correct locus was diagnosed by screening leucine prototrophs by a whole yeast PCR procedure, using primers against the *LEU2* gene and sequences adjacent to the *BRR2* gene. Tetrads from two diploids heterozygous for the gene disruption were dissected onto YEPD plates which were incubated at room temperature.

RESULTS

Identification of cs mutants defective in RNA splicing

We obtained the bank of 350 cs mutants of *S. cerevisiae* which had been generated by Moir et al. (1982) for the isolation of cdc mutants; this collection had not been rescreened subsequently for any other defect (J. Mulholland and D. Botstein, personal communication). To identify mutants which are cs because of defects in splicing, we shifted log phase cultures of each of the mutants from the permissive temperature of 30°C to the nonpermissive temperature of 16°C (see Figure 1). The duration of this temperature shift was 10 hours, about 1.5 doublings for the wild-type parental strain at this temperature. This interval was similar in terms of generation time to that used previously in a similar screen of ts mutants (Vijayraghavan et al., 1989); our hope was that a relatively short shift to the nonpermissive temperature would enrich for the detection of genes whose products participate directly in splicing and whose inactivation therefore results in a rapid decrease in splicing efficiency. We next prepared total cellular RNA from each of the cultures and evaluated the splicing efficiency of each of the mutants by Northern hybridization analysis, using oligonucleotide probes against the *CRY1* pre-mRNA (Larkin and Woolford, 1983), which encodes a ribosomal protein, and U5 snRNA (Patterson and Guthrie, 1987), which was used as an internal control for RNA loading. *CRY1* was chosen because of its sensitivity to RNA splicing defects in known mutants (S. Noble, unpublished observations).

We examined the resulting Northern blots for mutants with altered RNA profiles. The ratio of spliced mRNA to unspliced pre-mRNA is thought to provide the most reliable measure of splicing efficiency (Pikielny

and Rosbash, 1985); however, the unspliced pre-CRY1 species is normally undetectable by our assay. Therefore, we focused on those mutants that exhibited a decrease in *CRY1* mRNA relative to the internal control (U5) or an increase in unspliced pre-mRNA or lariat intermediate (which have similar electrophoretic mobilities in this assay). Ninety-eight candidates were identified which exhibited a difference in these ratios (data not shown). Eighty-two candidates exhibited a decrease in the ratio of mature *CRY1* mRNA to U5--with no apparent change in pre-mRNA levels--while fourteen exhibited an increase in the ratio of pre-mRNA to U5 snRNA. Two candidates demonstrated an increase in the ratio of mature *CRY1* mRNA to U5 snRNA, but the absolute level of U5 snRNA appeared to be very low. This pattern, while not indicative of a defect in splicing efficiency per se, is consistent with a defect in U5 synthesis or stability.

To determine which of these mutants exhibited general defects in splicing, we examined three other pre-mRNAs: *SNR17A*, *SNR17B*, and *RP51A*. *SNR17A* encodes the nucleolar U3 snoRNA, as does *SNR17B*. The two genes differ in the size and sequence of the introns that interrupt the coding sequence (Myslinski et al., 1990). By examining the splicing of a number of pre-mRNAs in known mutants, we have found that the levels of *SNR17A* and *SNR17B* pre-snoRNAs are highly sensitive indicators of defects in the first chemical step of splicing (S. Noble, unpublished). Likewise, the level of lariat intermediate for the *RP51A* transcript, which encodes a ribosomal protein (Teem and Rosbash, 1983), is a sensitive indicator for defects in the second chemical step of splicing (S. Noble, unpublished). Using a primer extension assay, we analyzed the ninety-eight candidates for their ability to splice *SNR17A*, *SNR17B*, and *RP51A*. Twenty candidates exhibited an increase in the levels of precursor or splicing intermediates for all three

gene transcripts; those meeting certain additional criteria (described below) are represented in Table 3. Seventeen of these exhibited first step defects with the three substrates (accumulation of precursor), whereas three exhibited a second step defect (accumulation of the *RP51A* lariat intermediate; these mutants also accumulated pre-mRNA). An example of this analysis for 12 candidate mutants, as well as wild-type and *prp18-1* negative and positive controls, is shown in Figure 2. Several of these mutants (DBY4172, 4217, 4234, 4236, 4276), show accumulation of *RP51A* pre-mRNA, while DBY4172 demonstrates in addition the accumulation of lariat intermediate. (Note that the fraction of mutants with splicing defects was uncharacteristically high on this gel, which represents some of the candidates with the strongest phenotypes with *CRY1* pre-mRNA.) In addition to these, 11 candidates displayed a decrease in mature *RP51A* message with no concomitant increase in precursor or intermediates (e.g. DBY4174 in Figure 2); as this phenotype could result from mutation of factors which stabilize mRNA, these candidates were not pursued further.

At least one ts RNA splicing mutant, *prp22*, has been reported to accumulate the excised lariat intron (in addition to unspliced pre-mRNA; Vijayraghavan et al., 1989). Because the primer extension assays performed above were not able to detect the accumulation of lariat intron (as the primers were directed against the second exon of *RP51A*), RNAs from the twenty candidates were examined by primer extension with a primer directed against the intron of *RP51A*. Using this primer, extension products from lariat intron comigrate with those from lariat intermediate. We found that only the three candidates which were previously shown to accumulate lariat intermediate also accumulate this mixed species band (Table 3)

Genetic analysis reveals nine complementation groups in which the cs growth defect is associated with the RNA splicing defect

To determine whether the cs alleles of the remaining candidates were dominant or recessive to wild-type, the twenty strains were crossed to a wild-type strain of similar genetic background, and the diploids were examined for growth at 16°C. All of the heterozygous diploids grew as well as a wild-type diploid, demonstrating that the cs mutants are recessive. One candidate was discarded because of its failure to mate.

To determine the number of genes represented by the remaining nineteen mutants, the heterozygous diploids were sporulated, the resulting ascospores dissected, and *MAT***a** and *MAT*α progeny sorted for complementation analysis. Certain candidates were backcrossed to the wild-type strain multiple times at this stage, either because of poor spore viability or the presence of multiple cs^- mutations; in cases of multiple independent cs- mutations, splicing was assessed for each of the segregants of a 4:0 cs^-:cs^+ tetrad. A complete matrix of crosses among the mutants, with testing of each diploid for growth at 16°C, revealed the presence of sixteen cs complementation groups.

Although these mutants were identified because of their defects in RNA splicing at the nonpermissive temperature, it remained possible that the cs growth defect was not associated with the RNA splicing defect. We therefore performed linkage analysis. Five tetrads from a heterozygous diploid (mutant crossed to wild type) representing each complementation group were examined. As in the initial screen, cells were shifted to the nonpermissive temperature and examined for splicing of U3 pre-snoRNA. Nine of the candidates exhibited perfect 2:2 cosegregation of the cs growth and splicing defects, indicating linkage between the two traits with 95% certainty;

these are the mutants described in Table 3. Cosegregation analysis for such a mutant (derived from DBY4340) is shown in Figure 3. In three of the candidates, the two phenotypes were unlinked, and in four candidates, the splicing defect was not reproduced; these seven mutants were not characterized further.

Four of the complementation groups correspond to *PRP8, PRP16, PRP22,* and *PRP28*

Cs alleles existed for three previously-identified splicing genes, *prp16-1*, *prp28-1*, and *slu7-1* (Couto et al., 1987; Strauss and Guthrie, 1991; Frank et al., 1992). These were crossed to members of each of the nine remaining complementation groups, and the resulting diploids were tested for growth at 16°C. *prp28-1* (YGS5) failed to complement cs strains derived initially from DBY4234, DBY4236 and DBY4489. *prp16-1* (YS111) failed to complement a descendant of DBY4406. Linkage of these mutations was confirmed by cosegregation analysis of growth phenotypes among the meiotic progeny.

As the remainder of previously-identified *prp* mutants existed only as ts alleles, we could not determine potential allelism by assessing the cold-sensitivity of the heterozygous diploids. We therefore isolated wild-type complementing plasmids for the remaining, unidentified candidate splicing factors and tested for their ability to complement the known ts splicing mutants. Complementing clones were obtained by transformation of the cs mutants with a genomic yeast library (Rose et al., 1987), followed by selection for growth of transformants at the nonpermissive temperature of 17°C. In all cases, one or more complementing plasmid(s) were isolated from yeast, reintroduced into the corresponding cs mutant, and shown to confer wild-type growth at the nonpermissive temperature (see Materials and Methods).

We transformed plasmids corresponding to the seven complementation groups into the previously identified ts splicing mutants and assessed transformants for growth at 37°C (relative to the same strains transformed with vector alone). The mutants tested (Table 1) were *prp2, prp3, prp4, prp5, prp6, prp8, prp9, prp10/11* (Hartwell et al., 1970; Rosbash et al., 1981); *prp17, prp18, prp19, prp20, prp21, prp22, prp24, prp27* (Vijayraghaven et al., 1989); *slu1, slu2, slu7* (Frank et al., 1992); *prp38, prp39* (Blanton et al., 1992; Lockhard and Rymond, 1995); *prp30, prp31, prp32, prp33, prp34* (J. Woolford, personal communication). Two mutants were complemented by clones from the screen: *prp8-7,* by the plasmid corresponding to the strain DBY4490 ; and *prp22-1,* by that corresponding to DBY4172 (Table 3).

Complementing clones for candidates derived from the strains DBY4483, DBY4340, DBY4217, DBY4475, and DBY4276 failed to complement any of the previously identified splicing mutants, suggesting that they correspond to novel genes . These were named *BRR1, BRR2, BRR3, BRR4,* and *BRR5,* respectively (*B*ad *R*esponse to *R*efrigeration; Table 3). Figure 4 displays the splicing defects of each of these mutants with respect to pre-U3 snoRNA. The defects of the previously identified splicing factors as well as of *brr2-1* and *brr3-1* are quite strong; those of *brr1-1* and *brr5-1* are moderate; and that of *brr4-1* is the weakest.

BRR2 encodes a helicase-like protein

As an initial step towards the molecular characterization of the *BRR* genes, we analyzed the *BRR2* gene, which exhibits a strong defect prior to the first chemical step of splicing. Only one complementing clone was recovered for *BRR2*; its insert was approximately 12 kb in length. In order to localize the *BRR2* sequence within the larger insert, the complementing plasmid was

subjected to partial restriction enzyme digestion with *Sau*IIIA, and libraries were created in the vector, pSE360 (*URA3-CEN*; pUN50 in Elledge and Davis 1988) . We created three libraries with inserts of increasing size (1-3.5 kb, 3-5 kb, and >5 kb) and used them to transform *brr2-1* mutant cells, followed by direct selection for growth at 17°C. To our surprise, fully cold-resistant transformants were not obtained from either of the libraries with smaller inserts, but several hundred were obtained from the library containing inserts greater than 5 kb. Of seven fully complementing plasmids, the smallest contained a 7.1 kb insert. Sequence analysis of the insert revealed that *BRR2* is located on *Chromosome* V (Swiss Protein database accession number P32639). Examination of the open reading frame (ORF) map for the 7.1 kb minimal complementing fragment revealed that almost the entire fragment is spanned by a long ORF of 2163 amino acids.

In order to determine whether the gene which complements *brr2-1* corresponds to bona fide *BRR2* (rather than being a low-copy suppressor), we created a *URA3*-marked version of the gene, which was integrated at its chromosomal locus in a wild-type strain. This strain was crossed to a cs *brr2-1* strain, followed by sporulation of the diploid, dissection of ascospores, and assessment of the cs and uracil auxotrophic phenotypes among the meiotic progeny. Among 11 four-spore tetrads, all cs spore progeny were also uracil auxotrophs, and all cold-resistant progeny were uracil prototrophs, confirming that the cloned gene which complements the *brr2-1* mutation is genetically linked to the *BRR2* locus.

Searches of the database with the Brr2 amino acid sequence identified two other yeast proteins with significant sequence homology, as well as two sequences from humans and one from *C. elegans* (Figure 5A). The two yeast proteins are Hfm1 and Ski2. One of the human genes encodes a close

homolog of Ski2, while the other human sequence and the worm sequence appear not to be close homologs of either yeast protein. Each of these proteins contains a 400 amino acid region more distantly related to the eIF4A family of RNA helicases, such that variants of the six motifs characteristic of the RNA helicase superfamily can be identified (Figure 5B; Gorbalenya et al., 1989). Interestingly, the approximately 1000 amino acid region of homology among the five proteins extends both upstream and downstream of this helicase-related domain (Figure 5A).

***BRR2* is essential for viability**

Because RNA splicing is essential for cell growth, we sought to determine whether the *BRR2* gene was required for cell viability. A disrupted allele was created in the cloned gene by replacing an 831 amino acid region of *BRR2*, including the entire helicase-like domain, with *LEU2* (Figure 6A). The *brr2::LEU2* fragment was liberated from vector sequences and used to transform a wild-type diploid strain, JO226, in a one-step gene replacement procedure. The resulting strain, which was heterozygous for the disruption, was sporulated and twenty tetrads dissected. The spore progeny germinated to yield 2:0 (13 cases), 1:0 (4 cases) or and 0:0 (2 cases) segregation of viability:inviability (Figure 6B). All surviving progeny were leucine auxotrophs (Figure 6B), indicating that *BRR2* is essential for viability.

DISCUSSION

Cold sensitivity as a route to novel RNA splicing genes

Previous efforts to identify genes involved in RNA splicing have relied heavily on screens of collections of ts yeast strains. This approach has been highly successful; however, the existence of genetic hotspots for temperature-sensitivity as well as the apparent inability of many essential genes to mutate to temperature-sensitivity (Harris and Pringle 1991; Diehl and Pringle 1991) suggests that a large number of splicing genes have been overlooked by this method. Reasoning that screening for splicing mutants based on a different conditional phenotype might circumvent these problems, we examined a collection of cs mutants (Moir et al., 1982). We chose for study those mutants that exhibited defects in the splicing of four cellular pre-mRNAs, *CRY1*, *SNR17A*, *SNR17B*, and *RP51A*. We identified nine complementation groups in which such a biochemical splicing defect was genetically linked to the cs growth defect. Two of these groups were found to be allelic to the previously isolated mutants, *prp28-1* and *prp16-1*, for which cs alleles were already available. By isolating genomic DNA complementing clones for the remaining candidates and introducing them into the available ts yeast splicing mutants, we determined that four of the complementation groups correspond to previously-identified genes (*PRP8*, *PRP16*, *PRP22*, *PRP28*), whereas five (*BRR1*, *BRR2*, *BRR3*, *BRR4*, *BRR5*) are genes which have not been previously identified in screens of ts mutants. In addition, sequence analysis of *BRR2* (this manuscript), *BRR1* (S. Noble and C. Guthrie, manuscript in preparation), *BRR5* (S. Noble, unpublished), and *BRR3* (T. Awabdy and C. Guthrie, unpublished) demonstrate them to be unrelated to splicing factors for which no cs or ts alleles are available.

These results compare favorably with the studies of Vijayraghavan et al., (1989), who screened 1000 ts mutants by Northern hybridization and identified ts alleles of eight genes that had not been identified previously. Our screen involved approximately one-third the number of mutants and identified five new genes. Our success in obtaining the *BRR* genes suggests that screening cs mutants represents an efficient alternative to identifying novel genes involved in RNA splicing. These results parallel those of Moir et al. (1982) who screened for cs *cdc* mutants. Like us, they identified nine complementation groups. Six (*CDC44, CDC45, CDC48, CDC49, CDC50, CDC51*) proved to be genes not identified previously in a screen of 1500 ts mutants by Hartwell et al. (1973), whereas one had been identified previously as *CDC11*. The other two complementation groups were not analyzed with respect to the ts collection of Hartwell et al.

Alleles of certain genes were represented in our screen more than once, the most frequent being the three alleles of *PRP28*. As mentioned above, *PRP28* was identified previously by a single mutation in a pilot screen of 18 cs strains (Strauss and Guthrie, 1991). Interestingly, no alleles of *PRP28* have been isolated in subsequent screens of ts collections (Vijayraghavan et al., 1989; B. Rymond, personal communication; J. Woolford, personal communication), despite the fact that this gene is essential for growth at all temperatures (Strauss and Guthrie, 1991). These data are consistent with the idea that hotspots (which reflect the propensity of a gene to mutate to a given phenotype) differ for ts and cs mutations.

In both our screen for cs splicing-defective mutants and previous screens of ts mutants, the majority of genes were represented as single alleles, demonstrating that the screens are far from saturating (Tables 3 and 4; Vijayraghavan et al., 1989; B. Rymond, personal communication, J. Woolford,

personal communication). Although we lack precise estimates for the number of genes involved in splicing, the fact that more than half of the genes identified in our screen were previously unknown suggests that a large number of genes required for splicing have yet to be discovered. This notion reinforces the need to identify new and effective strategies for locating the remaining genes. Here, we have established the utility of screening a cs collection of 340 strains. As has been shown in successive screens of ts collections, repetition of this type of screen would likely result in the identification of new genes. Moreover, alternative conditional phenotypes such as deuterium sensitivity (Bartel and Varshavsky, 1988) and suppressible ochre mutations (Riles and Olson, 1987) could potentially be exploited. Finally, with the sequencing of the yeast genome nearing completion, systematic gene disruption efforts will yield candidates for essential genes involved in splicing.

BRR2 encodes an essential member of a novel class of helicase-related proteins

A number of factors involved in RNA splicing are related in sequence. For instance, Prp2, Prp16 and Prp22 define a subfamily of helicase-related proteins (the so-called DEAH subfamily) that are required prior to the first chemical step of splicing, the second chemical step, and the spliceosome dissassembly reaction, repectively (reviewed by Schmid and Linder, 1992). Two members of a different subfamily (the DEAD subfamily), Prp5 and Prp28, are also required for splicing. Analysis of the *BRR2* gene revealed it to encode a large protein related to the RNA helicase superfamily; however, closer inspection of its sequence exposed differences with the DEAD and DEAH families (Figure 5B). These are most readily apparent in the conserved RNA

helicase motifs described by Gorbalenya et al. (1989). For example, helicase motif II (for which the DEAD family was named) consists in the Brr2 family of the consensus IFDE(I/V)HYI (Figure 5B), whereas the consensus is VLDEADML for the DEAD family. In addition, the homology within the Brr2 family extends both upstream and downstream of the 400 amino acid domain related to RNA helicases, such that the region of amino acid similarity spans approximately 1000 amino acids (Figure 5A). Taken together, these results demonstrate that Brr2 belongs to a novel subfamily of RNA helicase-like proteins which contain extensive sequence homology in regions unrelated to other RNA helicase family members.

Do the functions of the other genes in this family provide clues about the possible function of Brr2? Mutations in the *SKI2* gene reduce the resistance of *S. cerevisiae* to double-stranded RNA killer viruses (Ridley et al., 1984). It has been proposed that Ski2 normally functions in concert with the ribosome to discriminate against translation of uncapped cytoplasmic RNAs, such as cellular mRNAs which have been targetted for degradation as well as viral genomic RNAs (Widner and Wickner, 1993; Masison et al., 1995; Johnson and Kolodner, 1995). Human Ski2 has been demonstrated to possess a small amount of ATPase activity (Dangel et al., 1995); RNA-dependent ATPase activity is a known feature of several of the helicase-like factors required for splicing. Hfm1, the second yeast protein with homology to Brr2, was identified as a DNA binding protein with affinity for *GAL1-GAL10* operator sequences (R. W. West, manuscript in preparation). Unlike most canonical splicing factors, neither Hfm1 nor Ski2 is essential for viability. Thus, it seems likely that Brr2, Ski2, and Hfm1 are members of a helicase-like subfamily which participates in diverse cellular processes.

In the case of Brr2, the existence of dynamic RNA-RNA interactions in the spliceosome may be relevant to its function. With the important caveat that none of the six helicase-like factors (including Brr2) required for splicing has been demonstrated to possess helicase activity *in vitro*, the large number of known dynamic spliceosomal RNA duplexes is certainly intriguing (reviewed by Madhani and Guthrie, 1994). Of 12 RNA-RNA duplexes known to exist among splicing components, at least four must be remodeled for the splicing reaction to occur, and the remainder must be disrupted if snRNPs are to be recycled. Brr2 is the largest helicase-like factor yet identified in the splicing pathway, with large N- and C-terminal domains unrelated to other proteins. Because the *brr2-1* mutant inhibits splicing prior to the first step of splicing, it is a candidate for those rearrangements that occur at this time, including the destabilization of the extensive U4-U6 interaction and the formation of the putative catalytic core of the spliceosome, involving U2 and U6 snRNAs (Madhani and Guthrie, 1992). It is therefore interesting that *BRR2* has been identified independently by another group as a mutant which is synthetically lethal with a mutant U2 snRNA (D. Xu and J. Friesen, personal communication). Determining whether Brr2 functions as a helicase involved in spliceosomal RNA rearrangements will require its characterization *in vitro*.

Of the nine genes identified in this screen of about 300 cs mutants, four are homologous to RNA helicases. Previous screens of ts collections, involving more than 2000 strains, had also identified four factors sharing this homology. The enrichment for this class of factors in the cs screen suggests that helicase-like proteins may be particularly mutable to the cold-sensitive phenotype. In principle, a predisposition to cold-sensitivity could reflect an inherent property of certain classes of proteins (for instance, the ability to be

trapped in one conformation, such as a particular nucleotide-bound state) or of the process on which they act (for example, helix unwinding, which is more difficult at low temperatures). Regardless of the exact mechanism, screens of cs mutants might target processes which in some cases may be resistant to mutation to temperature-sensitivity. In addition, cs mutants can potentially be used as biochemical tools to trap structures such as the spliceosome in states corresponding to previously unrecognized intermediates. Precedents for this possibility come from studies of ribosome assembly and U2 snRNP, in which cs mutants (affecting 16S rRNA and U2 snRNA, respectively) have been shown to result in the stabilization of conserved, alternative RNA structures (Dammel and Noller 1993; Dammel and Noller, 1995; Zavanelli and Ares, 1991; Zavanelli et al., 1994).

ACKNOWLEDGEMENTS

We are grateful to J. Mulholland and D. Botstein for their collection of cold-sensitive lethal yeast strains. We thank J. Abelson for strains; I. Herskowitz for strains and helpful comments on the manuscript; J. J. Li for plasmids; B. Rymond for strains, plasmids and sharing unpublished information; and J. Woolford for strains and communicating results prior to publication. D. Xu, J. Friesen, and R. W. West also graciously shared results prior to publication. Thanks to H. Madhani and P. Raghunathan for their insightful comments on the manuscript. L. Esperas, C. Pudlow, and H. Roiha provided excellent technical assistance. This work was supported by a grant (GM21119) from the National Institutes of Health. S.M.N. was an American Heart Association Predoctoral Fellow during the course of this work and is also a trainee of the UCSF Medical Scientist Training Program. C.G. is an American Cancer Society Research Professor of Molecular Genetics.

340 cold-sensitive strains

↓

Shift of yeast cultures to nonpermissive temperature (16°C) for 1.5 wt generations

↓

RNA Preparation

↓

Assays for splicing efficiency
•Northern Hybridization *(CRY1)*
•Primer Extension *(SNR17A, 17B; RP51A)*

↓

Dominance/Recessiveness Analysis
Complementation Grouping: cs candidates
Cosegregation Analysis: splicing vs. cs defects

↓

Complementation Analysis (vs. *prp* mutants):

- Crosses to $prp16^{cs}$, $prp28^{cs}$
- Cloning of remaining candidates; transformation of complementing plasmids into known prp^{ts} strains

Figure 2 RNA profiles of candidate mutants.

Total RNA was extracted from the indicated cs mutants following a shift to 16°C (see Materials and Methods) and analyzed by a primer-extension method using an oligonucleotide complementary to the second exon of the yeast *RP51A* gene. The positions of fully unspliced pre-mRNA, lariat intermediate, and mRNA are indicated. The two *RP51A* mRNA species result from transcription initiation at more than one site. Also shown are levels of U5 snRNA, which was analyzed as a control for RNA loading in the same reactions. DBY473 is the parental wild-type strain and illustrates the normal ratios among splicing intermediates; *prp18-1* was included as a standard for a mutant that affects the second step of splicing, resulting in an increase in the ratio of lariat intermediate to mature mRNA.

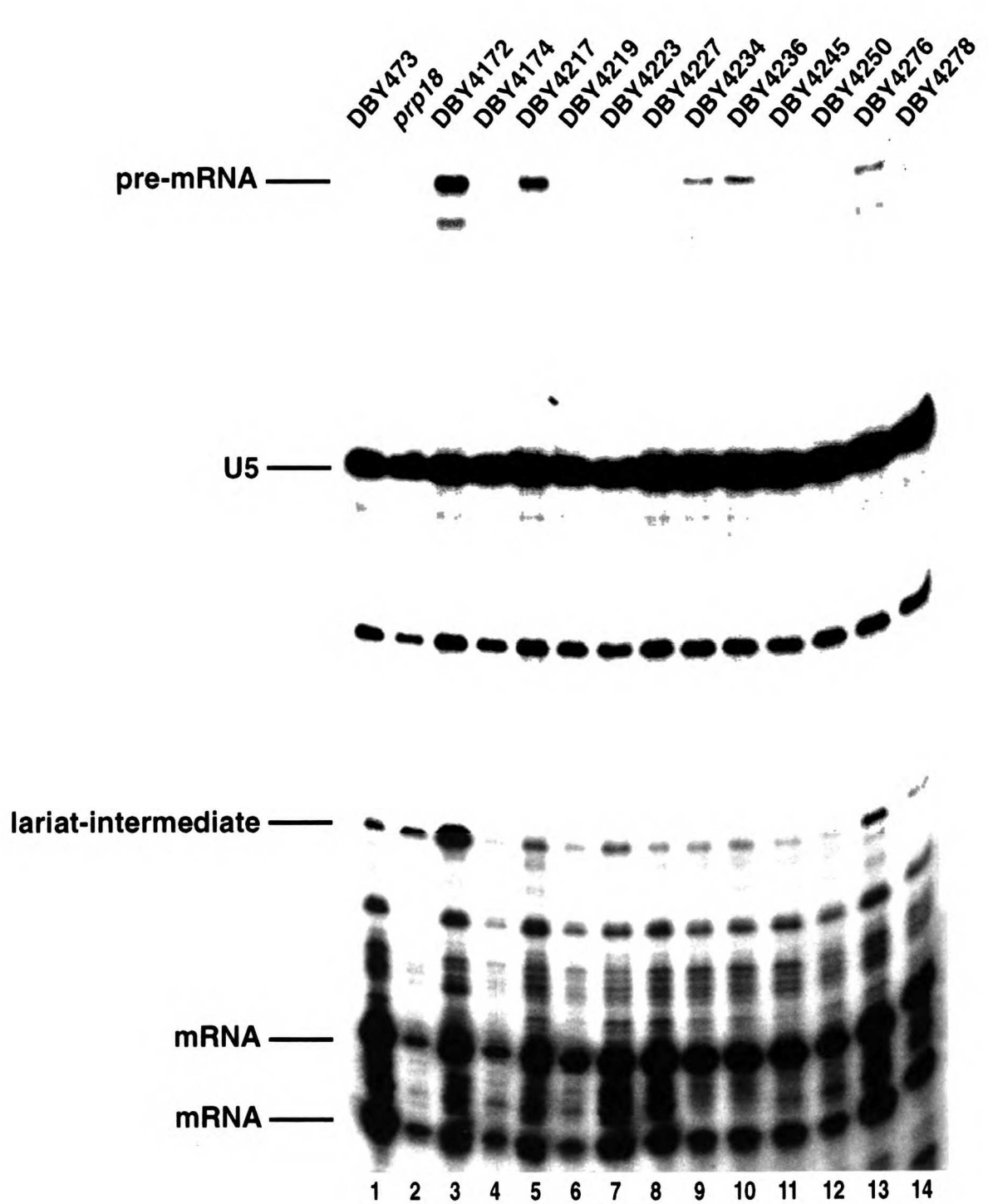

DBY473
prp18
DBY4172
DBY4174
DBY4217
DBY4219
DBY4223
DBY4227
DBY4234
DBY4236
DBY4245
DBY4250
DBY4276
DBY4278
pre-mRNA
U5
lariat-intermediate
mRNA
mRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 3 Cosegregation analysis of DBY4340 (*brr2-1*).

Shown is primer-extension analysis of RNA splicing efficiency among five tetrads derived from a cross of candidate DBY4340 to a wild-type strain. Bands corresponding to unspliced RNA from the *SNR17A* and *SNR17B* genes (encoding U3 snoRNA) are shown. The levels of U1 snRNA, which was analyzed in the same reactions as a control for RNA loading, is also shown. Indicated above each lane is the ability of the corresponding spore progeny to grow at 16°C ("-" indicates cold-sensitivity; "+" cold-resistance).

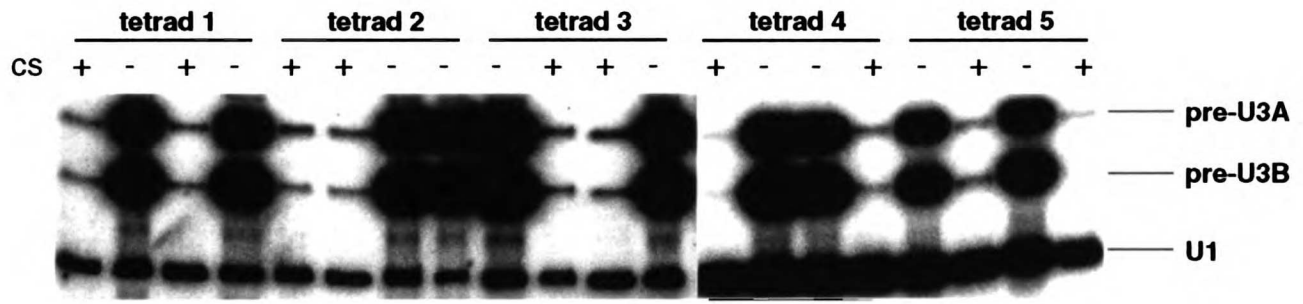

tetrad 1
tetrad 2
tetrad 3
tetrad 4
tetrad 5
CS + - + - + + - - - + + - + - - + - + - +
pre-U3A
pre-U3B
U1

Figure 4 Splicing defects among the nine cs complementation groups. A representative of each of the complementation groups identified in the screen as well as a wild-type sister spore were shifted to 16°C. Total RNA was prepared and primer extension was used to visualize the splicing of pre-U3 snoRNA, as in Figure 4. Bands corresponding to pre-U3A and pre-U3B, mature U3 snoRNA (which is overexposed in these autoradiograms to allow visualization of the pre-snoRNA species), and the U1 snRNA internal control are indicated. While only single alleles of *PRP28 (prp28-2)* and *PRP22 (prp22-2)* are shown, the other alleles of these genes were found to exhibit similar splicing defects.

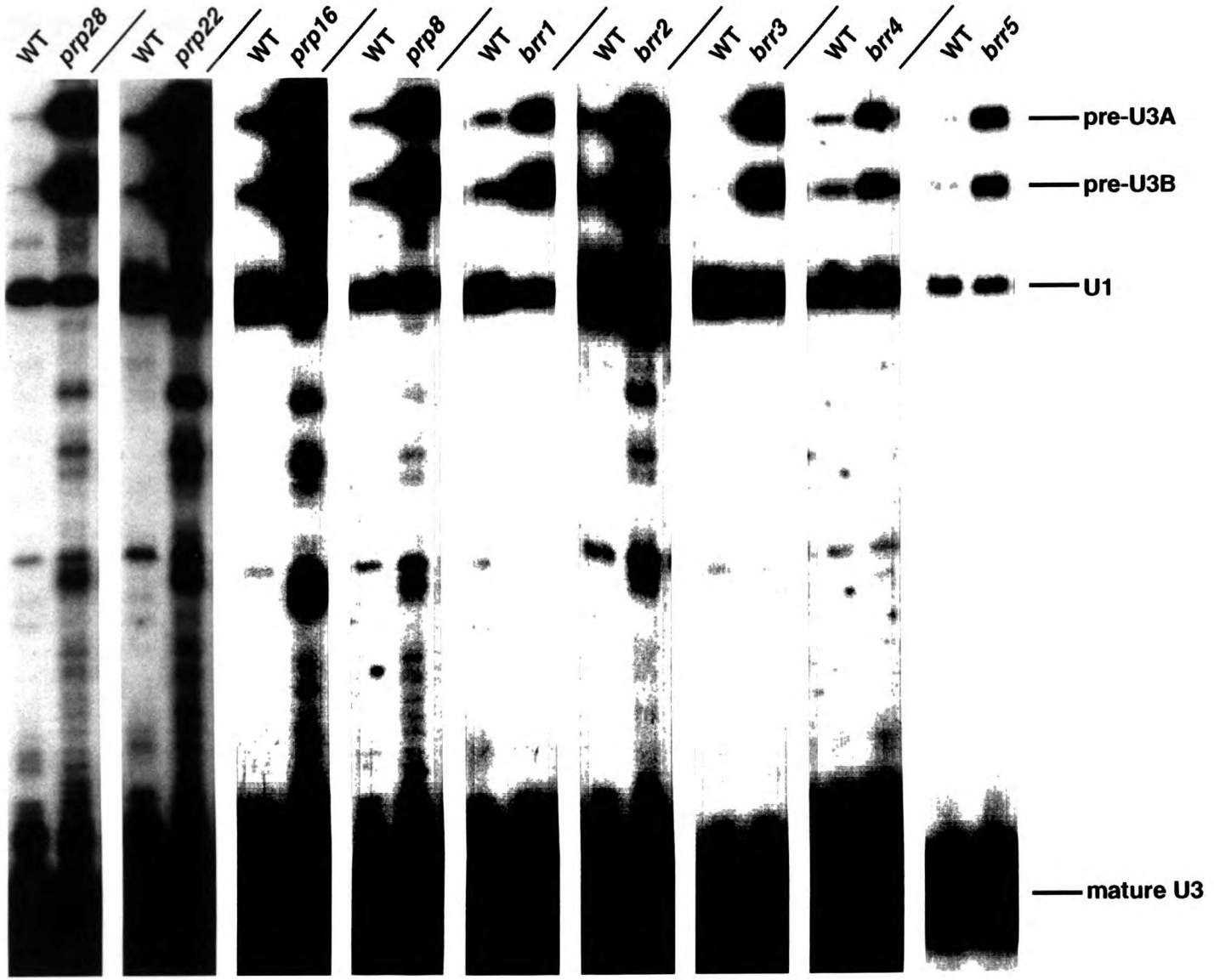
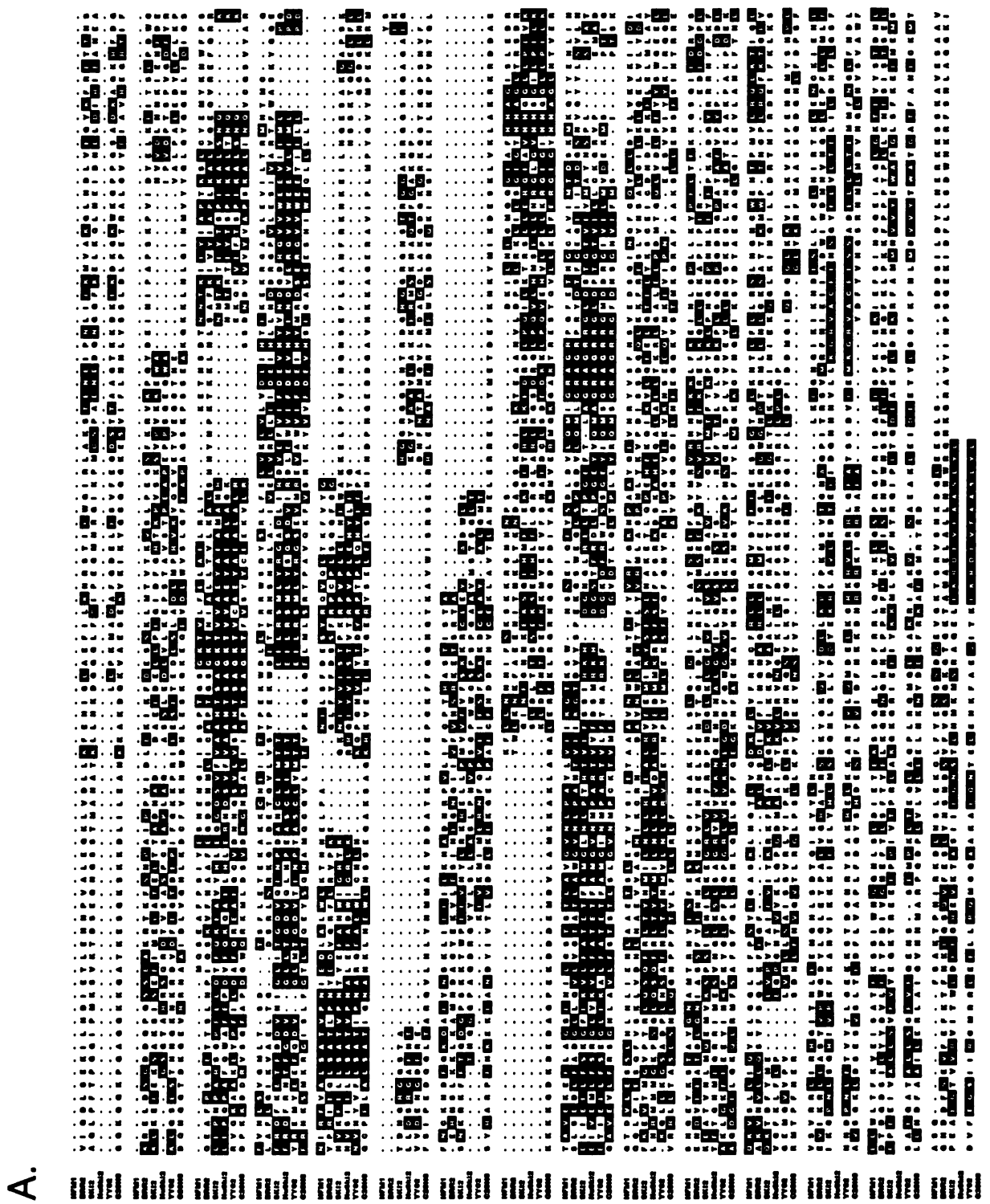

WT
prp28
WT
prp22
WT
prp16
WT
prp8
WT
brr1
WT
brr2
WT
brr3
WT
brr4
WT
brr5
pre-U3A
pre-U3B
U1
mature U3

Figure 5 Brr2 belongs to a novel subfamily of RNA helicase-related proteins. A. Alignment of Brr2 with related proteins. Shown is an alignment among the homologous sequences of Brr2; the yeast proteins, Hfm1 and Ski2; the hypothetical *C. elegans* protein, C28H8.3; and the two human proteins, HuSki2 and YY02. Amino acid identities are shown as filled boxes. The Brr2 amino acid sequence depicted begins at position 308 of the predicted full-length protein; the Hfm1, YY02, and HuSki2 sequences begin at the first amino acid; and the *C. elegans* sequence begins at amino acid 601. Accession numbers for these sequences are as follows: Brr2 (SwisProt P32639), Hfm1 (GenBank U22156), Ski2 (SwissProt 35207), HuSki2 (GenBank U09877), YY02 (SwissProt P42285), and C28H8.3 (SwissProt Q09475).

B. Conserved helicase motifs. The conserved helicase motifs described by Gorbalenya et al. (1989) as they occur in Brr2 and in the DEAD and DEAH subfamilies are shown.


A.

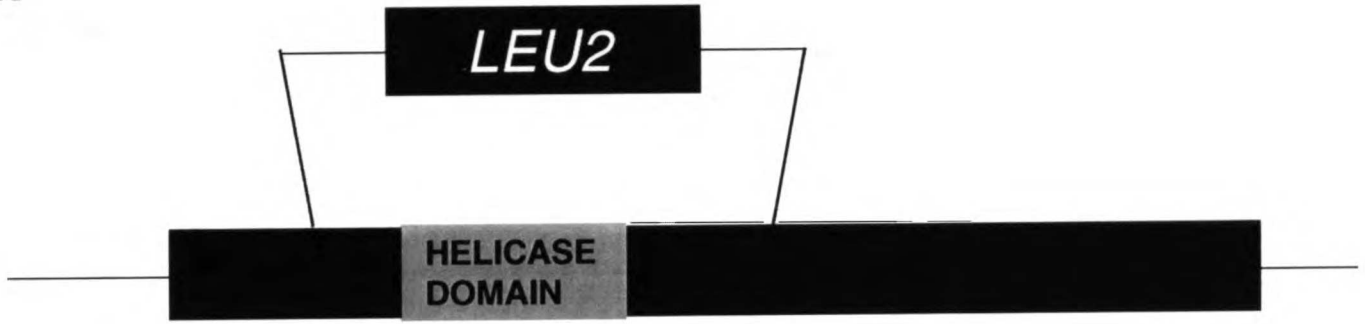
	MOTIF						
SUBFAMILY	I	IA	II	III	IV	V	VI
DEAD	AXTGXGKT	PTRELA	VLDEADXML	XSATXP	FXXT	TXVXARGXDXXV	HRIGRXGR
DEAH	GETGSGKT	PRRVAA	MIDEAHERT	TSATMN	FL/TG	TSL/TIDGIRYVI	QRXGRAGR
DE(I/V)H	AXTSAGKT	PXKALX	IFDE(I/V)HYI	LSATVP	FSXG	TETXAMGVNKPA	QMXGRAGR

Figure 6 *BRR2* is an essential gene.

A. Disrupted allele. A fragment of the *BRR2* gene which encodes amino acids 352 to 1185 was replaced with the yeast *LEU2* gene. The deleted portion includes the helicase-related domain, as well as other conserved sequences.

B. Analysis of gene disruption. The disrupted gene depicted in A was used to replace one of two endogenous copies of the *BRR2* gene in a wild-type diploid strain (see Materials and Methods); this strain is normally auxotrophic for leucine, prototrophic for tryptophan. The heterozygous strain was sporulated and the resulting ascospores were dissected onto a YEPD plate. Spore progeny were subsequently replica-plated to synthetic complete plates lacking either leucine (SC-LEU) or tryptophan (SC-TRP).

A.



BRR2

B.

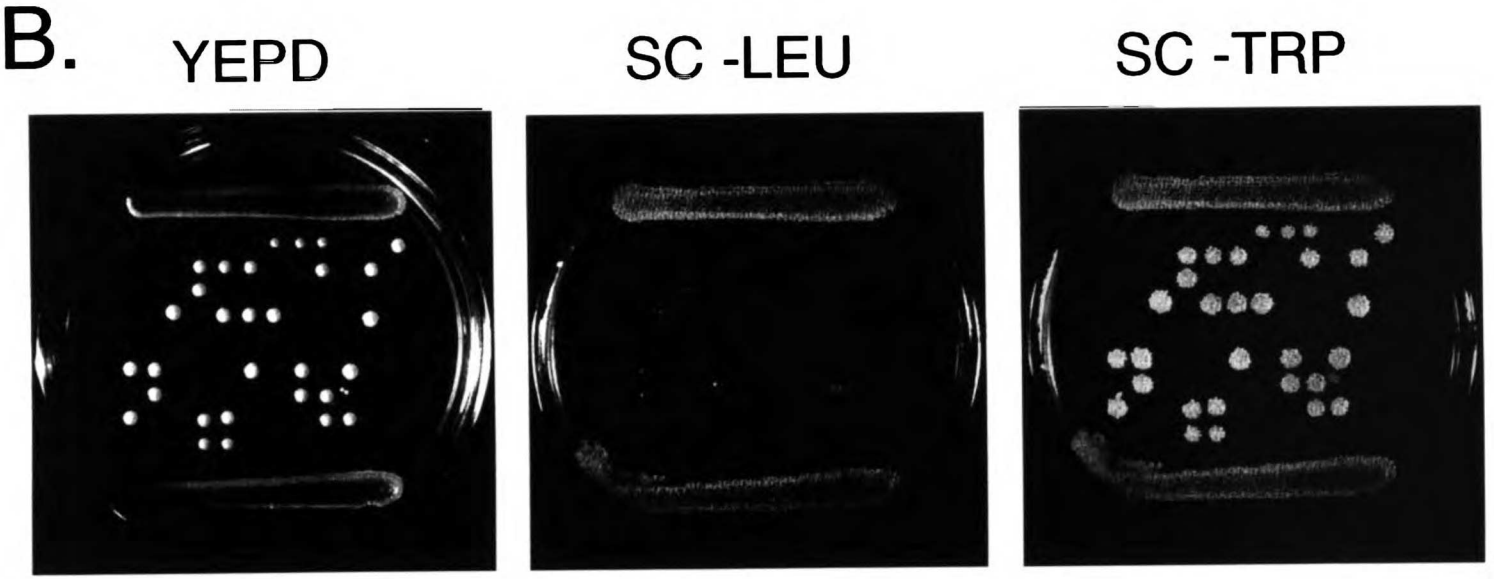


Table 1. *S. cerevisiae* strains used in this study.

Name	Relevant Genotype	Background Genotype	Source
DBY4157-4482	cold-sensitive mutants	derived from mutagenized DBY473 (see below)	D. Botstein
DBY4483-4497	cold-sensitive mutants	derived from mutagenized DBY640 (see below)	D. Botstein
DBY473	wild type strain	(S288C background) *MATα gal⁻ mal⁻ his4-619*	D. Botstein
DBY640	wild type strain	(S288C background) *MAT***a** *gal⁻ mal⁻ ade2*	D. Botstein
YGS1	wild type strain	(S288C background) *MAT***a** *ade2-100^{o} ura3-52 lys2-801^{a} his3-Δ200 leu2-Δ1*	I. Herskowitz
YGS5	*prp28-1^{cs}* on plasmid pHD26 (*HIS3, CEN*)	*MATα prp28:Δ:TRP1 trp1 ura3 his3 leu2*	this laboratory
YS111	*prp16-101^{cs}* on plasmid A7.5 (*TRP1, CEN*)	*MAT***a** *ade2-101 trp1 ura3-52 leu2 lys2-801 prp16Δ::LYS2 cup1Δ::URA3-52*	this laboratory

YEJS 35	*prp23-1 (=prp16-2)*	*MAT***a** *ade2-101 his3Δ200 ura3-52 tyr1*	J. Abelson
yEJS7	*prp2*	*MATα ade2-1 his3-532 trp1-289 ura3-1 ura3-2*	J. Abelson
yEJS8	*prp3*	*MAT***a** *his3 leu2 lys2 ura3-52*	J. Abelson
YEJS45	*prp4*	*MATα ade ura3-52 his leu2 lys2*	J. Abelson
YSR5.1a	*prp5-1*	*MAT***a** *ade2-101 his3-Δ200 tyr1*	S. Ruby
YEJS14	*prp6*	*MATα his3 lys2 ura3-52*	J. Abelson
YEJS75	*prp8-7*	*MATα leu2-3 leu2-112 tyr1 his ura3-52*	J. Beggs
YSR9-2	*prp9-1*	*ade2-101 his3-d200 tyr1 ura3-52*	S. Ruby
YBP74	*prp11*	*MAT***a** *ura3-52 leu2 his4-512*	this laboratory
YEJS23	*prp17*	*MATα ade2-101 his3Δ200 ura3-52 lys2-801*	J. Abelson
YEJS24	*prp18*	*MATα ade2-101 his3Δ200 ura3-52 lys2-801*	J. Abelson
YEJS26	*prp19*	*MATα ade2-101 his3Δ200 ura3-52 lys2-801*	J. Abelson

YEJS29	*prp20*	*MATα ade2-101 his3Δ200 ura3-52 tyr1*	J. Abelson
YEJS31	*prp21*	*MATα ade2-101 his3Δ200 ura3-52 lys2-801*	J. Abelson
YEJS33	*prp22*	MATα *ade2-101 his3Δ200 ura3-52 lys2-801*	J. Abelson
YEJS34	*prp23=prp16*	*MATα ade2-101 his3Δ200 ura3-52 tyr1 lys2-801*	J. Abelson
YEJS36	*prp24*	*MAT***a** *ade2-101 his3Δ200 ura3-52 lys2-801*	J. Abelson
YEJS41	*prp27*	*MATα ade2-101 his3Δ200 ura3-52 lys2-801 rho*$^-$	J. Abelson
JWY2439	*prp30-1*	*MAT***a** *his4 leu2-3,112 lys2-801 ura3-52*	J. Woolford
JWY2431	*prp31-1*	*MAT***a** *his4 lys2-801 ura3-52*	J. Woolford
JWY2433	*prp32-1*	*MAT***a** *his4 lys2-801 ura3-52*	J. Woolford
JWY2435	*prp33-1*	*MAT***a** *his4 lys2-801 ura3-52*	J. Woolford
JWY2437	*prp34-1*	*MAT***a** *his4 lys2-801 ura3-52*	J. Woolford

YSN4A	*slu1-1*	*MAT***a** *ura3 his3 trp1 lys2 ade2*	this laboratory
YBP97	*slu2-1*	*MAT***a** *ura3-52 his3Δ trp1Δ lys2-801^a ade2-101^o*	this laboratory
YDF50	*slu7-1*	*MAT***a** *trp1Δ63 his3Δ200 ura3-52 ade2-101 lys2-801 leu2Δ1*	this laboratory
YSN113	*prp28-2*	(from DBY4234) *MAT***a** *leu2-Δ1 ade2-100^o his3-Δ200 lys2-801^a*	this study
YSN150	*prp28-4*	(from DBY4489) *MAT***a** *his3-Δ200 lys2-801^a*	this study
YSN97	*prp22-2*	(from DBY4172) *MATα ura3-52 leu2-Δ1 ade2-100^o his3-Δ200*	this study
YSN160	*prp8-8*	(from DBY4490) *MATα ura3-52 leu2-Δ1 ade2-100^o lys2-801^a*	this study
YSN142	*brr1-1*	(from DBY4483) *MAT***a** *ura3-52 leu2-Δ1 ade2-100^o lys2-801^a*	this study
YSN239	*brr2-1*	(from DBY4340) *MAT***a** *ura3-52 leu2-Δ1 ade2-100^o lys2-801^a*	this study
YSN405	*brr2-1*	(from DBY4340) *MATα ura3-52 leu2-Δ1 ade2-100^o his3-Δ200 lys2-801^a*	this study

YSN103	*brr3-1*	(from DBY4217) *MAT***a** *ura3-52 ade2-100^{o} his3-Δ200*	this study
YSN137	*brr4-1*	(from DBY4475) *MAT***a** *ura3-52 leu2-Δ1 ade2-100^{o}*	this study
YSN402	*brr5-1*	(from DBY4236) *MATα ura3-52 leu2-Δ1 lys2-801^{a}*	this study
JO226	wild type	(S288C background) *MAT***a***/α his3Δ/his3Δ ura3/ura3 lys2/lys2 leu2/leu2 ade2/ade2*	I. Herskowitz
YSN404	*brr2Δ::LEU2/ BRR2*	(S288C background) *MAT***a***/α his3Δ/his3Δ ura3/ura3 lys2/lys2 leu2/leu2 ade2/ade2*	this study

Table 2 Complementing plasmids.

Gene	Plasmid Name	Original DBY strain	Mutant strain used for plasmid isolation
BRR1	pSN19	DBY4483	YSN142
BRR2	pSN21	DBY4340	YSN239
BRR3	pSN17	DBY4217	YSN103
BRR4	pSN24	DBY4475	YSN137
BRR5	pSN100	DBY4276	YSN402
PRP8	pSN25	DBY4490	YSN160
PRP22	pSN16	DBY4172	YSN97

Listed are genomic DNA clones isolated from a YCp50 library (Rose et al. 1987) by complementation of the indicated cs mutants.

Table 3 Cold-sensitive RNA splicing mutants isolated in this study.

Complementation Group	Gene Name	DBY strain	*CRY1* phenotype	U3 phenotype	*RP51A* phenotype (exon II primer)	*RP51A* phenotype (intron primer)
1	*PRP28*	4234	↓M	↑P	↑P	↑P
		4236	↓M	↑P	↑P	↑P
		4489	↑P	↑P	↑P; ↓M	↑P
2	*PRP22*	4172	↓M	↑P	↑P; ↑LI	↑P; ↑LI &L
		4423	↓M	↑P	↑P; ↑LI	↑P; ↑LI & L
3	*PRP16*	4406	↓M	↑P	↑P; ↑LI	↑P; ↑LI &L
4	*PRP8*	4490	↑P	↑P	↑P	↑P
5	*BRR1*	4483	↓U5 snRNA	↑P	↑P	↑P (weak)
6	*BRR2*	4340	↓M	↑P	↑P	↑P
7	*BRR3*	4217	↓M	↑P	↑P	↑P
8	*BRR4*	4475	↓M (weak)	↑P	↑P (weak); ↓M	↑P (weak)
9	*BRR5*	4276	↓M	↑P	↑P	↑P

In describing the phenotypes of the various mutants, the following symbols are used: ↓M stands for the loss of mature *CRY1* RNA relative to the U5 snRNA internal standard. ↑P stands for an increase in intron-containing precursor relative to an internal control (U1 snRNA in the U3 primer extensions; U5 snRNA in the *RP51A* primer extensions). ↑LI stands for an increase in the lariat intermediate relative to an internal control. ↑LI & L stands for an increase in the lariat intermediate plus excised lariat intron species in the *RP51A* intron primer extensions.

Table 4 Summary of previous screens for temperature-sensitive splicing mutants.

Screen	Mutagen	# screened, conditional phenotype	Assay	% defective in splicing*	Genes identified in screen†	# alleles
Hartwell et al. (1970)	nitroso-guanidine	400, ts	^{14}C pulse-labeling of RNA synthesis	5.8 %	*PRP2*	6
					PRP3	5
					PRP4	4
					PRP5	2
					PRP6	1
					PRP7	1
					PRP8	1
					PRP9	1
					PRP10=PRP11	2
Vijayraghavan et al. (1989)	ethylmethane sulfonate	1000, ts	northern blot (*ACT1, CRY1*) primer extension (*RP51A*)	3.3%	*PRP2*	6
					PRP3	7
					PRP6	6
					PRP9	2
					PRP17	2
					PRP18	3
					PRP19	1
					PRP20	1
					PRP21	1
					PRP22	1
					PRP16/23	1
					PRP24	1
					PRP25††	1
					PRP26††	3
					PRP27	1
J. Maddock, J. Roy, J. Woolford (personal communication)	ethylmethane sulfonate	426, ts	northern blot (*CRY1, ACT1*)	4.5 %	*PRP2*	5
					PRP3	2
					PRP6	1
					PRP16/23	1
					PRP18	2
					PRP19	1
					PRP26††	1
					PRP29	1
					PRP30	1
					PRP31	1
					PRP32	1
					PRP33	1
					PRP34	2
Blanton et al., 1992; Lockhart and Rymond,1995; S. Schulte, A. Srinivasan, S. Lockhart, B. Rymond (unpublished)	ethylmethane sulfonate	345, ts	northern blot (*RP51A*)	4.1%	*PRP2*	4
					PRP4	1
					PRP16	2
					PRP22	1
					PRP26††	1
					PRP38	1
					PRP39	1
					uncharacteriz-ed	2

* % represents those mutants which accumulate pre-mRNA or splicing intermediates at the nonpermissive temperature (as opposed to loss of mRNA only) and which exhibit linkage between their splicing and ts growth defects.

† Mutants identified for the first time are underlined.

†† The alleles of *prp25* and *prp26* are unlinked to the ts mutations.

CHAPTER 2

Brr1 is a Novel snRNP-Associated Protein Involved in the Manufacture of Spliceosomal snRNPs

ABSTRACT

In *Xenopus* oocytes and cultured mammalian cells, a conserved RNA sequence, the Sm site, is required in cis for the cytoplasmic assembly of the spliceosomal snRNAs into snRNPs, for the hypermethylation of their 5' cap structures, and for the nuclear import of the resultant particles. The Sm proteins are a complex of related proteins that bind to this site and which have been proposed to facilitate these events. Here, we describe a novel yeast protein, Brr1, which is unrelated to the Sm family yet which physically associates with Sm site-containing snRNAs and is important for their accumulation to normal levels *in vivo*. To define the pathway of snRNP biogenesis in *S. cerevisiae* and to determine whether Brr1 functions in this process, we have utilized a transcriptional pulse-chase method in which a pulse of snRNA synthesis is achieved using snRNA genes controlled by the sugar-regulated *GAL1-10* enhancer. We demonstrate that the rate of snRNA 3' end processing and the stability of newly synthesized snRNAs are diminished in the absence of Brr1, whereas the cap hypermethylation reaction is unaffected. In contrast, promoter shut-off experiments reveal that Brr1 is dispensable for the metabolic stability of mature snRNPs. Our data establish a role for a novel spliceosomal snRNP protein in the manufacture of Sm snRNPs. As overexpression of the yeast Sm D1 protein homolog, Smd1, can suppress the cold-sensitive growth defect of the *brr1-1* mutant, Smd1 may also function in this pathway.

INTRODUCTION

Small nuclear ribonucleoproteins (snRNPs) are essential mediators of many cellular RNA processing events. In particular, the Sm class of snRNPs has been demonstrated to play key roles in the splicing of mRNA precursors (reviewed by Rymond and Rosbash 1992, Moore et al. 1993, Madhani and Guthrie 1994). Although much has been learned in recent years of the mechanism by which spliceosomal snRNPs function in splicing, comparatively less is known about how they are manufactured and localized in the cell. In vertebrate cells, four of the five spliceosomal snRNAs (U1, U2, U4, and U5) associate independently with a complex of eight proteins that share antigenic determinants and a common sequence motif (reviewed in Lührmann 1988, Lehmeier et al. 1990; see also Hermann et al. 1995, Seraphin 1995, Cooper et al. 1995); these proteins are known as Sm proteins, and the RNA sequence to which they bind is the Sm binding site.

Experiments conducted in *Xenopus* oocytes and mammalian cells in culture have revealed that the pathway of snRNP biogenesis is complex and consists of many events in addition to assembly of the Sm ribonucleoprotein core (reviewed in Mattaj 1988, Zieve and Sauterer 1990). Initially, spliceosomal snRNAs containing the $PuAU_nGPu$ Sm sequence are exported to the cytoplasm. There, in a manner dependent upon the integrity of the Sm sequence, these snRNAs individually assemble with the Sm proteins, B, B', D1, D2, D3, E, F, and G. Once the Sm core has been assembled, the 5'-5' 7-methylguanosine (7MG) cap structure of the snRNAs is hypermethylated to yield 2,2,7-trimethylguanosine (TMG). In addition, varying numbers of nucleotides are trimmed from the 3' end of several of the snRNAs, and many internal nucleotides are modified. Proper Sm core assembly, cap

hypermethylation, and 3' end processing (reviewed by Mattaj 1988; Zieve and Sauterer 1990; see also Neuman de Vegvar and Dahlberg 1990) are important for the next step of biogenesis, nuclear import of the nearly mature snRNPs. In the nucleus, additional trimming of nucleotides at the 3' end may occur (shown for U1 snRNA by Yang et al. 1992). Finally, sometime just before or after nuclear import, more than 30 snRNP-specific proteins (enumerated by Ségault et al. 1995) associate with the individual snRNP precursors to complete biogenesis. Mature snRNPs are metabolically stable (Sauterer et al. 1988).

Although the study of snRNP biogenesis in yeast lags behind the studies in vertebrate systems, the available data suggest that general features of the process have been conserved. In particular, yeast contain homologs of the vertebrate spliceosomal snRNAs (reviewed in Guthrie and Patterson 1988) and most of the Sm proteins (Rymond 1993, Roy et al. 1995, Seraphin 1995). These components assemble into metabolically stable snRNPs (Wise et al. 1983). Yeast snRNAs, like the vertebrate species, contain a TMG cap (Wise et al. 1983). Furthermore, the human Sm protein, D1, can functionally substitute for its essential yeast counterpart, implying that its interactions with other snRNP components have been evolutionarily conserved (Rymond et al. 1993). Rymond (1993) and Roy et al. (1995) have demonstrated that *in vivo* depletion of the yeast proteins Smd1 or Smd3 results in a decrease in abundance of the snRNAs which contain Sm sites, with the residual snRNA population being unreactive with antibodies specific to the TMG cap structure. In agreement with hypotheses derived from the vertebrate work, these results are consistent with a role for yeast Smd1 and Smd3 in snRNP biogenesis, in the maintenance of snRNP stability, or both.

Many questions remain as to the universality and mechanism of snRNP biogenesis, as well as to the reasons for its surprising complexity. Specifically, do snRNPs in yeast undergo the same pathway that has been described for vertebrate cells: export of nascent transcripts to the cytoplasm, Sm ribonucleoprotein core assembly, cap hypermethylation, 3' end trimming, base modification, nuclear import, and addition of specific proteins? What factors mediate these events, and by what mechanism? What are the functions of individual Sm proteins, and what purpose is served by the extensive post-transcriptional alterations of the snRNAs?

In this manuscript, we have taken a genetic and biochemical approach to elucidate the pathway of snRNP biogenesis in *S. cerevisiae*. We describe a novel protein, Brr1, which is required for the normal events of snRNP biogenesis. *BRR1* was identified previously among eight other genes in a screen for mutants which are cold-sensitive for growth and defective in pre-mRNA splicing (Noble and Guthrie, submitted). In a subsequent secondary screen for snRNP proteins or factors involved in their biogenesis, we have found that *brr1-1* mutant cells possess lower levels of each of the spliceosomal snRNAs at steady state, whereas levels of non-Sm site containing snRNAs such as U3, snR5, snR14, and scR1 are unperturbed. This specific depletion of spliceosomal snRNAs is reminiscent of the effects of Sm protein depletion (Rymond 1993, Roy et al. 1995) and suggested to us that Brr1 might function in snRNP biogenesis. Examination of the Brr1 amino acid sequence has revealed it to be distinct from the Sm protein family. However, *BRR1* interacts genetically with *SMD1*, which encodes the yeast homolog of the mammalian Sm D1 protein; furthermore, immunoprecipitation experiments demonstrate that Brr1 associates physically with the Sm snRNAs. To test the hypothesis that Brr1 functions in

the biogenesis of spliceosomal ribonucleoproteins, we have utilized a transcriptional pulse-chase approach in which U2 and U4 snRNA genes have been placed under the control of the sugar-regulated *GAL1-10* transcriptional enhancer. We find that Brr1 is required for snRNPs to undergo efficient biogenesis *in vivo*; in the absence of the protein, a 3' processing event is delayed and nascent snRNAs are turned over more rapidly, whereas the cap hypermethylation reaction occurs normally. In contrast, for both U2 and U4, promoter shut-off experiments demonstrate that Brr1 is dispensable for the metabolic stability of mature snRNPs. These results define a pathway by which Sm ribonucleoproteins are formed in *S. cerevisiae*. Moreover, the data establish an important role for a novel kind of spliceosomal snRNP protein in the biogenesis of these particles.

RESULTS

A defect in Sm snRNA accumulation in the *brr1-1* mutant.

BRR1 was identified in a screen for cold-sensitive mutants which are defective in pre-mRNA splicing (Noble and Guthrie, submitted). In our initial characterization of mutants, we sought to determine whether any of the newly identified factors might correspond to snRNP proteins. Blanton et al. (1992) had previously reported that mutations in the U6 or U4/U6 snRNP-specific proteins, Prp4, Prp6, and Prp24, result in decreased stability of U6 snRNA; moreover, Jones and Guthrie (1990) had demonstrated that numerous mutations introduced into the Sm site of U5 snRNA result in decreased levels of that snRNA, presumably because of its failure to interact with Sm proteins. Pursuing the idea that mutations in snRNP proteins might destabilize their associated snRNAs, we screened the cold-sensitive mutants for reductions in the steady state levels of any of the spliceosomal snRNAs after a shift to the nonpermissive temperature. Northern analysis with snRNA-specific probes revealed that a single mutant, *brr1-1*, displays reduced levels of each of the spliceosomal snRNAs which contain Sm sites (U1, U2, U4, and U5; data not shown).

To quantitate these reductions, we repeated the temperature shift and Northern analysis procedure on ten *brr1-1* strains and ten isogenic wild-type strains, isolated as the meiotic progeny of a cross between a *brr1-1* strain and a wild-type strain of similar genetic background. The snRNA bands were quantitated using a PhosphorImager, and the average levels in wild-type and mutant cells are depicted graphically in Figure 1A. *brr1-1* shows a significant reduction in each of the spliceosomal snRNAs, most dramatically in U4

where the reduction is approximately ten-fold; U1, U2, and U5 are reduced about two-fold in abundance (Figure 1A). U6 snRNA is also reduced by about one third in the *brr1-1* mutant; this may result indirectly from the reduction in U4 snRNA levels, to which U6 is normally extensively base paired (see also below). In contrast, hybridization of the same Northern blots with oligonucleotide probes directed against the nonspliceosomal small RNAs, scR1, U3, snR5, and U14, indicated that these RNAs are not significantly affected by the *brr1-1* mutation (Figure 1B). Based on the mutational studies of the Sm site described above, the specific reduction of spliceosomal snRNA levels is consistent with the effects one would anticipate for mutation of a protein which interacts functionally with the Sm site, such as an Sm protein.

Brr1 encodes a novel protein required for growth at low temperatures

Our earlier studies identified a genomic clone which complements the *brr1-1* cold-sensitive growth defect (Noble and Guthrie, submitted). This initial complementing plasmid had an insert size of four kilobases (kb); by subcloning and complementation analysis, the Brr1 open reading frame (ORF) was delimited to a 1.4 kb region (see Materials and Methods). Sequencing of this region revealed Brr1 to be a novel protein with an estimated molecular weight of 40 kilodaltons (Figure 2A). Computer searches of currently available databases have revealed no sequence similarities to any other protein including the Sm proteins; the *BRR1* DNA sequence was recently deposited in Genbank as part of the Chromosome XVI sequencing project (Genbank accession number 805038). Linkage of the cloned *BRR1* gene to the *brr1-1* mutation was established by integrating a *URA3*-marked version of the *BRR1* gene into the chromosome of a wild-type haploid yeast strain, crossing that strain to the *brr1-1* strain, and demonstrating that the meiotic

progeny of the cross segregated exclusively as parental ditypes with respect to the uracil auxotrophic and cold-sensitive phenotypes (see Materials and Methods).

To determine whether the *BRR1* gene is essential for viability, we created a disrupted allele in which all but the last 75 nucleotides of the *BRR1* coding region was replaced with the yeast *LEU2* gene; the *brr1Δ::LEU2* allele was substituted for the chromosomal copy of *BRR1* by homologous recombination (see Materials and Methods). The Brr1 protein is not essential for viability, but cells lacking it are cold-sensitive for growth (Figure 2B), with a phenotype identical to that of *brr1-1*. Thus, the original *brr1-1* mutation behaves as a null mutation.

A suppressor of *brr1-1* encodes a yeast homolog of the human Sm D1 protein

In the process of cloning the wild-type *BRR1* gene, we obtained a second clone (pSN20), which only partially complements the cold-sensitive growth defect of the *brr1-1* mutant and the *brr1* disruption strain. Restriction digestion of this plasmid yielded a pattern of digestion products different from those of bona fide *BRR1*. In separate experiments to determine possible allelism between *brr1-1* and previously-identified splicing genes, a plasmid encoding wild-type Smd1 protein, the yeast homolog of the human D1 core snRNP protein, was introduced into *brr1-1* cells and found to be partially suppressing. By restriction site and PCR analysis, we determined that the insert from this plasmid shares sequences with pSN20 (data not shown). As both inserts encode several potential ORFs, we constructed a plasmid containing only the *SMD1* ORF and demonstrated that this gene alone suffices for the partial complementation phenotype; a frameshift mutation in this ORF eliminates suppression (Figure 2B).

Physical association of Brr1 with spliceosomal snRNAs

The observations that depletion of Brr1 protein specifically reduces the levels of the Sm snRNAs and that overexpression of a common Sm protein can partially alleviate the *brr1-1* cold-sensitive growth defect motivated us to ask whether Brr1 physically associates with these snRNAs. We constructed an epitope-tagged version of Brr1, with three copies of the influenza virus hemagglutinnin epitope (HA) introduced precisely at the C-terminus of the protein. The epitope-tagged *BRR1(HA)3* fully complements the cold-sensitivity of the *BRR1* knockout strain (data not shown), suggesting that the modified protein retains at least a portion of its wild-type activity. Using the 12CA5 monoclonal antibody which is specific for this epitope (Kolodziej and Young 1991), we performed immunoprecipitation experiments on *in vitro* splicing extracts made from strains containing *BRR1(HA)3* as the sole allele of *BRR1* and asked whether snRNAs were specifically coimmunoprecipitated with the epitope-tagged protein (see Materials and Methods). RNA extracted from the immunoprecipitated material was analyzed by Northern hybridization using probes specific for snRNAs. As shown in Figure 3A, each of the spliceosomal snRNAs is coimmunoprecipitated with epitope-tagged Brr1 (lane 9). Conversely, none of these snRNAs is immunoprecipitated from a control splicing extract prepared from an isogenic strain in which Brr1 is untagged (lane 4). The ability to coimmunoprecipitate snRNAs from the *BRR1(HA)3* extract is disrupted by including a hemagglutinin peptide in the reaction (lane 10), further demonstrating the specificity of the monoclonal antibody. No signal is seen when antibody is omitted (Figure 3A, lanes 3 and 8). Extract produced from the untagged strain contains a similar concentration of snRNAs (Figure 3A, lanes 1 and 6), and similar amounts of

snRNAs can be immunoprecipitated using polyclonal anti-TMG cap antibodies (Figure 3A, lanes 2 and 7). By a subsequent reprobing of this Northern blot, we found that the non-Sm small RNAs U14, scR1, and snR5 are not immunoprecipitated with Brr1(HA)3 (data not shown), arguing that the physical association is specific for the spliceosomal snRNAs.

We were intrigued by the result that some U6 snRNA, which lacks an Sm site, was coimmunoprecipitated with epitope-tagged Brr1. One possible explanation for this result is that U6 was recovered indirectly, by virtue of its extensive base pairing with U4 in the U4/U6 snRNP. Another is that Brr1 might associate independently with free (non-U4 associated) U6 snRNP, as has been reported recently for certain Sm-like proteins (Seraphin 1995, Cooper et al. 1995). To distinguish between these two possibilities, we performed immunoprecipitation reactions as above but this time separated the snRNAs in the immune pellets on a nondenaturing polyacrylamide gel (Li and Brow 1993), which allows the resolution of U4/U6 from free U6. The RNA complexes were visualized by hybridizing them in solution to a radiolabeled oligonucleotide complementary to U6 snRNA prior to loading them on the gel (Li and Brow 1993). As shown in Figure 3B, lane 10, only that U6 which is associated with U4 is detectably immunoprecipitated along with Brr1(HA)3 (compare the mobility of the immunoprecipitated material in lane 10 with that of the U4/U6 upper band (native RNA) in lanes 1 and 6 versus the free U6 band (denatured RNA) in lanes 2 and 7). Again, control reactions demonstrate that there is no nonspecific association of snRNAs in reactions without added antibody (Figure 3B, lanes 4 and 9) and that there is no nonspecific cross-reaction of the 12CA5 antibodies with snRNPs in an extract prepared with untagged Brr1 (lane 5); however, U4/U6 can be efficiently precipitated from both extracts using anti-TMG cap antibodies

(lanes 3 and 8; note that U6 snRNA does not bear a TMG cap). These data demonstrate that Brr1 is associated primarily with that fraction of U6 snRNA that is complexed with U4 snRNA.

Brr1 is dispensable for the stability of mature snRNP particles *in vivo*

In order to understand the function of the Brr1 protein, it was imperative that we determine why snRNA levels are diminished in the *brr1-1* mutant (and in the strain containing a disruption of *BRR1*; data not shown). In principle, such a phenotype could result either from a decrease in the rate of snRNP biogenesis, implying that Brr1 normally participates in the manufacture of snRNPs, or from a decrease in the stability of snRNPs once they are made, suggesting that Brr1 serves to stabilize mature snRNPs. In order to measure directly the stability of mature snRNPs in *brr1-1*, we utilized recombinant U2 and U4 snRNA genes whose coding sequences are different in size from their wild-type versions (to aid in detection) and whose transcription can be regulated by carbon source (Figure 4A; see Materials and Methods). Both of these genes can functionally replace their essential wild-type counterparts, suggesting that they assemble into normal snRNPs (Madhani and Guthrie 1992; data not shown). Mutant *brr1-1* and wild-type yeast strains containing both chromosomal, wild-type snRNA genes and plasmid-encoded, regulated genes (pGAL-U2 or pGAL-U4) were grown continuously for several generations in galactose-containing medium, where expression of the recombinant genes was induced and mature snRNPs allowed to accumulate. The cultures were then shifted to glucose-containing medium at 17°C, where further transcription of the regulated snRNAs is repressed. (In contrast to the cold-sensitive growth defect of *brr1* mutant cells on agar plates, the mutant cells divide with similar kinetics to that of wild-

type in minimal liquid culture medium at this temperature, despite the low abundance of snRNAs.) Samples were taken for a time period exceeding the doubling time of the strains, RNA was prepared, and the level of the induced snRNAs over time was determined by Northern analysis (for U2) or primer extension (for U4). The results are plotted in Figures 4B and 4C, on a logarithmic scale and normalized to initial snRNA levels as well as to an internal standard, the nucleolar snRNA U14. Comparison of the plots for wild type and the *brr1-1* mutant reveal no significant difference in apparent stabilities of the mature U2 or U4 snRNPs. (For U4, the ratio of apparent half lives in *brr1* relative to wild-type cells is 0.96; for U2, it is 0.94; see Materials and Methods for calculation.)

Transcriptional pulse-chase analysis of snRNP biogenesis

Because the metabolic stability of mature snRNPs is not dependent on Brr1, we infer that the lower steady state levels of snRNAs in the *brr1-1* strain is caused by a decrease in the rate of new snRNP synthesis. To test this hypothesis directly, we developed a method to create a pulse of snRNA synthesis followed by a chase in which synthesis is repressed (Figure 5). We utilized the *GAL1-10* enhancer-regulated U2 and U4 snRNA genes, this time cloned together into the single plasmid, pGAL-U2/GAL-U4. Our method is similar to that used by Parker and colleagues (Decker and Parker 1993) to study mRNA turnover in yeast. Briefly, cells containing pGAL-U2/GAL-U4 were grown on medium containing raffinose, which neither represses nor induces the *GAL* enhancer. A shift to galactose-containing medium resulted in the induction of snRNA transcription. Following this pulse of expression, transcription was repressed by the addition of glucose-containing media, which initiated the chase period. Because we desired to compare biogenesis

in wild-type and *brr1* mutant cells, these experiments were performed at 17 °C; at this temperature, cells have a generation time of 11 hours in the galactose-containing medium and 5 hours in the glucose-containing medium (see Materials and Methods for details). The duration of the pulse was 40 minutes (1/16th of a generation time); shorter pulses resulted in RNA yields inadequate for the analysis. During the six-hour chase, samples were obtained, and subsequently their RNAs were prepared and assayed by a variety of methods for the acquisition of properties characteristic of mature snRNPs.

Biogenesis of U4 snRNA in wild-type and *brr1* mutant cells

We first analyzed the induced U4 snRNA. Two methods were compared for detection of U4, primer extension and Northern analysis. Primer extension resulted in better resolution of the induced U4 band from the more abundant, endogenous U4 and also produced a lower background, enabling more precise quantitation of the induced band; therefore, this method was selected. Shown in Figure 6A is the profile of U4 snRNA in wild-type cells over the course of the transcriptional pulse chase; the results for *brr1* are displayed in Figure 6B. The radioactivity in the induced bands was quantitated using a PhosphorImager, normalized for RNA loading differences (see Materials and Methods), and plotted in Figure 7A on a logarithmic scale. Following the pulse, nearly identical amounts of the induced U4 snRNA are present in a wild-type strain and in the *brr1::LEU2* strain (Figure 7A). However, during the first two hours of the chase, the newly-synthesized RNA from the mutant cells decays rapidly; the kinetics for this early phase are too complex to estimate a half-life, but the overall reduction in induced U4 is about eight-fold (Figure 7A). During this same

period, a fraction of the induced U4 snRNA also decays in wild-type cells, but substantially more slowly, with an overall reduction of about two-fold. Following this initial period during the chase, the surviving fraction of RNA in both wild-type and in *brr1::LEU2* cells becomes much more stable, decaying with a half-life of about six hours.

In addition to measuring the appearance and stability of newly synthesized snRNAs, we sought to examine other aspects of snRNP biogenesis. As described in the Introduction, the 5′ cap structure of vertebrate snRNA transcripts is hypermethylated in the cytoplasm, in a manner dependent on an intact Sm site and assembly of the Sm core proteins. Yeast snRNAs also contain a TMG cap (Wise et al. 1983). To determine the kinetics of this event and the effect upon it of genetically removing Brr1, we immunoprecipitated the RNAs from the pulse-chase experiment with anti-TMG cap antibodies (Lührmann et al. 1982). These were then analyzed by primer-extension and the data were quantitated (see Materials and Methods). Examination of the absolute levels of TMG-capped U4 snRNA reveals a severe reduction in the *brr1* mutant (data not shown), as might be expected given the decrease in total U4 levels in these cells. However, if one normalizes these data to the maximal amounts of TMG-capped U4, observed near the end of the chase period, one finds that the overall kinetics of the reaction are similar in wild type and mutant cells (Figure 7B). Thus, cap hypermethylation of U4 snRNA is not detectably affected in the absence of Brr1.

Biogenesis of U2 snRNA in wild-type and *brr1* mutant cells

We next examined the induced U2 snRNA. Because of its large size and the position at which the induced U2 differs from the endogenous U2, we

analyzed this snRNA by Northern hybridization instead of primer-extension (Figures 8A and 8B). At the beginning of the chase, in both wild-type and mutant cells, two hybridizing bands can be seen in the size range expected for the induced U2 snRNA. The lower one is of the expected size, whereas the upper is larger than expected. Primer extension analysis revealed no differences in the 5' ends of these snRNAs, and RNase protection mapping and oligonucleotide-directed RNase H digestion experiments indicated that the larger form of U2 contains an approximately 20 nt extension at its 3' end (data not shown). During the course of the chase in both wild-type and mutant cells, the upper band is chased into the lower (Figures 8A and 8B), suggesting that the longer form is a precursor form which must undergo a 3' processing event. These data were quantitated and are plotted in Figure 8C. In wild-type cells, this processing event occurs very rapidly, as more than 50% of the induced U2 snRNA is of the faster mobility immediately after the pulse. In contrast, in the *brr1::LEU2* mutant, 3' end processing is delayed such that, at the beginning of the chase, only 20% of the induced U2 is processed, and at 30 minutes, when 50% of the induced U2 snRNA is processed in the *brr1::LEU2* strain, processing in wild-type cells is nearly complete (85%). In sum, the induced U2 undergoes a rapid 3' end processing reaction during biogenesis, and this step (or a previous step, upon which processing depends) is defective in the *brr1::LEU2* mutant.

In assaying the cap hypermethylation event, we were unable to reliably quantitate the rate of hypermethylation, as we were for U4, because of a combination of incomplete immunoprecipitation efficiency and a higher background signal associated with the Northern blot procedure. Nevertheless, we were able to assess the methylation state of the processed and unprocessed forms of U2 by analyzing RNA prepared from pGAL-U2-

containing cells which had been grown for several hours in galactose medium, in order to accumulate greater quantities of the induced U2 species. In probing for hypermethylated species, we subjected these RNAs to analysis similar to that described above for U4, performing immunoprecipitations with antibodies against the TMG cap and analyzing the RNAs derived from the immune pellets by Northern hybridization (Figure 9A). Interestingly, in both wild-type and *brr1::LEU2* cells, the unprocessed induced U2 snRNA is inefficiently immunoprecipitated with these antibodies (Figure 9A; note the small amount of the more slowly migrating band in the immune pellets, lanes 2 and 4, relative to the large amount of unprocessed material remaining in the supernatants, lanes 6 and 8). Control pellets from reactions lacking added antibody are in lanes 1 and 3; the supernatants in lanes 5 and 7 reflect the proportion of the two forms of U2 present in the original RNA samples. In wild-type cells, it is mainly the fully processed material which contains a TMG cap, with a very small amount of unprocessed material also being trimethylated. In the *brr1::LEU2* mutant, fully processed material is also efficiently immunoprecipitated; in addition, a slightly larger amount of the unprocessed material reacts with the anti-TMG antibodies.

These data do not address the structure of the 5′ cap of the bulk of the unprocessed U2 species. Although capping is thought to occur cotranscriptionally in eukaryotic organisms, this has not been directly assayed for snRNAs in yeast. We therefore performed immunoprecipitation experiments analogous to those described above, this time using antibodies specific for the 7-methylguanosine cap found at the 5′ end of polymerase II transcripts (Munns et al. 1982). In this case, for both wild-type and *brr1* mutant cells, only the unprocessed band was immunoprecipitated (Figure 9B, lanes 2 and 4.). Thus, conversion of the induced U2 snRNA from an

unprocessed to a processed species is accompanied by hypermethylation of its 7-methylguanosine cap to a TMG cap. This relationship is not significantly affected by the *brr1::LEU2* mutant.

DISCUSSION

As described in the Introduction, studies in vertebrate systems have defined a pathway of events which leads to the production of mature snRNPs. An understanding of the mechanism underlying these events and the purpose of their complexity will require isolating the factors which mediate them and determining the specific functions of these factors. In this study, we have identified a yeast protein, Brr1, that exhibits many of the characteristics of the common snRNP proteins but is unrelated to the Sm family at the primary sequence level. We have shown that Brr1 is required for the accumulation of Sm snRNPs and physically associates with them. Using a transcriptional pulse-chase method, we have been able to examine the pathway of biogenesis for the first time in yeast. We present direct evidence that Brr1 functions in the manufacture of snRNPs but is dispensable for the stability of mature Sm snRNPs. These data establish a transient role for Brr1 in snRNP metabolism.

Brr1 is a novel Sm snRNP protein required for Sm snRNA accumulation

Brr1 was identified as a candidate snRNP biogenesis factor because of the effect of the *brr1-1* mutation in decreasing steady state levels of the spliceosomal snRNAs but not other small RNAs such as U3, snR5, snR14, and scR1. Cloning and sequencing of the *BRR1* gene revealed it to encode a novel protein which is larger than the canonical Sm proteins and which lacks the recently reported Sm motif (Seraphin 1995, Hermann et al. 1995, Cooper et al. 1995). Despite this absence of primary sequence relatedness, Brr1 exhibits certain properties characteristic of the Sm family of snRNP proteins. First is the effect of depletion of Brr1 in lowering steady state Sm snRNA

levels, a phenotype which has been reported independently for cells depleted of the yeast Sm D1 and Sm D3 homologs (Rymond 1993, Roy et al. 1995). Second, Brr1 is physically associated with each of the spliceosomal Sm site-containing snRNAs, as revealed by immunoprecipitation experiments using a monoclonal antibody against an epitope-tagged version of Brr1. Like canonical Sm proteins, Brr1 associates with U6 snRNA by virtue of base-pairing between U6 and the Sm-site containing snRNA, U4; however, unlike the recently identified yeast U6-specific Sm-like proteins (Cooper et al. 1995, Seraphin 1995), Brr1 does not associate detectably with free U6.

The relatively inefficient immunoprecipitation of snRNAs with antibodies against epitope-tagged Brr1 (Figure 3A; compare the intensity of the signal in lane 7, where antibodies against the TMG cap were used, to that in lane 9, where an equal amount of antibodies against epitope-tagged Brr1 were used) may reflect a loose or transient interaction of Brr1 with snRNPs or, alternatively, may result from partial inaccessibility of the epitope in snRNP-associated Brr1. If the former possibility is correct (as is suggested by the sensitivity of the interaction to high salt concentrations; data not shown), then Brr1 would resemble p69, a human protein which associates directly with the Sm ribonucleoprotein core structure but which was not identified in earlier studies because of its relatively low affinity for snRNPs (Hackl et al. 1994). p69, like Brr1, is distinct from the canonical Sm proteins in its large size and in lacking the Sm amino acid motif (R. Lührmann, personal communication); however, p69 bears no sequence similarity to Brr1 (R. Lührmann, personal communication).

A final piece of evidence relating Brr1 to the Sm family of proteins is the genetic interaction between *BRR1* and the gene encoding the yeast Sm D1 homolog, Smd1. *SMD1* was recovered as a suppressor of *brr1-1* in initial

attempts to clone the *BRR1* gene. Overexpression of wild-type *SMD1*, but not an allele containing a frameshift mutation, suppresses the cold-sensitive growth defect of *brr1-1* and of the *BRR1* gene disruption. Thus, Brr1 is a protein which physically associates with Sm snRNPs, which is required for their accumulation *in vivo* to normal levels, which interacts genetically with at least one member of the canonical Sm family, but which is distinct in amino acid sequence and, possibly, in its affinity for snRNPs.

Brr1 functions in the manufacture of spliceosomal snRNPs

Prompted by the effects of the *brr1-1* mutation in lowering steady state snRNA levels, we considered two general possibilities for the function of wild-type Brr1 protein. The first possibility was that Brr1 normally mediates the biogenesis of snRNPs; in its absence, fewer new snRNPs are made and the steady state level of snRNPs is reduced. The second was that Brr1 stabilizes mature snRNPs such that, in its absence, they lose their structural integrity and are degraded more rapidly than usual. This latter possibility was buttressed by the studies of Blanton et al. (1992), who found that inactivation *in vivo* of several snRNP-associated proteins results in degradation of the associated snRNA. Of course, these models are not mutually exclusive.

Transcriptional pulse-chase analysis of snRNP biogenesis in wild-type yeast and *brr1* mutant cells

To test the first hypothesis, we developed a method to examine the kinetics of several events of snRNP biogenesis. By following a short pulse, in which transcription of U2 and U4 snRNAs was induced, with a chase period, in which additional transcription was repressed, we were able to monitor the appearance and stability of newly synthesized snRNAs during the course of

snRNP biogenesis. Because levels of U4 snRNA were nearly identical in wild-type and *brr1* mutant cells at the end of the pulse period, the transcription reaction seems to be generally unaffected by depletion of Brr1; the suggestion that Brr1 is probably not an snRNA-specific transcription factor is further supported by the observation that, at steady state, U4 and U2 snRNAs accumulate to lower levels in *brr1* cells even when their expression is regulated by the exogenous *GAL1-10* enhancer (S. Noble, unpublished). However, for a window of time after transcription, the newly synthesized U4 snRNA is greatly destabilized in a strain lacking Brr1 compared to an isogenic wild-type strain. In the mutant cells, only a small fraction of the pulsed U4 survives long enough to form metabolically stable snRNPs, accounting for the severe defect in U4 snRNA accumulation at steady-state. We were able to monitor the cap hypermethylation reaction, utilizing the ability of antibodies specific for the TMG cap to precipitate U4 snRNA. Although at any given time after the pulse there is less anti-TMG precipitable material in *brr1* cells than in wild-type cells, the kinetics of the reaction are very similar between mutant and wild type, suggesting that this event is not affected by the absence of Brr1. Thus, the defect in U4 snRNP biogenesis is unlikely to result from failure of the hypermethylation reaction.

The results for U2 snRNA differed somewhat from those for U4. First, less induced U2 was present in *brr1* cells than in wild-type cells at the end of the pulse. We believe that this difference does not reflect a difference in transcription efficiency, because of the arguments made above. Instead, we favor the idea that biogenesis of U2 is more rapid than that of U4, such that nascent U2 snRNA is destabilized soon after transcription, within the period of the pulse. Unfortunately, a shorter pulse does not produce sufficient material to monitor during the chase period (data not shown). A second

difference between the results for U2 and U4 snRNAs is that U2 snRNA undergoes a processing event early in its biogenesis. At early times during the chase period, we were able to detect two forms of U2 snRNA in both wild-type and mutant cells. In wild-type cells, the longer, 3' end-extended form was rapidly chased to the shorter form; in fact, about 60% of the material had already been processed by the end of the pulse. However, in cells lacking Brr1, an hour of chase was required for the same fraction of U2 to be processed.

For U2 snRNA, we were unable to quantitate the rate of cap hypermethylation over the period of the chase, because of a combination of incomplete immunoprecipitation efficiency and a relatively high background signal associated with the method of detection (Northern hybridization). Nevertheless, by examining U2 from cells which had undergone a longer period of induction, we were able to assess the methylation state of the cap structures of the two forms of U2. Interestingly, immunoprecipitation experiments revealed that, for the majority of transcripts, U2 3' end processing and cap hypermethylation hold a largely constant relationship: in wild-type cells, the bulk of the unprocessed U2 precursor bears a 7MG cap, whereas processed material bears a TMG cap. That this relationship is maintained in *brr1* mutant cells, which are defective for 3' end processing, suggests that the two reactions are either coupled or that both reactions depend upon an upstream event which is defective in the *brr1* mutant. If the former possibility is true, then our ability to detect some TMG-capped, unprocessed material but no 7MG-capped, processed U2 in both wild-type and *brr1* mutant cells would indicate that coupling of the two events is not absolute, such that a small amount of cap hypermethylation can occur without simultaneous 3' end processing. Alternatively, if the two reactions are dependent on an upstream, Brr1-mediated event, then our detection of a

TMG-capped, unprocessed intermediate is consistent with the possibilities either that cap hypermethylation precedes 3' end processing or that the two reactions occur without an obligate order, with cap hypermethylation being the slightly faster reaction.

To summarize the results for the transcriptional pulse-chase, we obtained different kinds of information for U4 and U2 snRNAs. For U4 in *brr1* mutant cells, we observed a dramatic decrease in the initial stability of newly synthesized snRNAs, cap trimethylation of normal kinetics, and acquisition over time of wild-type stability for those snRNPs which achieved maturity. For U2 in *brr1* mutant cells, we observed a decrease in initial snRNA levels and a decrease in the rate of 3' end processing. An important unresolved question is whether the defects seen for U2 and U4 snRNAs are related; in other words, does Brr1 affect both snRNAs because it mediates a single reaction which is common to the two snRNPs or because it is required for more than one type of reaction? We have not able to identify longer, unprocessed forms of U4 by Northern analysis (in which we could have detected precursors as little as five nucleotides longer; S. Noble, unpublished); thus, to our knowledge, U4 snRNA does not undergo 3' end processing in yeast. In addition, whereas the efficiency of cap hypermethylation of U4 snRNA appears to be unaffected by the *brr1* mutation, bulk hypermethylation of U2 snRNA is presumably slowed in the mutant as this reaction appears to occur in conjunction with the (delayed) processing event. The simplest model to account for these differences in phenotype is that Brr1 functions in an early, common event in the manufacture of U2 and U4 snRNPs; this event is a prerequisite for the 3' end processing of U2 but is not required for cap hypermethylation of U4.

Brr1 is dispensable for the stability of mature snRNPs

We tested the hypothesis that Brr1 is required for the stability of mature snRNPs by means of promoter shut-off experiments. These were performed by continuously expressing U2 and U4 snRNAs from *GAL1-10* enhancer-regulated snRNA genes, and then, after several cell generations, repressing expression with glucose-containing medium. When we compared the apparent rates of decay of mature snRNPs in a wild-type strain and in an isogenic *brr1* knockout strain, we observed no differences in the stabilities of either U2 or U4 (Figure 4). Thus, the Brr1 protein plays a transient role in snRNP metabolism, being required for efficient construction of new snRNPs but dispensable for the maintenance of snRNPs once they are mature. Of course, Brr1 may have additional functions, for example in snRNP-snRNP interactions, which are independent of its role in biogenesis. Indeed, *brr1-1* was isolated because it is defective in the first step of splicing *in vivo*. As snRNAs in yeast are present in great excess over the levels necessary for splicing (Liao et al. 1990), the splicing defect of the *brr1-1* mutant may not result solely from a decrease in abundance of the Sm snRNPs but also from reduced function of those snRNPs that are formed.

A model for Brr1 function

Integrating our observations of the effects of Brr1 depletion in yeast with knowledge of snRNP biogenesis in vertebrate systems (Figure 10), we can speculate as to what may be the primary function of Brr1. The transcriptional pulse-chase analysis of U4 snRNA indicates that Brr1 functions downstream of transcription. Further, our studies of U2 snRNA suggest that Brr1 functions at or upstream of snRNA 3' end processing. If we assume that the pathway of biogenesis is largely conserved between yeast and vertebrates and that Brr1 functions at a step which is common to the

biogenesis of U4 and U2 snRNPs, then the steps of snRNA export from the nucleus (which has not yet demonstrated to occur in yeast) and Sm core assembly in the cytoplasm present themselves as obvious candidates for the Brr1-facilitated reaction. If pre-export snRNAs and cytoplasmic snRNAs which are unassembled with Sm proteins are less stable than their cytoplasmic, fully assembled counterparts, then a defect in either of these steps could account for the destabilization of nascent snRNAs observed in *brr1* mutant cells. Consistent with this notion, snRNA export is known to occur upstream of 3' end processing in vertebrates, and Sm core assembly--although not strictly ordered in the pathway--can occur before processing (Neuman de Vegvar and Dahlberg 1990). Finally, both decreased efficiency of snRNA export and of Sm core assembly could plausibly be complemented by overexpression of Smd1. In the first case, that fraction of snRNAs which manages to exit to the cytoplasm in the absence of Brr1 could be stabilized by the overexpressed Smd1, either by direct binding or promotion of Sm core assembly. Alternatively, depletion of Brr1 may inhibit the rate of Sm core formation per se, with overexpression of Smd1 compensating as above. In the future, these models can be distinguished by developing assays for nuclear export of yeast snRNAs as well as quantitative methods for assessing *de novo* assembly of yeast snRNP core particles *in vitro*.

MATERIALS AND METHODS

Yeast and molecular biology methods

Cultivation and genetic manipulation of*S. cerevisiae* were done using standard methods (Guthrie and Fink 1991). Molecular biological methods are described in Sambrook et al. (1989).

Northern analysis of snRNA levels in *brr1* versus wild-type cells

The original cs isolate of *brr1-1* (DBY483; Noble and Guthrie, submitted) was crossed to a wild-type parental strain (DBY473). Ten CS and ten cs meiotic progeny were grown in YEPD at 30 °C to saturation. The cultures were diluted into 25 ml of YEPD, to an optical density (O.D.) at 600 nm of between 0.15 and 0.2, and allowed to recover for 3 hours (long enough to double once). The cultures were then shifted to 16 degrees for 12 hours (approximately two doubling times for wild-type cells). Total cellular RNA was prepared. Twenty ug of each RNA sample was electrophoresed through a 6% denaturing polyacrylamide gel for 1 hour at 15 Watts. The gel was electroblotted onto Hybond N (Amersham) paper in 25 mM sodium phosphate buffer, pH 6.5, for 3 hours at 40 volts. RNA was crosslinked to the membrane with UV light using a Stratalinker (Stratagene). Northern analysis was performed using ^{32}P-labeled oligonucleotide probes against the spliceosomal snRNAs and other small cellular RNAs (see below for oligonucleotide sequences). The intensity of each band in the resulting blot was quantified using a PhosphorImager (Molecular Dynamics).

Oligonucleotides used in this study

U1-19G: CAG TAG GAC TTC TTG ATC

U2-0SeqC: TAG TGA GAC CTG ACA TTA GC

U2-L15: CAG ATA CTA CAC TTG

U4-14B: AGG TAT TCC AAA AAT TCC CTA C

U5-7wtsmnr: AAG TTC CAA AAA ATA TGG CAA GC

U6-6D: AAA ACG AAA TAA ATC TCT TTG

scR1: GGG ATG GAG TGT GTC CTG

U3: CCA AGT TGG ATT CAG TGG CTC

snR5A: AGAA GTG AAG ATA TGT ACA CC

U14: ACG ATG GGT TCG TAA GCG TAC TCC TAC CGT GG

PRP6-6: CCC CCC TCG AGG TCG AC

MCP-2: ACC TTC TCT CAA TAT TGT AG

LEU2-1: AGC CAT TAA GGT TCT TAA AG

SMD-1: CC GAATTC TAT TAC ATA AAG TAT TCC CC

SMD-2: CC GTCGAC CAT TGA TGA ATT AGT CGA TT

HA-C: ATC TTT TAC CCA TAC GAT GT

BRR1SeqC: ATA ACA CTT TTT AC

Yeast strains used in this study

DBY4483 (S288C derivative) *brr1-1* (original isolate) *MAT***a** *gal*$^-$ *mal*$^-$ *ade2*$^-$

DBY473 (S288C derivative) *MATα gal*$^-$ *mal*$^-$ *his4-619*

YGS1 (S288C derivative) *MAT***a** *ade2-100*^{o} *ura3-52 lys2-801*^{a} *his3-Δ200 leu2-Δ1*

YJO226 (S288C derivative) *MAT***a**/*α his3 ura3 lys2 leu2 ade2*

BJ2168 *MAT***a** *leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407 gal2*

YSN195 *BRR1(HA)3* (chromosomal gene replacement) *MAT***a** *leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407 gal2*

YSN186 *brr1-1 MATα ura3-52 ade2-100*^{o} *his3-Δ200 lys2-801*^{a}

YSN226 (haploid segregant of YJO226 *brr1* disruption strain) *brr1::LEU2* *MAT***a** *ura3 leu2 ade2 his3 lys2*

Whole yeast PCR reactions

Single yeast colonies were introduced into a 50 ul reaction containing Hot Tub Polymerase (Amersham), 200 uM dNTPs, 1 uM primers, in the manufacturer's supplied buffer. PCR parameters consisted of a 5 minute denaturation at 95°C, followed by 30 cycles of a 1 minute denaturation at 95°C, a 2 minute annealing at 40 °C, and a 4 minute extension at 65°C. Terminal products were extended in a final five minutes at 65°C.

Molecular analysis of *BRR1*

Subcloning and sequence analysis of the BRR1 gene

A fully complementing genomic clone had been isolated (Noble and Guthrie, submitted) from a YCp50 library. The 4 kb insert of this plasmid (pSN 19) was separated into two fragments, a 1.6 kb *Eco*RI-*Eco*RI fragment and a 2.4 kb *Eco*RI-*Sal*I fragment. These were subcloned into the centromere, ARS-containing vector, pSE362 (*HIS3*; called pUN90 in Elledge and Davis 1988) as pSN27 and pSN28, respectively. The two plasmids, along with pSN19 and pSE362 controls, were introduced into *brr1-1* cells, and the transformants were assessed for growth after three days at 17°C. As pSN28 gave full complementation, its insert was selected for DNA sequencing. A candidate ORF was found to reside within the 1.4 kb region defined by*Xho*I and *Hin*dIII restriction sites; subcloning of this region into pSE360 (*URA3*; called pUN50 in Elledge and Davis 1988) to create pSN68 and transformation of *brr1-1* cells confirmed that this ORF corresponded to the complementing activity.

Linkage analysis and gene disruption

For linkage analysis, a 2.5 kb *Eag*I-*Sal*I fragment containing the *BRR1* gene was subcloned into the *URA3*-marked integration vector pRS306 (Sikorski and Hieter 1989). The resulting plasmid, pSN34, was linearized with *Hin*dIII (which cuts just adjacent to the 5′ end of the coding sequence of *BRR1*) and transformed into YGS1, a *BRR1* strain with a similar strain background to that of *brr1-1*. Ura$^+$ transformants were screened for integration of the pSN34 fragment to the correct chromosomal locus by a PCR technique. Genomic DNA was prepared from several candidates as well as from the parental strain. Sequences within the DNA of correct integrants were amplified by using oligonucleotides PRP6-6 (which overlaps the pRS306 polylinker) and MCP-2 (which was designed against sequences which lie just downstream of *BRR1*). Eight of ten candidates were correct. Two such integrants were crossed to a *ura3, brr1-1* strain, and ten tetrads were dissected from each diploid. The meiotic progeny were assessed for growth on plates lacking uracil and for growth on YEPD plates at 17 °C; all uracil prototrophs were cold-resistant, whereas all uracil auxotrophs were cold-sensitive, confirming that the cloned complementing gene is tightly linked as would be expected for the wild-type version of the *BRR1* gene.

For disruption of the *BRR1* gene, a construct was made in which DNA from nt 4 to 945 of the 1020 nt *BRR1* ORF was removed and replaced by the *LEU2* gene. The cloning was accomplished by deleting the *Not*I-*Sac*II fragment of *BRR1* from pSN88 (in which a NotI restriction site has been introduced in frame and just after the first ATG of *BRR1*; its construction parallels that of pSN92, described below), blunting the ends with T4 DNA Polymerase (USB), and ligating to a 2.2 kb blunt-ended *LEU2* fragment, to create pSN99. The disruption fragment was released with *Nco*I and *Bam*HI

and transformed into wild-type diploid strain YJO226, which is *leu2* and which has a similar genetic background to that of *brr1-1*. Correct disruptants were identified by a PCR method similar to that described above: PCR reactions were performed on whole yeast with the MCP-2 primer and with the LEU2-1 primer. One disruptant identified in this manner, YSN228 (which is heterozygous for the disruption), was sporulated and the meiotic products assessed for viability at 16, 25, 30 and 37°C as well as for segregation of the *LEU2* marker for the disruption.

SMD1 constructions and complementation analysis

The *SMD1* coding region was amplified from pSN20 by PCR, using the SMD-1 (upstream) and SMD-2 (downstream) primers. The ends of the PCR fragment were cleaved with *Eco*RI and *Sal*I and subcloned into the 2 micron vector, pRS426 (*URA3*; Christianson et al. 1992), to create pSN72. A version of this *SMD1* construct containing a frameshift mutation, pSN98, was created by filling in a BstEII restriction site located at nucleotide 41 of the 441 nt coding region, using T4 DNA polymerase. Both plasmids, along with a vector control, were transformed into *brr1-1* and the transformants scored for growth at 17°C.

Epitope tagging and immunoprecipitation of Brr1

A *Not*I restriction site was introduced in-frame and just before the TAA stop codon of *BRR1* by means of oligonucleotide-directed mutagenesis of a uracil-containing single-stranded template produced in a *dut1⁻ung1⁻* bacterial strain, CJ236 (Kunkel 1987). A 111 base pair fragment encoding three tandem repeats of the hemagglutinin epitope, YPYDVPDYA (Kolodziej and Young 1991), was inserted at the C-terminal coding segment of *BRR1* by

means of this NotI site, to generate pSN95. The epitope-tagged *BRR1* fragment of pSN95 was liberated with *Eco*RI and transformed into the protease-deficient haploid yeast strain, BJ2168, in a one-step gene replacement procedure. Bona fide gene replacements were identified in a whole yeast PCR procedure as described for the *BRR1* gene disruption, using oligonucleotide primers HA-C (located within the triple epitope and pointing downstream, toward the C-terminus of *BRR1*) and BRR1SeqC (located downstream from the *BRR1* gene and pointing upstream, toward its C-terminus). Strain YSN195, containing *BRR1(HA)3* in place of the normal *BRR1* gene, was chosen for subsequent work.

Splicing extracts from BJ2168 and YSN195 were prepared as described by Lin et al. (1985). Immunoprecipitation reactions were carried out as follows: 48 ul of each extract were diluted to 120 ul in standard splicing buffer (lacking added ATP or DTT; i.e. final concentrations of 2.5 mM $MgCl_2$, 3% (w/v) PEG 8000, 60 mM potassium phosphate pH 7.0, 20 mM KCl, 8 mM HEPES-KOH pH 7.9, 8% (v/v) glycerol, 80 uM EDTA, 0.05% NP40 and 80 mM NaCl (100 mM total salt, including KCl). These reactions were incubated on ice for 1 hour with either 10 ul of PBS (no antibody control), 20 ug of anti-TMG antibody (anti-TMG control for immunoprecipitability of snRNPs), 11.5 ug of 12CA5 anti-HA antibody, or 11.5 ug of 12CA5 plus HA peptide to a final concentration of 250 uM. At the end of the incubation, the samples were centrifuged in a microfuge for 10 min to pellet aggregated material. The supernatants were transferred to new tubes and incubated with 40 ul of ProteinA-Sepharose resin (Pharmacia) which had been equilibrated in splicing conditions buffer containing 100 mM salt (composition same as described above) and blocked with bovine serum albumin (10 mg/ml). The samples were gently agitated with the resin at 4 °C for 15 minutes and then

centrifuged briefly in a microfuge. The supernatants were removed and the pellets washed five times with 1 ml of NET-100 (50 mM Tris-HCl pH 7.4, 0.05% Nonidet P-40, 100 mM NaCl). On the last wash, the contents were transferred to new tubes. Two hundred ul of a solution containing proteinase K (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% SDS, 0.2 mg/ml proteinase K) were added to each tube and the reactions incubated for 15 min at 37 °C. The reactions were extracted once with phenol:chloroform (1:1 v/v) and precipitated with ethanol, using glycogen (20 ug) as a carrier. The RNA pellets were resuspended and electrophoresed through a 6% acrylamide, 7M urea gel (for snRNA analysis); the gel was prepared for Northern analysis as described above. For discrimination of Brr1-associated U4/U6 from free U6, reactions were set up as described above, but the RNA pellets were electrophoresed on a 9% nondenaturing gel and the complexes were visualized by hybridization in solution (Li and Brow 1993)

Promoter shut-off experiments

A sugar-regulated version of the U4 gene was created in the following manner: We began with plasmid GAL-U2 (pHM103), which contains a truncated U2 gene placed just downstream of a 365 bp *Sau*IIIA-*Dde*I fragment of the *GAL1-GAL10* intergenic region (Schneider and Guarente 1991); the vector for pHM103 is pSE362 (*HIS3*, identical to pUN90 in Elledge and Davis 1988). We deleted the U2 gene with the enzymes *Dra*III and *Eco*RI, leaving the vector with the *GAL1-GAL10* enhancer sequences just upstream of the *Dra*III site. We next used PCR to amplify the U4 gene in two pieces, using oligonucleotide primers to introduce a *Dra*III site just upstream of the natural promoter of the U4 gene and an EcoRI site 114 bp downstream of the 3' end of the U4 coding sequence. The gene was amplified in two pieces in order to

introduce a 20 bp insertion into U4, at position +76 which is downstream of the U4/U6 interaction region of U4 and upstream of the Sm sequence. The 20 bp insertion consists of a stem-loop sequence of tandem *Xba*I, *Eco*RI, and *Xba*I sites (the sequence of the insertion is 5' TCT AGA GGA ATT CCT CTA GA 3'), chosen to disturb the normal structure of U4 snRNA minimally. The vector and inserts were ligated in successive DraIII-EcoRI and *Eco*RI-*Eco*RI two-way ligations. The insert of the resulting plasmid, pGAL-U4 or pSN138, was sequenced to rule out the presence of PCR errors. We tested pSN138 for ability to complement a disruption of the *SNR14* gene by using it to transform YKS2, which carries an *SNR14* gene disruption along with wild-type U4 gene expressed from a *URA3*-containing plasmid (Shannon and Guthrie 1991); after passaging the transformants on 5-fluoroorotic acid-containing medium, which selects against *URA3*-encoding plasmids, the pSN138-containing strains grew as well as transformants with wild-type U4, demonstrating that the recombinant U4 molecule is functional.

For the promoter shut-off experiments, strain YSN186 (*brr1-1*) was cotransformed with the pGAL-U4 plasmid (marked by *HIS3*) and either pSN68 (encoding wild-type Brr1, as described above, and marked by *URA3*) or the pSE360 vector (also marked by *URA3*). Transformants were grown to saturation in 20 ml medium lacking uracil and histidine and containing 2% glucose (which allows the cells to grow robustly). The cultures were then centrifuged, washed with 2% raffinose (which neither represses nor induces expression mediated by the *GAL1-GAL10* enhancer), 0.2% galactose (which induces expression) but lacking uracil and histidine, and then inoculated to O.D. 600 nm of 0.2 in 110 ml of the same medium. The cells were grown for 24 h at 30°C, to allow multiple cell doublings and accumulation of snRNPs containing the induced U4 molecule. They were then centrifuged and

inoculated into chilled synthetic complete medium containing 2% raffinose 0.2% galactose but lacking histidine and uracil and incubated overnight at 17°C, to allow for maximal expression of the *brr1-1* phenotype on snRNPs. After growing for 10 h, the exponentially growing cells were centrifuged and resuspended in 110 ml chilled synthetic complete medium containing 2% glucose but lacking histidine and uracil, to repress any new expression from the GAL-U4 construct. The cultures were grown at 17°C in the glucose-containing medium for 48 h, with periodic dilution with prechilled medium to maintain them in the exponential state. Twenty-five ml samples were taken for optical absorbance determination and RNA analysis at t=0 and every 6-12 h thereafter. Total cellular RNA was prepared from the samples, and 20 ug of RNA was subjected to primer extension analysis, using the 14B primer to detect U4 species and the U14 primer to detect U14 snoRNA, which serves as an internal control. The resulting data were quantitated using a PhosphorImager. The endogenous U4 in cells containing vector alone was about 5-fold lower than the U4 in cells containing wild-type Brr1 in this experiment, when the bands were normalized to U14 levels. The induced U4 bands were quantitated and normalized either to endogenous U4 (corrected by a factor of 5 in the case of the vector alone control strain) or to U14. Whereas the results were generally the same by either mode of standardization, the curve for recombinant U4 was smoother when endogenous U4 was used as the internal standard; this is the curve which is presented in Figure 4B. Relative stability of U4 in *brr1* versus wild-type cells was calculated as follows: relative stability= (U4 half-life in *brr1*/doubling time of *brr1*)/(U4 half-life in wild type/doubling time of wild type).

U2 snRNA stability was determined in a similar manner, except that cells were shifted directly from galactose, raffinose medium at 30°C to glucose

medium at 16°C. U2 snRNA was detected by Northern analysis; endogenous U2 and U14 were used as internal controls for RNA loading.

Transcriptional pulse-chase

For this experiment, a plasmid was created in which recombinant, galactose-regulated versions of both U2 and U4 snRNAs are expressed from the same plasmid. This was accomplished by subcloning the *GAL1-GAL10* enhancer region and the adjacent U2 gene (U2Δ6, which lacks about 600 bp of yeast-specific sequences; Shuster and Guthrie 1988) from plasmid pHM103 (pGAL-U2) into pSN138 (pGAL-U4). The GAL1-GAL10-U2Δ6 fragment was released from pGAL-U2 in two steps: first, it was cut with *Sac*I (just downstream of U2Δ6) and ligated to an oligonucleotide adaptor with *Sac*I and *Sal*I sticky ends; next, it was cut with *Sal*I (just upstream of the enhancer sequence). The resultant *Sal*I-*Sal*I fragment was subcloned into the SalI site of pGAL-U4 to generate pSN166 or pGAL-U2/GAL-U4, which has the galactose-regulated U2 and U4 genes organized in a head to tail orientation. As for the parent constructs, pGAL-U2/GAL-U4 was tested for the ability to complement disruption of the U2 and U4 genes and behaved identically to the wild-type complementing plasmids.

Strain YSN226 (containing the cold-sensitive *brr1* gene disruption) was cotransformed with pGAL-U2/GAL-U4 and either pSN68 or pSE360. Cultures were grown to saturation in synthetic complete medium containing 2% sucrose but lacking histidine and uracil. Sucrose is preferred as a carbon source to raffinose and should neither induce nor repress transcription regulated by the *GAL1-GAL10* enhancer; in practice, though, it can diminish the rate of induction by galactose (R. Parker, personal communication). Raffinose was therefore used before and during the galactose induction phase

of the experiment. The saturated cultures were diluted into 100 ml fresh 2% raffinose medium lacking histidine and uracil and grown overnight at 30°C. To maximize the mutant phenotype, the cells were transferred to 300 ml fresh medium and grown for another day at 17°C. Finally, the 17° C, exponentially growing cultures were diluted slightly to an O.D. 600 of about 0.4, allowed to recover for an hour, and then pulsed for snRNA expression by the addition of galactose (to a final concentration of 0.2%). After 40 min of growth in galactose, transcription was quenched by addition of glucose (to a final concentration of 4%). Samples were taken for absorbance measurements and RNA analysis just before the pulse, at the end of the pulse, and at 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, and 360 min into the chase.

RNA preparation and primer extension analysis of U4 were performed as described above. Northern analysis of U2 was performed as above, except that 4% acrylamide denaturing gels were used for better resolution of the unprocessed and processed U2 species. The internal control for the U2 gels was scR1 RNA; once again, although normalizing the induced U2 to either endogenous U2 or scR1 gave generally similar results, the plots of induced U2 levels were smoother over time when standardized to the snRNA, and these are the data presented.

Immunoprecipitation of TMG-capped snRNAs

Fifteen ug total RNA from each sample were incubated with 15 ug of anti-TMG cap antibody (2 ul phosphate-buffered saline (PBS) for the control reaction which lacks antibody) in 50 ul 20 mM HEPES pH7.9, 150 mM NaCl, 0.05% Triton X-100 on ice for 2 hrs. Fifty ul of a Protein A-Sepharose (Pharmacia) slurry (preequilibrated 1:10 w/w in the same buffer) was added and the mixture agitated gently in the cold room for one hour. The tubes

were then centrifuged at 4°C for a few seconds, the supernatants removed to new tubes, and the pellets washed five times with 1 ml of the same buffer. After the final wash, the immune pellets were treated with 200 ul of a proteinase K solution, as above, for 15 min at 37°C, then phenol/chloroform extracted and ethanol precipitated, using glycogen as a carrier. For analysis of trimethylated U4, the trimethylated snoRNA snR8 was used as an internal control. The graph depicted in Figure 7B reflects induced U4 snRNA normalized to endogenous U4.

In the analysis of the cap structure of the processed and unprocessed forms of U2, immunoprecipitations were performed with anti-TMG as above with the exception that only 5 ug of RNA were used and the RNA analyzed was prepared from cells grown for 3 h in galactose-containing medium, in order to accumulate more of both the processed and unprocessed species. For detection of the 7MG-capped species, we followed a protocol provided by Roy Parker (personal communication) in which, for each reaction, 15 ul of the anti-7MG ascites fluid (Munns et al. 1982) were prebound to 20 ul of a Protein A-Sepharose slurry for 5 h and then incubated overnight with denatured RNA samples. The remainder of the protocol is similar to that described for immunoprecipitations with anti-TMG antibodies.

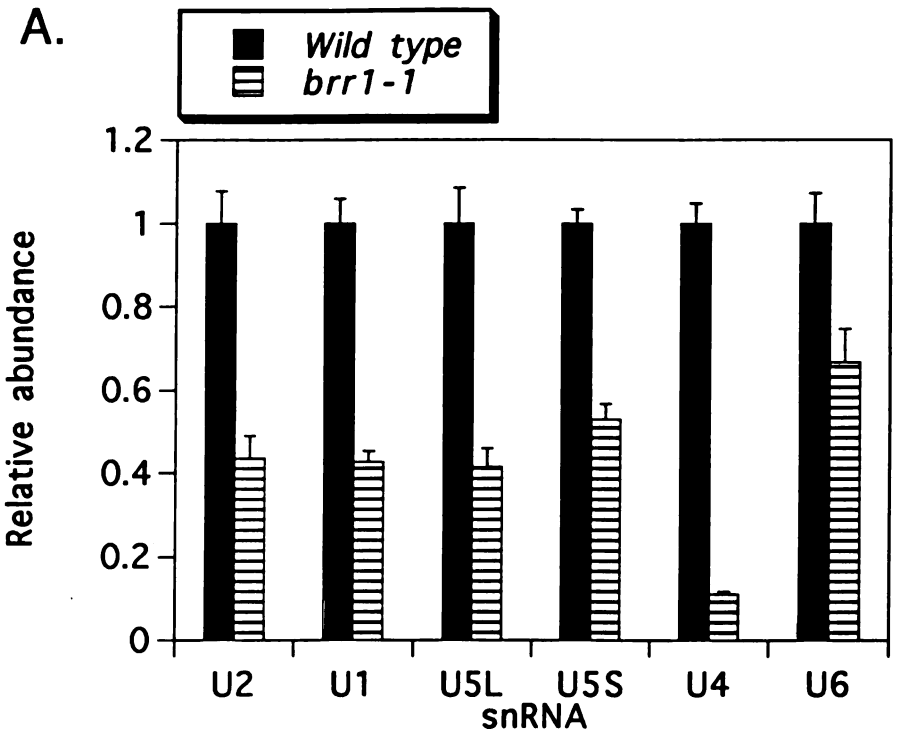
ACKNOWLEDGMENTS

We are very grateful to David Botstein for providing strains, to Joachim Li and Brian Rymond for plasmids, to Reinhard Lührmann, Ted Munns, and Elsebet Lund for antibodies, to Roy Parker for antibody protocols and helpful discussions, and to Ira Herskowitz and members of his lab for strains. We thank Hiten Madhani, Jim Umen, Dan Herschlag, and Ira Herskowitz for helpful comments on the manuscript. Thanks also to Lucita Esperas, Carol Pudlow, and Heli Roiha for their expert technical assistance. S.M.N. is a trainee of the UCSF Medical Scientist Training Program and was supported during the period of this research by a predoctoral fellowship from the American Heart Association. C.G. is an American Cancer Society Research Professor of Molecular Genetics. This work was supported by a grant GM21119 from the National Institutes of Health.

Figure 1. Levels of small RNAs in wild-type and *brr1-1* strains.

A. Spliceosomal snRNA levels. Equal amounts of total RNA prepared from ten wild-type and ten *brr1-1* strains (derived from a single cross between a *brr1* strain and a wild-type strain) which had been shifted to a 16°C were analyzed by Northern hybridization using probes directed against the spliceosomal snRNAs. Blots were quantitated using a PhosphorImager. The levels in wild-type cells are assigned a value of 1. Values are displayed in the bar graph as means; error bars represent the standard error of the mean.

B. Non-spliceosomal RNA levels. Northern blots analyzed in A were stripped of spliceosomal snRNA probes and rehybridized with probes complementary to non-spliceosomal RNAs. Analysis of levels was performed as in A.


A.
Wild type
brr1-1
Relative abundance
1.2
1
0.8
0.6
0.4
0.2
0
U2
U1
U5L
U5S
U4
U6
snRNA

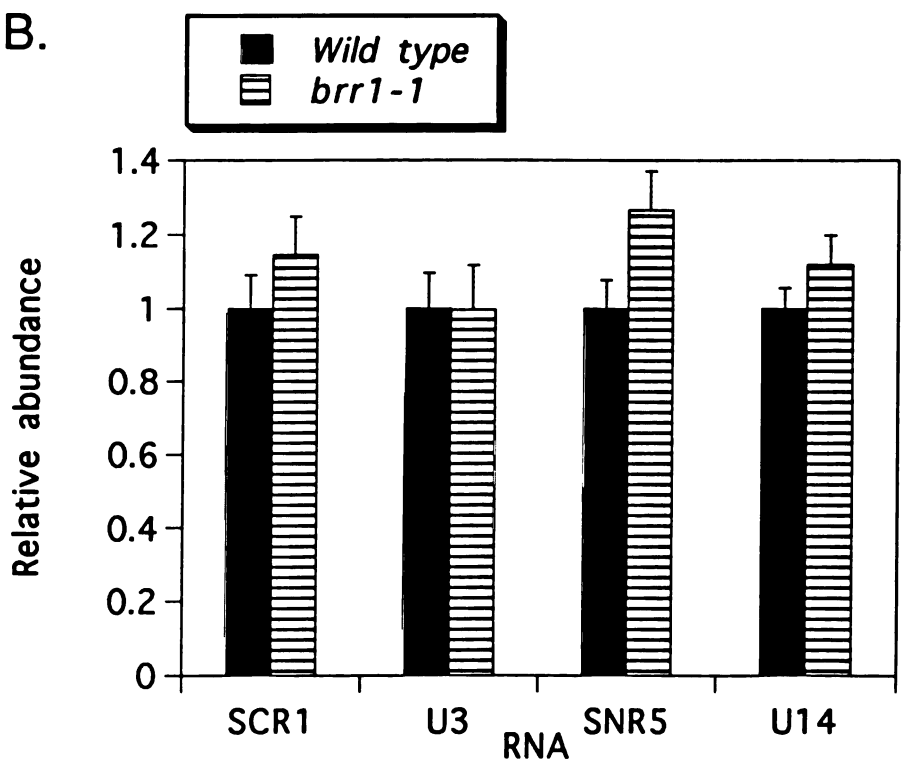

B.
Wild type
brr1-1
Relative abundance
1.4
1.2
1
0.8
0.6
0.4
0.2
0
SCR1
U3
SNR5
U14
RNA

Figure 2. Analysis of the *BRR1* gene.

A. Amino acid sequence of Brr1.

B. Suppression of *brr1-1* by *SMD1*. Shown is a plate containing a *brr1* disruption strain (YSN226) transformed with either a vector control (pRS426; Christianson et al. 1992), the wild-type *BRR1* gene on a centromere (*CEN*) plasmid (pSN68), the wild-type *SMD1* gene on a high copy (2μ) plasmid (pSN72), or a mutant *smd1* allele containing an engineered frameshift (fs) mutation (pSN98). The plate was incubated at 17°C.

A.

MKRGESQAPDAIFGQSRAFALSDSSVNPDVIEYLKS
VRQEALRTNAISIKNHMNLQKRTRHKSSMYDDEDEG
ALKRHAISPSLIRLQRNVEIWVRWFNSVKATVLTNA
YEFTGYEDETLDLLLLFLKNYLEDMPSKCTTVEKII
SVLNQHSFPEKAEEKEENLQIDEEWAKNILVRLEKT
KIDSVEDVKKVITEGDKHELVGYNQWFQYLINNEPQ
HTTFHEKITSKQLWVLIKYMSNTWIKEIHKKGRHYR
RLQDWLFYILVHTPERVTAEYTSILRDLGKKCLELI
QKKPVEAHENKITLPKEMAELNVEIPAAVENMTITE
LTVSVIAVNYGQKDLIE

B.

Figure 3. Immunoprecipitation analysis of Brr1 protein assocation with snRNAs.

A. Denaturing gel analysis of snRNAs coimmunoprecipitated with Brr1. Samples in lanes 1-5 were derived from splicing extract prepared from a strain containing the wild-type (untagged) *BRR1* allele (BJ2168); those in lanes 6-10 were derived from extract prepared from a strain containing epitope-tagged *BRR1 (HA)3* (YSN195). Following the indicated treatments, RNA was extracted and analyzed by Northern hybridization as in Figure 1.

B. Native gel analysis of U6 snRNA coimmunoprecipitated with Brr1. The indicated samples were processed under nondenaturing conditions and electrophoresed on a nondenaturing polyacrylamide gel. Prior solution hybridization of the samples was performed with a probe complementary to U6 snRNA. The positions of the U4-U6 complex and free U6 snRNA are indicated. A small amount of material precipated by anti-TMG antibodies that migrates with intermediate mobility is also seen; it may represent a degradation product of the U4-U6 complex.

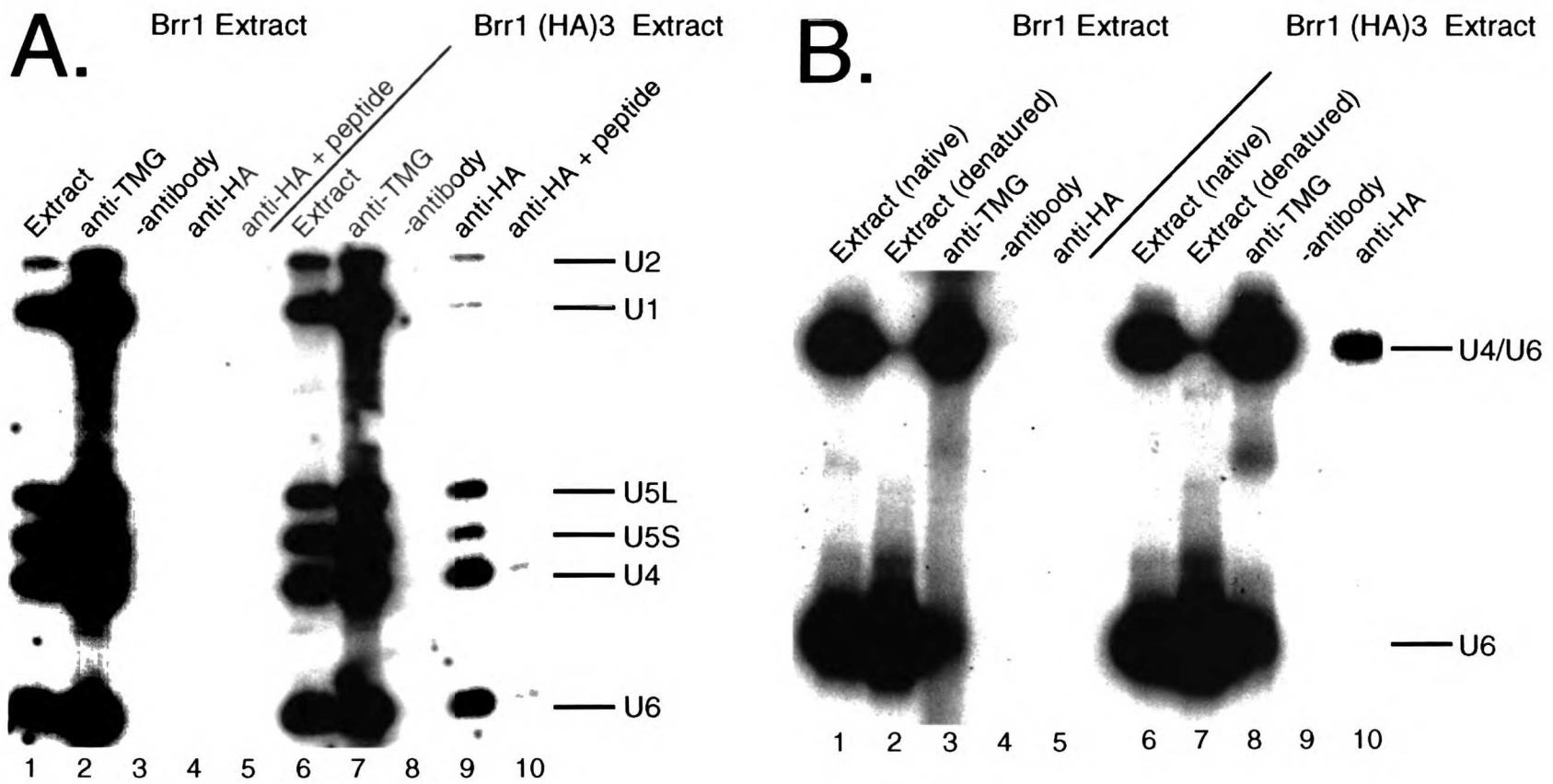

A.
Brr1 Extract
Brr1 (HA)3 Extract
Extract
anti-TMG
-antibody
anti-HA
anti-HA + peptide
Extract
anti-TMG
-antibody
anti-HA
anti-HA + peptide
U2
U1
U5L
U5S
U4
U6
1 2 3 4 5 6 7 8 9 10
B.
Brr1 Extract
Brr1 (HA)3 Extract
Extract (native)
Extract (denatured)
anti-TMG
-antibody
anti-HA
Extract (native)
Extract (denatured)
anti-TMG
-antibody
anti-HA
U4/U6
U6
1 2 3 4 5 6 7 8 9 10

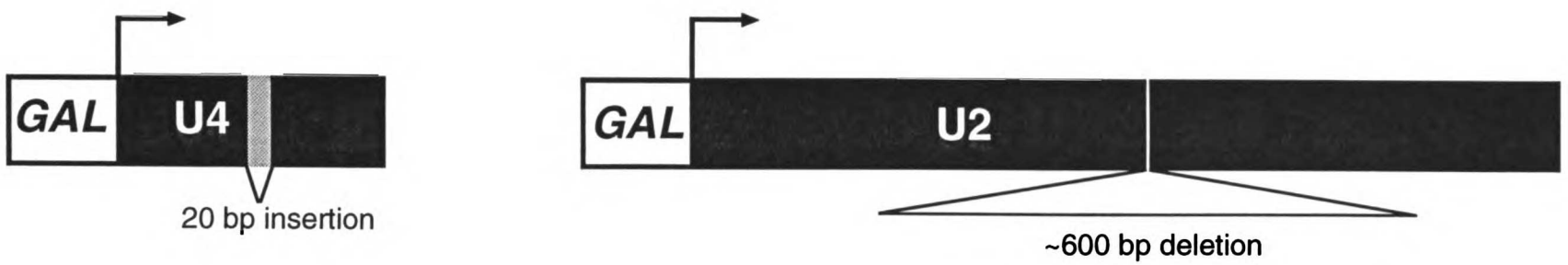
Figure 4. Stability of mature snRNPs in wild-type and *brr1-1* strains containing *GAL*-snRNA constructs.

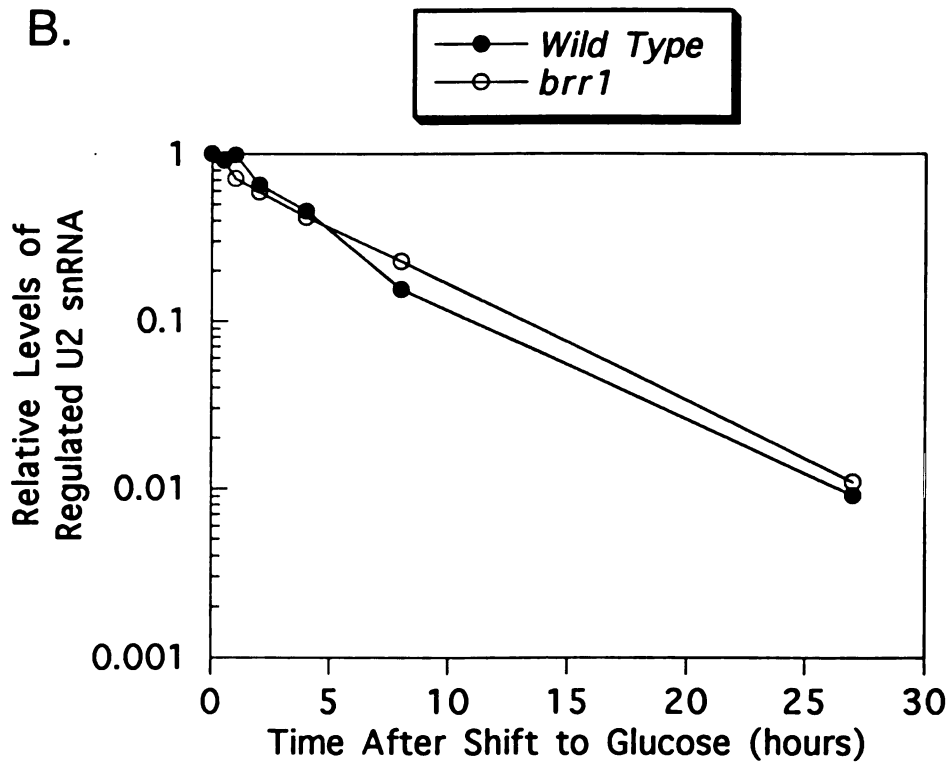
A. Schematic diagram of *GAL1-10* enhancer-snRNA gene fusion constructs. Sequences upstream of the TATA boxes of U2 and U4 snRNA derivatives were replaced with the *GAL1-10* enhancer. These genes complement the lethal phenoytpe of U2 and U4 snRNA gene disruptions.

B. Stability of mature U2 snRNPs. A *brr1-1* strain (YSN186) was tranformed with either a wild-type *BRR1* plasmid (pSN68; "Wild Type") or with a vector (pSE360; Elledge and Davis, 1988; "*brr1*") as well as pGAL-U2 and grown continuously in galactose-containing medium for several generations to accumulate mature U2 snRNP. The cultures were then shifted to glucose-containing medium to repress expression. Plotted are the relative levels (determined by Northern hybridization and normalized as described in the Materials and Methods) of the regulated U2 snRNA after the shift to glucose-containing medium.

C. Stability of mature U4 snRNPs. This experiment was performed in similar fashion to A except that strains contained the pGAL-U4 construct; levels of induced U4 were analyzed using a primer-extension assay and quantitated as in B.

A.




B.
Wild Type
brr1
Relative Levels of Regulated U2 snRNA
1
0.1
0.01
0.001
0
5
10
15
20
25
30
Time After Shift to Glucose (hours)

C.

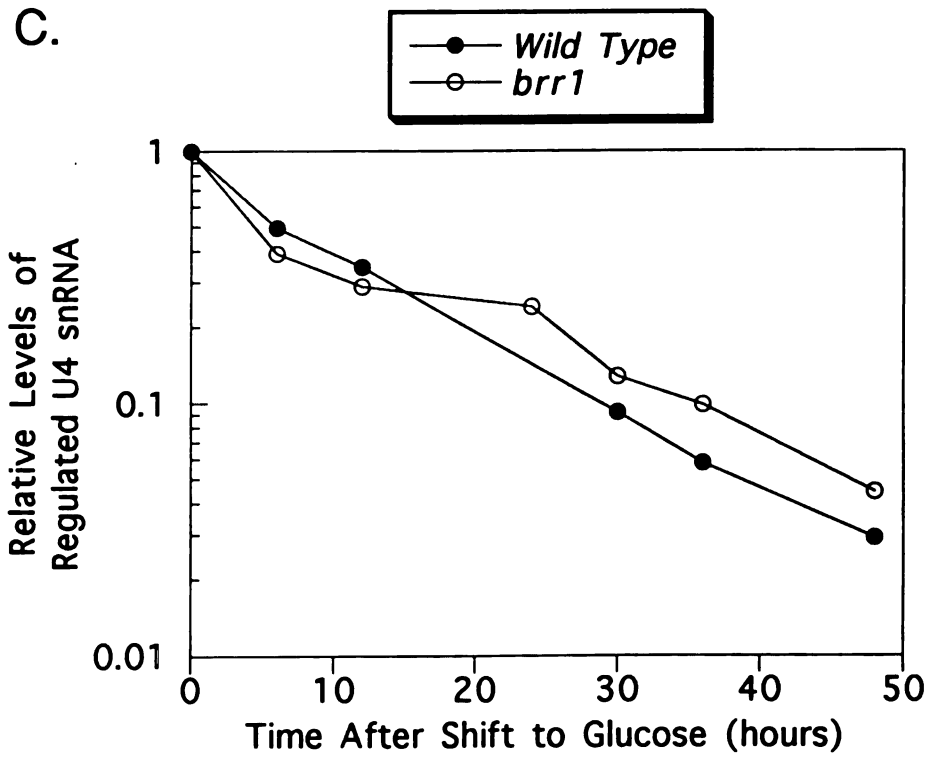
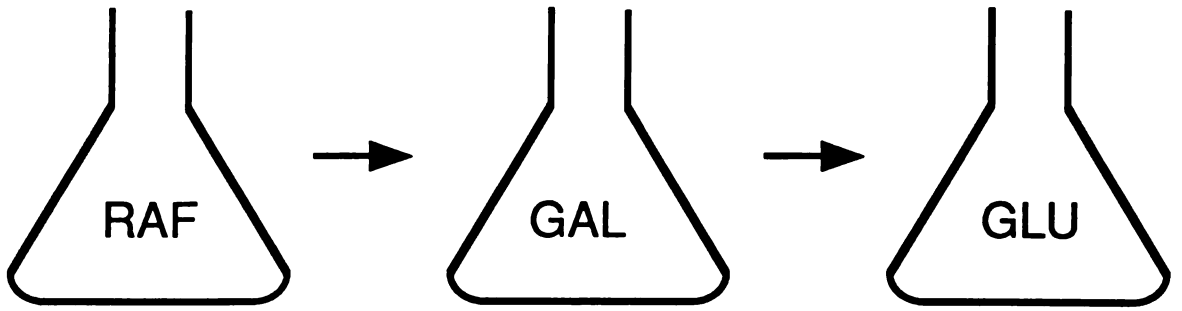

Wild Type
brr1
Relative Levels of Regulated U4 snRNA
1
0.1
0.01
0
10
20
30
40
50
Time After Shift to Glucose (hours)

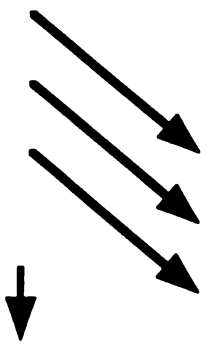
Figure 5. Transcriptional pulse-chase experiment.

Wild-type and *brr1* cells containing the *GAL1-10* enhancer-regulated snRNA gene constructs are grown in raffinose (RAF)-containing medium, which results in neither activation nor repression of snRNA gene expression. A pulse of snRNA transcript synthesis is achieved in galactose (GAL)-containing medium. A chase is achieved by repression of the *GAL1-10* enhancer in glucose (GLU)-containing medium. Cells are harvested at various points in time during the chase, and RNA is isolated for analysis.



OFF **ON** ***(Pulse)*** **REPRESSED** ***(Chase)***

Harvest Cells During Chase



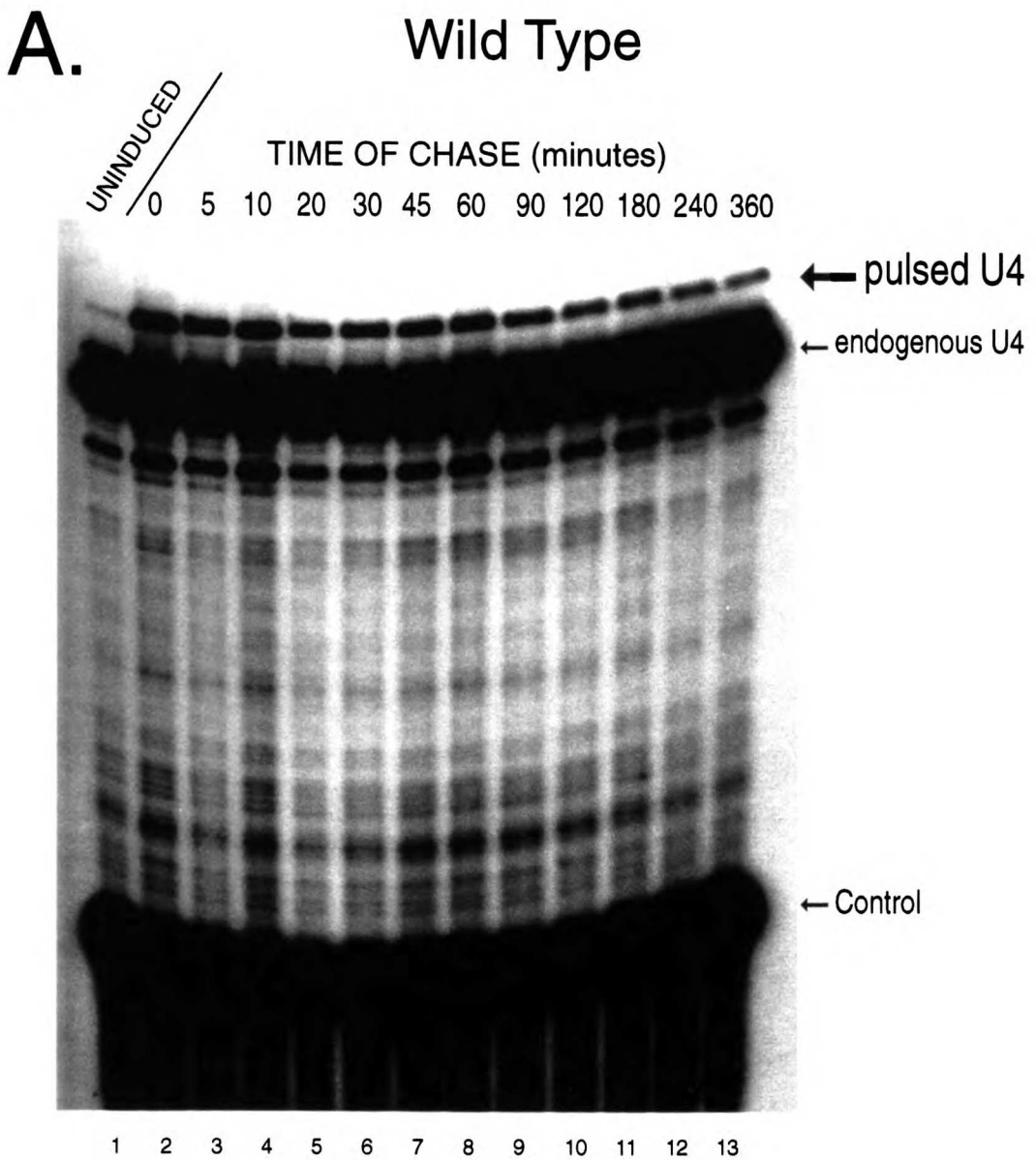
Prepare RNA

Assays for Biogenesis

Figure 6. Transcriptional pulse-chase analysis of U4 snRNA biogenesis. The transcriptional pulse-chase described in Figure 5 was performed. Shown are the results of primer-extension assays using a probe complementary to the 3' end of U4 snRNA. The positions of the endogenous U4 snRNA, the induced U4 snRNA, and an internal control (U14 snoRNA) are shown.

A. "Wild-type" cells (YSN226, transformed with a fully-complementing *BRR1* plasmid, pSN68) which contain pGAL-U2/GAL-U4.

B. *brr1::LEU2* cells (YSN226, transformed with the vector, pSE360; Elledge and Davis, 1988) which contain pGAL-U2/GAL-U4.


A.
Wild Type
UNINDUCED
TIME OF CHASE (minutes)
0 5 10 20 30 45 60 90 120 180 240 360
pulsed U4
endogenous U4
Control
1 2 3 4 5 6 7 8 9 10 11 12 13

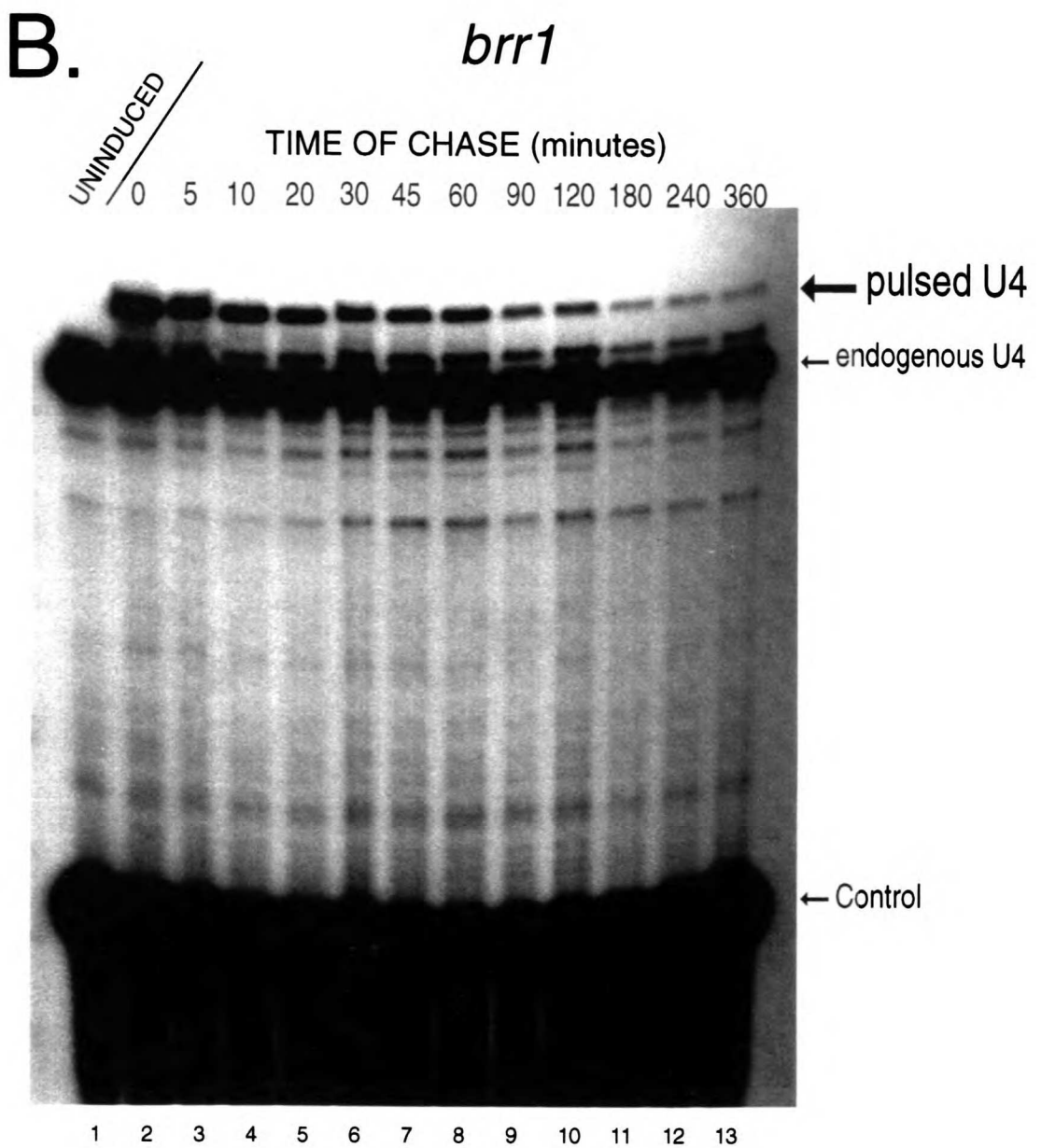
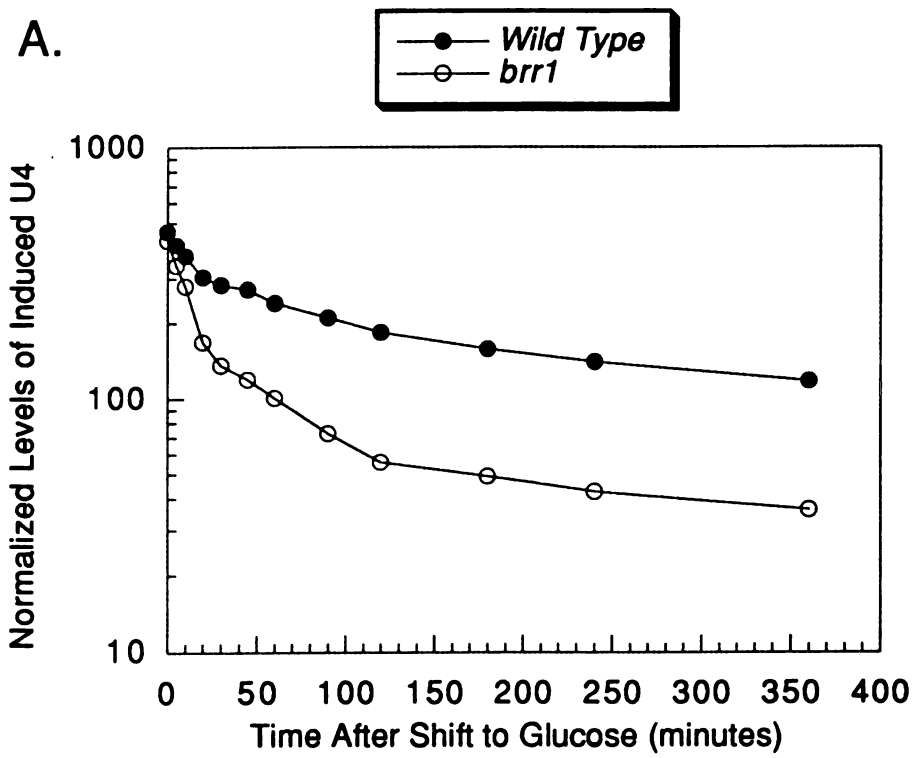

B.
brr1
UNINDUCED
TIME OF CHASE (minutes)
0 5 10 20 30 45 60 90 120 180 240 360
pulsed U4
endogenous U4
Control
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 7. Levels of total and hypermethylated U4 snRNA during the chase period.

A. Quantitation of total U4 snRNA levels. The autoradiograms shown in Figure 6 were analyzed using a PhosphorImager. Levels are normalized to an internal control and plotted during the chase.

B. Cap hypermethylation during the chase. The RNAs analyzed in Figure 6 were immunoprecipitated with antibodies specific for the TMG cap. Their levels were determined by primer-extension and the resulting autoradiograms were analyzed using a PhosphorImager. Plotted is the amount of TMG-capped material detected at various times during the chase, normalized to the maximal amount of TMG material apparent at late times in the chase as well as to an internal control.


A.
Wild Type
brr1
Normalized Levels of Induced U4
1000
100
10
0
50
100
150
200
250
300
350
400
Time After Shift to Glucose (minutes)

B.

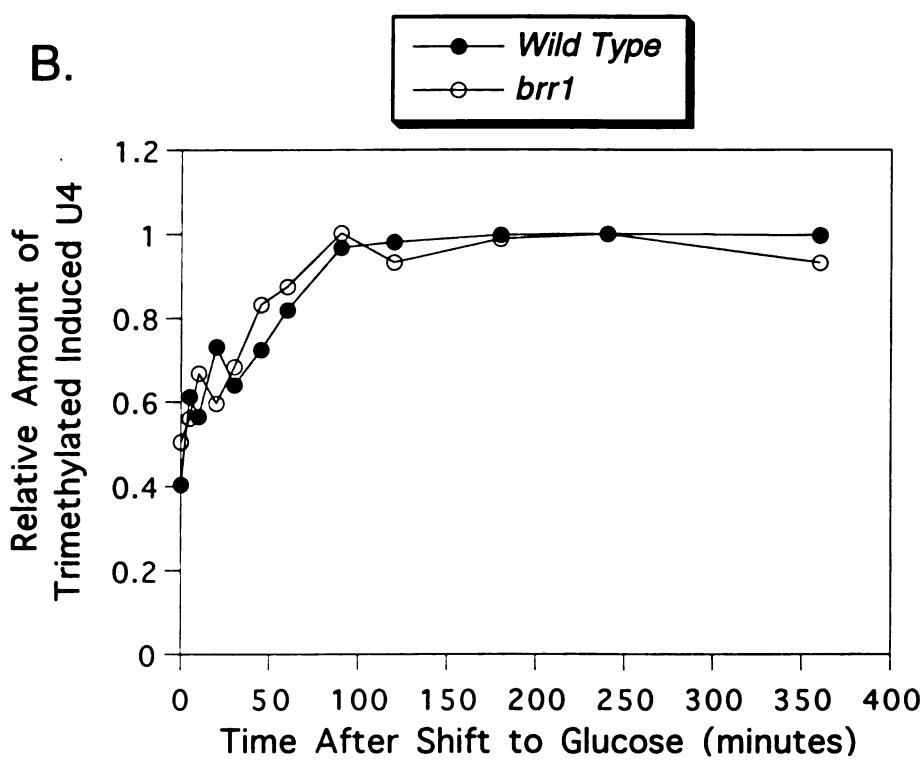

Wild Type
brr1
Relative Amount of Trimethylated Induced U4
1.2
1
0.8
0.6
0.4
0.2
0
0
50
100
150
200
250
300
350
400
Time After Shift to Glucose (minutes)

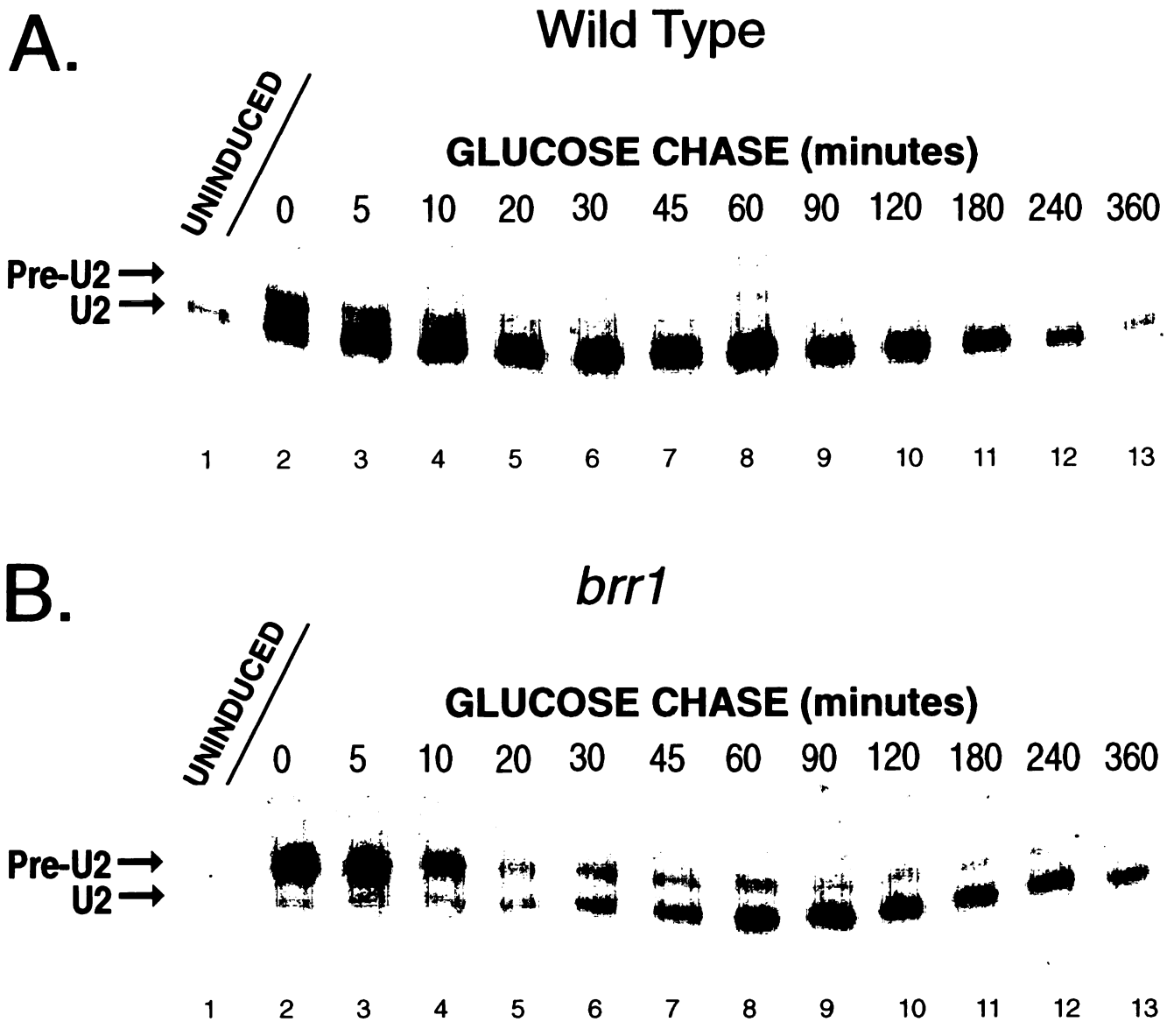
Figure 8. Transcriptional pulse-chase of U2 snRNA biogenesis.

The RNAs analyzed in Figure 6 were examined by Northern hybridization using a probe complementary to U2 snRNA.

A. Levels of regulated pre-U2 and mature U2 snRNA during the chase in wild-type cells.

B. Levels of regulated pre-U2 and mature U2 snRNA during the chase in *brr1::LEU2* cells.

C. Quantitation of the kinetics of processing of the regulated U2 snRNA during the chase. The Northern blots shown in A and B were analyzed using a PhosphorImager. Plotted is the fraction of the regulated U2 snRNA that is processed during the chase in wild-type and *brr1::LEU2* cells.


A.
Wild Type
UNINDUCED
GLUCOSE CHASE (minutes)
0 5 10 20 30 45 60 90 120 180 240 360
Pre-U2
U2
1 2 3 4 5 6 7 8 9 10 11 12 13
B.
brr1
UNINDUCED
GLUCOSE CHASE (minutes)
0 5 10 20 30 45 60 90 120 180 240 360
Pre-U2
U2
1 2 3 4 5 6 7 8 9 10 11 12 13

C.

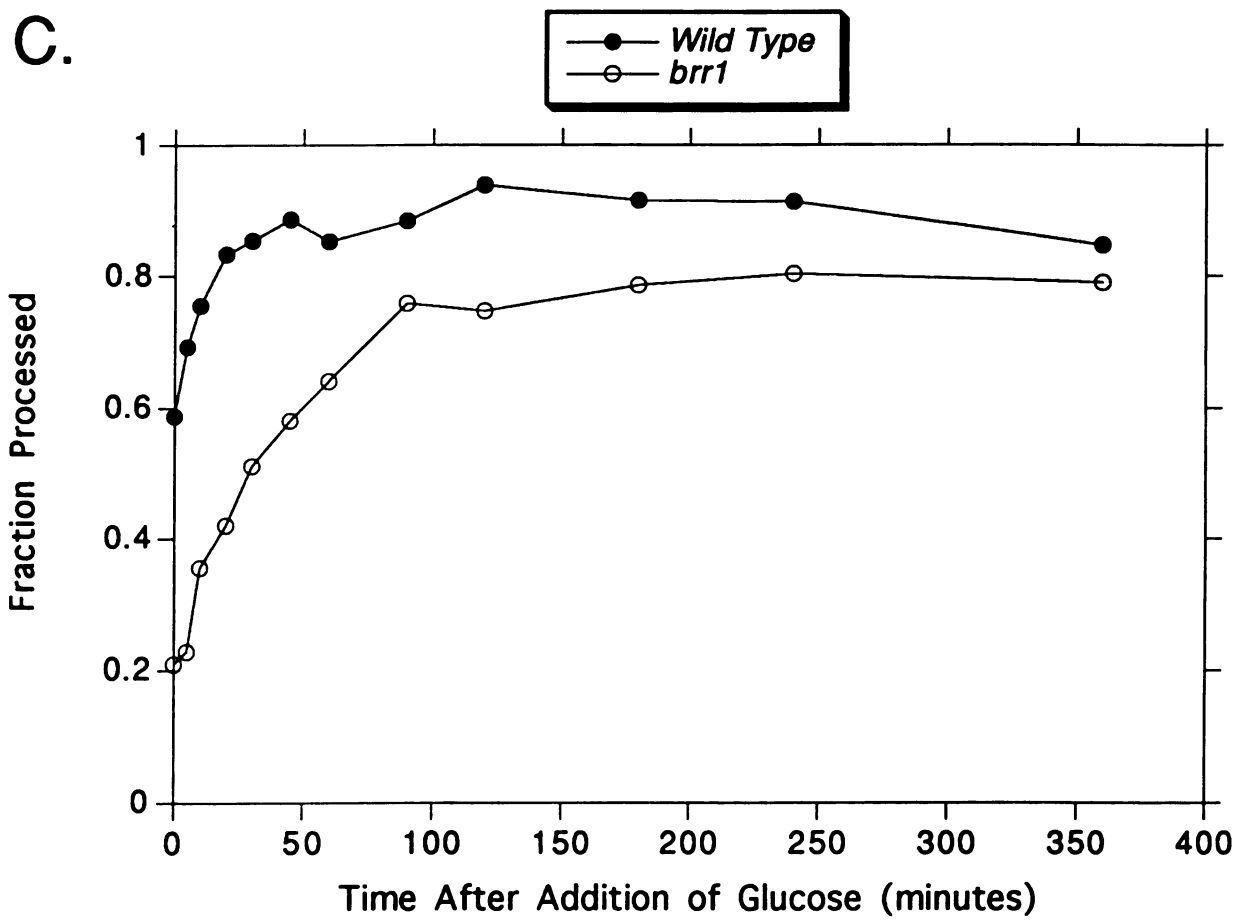
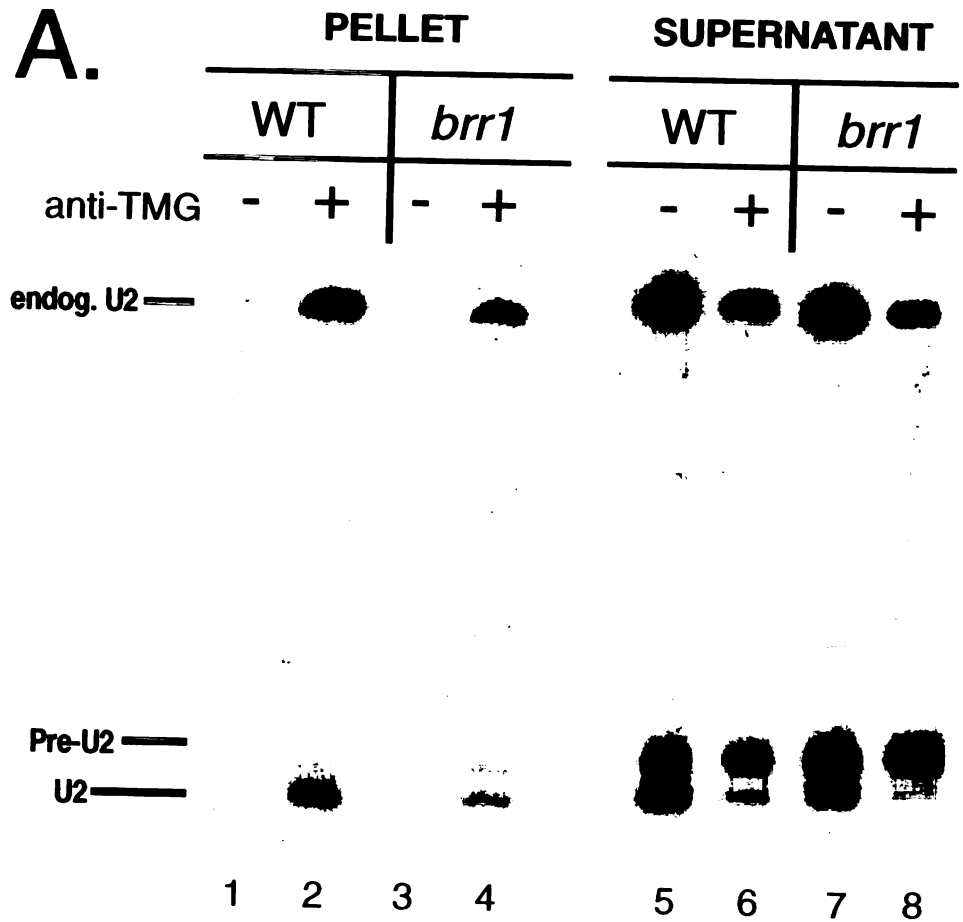

Wild Type
brr1
Fraction Processed
1
0.8
0.6
0.4
0.2
0
0
50
100
150
200
250
300
350
400
Time After Addition of Glucose (minutes)

Figure 9. Relationship between processing of U2 snRNA and cap hypermethylation.

Total RNA was extracted from wild-type (YSN186 containing the *BRR1* plasmid, pSN68) and *brr1-1* (YSN186 containing the vector, pSE360; Elledge and Davis, 1988) cells which bore the pGAL-U2 construct and which had been grown in galactose-containing medium for several cell generations. RNAs were analyzed by immunoprecipitation using antibodies specific for either the TMG cap or 7MG cap. Pellet and supernatant fractions were then analyzed by Northern hybridization using a probe complementary to U2 snRNA.

A. Analysis of induced U2 snRNA with anti-TMG cap antibodies.

B. Analysis of induced U2 snRNA with anti-7MG cap antibodies.


A.
PELLET
SUPERNATANT
WT
brr1
WT
brr1
anti-TMG
- + - + - + - +
endog. U2
Pre-U2
U2
1 2 3 4 5 6 7 8

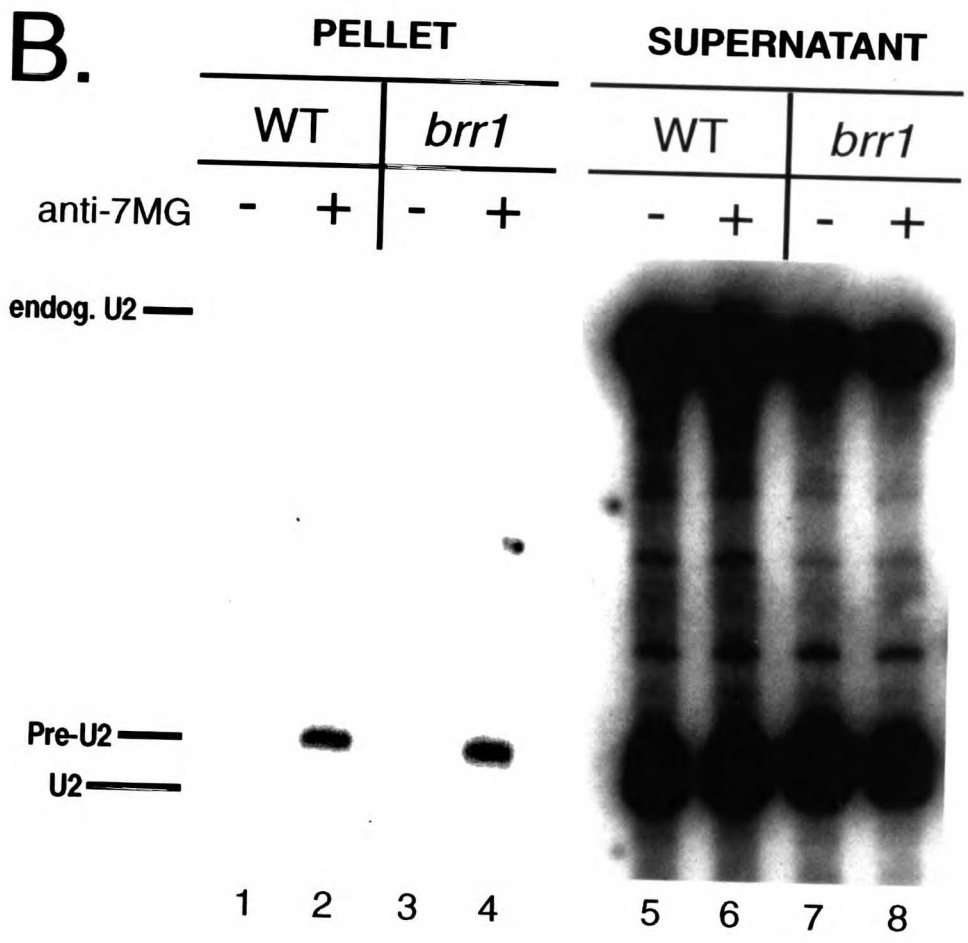
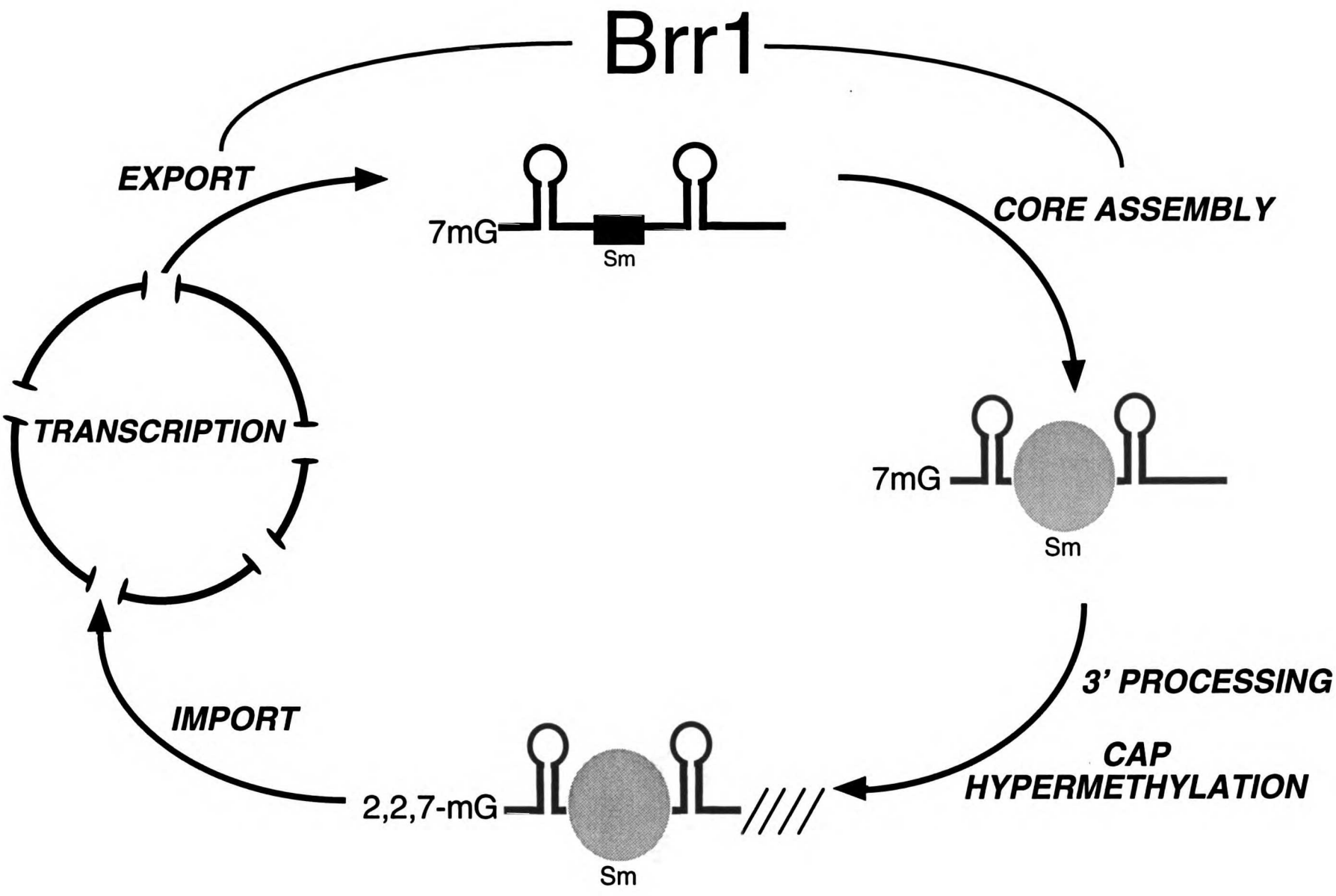

B.
PELLET
SUPERNATANT
WT
brr1
WT
brr1
anti-7MG
-
+
-
+
-
+
-
+
endog. U2
Pre-U2
U2
1
2
3
4
5
6
7
8

Figure 10. Model for Brr1 Function.

Depicted are the major events of vertebrate snRNP biogenesis, described in the Introduction. Assuming that Brr1 functions at a step which is common to biogenesis of U4 and U2 snRNPs and that the general events of the vertebrate pathway are conserved in yeast, we would predict that Brr1 functions either in nuclear export of snRNAs or in assembly of the Sm core. Defects in either of these steps in the *brr1* mutant would be expected to leave transcription intact (which we observe for U4 snRNA) and to inhibit 3' end processing (which we observe for U2 snRNA).


Brr1
EXPORT
7mG
Sm
CORE ASSEMBLY
TRANSCRIPTION
7mG
Sm
3' PROCESSING
CAP
HYPERMETHYLATION
IMPORT
2,2,7-mG
////
Sm

CHAPTER 3

A Yeast Homolog of the 100 kD Subunit of the Human Polyadenylation Signal Binding Protein is Required for Efficient Pre-mRNA Splicing and 3' End Formation *in Vivo*

ABSTRACT

Studies of vertebrate cells have demonstrated that pre-mRNA splicing and 3′ cleavage and polyadenylation are normally coupled reactions, such that the splicing of a terminal intron influences the efficiency of 3' end formation, and vice versa. The mechanisms that promote this coupling are unknown. Pre-mRNA splicing in yeast is largely conserved with that of metazoan systems in terms of intron sequence requirements, as well as components of the catalytic machinery. This unambiguous conservation is not the case with 3' end formation, where significant differences have been observed in both the substrates and the machinery for processing. In a screen for novel factors involved in pre-mRNA splicing, we identified the *BRR5* gene, which we have shown to be essential. Analysis of *BRR5* revealed sequence homology between the encoded protein and the 100 kD subunit of the mammalian cleavage and polyadenylation specificity factor (CPSF). Mutant *brr5-1* cells exhibit defects not only in pre-mRNA splicing, but also in 3′ end processing, suggesting a possible role for Brr5 in the coupling of these two essential RNA processing reactions. The existence of a yeast homolog of CPSF and the association between yeast splicing and 3' end formation described here suggest that the cleavage/polyadenylation machinery in yeast and mammals may be more similar than previously thought.

INTRODUCTION

Eukaryotic mRNAs are initially transcribed in a form which is not competent for translation. The primary transcripts are elongated at their 3' ends and must undergo cleavage and polyadenylation reactions before they can be efficiently translated; furthermore, coding sequences frequently contain introns which must be removed by RNA splicing. A large body of evidence derived from studies of vertebrate cells indicates that splicing and 3' end formation are coupled (Batt et al., 1994; Chiou et al., 1991; Furth et al.,1994; Huang and Gorman, 1990; Liu and Mertz, 1993; Luo and Carmichael. 1991a; 1991b; Lutz and Alwine, 1994; Nesic et al., 1993; Nesic and Maquat, 1994; Niwa et al., 1990; 1992; Niwa and Berget, 1991; Pandey et al., 1990; Villarreal and White, 1983). For example, the 3' ends of histone mRNAs are generated by an unusual mechanism involving recognition of 3' sequences by the U7 snRNP; strikingly, introduction of an intron into a chimeric gene with histone 3' termination sequences causes substantial bypass of the usual mechanism of 3' end formation in favor of cleavage and polyadenylation (Pandey et al., 1990). Other studies have demonstrated that selection *in vivo* of a polyadenylation site can occur only prior to removal of the 3' terminal intron (Liu and Mertz, 1993). Conversely, splicing of the terminal intron of mammalian pre-mRNAs is influenced by the integrity of the downstream polyadenylation sequences; mutation of the highly conserved AAUAAA sequence decreases the efficiency of splicing *in vitro* (Niwa and Berget, 1991).

Although the phenomenon is well-established, the mechanism of coupling remains obscure. Presumably, components of the splicing and cleavage/polyadenylation machineries mediate communication between the two systems. From the splicing perspective, U1 snRNP appears to be a good

candidate for such a factor. U1 snRNA and the U1A snRNP protein can be crosslinked to different polyadenylation efficiency elements in mammalian cell extracts (Wassarman and Steitz, 1993; Lutz and Alwine, 1994), and U1A can stimulate polyadenylation in the test tube (Lutz and Alwine, 1994). In addition, antiserum against U1 snRNP inhibits the mammalian cleavage/polyadenylation reaction *in vitro* (Moore and Sharp, 1984). Finally, the human U1A protein has been shown to interact physically with the C-terminus of poly(A) polymerase, albeit in an inhibitory fashion (Gunderson et al., 1994). In contrast to these examples of splicing factors which influence 3' end formation, no cleavage/polyadenylation factors are known to contribute to splicing.

In the budding yeast *S. cerevisae,* genetic and biochemical studies have allowed the identification of many components of the splicing apparatus as well as several proteins involved in 3′ end processing. Whereas the spliceosome appears to be fundamentally conserved from yeast to mammals, the similarity is less certain for the cleavage/polyadenylation machinery. The sequence AAUAAA is present near the 3′ end processing sites of virtually all metazoan pre-mRNAs (histone pre-mRNAs being a notable exception) but is not found in the 3' processing signals of most yeast pre-mRNAs (reviewed by Keller, 1995). Moreover, although candidate yeast homologs exist for mammalian poly(A) polymerase as well as two components of the cleavage stimulatory factor (CStF), the activities of the yeast proteins differ from those of the mammalian ones. In mammals, poly(A) polymerase is required for both cleavage and polyadenylation, whereas CStF is required only for cleavage; in contrast, yeast Pap1 (the poly(A) polymerase homolog) is required *in vitro* only for polyadenylation, and Rna14 and Rna15 (the putative CStF homologs) participate in both steps (reviewed by Keller, 1995).

Here we describe an essential yeast gene, *BRR5*, which is required for efficient pre-mRNA splicing *in vivo*. Surprisingly, the Brr5 protein is homologous through its entire length to the 100 kD subunit of the mammalian cleavage and polyadenylation specificity factor (CPSF; Jenny et al., 1994). CPSF is the factor that recognizes the AAUAAA signal for cleavage and polyadenylation; like the poly(A) polymerase, it is required for both steps in mammals. We show that *brr5-1* cells are defective in 3′ end processing *in vivo* and exhibit similar kinetics of 3' end formation and splicing defects. The requirement for Brr5 in both splicing and cleavage/polyadenylation suggests that the two reactions may be coupled in yeast, as they are in mammals, and that Brr5 may function in this coupling. If true, this would be the first demonstration of a role for a 3' end processing factor in splicing. Finally, the potential for coupling between splicing and 3' end formation in yeast and the relationship of Brr5 to a subunit of CPSF imply that the differences observed between yeast and mammalian 3′ end formation may not be as significant as previously thought.

RESULTS

brr5-1 is a cold-sensitive (cs) mutant of *S. cerevisiae* identified in a screen for novel splicing factors (Noble and Guthrie, submitted). Upon shift to the nonpermissive temperature of 16°C, *brr5-1* cells accumulate intron-containing precursors of at least four different pre-mRNAs, reflecting a defect prior to or at the first chemical step of splicing (Noble and Guthrie, submitted). *BRR5* appears to be a novel splicing gene, in that a genomic clone which fully complements the cs growth defect of *brr5-1* cells failed to suppress any of the previously-identified temperature-sensitive splicing mutants (Noble and Guthrie, submitted). We therefore undertook the molecular characterization of the *BRR5* gene. In order to delimit the boundaries of *BRR5* within the 8 kb insert of the original complementing clone, we prepared libraries by partial restriction enzyme digestion of the original plasmid. These were introduced into *brr5-1* mutant cells and transformants were selected at 16°C. Ultimately, we identified a minimal complementing fragment of 2.8 kb. Sequencing of this insert revealed the presence of a single open reading frame (ORF) of 779 amino acids. We confirmed that this ORF corresponded to Brr5, and not to a suppressor, by creating a wild type strain with a chromosomal version of the gene which is marked by *URA3* (see Materials and Methods). This strain was crossed to a *ura3, brr5-1* strain, the diploid sporulated, and the meiotic progeny assessed for the cs and uracil auxotrophic markers. Among 18 tetrads, all of the cs spores were also uracil auxotrophs, whereas all cold-resistant spores were uracil prototrophs, indicating linkage of the markers.

Searches of the available sequence databases with the Brr5 amino acid sequence revealed significant homology between Brr5 and the 100 kD subunit

of the mammalian cleavage and polyadenylation specificity factor (CPSF 100kD; Jenny et al., 1994; Figure 1), through the entire length of both proteins. Overall, the proteins exhibit 29% sequence identity and 39% similarity; in addition, they are virtually identical in size (779 amino acids for Brr5 versus 782 amino acids for CPSF 100kD). CPSF is a key component of the mammalian cleavage and polyadenylation machinery (Keller et al., 1991). Besides the 100 kD protein, CPSF consists of two to three additional subunits, (Murthy and Manley, 1992; Jenny et al., 1994), one or two of which are thought to mediate its direct binding to the AAUAAA processing signal (Jenny et al., 1994). In mammalian *in vitro* systems, CPSF is required for both the cleavage and polyadenylation reactions (Keller et al., 1991).

To determine whether the *BRR5* gene is essential, we prepared a disrupted version by replacing almost the entire coding sequence with the yeast *LEU2* gene. This disrupted allele was used to replace one copy of *BRR5* in a wild-type diploid strain, JO226, by homologous recombination. A diploid heterozygous for the *BRR5* disruption was sporulated and resulting ascospores were dissected (Figure 2). Each of the 12 asci dissected gave rise to only two colonies, and surviving spores were leucine auxotrophs. These results indicate that the *BRR5* gene is essential for viability.

We wished to determine whether the *brr5-1* mutation affects 3' end formation, as would be suggested by its homology to a component of CPSF. Moreover, we desired to know whether splicing and 3' end formation are physically coupled or whether a defect in one process might precipitate defects in the other only indirectly; for example, a primary defect in the cleavage/polyadenylation reaction could potentially elicit a splicing defect by destabilizing the mRNA for a short-lived splicing factor. Presumably, an indirect relation would result in a lag in the occurence of one defect with

respect to the other. We therefore performed a temperature shift experiment in which the efficiency of splicing and 3′ end formation were examined in *brr5-1* and isogenic "wild-type" cells (containing the wild-type complementing plasmid). Following a shift to 15°C, samples were isolated periodically over a 10 hour interval (approximately one doubling time for wild-type cells and one third of a doubling for *brr5-1* cells; Figure 3A) and assayed for splicing and 3' end formation.

To assess splicing, we performed primer extension reactions using primers against the second exons of three different intron-containing precursors. These were*SNR17A* and *SNR17B* , which encode the nucleolar snoRNA, U3 (Mylinski et al., 1990) and which are identical except for the length and sequence of their introns, as well as*RP51A*, which encodes a ribosomal protein (Teem and Rosbash, 1983). The resulting autoradiograms are shown in Figure 3B and 3C. For each spliced substrate, comparison of the precursor bands between wild type and mutant reveals the appearance of splicing defects in *brr5-1* cells by three hours of the shift (1/10 of a generation). Quantitation of the data for U3 snoRNA (Figure 3D) revealed that the *brr5-1* splicing defect increased during the course of the shift, peaking at six hours and diminishing somewhat at the end, perhaps because of downregulation of transcription in the dying cells.

To assess 3' end formation, we performed Northern analysis using a radiolabeled probe directed against *CUP1* mRNA. *CUP1* has been shown previously to be sensitive to defects in 3' end processing (Forrester et al., 1992). Results for *brr5-1* are presented in Figure 4. We observed an accumulation of elongated transcripts in the *brr5-1* strain, which suggests that 3' end formation is defective in these cells. Furthermore, the appearance of these aberrant transcripts paralleled that of the observed splicing defects, consistent with the

notion that the splicing defects are not an indirect, delayed consequence of a primary disturbance of 3' end formation or vice versa. In contrast, an isogenic wild-type strain does not display an accumulation of the elongated forms under the same conditions (although a small amount is present throughout the experiment, Figure 4).

DISCUSSION

In this manuscript, we have described the analysis of Brr5, a novel yeast protein identified in a screen for components of the pre-mRNA splicing machinery. Surprisingly, Brr5 exhibits significant sequence homology to the 100 kD component of the mammalian 3' end processing factor, CPSF. We demonstrate that *BRR5* is an essential gene and that the mutant, *brr5-1*, exhibits defects at the nonpermissive temperature in both pre-mRNA splicing and 3' end formation. During a shift to the nonpermissive temperature, these two kinds of defects are detected with similar kinetics. Together, the results suggest that pre-mRNA splicing and 3' end formation may be coupled events in yeast, as they are in mammals, and that this coupling could occur via an integral component of the 3' end processing machinery.

In yeast, our understanding of 3' end processing has lagged behind that in mammals, and the extent of conservation between the two systems is still in question. Although yeast transcripts are processed by the same two-step pathway as in mammals--endonucleolytic cleavage followed by polyadenylation--the sequences on the RNA substrate and the character of the enzymes which mediate the reaction are divergent. As described in the Introduction, yeast pre-mRNA cleavage/polyadenylation signals do not retain the almost invariant AAUAAA sequence which is the single most important determinant of 3' end processing of vertebrate pre-mRNAs (e.g. Sheets et al., 1990). Poly(A) polymerase is conserved in yeast but functions somewhat differently, being required for both cleavage and polyadenylation in the mammalian system but only for polyadenylation in yeast (reviewed by Keller, 1995). The products of two yeast genes, *RNA14* and *RNA15*, are

related in sequence to two components of mammalian CStF (Takagaki and Manley, 1994). However, whereas CStF plays a role in the cleavage reaction only (Takagaki and Manley, 1992; 1994), Rna14 and Rna15 are absolutely required for both cleavage and polyadenylation *in vitro* (Minivielle-Sebastia et al., 1994). These observations have raised doubts about the fundamental conservation of the cleavage/polyadenylation machinery in eukaryotes, both in terms of protein-RNA and protein-protein interactions. It is therefore significant that we have identified a yeast factor, Brr5, which shares sequence homology throughout its length with a component of mammalian CPSF and which affects 3' end formation *in vivo*.

The dual requirement for Brr5 in both splicing and 3' end formation makes it and its mammalian homolog, the 100 kD subunit of CPSF, plausible candidates for factors involved in the coupling of these two processes. The similar kinetics of splicing and cleavage/polyadenylation defects observed in cold-shifted *brr5-1* cells are consistent with the possibility that the two processes are directly coupled in yeast; however, an indirect association cannot be ruled out by these results alone. The best test of the model that Brr5 couples splicing and 3' end processing by functioning independently in each process would be to assess the splicing and 3' end processing reactions directly in yeast cell extracts depleted of Brr5 activity, either by inactivation of the cs allele or by immunodepletion of the wild-type protein. Inhibition of both reactions would argue that the model is correct, whereas inhibition of 3' end processing but not of splicing could result either if splicing *in vivo* depends indirectly on 3' end processing or, alternatively, if the two processes are indeed coupled, but through a factor other than Brr5. If the latter possibility is true, then inactivation of additional components of the

cleavage/polyadenylation machinery should result in splicing defects, and possibly vice versa.

Mammalian CPSF contains three (Murthy and Manley, 1992) or four (Jenny et al., 1994) subunits, of 160 kD, 100 kD, 73 kD, and perhaps 30 kD. Two of these, the 100 kD and 30 kD subunits, have been implicated in recognition of the metazoan AAUAAA 3' end processing signal (Jenny et al., 1994). No specific function is known for the 100 kD subunit (Jenny et al., 1994). We suggest that, whatever its role in 3' end processing per se, the 100 kD subunit may mediate a physical link between the spliceosome and the polyadenylation machinery. Given the findings that components of U1 snRNP interact with mammalian 3' end processing signals as well as the the poly(A) polymerase and can influence the efficiency of processing *in vitro* (as described in the Introduction), an intriguing possibility is that the spliceosomal target of CPSF is U1 snRNP. If the coupling hypothesis is correct, the availability of a cold-sensitive mutant in the *BRR5* gene offers the potentially powerful approach of pseudoreversion (Moir et al., 1982) and other genetic analyses to identify additional factors involved in this phenomenon.

MATERIALS AND METHODS

Yeast and molecular methods

Molecular cloning and yeast manipulations were carried out by standard techniques described in Sambrook et al. (1989) and Guthrie and Fink (1991), respectively.

Oligonucleotides used in this work

Brr5 Dx: GGT AAG CAG TTA TTC TGG

T7: AAT ACG ACT CAC TAT AG

LEU2-1: AGC CAT TAA GGT TCT TAA AG

U3 exon 2: CCA AGT TGG ATT CAG TGG CTC

RP51A exon 2: CGC TTG ACG GTC TTG GTT C

U14: ACG ATG GGT TCG TAA GCG TAC TCC TAC CGT GG

U5-7wtsmnr: AAG TTC CAA AAA ATA TGG CAA GC

SCR1: GGG ATG GAG TGT GTC CTG

Yeast strains used in this study

YSN173 *brr5-1 MATα ura3-52 leu2-Δ1 his3-Δ200 lys2-801*^{a}

YGS1 *MAT**a** ade2-100*^{o} *ura3-52 leu2-Δ1 his3-Δ200 lys2-801*^{a}

JO226 *MAT**a**/α his3 ura3 lys2 leu2 ade2*

YSN378 *brr5Δ::LEU2/BRR5 MAT**a**/α his3 ura3 lys2 leu2 ade2*

BRR5 subcloning and sequencing

The Brr5 ORF was defined within a 8 kb insert of the original complementing clone (pSN100; Noble and Guthrie, submitted) by cloning size-fractionated, *Sau*IIIA-digested fragments from this plasmid into pSE360 (*URA3, CEN*; pUN50 in Elledge and Davis, 1988). By pooling DNA from this library and

using it to transform *brr5-1* cells (YSN173), a minimal complementing fragment of 2.8 kb was defined on the plasmid, pSN104. This insert was sequenced and found to contain a single open reading frame of 779 amino acids.

Linkage analysis

To confirm that the cloned gene corresponds to *BRR5* and not to a suppressor, the insert from pSN104 was subcloned into the integrating vector, pRS306 (*URA3*; Sikorski et al., 1989), to create the plasmid pSN212. pSN212 was linearized with *Bgl*II, which cuts midway through the insert, and transformed into YGS1, a wild-type strain of similar genetic background to *brr5-1*. Integration at the correct chromosomal locus was detected by a PCR screening procedure, in which whole yeast colonies were subjected to PCR with primers from within pSN212 (T7) and from chromosomal sequences upstream of *BRR5* gene (Brr5 Dx). One such integrant was crossed to *brr5-1* (YSN173, *ura3*) and the resulting diploid sporulated and dissected. Analysis of fifteen four-spored tetrads revealed 2:2 segregation of the cs and uracil prototrophic phenotypes, with all cs spore progeny being uracil auxotrophs and all cold-resistant spore progeny being uracil prototrophs.

***BRR5* gene disruption**

To determine whether *BRR5* is an essential gene, a disrupted version was created as follows: the *LEU2* gene (2.2 kb *Sal*I-*Sal*I fragment blunted with T4 DNA polymerase) was used to replace a 1.3 kb *Eco*RI-*Bgl*II fragment (blunted with T4 DNA polymerase) from the *BRR5* gene of pSN212, to create pSN213. The disruption removes amino acids 39-429 of the ORF. The disruption fragment was liberated from pSN213 with *Xma*I and *Sal*I and used

to transform the wild-type diploid strain, JO226, in a one-step gene replacement procedure. Accurate disruptants were detected by a PCR procedure similar to that described for the linkage analysis section, using primers within the *LEU2* gene (LEU2-1) and upstream of the *BRR5* gene (BRR5 Dx) One such diploid (YSN378) was sporulated and the resultant ascospores were dissected. Progeny from 12 asci were incubated at 25 °C. Spores which germinated were assessed for growth on synthetic complete plates lacking leucine.

Temperature shift of *brr5-1* cells

brr5-1 cells (YSN173) containing either YSN104 (*BRR5* on pSE360) or pSE360 alone were grown to midlog phase at 30°C in synthetic complete medium lacking uracil. The cultures were then shifted to 15°C, and 20 ml samples were taken immediately and periodically thereafter for optical density measurements and RNA preparation.

Primer extension analysis of intron-containing RNAs

Primer extension was performed as in Patterson and Guthrie (1991), using oligonucleotide primers against the second exon of the *SNR17A* and *SNR17B* genes (primer U3 exon II) or that of *RP51A* (RP51A exon II). Primers against U14 snoRNA (U14) and U5 snRNA (U5-7wtsmnr) were used as internal controls for *SNR17* and *RP51A*, respectively. Quantitation was performed using a PhosphorImager (Molecular Dynamics).

Northern analysis of *CUP1* transcripts

20 ug of total RNA from each sample was loaded onto a 6% acrylamide/7M urea gel and electrophoresed for 1 h at 15 W. The gel was

electroblotted to a nylon membrane (Hybond-N; Amersham) at 10 V overnight. After crosslinking UV light using a Stratalinker (Stratagene), the blot was prehybridized at 42 °C under standard conditions, then hybridized to uniformly labeled single stranded DNA, generated from the *CUP1*-containing *Xba*I-*Kpn*I fragment of pWF1 (Forrester et al., 1992).

Figure 1. Alignment of Brr5 and CPSF 100kD.

Solid boxes represent amino acid identities, and box outlines represent chemical amino acid similarities.

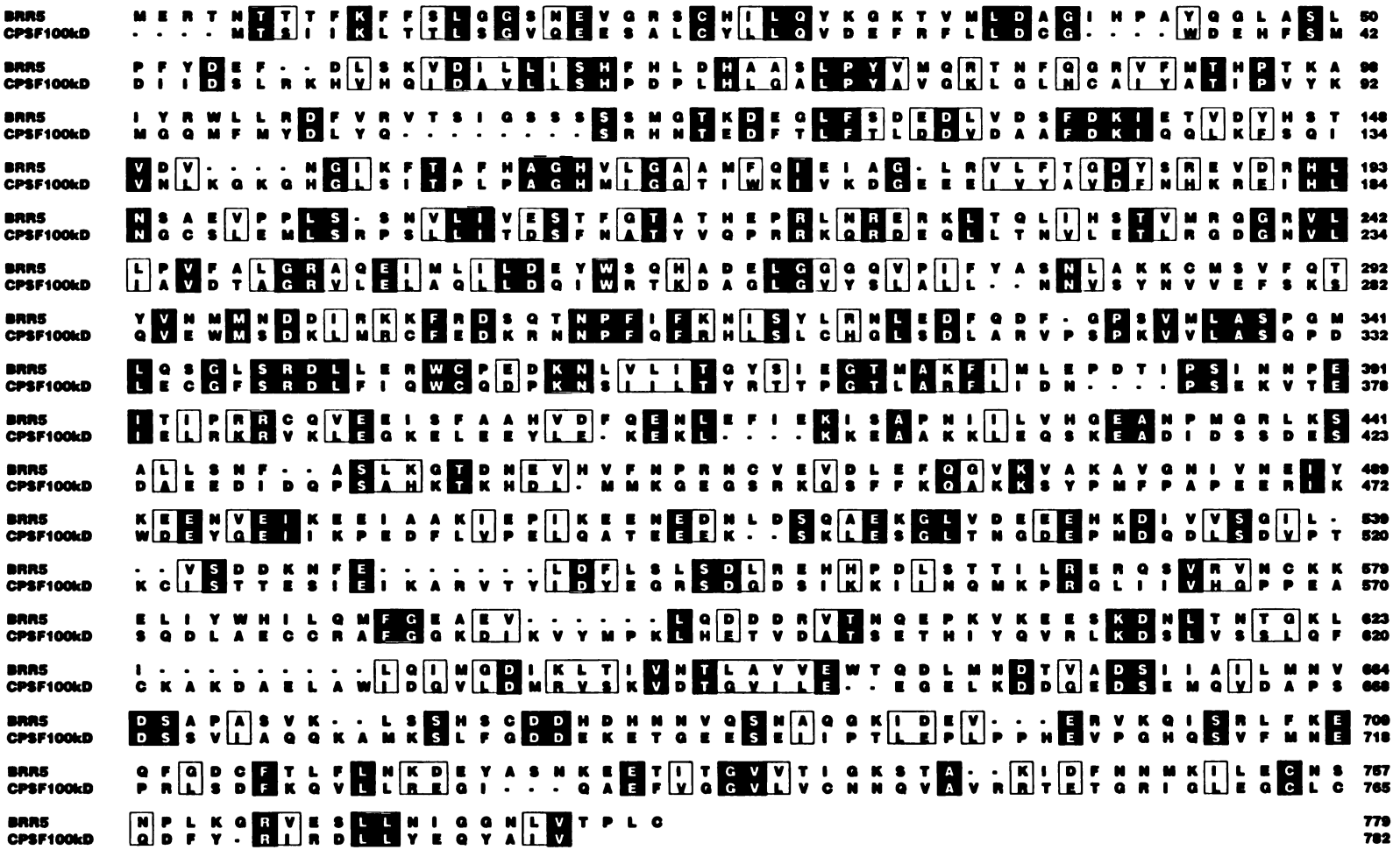

BRR5
CPSF100kD
M E R T N T T T F K F F S L Q G S N E V Q R S C H I L Q Y K Q K T V M L D A G I H P A Y Q Q L A S L 50
. . . . M T S I I K L T T L S G V Q E E S A L C Y L L Q V D E F R F L L D C G . . . . W D E H F S M 42
P F Y D E F . . D L S K V D I L L I S H F H L D H A A S L P Y V M Q R T N F Q Q R V F M T H P T K A 98
D I I D S L R K H V H Q I D A V L L S H P D P L H L Q A L P Y A V Q K L Q L N C A I Y A T I P V Y K 92
I Y R W L L R D F V R V T S I Q S S S S S M Q T K D E Q L F S D E D L V D S F D K I E T V D Y H S T 148
M Q Q M F M Y D L Y Q . . . . . S R H N T E D F T L F T L D D V D A A F D K I Q Q L K F S Q I 134
V D V . . . . N G I K F T A F H A G H V L G A A M F Q I E I A G . L R V L F T Q D Y S R E V D R H L 193
V N L K Q K Q H G L S I T P L P A G H M I G Q T I W K I V K D G E E E I V Y A V D F N H K R E I H L 184
N S A E V P P L S . S N V L I V E S T F Q T A T H E P R L N R E R K L T Q L I H S T V M R Q G R V L 242
N Q C S L E M L S R P S L L I T D S F N A T Y V Q P R R K Q R D E Q L L T N V L E T L R Q D G N V L 234
L P V F A L G R A Q E I M L I L D E Y W S Q H A D E L G Q Q Q V P I F Y A S N L A K K C M S V F Q T 292
I A V D T A G R V L E L A Q L L D Q I W R T K D A Q L G V Y S L A L L . . N N V S Y N V V E F S K S 282
Y V N M M N D D I R K K F R D S Q T N P F I F K N I S Y L R N L E D F Q D F . Q P S V M L A S P Q M 341
Q V E W M S D K L M R C F E D K R N N P F Q F R H L S L C H Q L S D L A R V P S P K V V L A S Q P D 332
L Q S G L S R D L L E R W C P E D K N L V L I T Q Y S I E G T M A K F I M L E P D T I P S I N N P E 381
L E C G F S R D L F I Q W C Q D P K N S I I L T Y R T T P G T L A R F L I D N . . . . P S E K V T E 378
I T I P R R C Q V E E I S F A A H V D F Q E N L E F I E K I S A P N I I L V H Q E A N P M Q R L K S 441
I E L R K R V K L E Q K E L E E Y L E . K E K L . . . . K K E A A K K L E Q S K E A D I D S S D E S 423
A L L S N F . . A S L K Q T D N E V H V F N P R N C V E V D L E F Q Q V K V A K A V Q N I V N E I Y 489
D A E E D I D Q P S A H K T K H D L . M M K Q E Q S R K Q S F F K Q A K K S Y P M F P A P E E R I K 472
K E E N V E I K E E I A A K I E P I K E E N E D N L D S Q A E K G L V D E E E H K D I V V S Q I L . 539
W D E Y Q E I I K P E D F L V P E L Q A T E E E K . . S K L E S G L T N Q D E P M D Q D L S D V P T 520
. . V S D D K N F E . . . . . L D F L S L S D L R E H H P D L S T T I L R E R Q S V R V N C K K 579
K C I S T T E S I E I K A R V T Y I D Y E Q R S D Q D S I K K I I N Q M K P R Q L I I V H Q P P E A 570
E L I Y W H I L Q M F G E A E V . . . . . L Q D D D R V T N Q E P K V K E E S K D N L T N T Q K L 623
S Q D L A E C C R A F G Q K D I K V Y M P K L H E T V D A T S E T H I Y Q V R L K D S L V S S L Q F 620
I . . . . . L Q I M Q D I K L T I V N T L A V V E W T Q D L M N D T V A D S I I A I L M N V 664
C K A K D A E L A W I D Q V L D M R V S K V D T Q V I L E . . E Q E L K D D Q E D S E M Q V D A P S 660
D S A P A S V K . . L S S H S C D D H D H N N V Q S N A Q Q K I D E V . . . E R V K Q I S R L F K E 709
D S S V I A Q Q K A M K S L F Q D D E K E T Q E E S E I I P T L E P L P P H E V P Q H Q S V F M N E 718
Q F Q D C F T L F L N K D E Y A S N K E E T I T G V V T I Q K S T A . . K I D F N N M K I L E C N S 757
P R L S D F K Q V L L R E Q I . . . Q A E F V Q G V L V C N N Q V A V R R T E T Q R I Q L E Q C L C 765
N P L K Q R V E S L L N I Q Q N L V T P L C 779
Q D F Y . R I R D L L Y E Q Y A I V 782

Figure 2. *BRR5* is an essential gene.

Shown are the spore progeny from 12 tetrads of a sporulated *brr5::LEU2/BRR5* diploid strain (YSN378). This YEPD plate was incubated for 4 days at room temperature.

Figure 3. Timecourse of RNA splicing defects in *brr5-1* cells.

A. Growth curve.

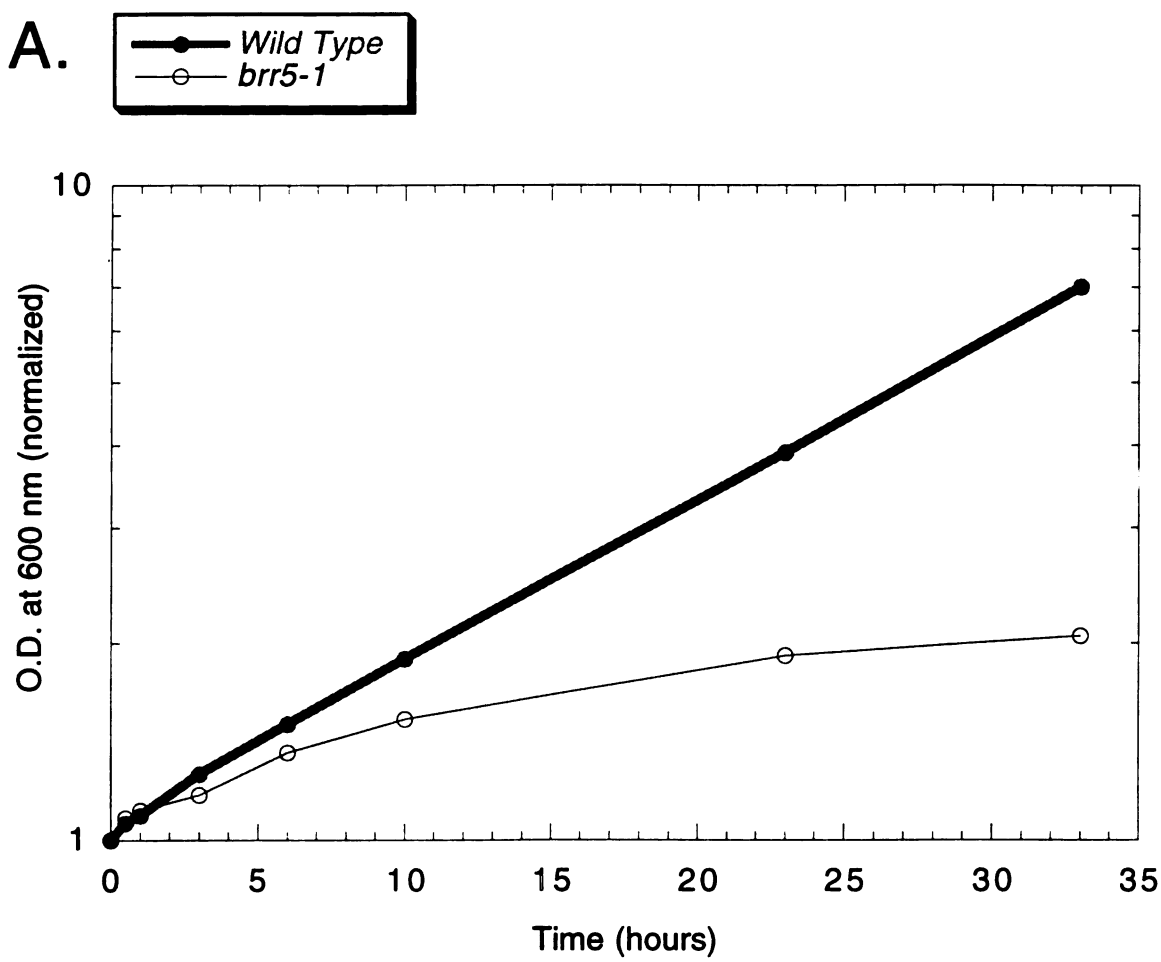
B. Primer-extension analysis of pre-U3 snoRNA splicing.

Total RNA was prepared from wild-type or *brr5-1* cells at the indicated times after a shift of to 15°C. This was analyzed by primer-extension using a ^{32}P-labeled oligonucleotide complementary to the second exon of U3 snoRNA.

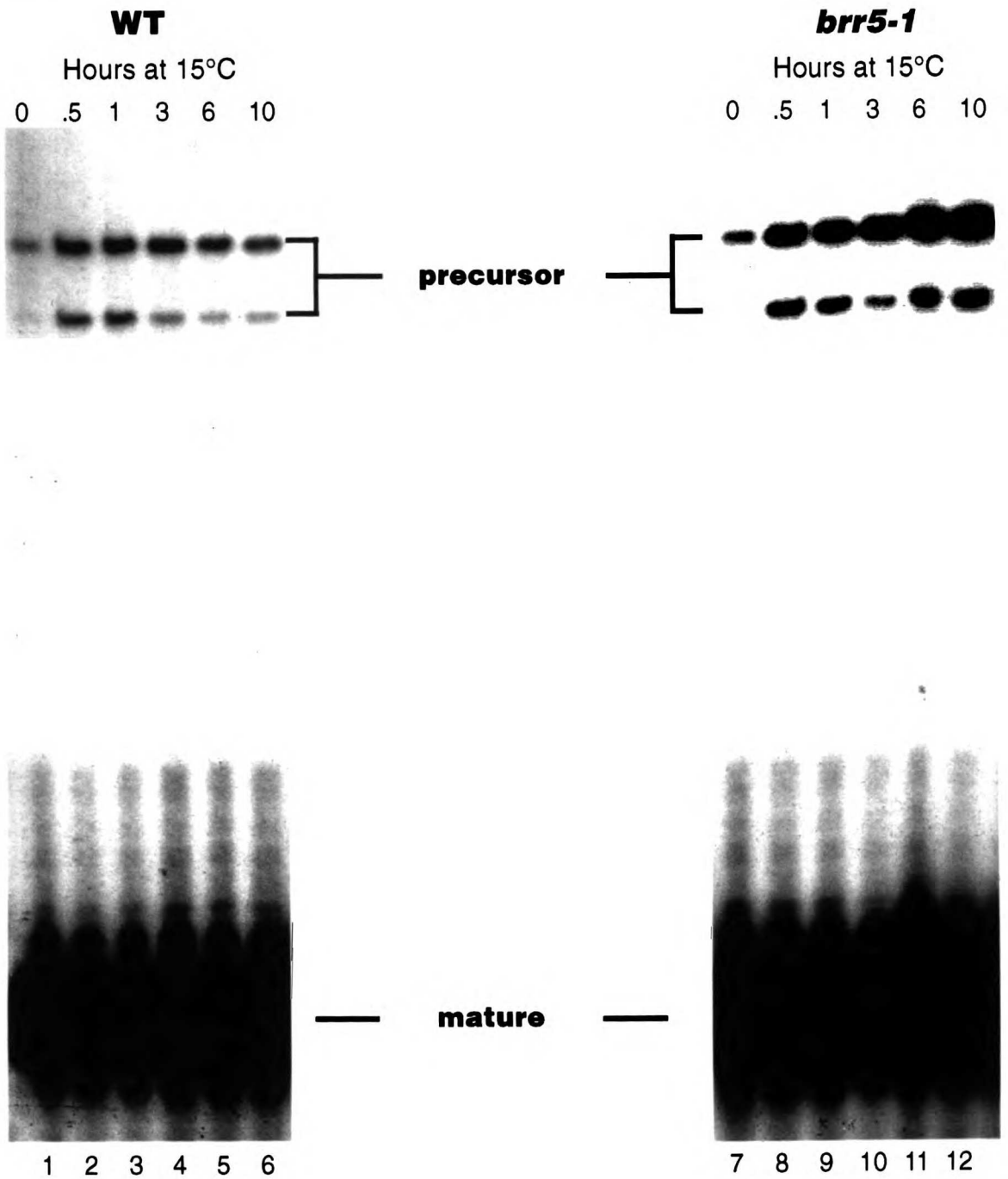
C. Primer-extension analysis of pre-*RP51A* splicing.

RNAs described in B were analyzed by primer extension using a primer complementary to the second exon of the *RP51A* gene.

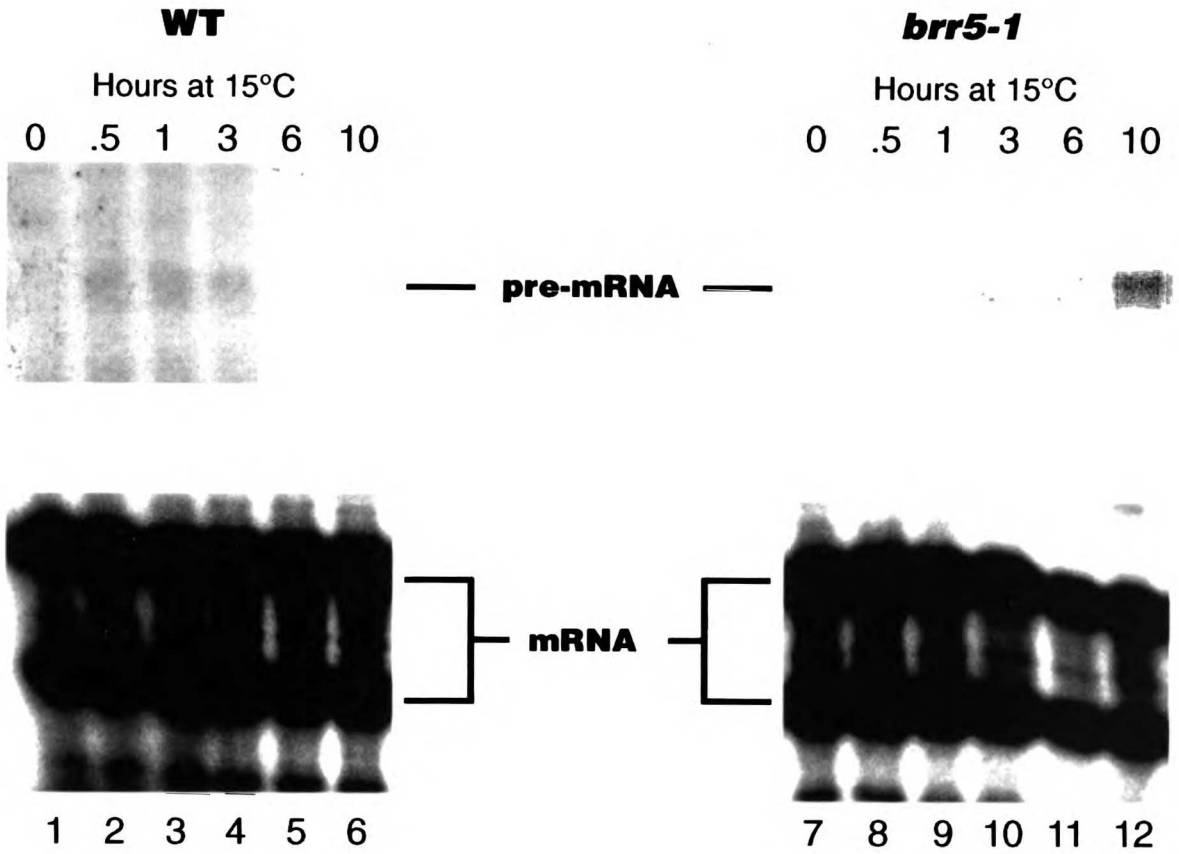
D. Quantitation of pre-U3 snoRNA splicing defect.


A.
Wild Type
brr5-1
10
1
O.D. at 600 nm (normalized)
0
5
10
15
20
25
30
35
Time (hours)

B.


WT
Hours at 15°C
0 .5 1 3 6 10
brr5-1
Hours at 15°C
0 .5 1 3 6 10
precursor
mature
1 2 3 4 5 6
7 8 9 10 11 12

c.


WT
Hours at 15°C
0 .5 1 3 6 10
brr5-1
Hours at 15°C
0 .5 1 3 6 10
pre-mRNA
mRNA
1 2 3 4 5 6
7 8 9 10 11 12

D.

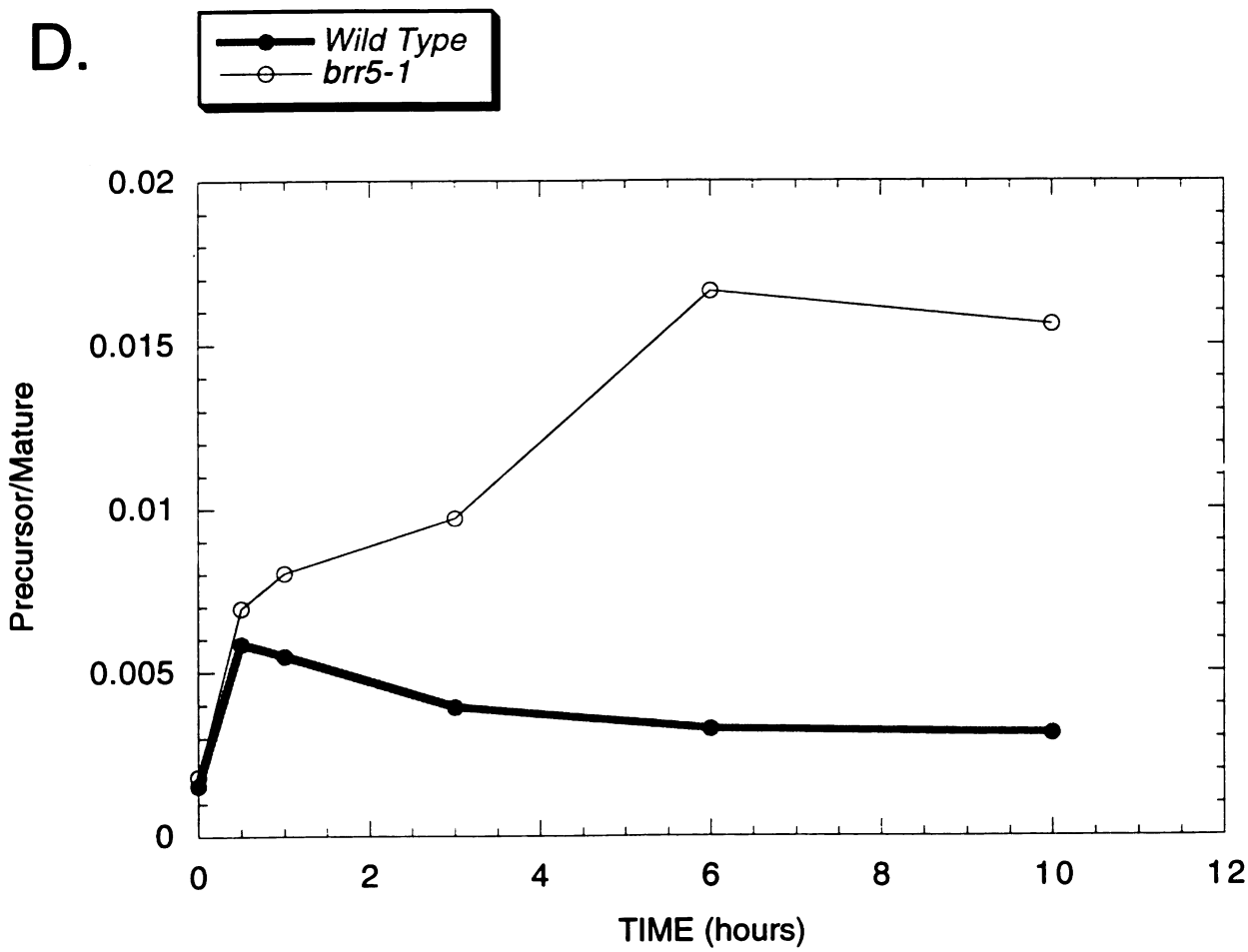
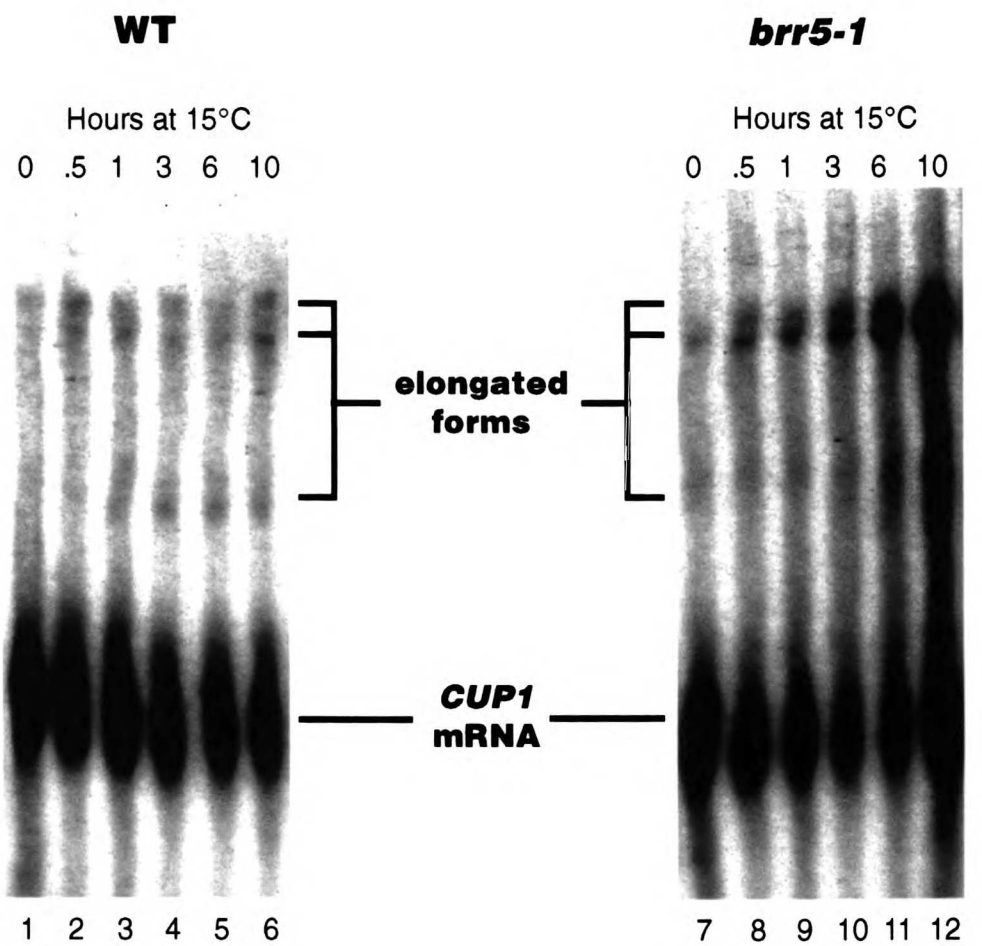

Wild Type
brr5-1
0.02
0.015
0.01
0.005
0
Precursor/Mature
0
2
4
6
8
10
12
TIME (hours)

Figure 4. Timecourse of 3' end processing defects in *brr5-1* cells.
Total RNA prepared from wild-type and *brr5-1* cells at the indicated times after a shift to 15°C was analyzed by Northern hybridization using a ^{32}P labeled probe complementary to the *CUP1* mRNA. Indicated are the positions of *CUP1* mRNA as well as elongated transcripts (Forrester et al., 1992).


WT
brr5-1
Hours at 15°C
0 .5 1 3 6 10
Hours at 15°C
0 .5 1 3 6 10
elongated forms
CUP1 mRNA
1 2 3 4 5 6
7 8 9 10 11 12

CHAPTER 4

EPILOGUE

Cold-sensitive mutants as a route to new types of components involved in RNA splicing

As I had hoped in embarking upon a screen for cold-sensitive pre-mRNA splicing mutants, the method proved to be efficient in identifying novel genes involved in this process. From a screen of 340 cs yeast mutants, I was able to identify single mutants in five novel genes, as well as seven new alleles of four previously identified *PRP* genes. One of the known genes, *PRP28*, has been isolated only in the cs form (underscoring that there are different hotspots for mutation to cold-sensitivity versus temperature-sensitivity), whereas the new alleles of *PRP22* and *PRP8* are the first reported cs alleles and may be useful biochemical tools in the further characterization of these splicing factors (for example, in characterizing novel intermediates which accumulate at low temperature). The success of the approach parallels that of Moir et al. (1982) in isolating cold-sensitive *cdc* mutants in yeast. Thus, it seems that the utilization of cs mutants is a generally useful strategy for identifying components of essential cellular processes.

The observation that *BRR1-BRR5* were isolated as single alleles suggests that the cs screen was nonsaturating and that further such screens would likely yield additional candidate splicing factors. Alternative methods for identifying such factors include screens of mutants with unconventional conditional lethal phenotypes such as deuterium sensitivity (Bartel and Varshavsky, 1988) or suppressible ochre mutations (Riles and Olson, 1988); such screens may enrich for different subsets of splicing factors. Directed screens for factors which interact genetically with known splicing components have been successful in identifying several new splicing genes as well as helping to define interactions among known *PRP* genes; in this

regard, the cs *brr* mutants are obvious targets for pseudoreversion screens, in which ts or high copy suppressors of the cs mutants could be sought. Finally, sequencing of the yeast genome will allow both the identification of yeast homologs for those mammalian splicing factors which have already been cloned as well as the testing of random ORFs for a potential role in splicing.

A novel family of RNA helicase-like proteins

Four of the genes identified in the cs screen encode splicing factors with strong homology to a superfamily of RNA helicase-like proteins, whose prototype is the translation initiation helicase, eIF4A (Rozen et al., 1990). Perhaps because of their structure (e.g. multiple conformational states, different states of nucleotide binding) or the types of processes in which they participate (e.g. helix unwinding, macromolecular assembly), these molecules appear to be particularly vulnerable to mutation to the cold-sensitive phenotype. Sequence examination of Brr2, the one novel protein in this class, revealed similarities with two other yeast proteins (Hfm1 and Ski2), two human proteins (HuSki2 and YYO2), and a *C. elegans* sequence (C28H8). The similarities extend well beyond the canonical helicase domain shared among all eIF4A superfamily members to include sequences both upstream and downstream of this region; together, these proteins define a new subfamily of helicase-like proteins, the DE(I/V)H family. As these proteins apparently participate in diverse cellular processes, the functional significance of their strong homology remains to be determined.

Brr2 is an essential gene which is required for the first step of splicing. Like the five previously characterized Prp proteins with homology to RNA helicases, Brr2 may function to mediate one of the multiple RNA-RNA rearrangements which are known to take place during the splicing cycle;

however, it is important to note that none of these factors has been demonstrated to possess helicase activity (see below). The most dramatic of these spliceosomal RNA rearrangements is the disruption of U4/U6 to allow formation of U2/U6, the putative catalytic center of the spliceosome (Madhani and Guthrie, 1992). Mutants of the previously characterized helicase superfamily members interfere with splicing at steps other than the U4/U6 to U2/U6 isomerization reaction and are therefore unlikely to mediate this important rearrangement (Cheng and Abelson, 1987; Company et al., 1991; Schwer and Guthrie, 1991; Strauss and Guthrie, 1991, Ruby et al., 1993). However, the independent identification of the *BRR2* gene by Xu and Friesen (personal communication) as a mutant which is synthetically lethal with a mutant U2 snRNA may place Brr2 precisely at this crucial transition point.

Much remains to be learned about Brr2. To test the hypothesis that Brr1 may be involved in U4/U6 helix destabilization, one must examine splicing and spliceosome assembly in the extracts depleted of Brr2 activity. For example, do spliceosomes assemble normally, or is there an aberrant intermediate, possibly similar to the active spliceosome but with U4 and U6 still base-paired? As for any helicase-like protein, one must characterize the properties of recombinant Brr2. Does it display RNA-dependent ATPase or helicase activities, as suggested by its sequence homology? Since none of the other helicase-like splicing factors has been demonstrated to possess helicase activity on generic RNA helices, the prevailing hypothesis is that these factors require a specific substrate and/or a protein cofactor for activity. To search for such cofactors and substrates, the cs allele can be used in general pseudoreversion screens (for example, looking for ts revertants or high copy suppressors of the cs growth phenotype) or in a more specific screen for potential snRNA substrates, employing pools of mutagenized snRNAs.

Mechanism of snRNP biogenesis in yeast

Brr1 is a novel mediator in the manufacture of spliceosomal snRNPs

As a secondary screen to help characterize the novel *BRR* genes, I examined snRNA levels at steady state in each of the mutants. The *brr1-1* mutant exhibited a striking decrease in abundance of each of the spliceosomal snRNAs, with no detectable change in levels of other small cellular RNAs. This observation led me to hypothesize that the natural function of wild-type Brr1 may be in the manufacture of snRNPs, with the observed first step splicing defect of *brr1-1* occurring as a secondary consequence either of snRNP depletion per se or of suboptimal activity of the residual snRNPs. The observation that overexpression of the common snRNP protein, Smd1, could suppress the *brr1* growth defect reinforced a possible connection to snRNP biogenesis, as did the finding that epitope-tagged Brr1 physically associates with each of the spliceosomal snRNAs. Ultimately, direct measurement of the stability of mature snRNPs (unchanged in *brr1-1* relative to wild-type cells) ruled out the alternative possibility that Brr1 serves only to maintain the stability of mature snRNPs.

Until recently, the study of snRNP biogenesis was performed solely in vertebrate systems (reviewed by Mattaj et al., 1993), where the greater abundance of snRNPs (approximately 100 to 1000 times more snRNPs per cell than in yeast) has made biochemical studies more straightforward than in yeast. A particular advantage of the *Xenopus* oocyte system is the ability to recapitulate snRNP biogenesis with radiolabeled snRNAs which are microinjected into the nucleus or cytoplasm of the oocyte. From a combination of pulse-chase, cell fractionation, and microinjection experiments, a pathway of biogenesis has emerged from the vertebrate

systems: first, snRNAs U1, U2, U4, and U5 are transcribed by RNA polymerase II in the nucleus. Next, in a manner dependent upon the 7methylG (7MG) cap and the Cap Binding Complex (proteins of 20 and 80 kD which bind to 7mG capped species in the nucleus; Izaurralde et al., 1995), the snRNAs are exported to the cytoplasm. In the cytoplasm, the snRNAs undergo several events, including association with the eight so-called Sm or common snRNP proteins, trimming of nucleotides at the 3' end (known to occur for U1, U2, and U4), and hypermethylation of the 7MG cap to a trimethyl guanosine (TMG) species. Extensive modifications of internal bases of the snRNAs also occurs at some point during the biogenesis cycle. Subsequently, the RNPs are imported into the nucleus, with snRNP-specific proteins adding just before, during, or shortly after nuclear import. All three cytoplasmic events play some role in building a signal for nuclear import, as snRNAs which fail to undergo association with Sm proteins (Mattaj and De Robertis, 1985), which are not hypermethylated (Hamm et al., 1990), or whose 3' ends are not trimmed (Neuman de Vegvar and Dahlberg, 1990) remain cytoplasmic.

In vertebrate cells, there has been little progress in identifying the protein factors which mediate the cytoplasmic events, the functions of individual Sm proteins, and the purpose of the many cytoplasmic reactions, beyond creating a snRNP which is generally competent for nuclear transport. The last point is more of an issue now that U6-specific Sm-like proteins have been identified, since U6 snRNP does not normally undergo a cytoplasmic phase and therefore does not undergo nuclear import (Séraphin, 1995; Cooper et al., 1995). In yeast, the study of snRNP biogenesis has been initiated only recently, as a consequence of genome sequencing efforts which have led to the identification of yeast homologs for several of the mammalian Sm proteins,

as well as two U6-specific Sm-like proteins (Séraphin, 1995; Cooper et al., 1995; Rymond, 1993; Roy et al., 1995). Although the ability of human Sm D1 protein to substitute for yeast Smd1 suggests that certain features of biogenesis must be conserved, until this study there had been no examination of the yeast pathway.

Transcriptional pulse-chase analysis of the snRNP biogenesis pathway

To determine how Brr1 might function in snRNP biogenesis, as well as to characterize the biogenesis pathway in yeast, I developed a method for monitoring newly synthesized snRNAs. This method involved expressing versions of the U2 and U4 snRNA genes that are under the control of the inducible and repressible *GAL1-GAL10* enhancer region. The encoded snRNAs could be distinguished from the continuously expressed endogenous snRNAs because of differences in size. By comparing the reactions of transcription, cap hypermethylation, and 3' end trimming (which had not been described previously in yeast) of snRNAs in wild-type and *brr1* mutant cells, I was able to determine that Brr1 is not required for the transcription or cap hypermethylation reactions per se (U4 snRNA), but that Brr1 function is required for efficient 3' end trimming (U2 snRNA). Thus, the transcriptional pulse-chase experiment offers a direct demonstration of a role for Brr1 in the manufacture of spliceosomal ribonucleoproteins, the first such demonstration for any factor in yeast.

Assuming that snRNP biogenesis occurs similarly in yeast and vertebrates (an assumption which may be flawed; see below) and that Brr1 functions at a step common to the biogenesis of U2 and U4 snRNPs, the obvious candidates for a Brr1-mediated reaction are snRNA export from the nucleus and Sm core assembly. Testing of these models should be performed

at several levels. Firstly, there has been no demonstration that snRNP biogenesis occurs cytoplasmically in yeast; the section below describes preliminary efforts to address that question. If yeast snRNA export does occur, then ideally one would test the hypothesis that depletion of Brr1 inhibits this reaction by comparing the rate of snRNA export in the *brr1::LEU2* disruption strain relative to wild type. This assay is likely to be very difficult technically, as precise nuclear-cytoplasmic yeast cell fractionation techniques have not yet been developed. Likewise, *in situ* hybridization of nascent snRNAs with specific probes is not yet an option. Direct assessment of snRNP assembly events should be easier to accomplish. The hypothesis that Brr1 functions to assemble the Sm core onto nascent snRNAs could be tested by comparing the rates of *de novo* snRNP assembly with *in vitro* transcribed snRNAs in *brr1* and wild type extracts. A simple test for a potential role in Sm core assembly is to determine whether Sm proteins such as Smd1 are associated with the unprocessed form of U2. If Brr1 is an assembly factor, one would predict that Sm proteins such as Smd1 (which I have epitope-tagged at its C-terminus) should be associated only with the processed U2. The opposite result, that accumulated unprocessed U2 is fully associated with Smd1, would suggest that Brr1 functions downstream of Smd1 assembly. One could perform a similar experiment--inducing U2 snRNA transcription for a substantial period of time (e.g. a few hours), in order to accumulate large amounts of the unprocessed and processed forms of the induced U2--to ask whether Brr1 and Smd1 are associated with the same or different populations of U2 snRNA. Finally, to pursue a possible conserved role for Brr1 in vertebrate snRNP biogenesis, we are collaborating with Reinhardt Lührmann to produce radiolabeled Brr1 protein (by *in vitro* transcription and translation); this reagent will be microinjected into

Xenopus oocytes in order to examine the ability of Brr1 to associate with vertebrate snRNPs.

A possible difference between snRNP biogenesis in yeast and vertebrates

Underscoring the possibility that some aspects of biogenesis may differ between yeast and vertebrates is my observation that one event of yeast biogenesis, 3' end trimming of U2 snRNA, appears to be unaffected by temperature inactivation of the mutant, *prp20-1* (S. Noble, unpublished). Cheng et al., (1995) have recently demonstrated that the mammalian homolog of Prp20, Rcc1, is required for export of snRNAs to the cytoplasm. As an indirect test of the hypothesis that yeast snRNP biogenesis occurs in the cytoplasm, I performed a heat shift of the temperature-sensitive mutant, *prp20-1*, and asked whether 3' end processing of U2 snRNA was inhibited. The protocol involved a pre-shift to 37°C for one hour, in order to inactivate cellular Prp20 molecules, followed by a continuous induction of galactose-regulated U2 snRNA, with samples being taken over the course of an additional 2 hours at 37°C. The surprising result was that mature, processed U2 snRNA accumulated in the *prp20-1* cells, although to a lower level than in wild-type control cells; further, there was no apparent increase in the ratio of unprocessed U2 to processed U2 in the mutant relative to wild type. There are two important caveats to this (negative) result. First, the levels of induced U2 were reduced in the *prp20-1* mutant relative to a wild-type control throughout the course of the experiment. This reduction may result from pleiotropic effects of the *prp20-1* mutation on transcription (Forrester et al., 1992), but one cannot rule out the possibility that a proportion of U2 is not processed and is degraded in the mutant. Second, although others have reported that *prp20* mutants are inactive for RNA transport within 30

minutes of a shift to 37°C (Forrester et al., 1992), I performed no secondary assays in the experiment to ascertain whether Prp20 had been inactivated at the start of the induction. A small proportion of functional Prp20 could have permitted sufficient transport to account for the modest level of processed U2 observed in *prp20*.

I controlled for the latter concern in a subsequent temperature shift experiment. In this experiment, *prp20-1* and control wild-type cells were shifted for 1.5 hours to 37°C before induction of U2 snRNA. Samples were taken before the temperature shift, at 1.5 hours after the shift, and 0.5 and 1 hour after induction of U2. The samples were then analyzed for localization of bulk polyadenylated mRNA by *in situ* hybridization with fluorescein-conjugated oligo-dT. The results were that, after 1.5 hours at 37°C, virtually 100% of the cells exhibited a dramatic mislocalization of polyadenylated message to one or a few spots in the nucleus, indicating that a shift of this duration is sufficient to inactivate the majority of mutant Prp20 molecules. In contrast, a *nup116* deletion strain which was shifted in parallel had only begun to exhibit mRNA localization defects by the end of the shift, underlining the importance of this control.

Although these experiments were imperfect, the suggestion that Prp20 may be dispensible for processing of U2 snRNA in yeast is consistent with the possibility that yeast snRNPs do not undergo a cytoplasmic phase in their biogenesis. In the future, one should initially repeat these experiments, utilizing RNA transport mutants which do not affect transcription; *prp20-1* remains a useful control, however, as the analogous mutant (*rcc1*) is the only mutant demonstrated (in mammals) to inhibit snRNA export. Ideally, the same samples should be tested both for extent of processing and for RNA localization. Because clean nuclear-cytoplasmic fractionation of RNA species

is not feasible at this time in yeast, it will be difficult to demonstrate directly that snRNP biogenesis is nuclear, if this is in fact the case. Potentially, one could attempt a pulse-chase type of experiment, using *in situ* hybridization with probes specific to the induced snRNA to monitor localization over the course of biogenesis; however, this method would likely suffer from problems of signal detection. An alternative would be to adapt the permeabilized cell system developed by Schlenstedt et al. (1993) for snRNP biogenesis in yeast. A demonstration that biogenesis occurs at the same rate in semi-intact cells with or without supplemental cytoplasm would argue that snRNP biogenesis is a nuclear process in yeast.

Overexpression suppressors of brr1-1

With the idea of identifying additional factors involved in the snRNP biogenesis pathway, I performed a screen for overexpression suppressors of *brr1-1*. In addition to *BRR1* itself, I obtained three genes. The first of these was the yeast homolog of the human autoantigen, La. Mammalian La is thought to participate in transcriptional termination of RNA polymerase III transcripts, as well as polymerase release and recycling (Maraia et al., 1994). In addition, La may be involved in internal initiation of translation of poliovirus RNA and in inhibition of translation of HIV-1 transcripts (Svitkin et al., 1994a; 1994b). La binds to RNA sequences which end in polyuridine-OH (Stefano, 1984; interestingly, each of the yeast spliceosomal snRNAs ends in one [U5] or several U residues [U2, U4, U5, U6]). In addition, La has been shown to shuttle between the nucleus and cytoplasm, although at steady state it is mainly a nuclear protein (Bachmann et al., 1990).

In terms of RNA splicing, the most relevant information from the mammalian work is that La is associated with 3' elongated, cytoplasmic

precursors of U1 snRNA (Madore et al., 1984). This observation suggests that La may play a role in snRNP biogenesis, perhaps in shuttling snRNPs from the nucleus to the cytoplasm. If the step at which La acts is rate-limiting in snRNP biogenesis, then suppression of *brr1-1* could occur by increasing the flow of snRNAs through the biogenesis pathway. Alternatively, the less interesting possibility is that La suppresses indirectly, by binding fortuitously to the 3' ends of snRNAs and sequestering them from degradation enzymes. Although *LHP1* and the partially homologous *YCL37* are nonessential genes (either alone or in combination), it may be possible to detect a subtle role for La in yeast snRNP biogenesis. Three experiments can easily be performed. The first would be to examine snRNA levels in *brr1* cells in the presence and absence of overexpressed La protein; if suppression is acting via snRNP biogenesis, then snRNAs levels should be at least partially restored in the presence excess La. The second is to examine snRNA levels in a *LHP1/YCL37* gene disruption strain; snRNA levels may be diminished if La normally participates in snRNP biogenesis. The third is to induce expression of the *GAL*-U2 gene in a wild-type strain bearing an epitope-tagged version of La. If La participates in snRNP biogenesis, then it may be physically associated with the pre-U2 species.

The second suppressor, *SUB2*, suppresses only moderately well and bears homology to a mammalian member of the RNA helicase-like superfamily. The particular consensus sequence of this homolog represents another class of variants and defines the DECD subfamily. No functional information is known about the mammalian protein (p47 from rat), although it has been localized to the nucleus (Nair et al., 1992). Recently, a *Drosophila* homolog has been identified as an overexpression suppressor of a temperature-sensitive, doubly mutant strain of *S. pombe* which contains

defective copies of both the *WEE1* and *MIK1* genes (two inhibitors of mitosis); the mechanism of suppression is unknown (Warbrick and Glover, 1994). Given its localization in mammals, the ability of *SUB2* to suppress *brr1-1* could potentially occur at the level of nuclear export of snRNAs or import of snRNPs; many other less interesting possibilities are also plausible. To characterize *SUB2* further, an efficient approach would be to utilize a fragment of the *GAL*-regulated cDNA in a one-step gene disruption procedure, to produce an otherwise wild-type strain in which the endogenous copy of *SUB2* is disrupted but which contains a second, full length *SUB2* gene under the regulation of the *GAL* enhancer. As a quick assay for a potential role for Sub2 in snRNP biogenesis, such a strain could be shifted from galactose to glucose medium to deplete the Sub2 protein and then assessed for the abundance of spliceosomal snRNAs.

The third suppressor, *SUB3*, is homologous to one component of the mammalian basal transcription factor, TFIIH (specifically, the 34 KD subunit of BTF2). TFIIH in human cells is active not only in initiation of transcription but also in DNA mismatch repair and, perhaps, in translation (reviewed by Seroz et al., 1995). Although such a complex may have a fourth role in snRNP biogenesis, I suspect that suppression of *brr1-1* occurs at the level of transcription, increasing the abundance either of snRNAs or of mRNAs for biogenesis factors. A potential role in biogenesis could be elicited in the same manner as described above for *SUB2*.

Coupling of splicing and polyadenylation

Brr5, a yeast homolog of the mammalian AAUAAA recognition factor, CPSF

Sequence analysis of Brr5 revealed an unexpected homology to the 100kD component of the mammalian cleavage and polyadenylation

specificity factor, CPSF. The homology is moderate in strength (29% sequence identity; 39% similarity) but gains additional significance from the facts that the two proteins are almost identical in length and that the homology extends through their entire sequences. The *BRR5* gene was disrupted and found to be essential for viability, consistent with a role in splicing and/or polyadenylation.

The process of mRNA 3' end formation has been best studied in mammals (reviewed by Keller, 1995), in which pre-mRNAs bearing the AAUAAA sequence undergo endonucleolytic cleavage and polyadenylation reactions to form their mature 3' ends. *In vivo*, these reactions are tightly coupled; however, *in vitro*, they can be separated and the requirements for each step assessed independently. Three factors are required for the cleavage reaction: CPSF (which consists of four polypeptides) binds to the AAUAAA sequence; CStF (cleavage stimulatory factor, which consists of three polypeptides); and the poly(A) polymerase. After cleavage, the downstream cleavage product is rapidly degraded, CStF is thought to fall away from the assemblage, and CPSF stimulates the poly(A) polymerase to add about 10 adenosine residues to the 3' end of the upstream cleavage product. At this point, a new component, polyadenylate binding protein II (PAB II), binds to the oligoadenylate sequence and stimulates the CPSF-poly(A) polymerase complex to undergo a conversion from relatively low activity to very high and processive activity. Rapid polyadenylation continues until the poly(A) sequence is about 250 nucleotides in length, at which point the enzyme loses its processivity.

In yeast, the 3' end processing machinery is described less fully (reviewed by Keller, 1995), although the reaction has been reconstituted *in vitro* and seems to occur by the same general mechanism (i.e. via

endonucleolytic cleavage and polyadenylation reactions; Butler and Platt, 1988; Butler et al., 1990). Yeast do not retain the AAUAAA cleavage and polyadenylation sequence. Also, although yeast homologs exist for the poly(A) polymerase and for two components of CStF, the activities of these enzymes differ between the two systems. In mammals, poly(A) polymerase is required *in vivo* and *in vitro* for both steps of 3' end formation, whereas CStF is required only for cleavage; in yeast, poly(A) polymerase is required *in vitro* only for the polyadenylation reaction, and the CStF homologs (Rna14 and Rna15) are required for both steps.

BRR5 was identified because of a defect in the first step of splicing caused by the cs mutant allele, *brr5-1*. To determine whether *brr5-1* also results in defects in 3' end processing, I assessed the length of *CUP1* mRNAs in cells which had been shifted to low temperatures. This analysis was performed over a period of time, so that the onset of potential 3' end processing defects could be compared to the onset of splicing defects. The result was that *brr5-1* cells accumulate greatly elongated *CUP1* transcripts; these have been shown previously (Forrester et al., 1992) to be elongated at their 3' ends. Moreover, the onset of the 3' end formation defect paralleled that of the splicing defect, suggesting that the defects may be intimately coupled (as opposed to a primary 3' end processing defect leading to depletion of a splicing factor, or vice versa).

Potential Coupling Mechanisms

Connections between the pre-mRNA splicing apparatus and the polyadenylation machinery are well-documented in mammalian systems, though poorly understood. It is known that the presence of an upstream intron or 3' splice site can stimulate polyadenylation *in vivo* and *in vitro*;

conversely a downstream polyadenylation sequence stimulates splicing of an upstream, terminal intron. The mechanism of this coupling is not known, although components of U1 snRNP have been implicated (see Nesic and Maquat, 1994, and references therein).

No such association between polyadenylation and splicing had been previously recorded in yeast, and *BRR5* represents the first gene known to be required for both processes. Brr5 is therefore a reasonable candidate for a factor involved in coupling of splicing and polyadenylation. As described in Chapter 3, many experiments must be performed to ascertain whether coupling truly occurs in yeast and to understand what might be the role of Brr5 in this process. First, one must determine whether Brr5 is required for efficient splicing *in vitro*, as would be expected if the coupling model is correct. If such a defect is observed, then one can ask questions which address possible mechanisms of coupling: Is spliceosome assembly affected? Does Brr5 associate with U1 snRNP? Although the homology of Brr5 with CPSF 100 kD and the appearance of elongated *CUP1* transcripts in *brr5-1* cells argue fairly strongly for a role for Brr5 in 3' end formation, one would also like determine the role of Brr5 *in vitro*. Is processing inhibited in a *brr5-1* (or immunodepleted) extract, and at what step? Inhibition of both cleavage and polyadenylation would parallel the effects of CPSF depletion in mammalian extracts and would resemble the *in vivo* defects in yeast; however, as described above, the candidate yeast homologs for other mammalian 3' end processing factors do not behave as expected. If there is a processing defect, can it be complemented by one of the known processing factors? Is Brr5 present in one of the previously defined biochemical fractions? To address the specificity of the coupling, one should examine known splicing mutants for 3' end processing defects and other processing mutants for splicing defects

in vivo. Finally, there exist mutant versions of several yeast components which may interact synergistically with a defective splicing/polyadenylation factor. These include mutations in *SNR19* (encoding U1 snRNA), in *MUD1* (encoding a nonessential U1 snRNP protein which has been shown to interact with the polyadenylation apparatus in mammals), in *PAP1* (encoding the poly(A) polymerase), and in *RNA14* and *RNA15* (encoding the yeast CStF homologs). Interactions between *brr5-1* and any of these mutations would strengthen the idea that Brr5 normally functions to couple these related processes.

Conclusion

What began as a search for new factors involved in pre-mRNA has both met and exceeded my expectations. The cs screen proved to be efficient at retrieving mutations in splicing factors, new and previously known. All of these alleles are useful potential tools for both biochemical and genetic dissection of the processes in which they participate. Of the novel genes, *BRR2* is most like what I had originally hoped to identify. Brr2 probably functions within the spliceosome and is a candidate for the factor which mediates the U4/U6 to U2/U6 isomerization reaction. My thesis work has mostly focused on the Brr1 protein, which is the first factor in yeast to play a demonstrated role in the process of snRNP biogenesis. Brr5 relates splicing to yet another nuclear process, 3' end formation of mRNAs. Finally, mutants in the *BRR3* and *BRR4* genes have been isolated independently in a new screen of the cs collection of Moir et al. (1982), for mutants which accumulate bulk polyadenylated RNAs in the nucleus (de Bruyn Kops and Guthrie, unpublished). Thus, Brr3 and Brr4 may relate splicing to a fourth process, nuclear RNA export. This progression from splicing to other events in

nuclear RNA metabolism in my own work has paralleled exciting developments in each of these fields by our own and other labs, so that we now know enough about the basic processes to imagine what may be connecting them.

APPENDIX

A Cold-Sensitive Mutation in the Gene Encoding the snRNP Biogenesis Factor Brr1 is Suppressed by Overexpression of the Yeast La Protein Homolog, a Conserved Helicase-Like Protein, and a Putative Component of TFIIH.

ABSTRACT

Brr1 is a yeast protein involved in the manufacture of spliceosomal snRNPs. In the cold-sensitive (cs) mutant, *brr1-1*, steady state levels of snRNPs are reduced from 2-fold (U1, U2, U5, U6) to 10-fold (U4). The cs growth defect of *brr1-1* is partially alleviated by overexpression of the common snRNP protein, Smd1. This observation prompted us to design a screen for additional factors which interact with Brr1, reasoning that overexpression of other participants in the poorly characterized pathway of yeast snRNP biogenesis might also suppress the *brr1-1* growth defect. Utilizing a yeast library in which individual cDNAs are under control of the highly inducible *GAL1* promoter, we have isolated three suppressors of the *brr1-1* mutant (*SUB1*, *SUB2*, *SUB3*). *SUB1* is identical to *LHP1*, a nonessential yeast homolog of the human La autoantigen, which is thought to be an RNA polymerase III transcription termination factor. *SUB2* encodes a putative homolog of p47, a mammalian helicase-like protein of unknown function that exhibits nuclear localization. A *Drosophila* homolog of Sub2 and p47 has been identified as a high copy suppressor of a cell cycle mutant. Together, the three define a novel subfamily (DECD) of helicase-like proteins. *SUB3* encodes a protein homologous to the p34 chain of BTF2, a component of the mammalian basal transcription factor, TFIIH. The suppression of *brr1-1* by the overexpression of the *SUB* genes may be due to increases in 1) flux through the snRNP biogenesis pathway, 2) transcription of snRNA genes, or 3) other rate-limiting steps.

RESULTS AND DISCUSSION

The *brr1-1* cs mutant (YSN183) was transformed with a *URA3*-marked yeast cDNA library in which gene expression is regulated by the *GAL1* promoter (Liu et al, 1992). Approximately 10^6 transformants were plated on prechilled synthetic complete medium which included galactose and sucrose as the carbon sources and which lacked uracil. The number of transformants was calculated to represent the cDNA from a single copy message about 20 times. Plates were incubated at 17°C for 5 days. On each day of incubation, growth of transformants with the cDNA library was compared to that of control *brr1-1* cells which had been transformed with the vector, pSE360 (*URA3, CEN*; Elledge and Davis, 1989). Possibly because of the high density of transformants (10^4-10^5 per plate), it was difficult to distinguish faster growing colonies which exhibited true suppression from those which arose from normal variegation in transformant colony size (as represented by the pSE360 transformants). Therefore, the colonies were replica plated to fresh, prechilled plates and incubated at 16°C. After 3 days, patches which appeared to be growing faster than the rest were marked. Plates were incubated for 2 days longer, and at this time 100 faster growing colonies were streaked to prechilled YEP plates containing galactose and sucrose, to confirm the phenotype.

Particular attention was paid to those patches which were only moderately cold-resistant, as this is the phenotype of *brr1-1* containing cells which bear a *GAL* enhancer-regulated version of *SMD1* (S. Noble, unpublished. Interestingly, the ability of overexpressed *SMD1* to suppress the *brr1-1* growth defect depends on the extent of overexpression, with moderately overexpressed *SMD1*, driven by its own promoter on a *CEN*

vector, suppressing much better than the presumably higher level of expression produced by the *GAL-SMD1* construct). Patches containing fast-growing papillations were considered to be likely revertants and were not studied further. In total, 28 of the fast growing (like wild type) candidates (Overexpression Suppressor (OS) candidates 1-28) retained the cold-resistant phenotype upon retesting, as did 24 moderately fast growing (OS29-52) and 4 marginally fast growing candidates (OS53-56).

I determined whether the introduced genes were responsible for the cold-resistant phenotypes of the candidates by selecting for mitotic loss of the cDNA-containing plasmid. Because the vector used for overexpression contains the *URA3* gene, candidates OS1-56 were streaked to plates containing 5-fluoroorotic acid (5-FOA) in order to select for cells that lack *URA3* (Boeke et al., 1987). Colonies arising from these plates were assessed in parallel with their uracil prototrophic parents on galactose-containing media for growth at 18°C. Thirty-four candidates displayed clear plasmid-dependent suppression (i.e. they lost their cold-resistance upon passage on 5-FOA medium). These included 26 of the strong candidate suppressors, 8 of the moderate candidates, and one of the weak candidates.

Plasmids were isolated from each of the remaining candidates and retested for suppression of *brr1-1*. Plasmids rescued from 25 of the strongly cold-resistant candidates were able to confer cold-resistance at 17°C (the twenty-sixth could not be separated from a second plasmid contaminant and was discarded), as were 6 of the moderate suppressors and the remaining weak suppressor. A combination of restriction enzyme analysis and diagnostic polymerase chain reaction analysis were used to identify plasmids which encoded the *BRR1* gene, as well as to identify multiple isolates of suppressor cDNAs. cDNAs corresponding to *BRR1* were isolated 4 times

(OS8, 10, 11, 21; all strong suppressors); those corresponding to a second gene appeared 26 times (OS1, 2, 3, 4, 5, 9, 12, 13, 14, 15, 16, 17, 18, 20, 23, 24, 25, 26, 27, 28, 29, 30, 31, 36, 41, 52; mostly strong suppressors; named *SUB1* for SUppressor of *brr1*); a third gene was represented once (OS54; weak suppressor; named *SUB2*); and a fourth gene was also represented once (OS22; strong suppressor; named *SUB3*). Estimates of plasmid insert sizes were made from the restriction enzyme analysis: *BRR1* inserts were about 1.2-2.1 kb in length; *SUB1* inserts ranged from .95 (most common) to 4.5 (one example) kb; *SUB2* was about 1.5 kb; and *SUB3* about 2.4 kb.

The observation that almost all of the candidates which survived retesting came from the group of strongest suppressors, in addition to the fact that very few of the class proved to be revertants, suggests that it would have been wise to focus on this class and to have selected all of the strongly cold-resistant colonies for further analysis. The preponderence of *SUB1* isolates in this screen does not have an obvious significance; although this gene suppresses *brr1-1* very well (so that *SUB1* transformants would unlikely be missed), its frequent isolation could reflect a high mRNA copy number.

Candidates from each group were assessed for growth at 17°C on YEP plates containing either glucose or galactose plus sucrose as the carbon source. Interestingly, two of the cDNA classes (corresponding to *BRR1* and to *SUB3*) suppress the *brr1-1* cs growth defect even on plates containing glucose, whereas the remaining two classes (*SUB1* and *SUB2*) required galactose for their suppressor activity. Apparently, there must be sufficient expression of *GAL-BRR1* and of *GAL-SUB3* even on glucose medium to suppress.

The inserts for *SUB1*, *SUB2*, and *SUB3* were sequenced, and each of their ORFs was found to have homology with proteins previously described in the literature (Table 1). *SUB1* is identical to *LHP1*, which encodes the yeast

homolog of the human La protein (Yoo and Wolin, 1994). La is an autoantigen in a significant proportion of patients suffering from the rheumatologic diseases Systemic Lupus Erythematosis and Sjogren's Syndrome. Its normal cellular function is somewhat controvertial. Good evidence exists to implicate La in transcriptional termination of RNA polymerase III transcripts, as well as release of that enzyme for new rounds of synthesis (Maraia et al., 1994). However, La has also been implicated in several other processes. In the cytoplasm, La associates with a region of poliovirus RNA involved in the internal initiation of translation (Svitkin et al., 1994a). In addition, La associates with the leader sequence of HIV-1 RNA and alleviates translational repression (Svitkin et al., 1994b). The observation that La associates with 3' elongated, cytoplasmic precursors of U1 snRNA (Madore et al., 1984) is particularly intriguing in light of our observation that overexpression of a yeast La homolog suppresses *brr1-1*, a mutant in the snRNP biogenesis pathway.

Sub1/Lhp1 shares sequence homology with a second yeast protein, Ycl37, a protein of unknown function, which was identified as part of the yeast genome sequencing project (Swiss Prot P25567). The homology extends through the N-terminal third of Sub1/Lhp1, including the region just preceding its RNA recognition motif; this region has been shown to be important for specific RNA binding to oligouridylate among La homologs (Kenan et al., 1991). Because the *SUB1/LHP1* gene is not essential (Yoo and Wolin, 1994), we sought to make a double mutant with a *YCL37* disruption, to test the hypothesis that the two genes are functionally redundant. We used the *LEU2* gene to replace almost the entire open reading frame of *YCL37* in the plasmid pSN147. One-step gene replacement was accomplished by tranformation of the wild type diploid strain, JO226, with the linearized

disruption fragment. Integration to the correct chromosomal locus was diagnosed by screening leucine prototrophs via a whole yeast PCR procedure, using oligonucleotide primers against the *LEU2* gene and sequences flanking the *YCL37* gene. A *ycl37::LEU2/YCL37* diploid strain was sporulated and the resulting ascospores were dissected onto YEPD plates and incubated at 30°C (11 tetrads) or 25°C (10 tetrads). At both temperatures, the majority of ascospores germinated in a 4:0 alive:dead pattern (9 at 30°C and 8 at 25°C); concomitantly, leucine prototrophy:leucine auxotrophy segregated in a 2:2 pattern, demonstrating that the *YCL37* gene is not essential for viability. Subsequent examination of growth of the spore progeny at 17°C, 25°C, 30°C, and 37°C, however, revealed a subtle growth defect in the *ycl37* disruptants at all temperatures tested.

A *SUB1/LHP1* gene disruption construct was made, using a *URA3* gene flanked by direct repeats of bacterial *hisG* sequences to replace the majority of the coding sequence (Alani et al., 1987). This construct was used to replace one copy of *SUB1/LHP1* in diploid strain, YSN266, which is heterozygous for the *YCL37* disruption (*ycl37::LEU2/YCL37*). Integration of the disruption fragment at the correct chromosomal locus was diagnosed with a PCR procedure. Diploids were sporulated, dissected, and incubated at 30°C and 25°C. Most of the tetrads arose in 4:0 pattern of life:death, suggesting that the *sub1/ycl37* double disruption is viable. Spore progeny were streaked to YEPD plates and incubated at 17°C and 37°C; in each four-spore tetrad, progeny from two of the spores grew somewhat worse than the other two, and the severity of the defect was identical to that of the *ycl37* disruption alone. Thus, there are no apparent genetic interactions between the disrupted alleles of *SUB1/LHP1* and *YCL37*.

An important question is what is the mechanism of *brr1-1* suppression by overexpressed *SUB1/LHP1*. From the point of view of our initial goals in performing the screen, the interesting possibility is that Sub1 may normally participate in some aspect of snRNP biogenesis (although this role appears not to affect cell growth). By increasing the efficiency of snRNP manufacture at one point in the pathway, Sub1 could potentially compensate for the 2-10 fold decrease in spliceosomal snRNP species observed in the *brr1-1* mutant. Such suppression would not necessarily result in fully wild type levels of snRNPs, as depletion of individual snRNPs such as U1 is not detrimental to cells until levels are quite low (10% of wild type for U1; Liao et al., 1990). A simple experiment for the future is to examine snRNA levels of *brr1-1* cells which contain the *SUB1* suppressor.

A second straightforward experiment which could reveal a role for Sub1/Lhp1 in snRNP biogenesis would be to look for physical association of the protein with spliceosomal snRNAs. I have already constructed two different epitope-tagged versions of Sub1/Lhp1 for this purpose (see Materials and Methods). Because we suspect that Sub1/Lhp1 may associate transiently with elongated precursors of snRNAs (as has been demonstrated for U1 snRNA in HeLa cells; Madore et al., 1984), one should perform immunoprecipitation reactions using splicing extracts prepared from cells which contain an abundance of easily identified snRNA precursors. This can be accomplished by utilizing a *GAL1-GAL10* enhancer-regulated U2 snRNA which is different in size from the endogenous U2 (Madhani and Guthrie, 1992; Noble and Guthrie, manuscript in preparation).

Sub2 is homologous to p47, a rat helicase-like protein which localizes to the nucleus (Nair et al., 1992). A *Drosophila* homolog of Sub2 and p47 was isolated as a high copy suppressor of the *S. pombe* mitotic inhibitor double

mutant *wee1/mik1* (Warbrick and Glover, 1994). How this *Drosophila* sequence suppresses the cell cycle defect is unknown. These sequences are aligned in Figure 1. These closely related proteins define a new subfamily of the RNA helicase family, characterized by the variation of the DEAD box motif, DECD (Figure 1). This brings the number of eIF-4A-like helicase subfamilies to at least four, including the DEAD (reviewed in Schmid and Linder, 1992), DEAH (Company et al., 1991), DE(I/V)H (Noble and Guthrie, submitted), and now the DECD families; an alignment of the characteristic motifs of each subfamily is presented in Figure 2. Given the nuclear localization of p47, an intriguing possibility is that snRNP trafficking is defective in the *brr1-1* mutant, and that Sub2 suppresses the defect by increasing the rate of transport (alternatively, transport may be normal in *brr1-1* cells, but snRNPs may be more stable in the nucleus, so that increasing the rate of snRNP uptake into the nucleus effectively increases snRNP abundance).

Sub3 is homologous to the p34 subunit of human BTF2/TFIIH, a basal transciption factor for RNA polymerase II as well as a participant in the nucleotide excision repair apparatus (Humbert et al., 1994; an alignment is shown in Figure 3). The overall sequence identity is 29% over a 307 amino acid overlap. As the identity occurs through the length of the two proteins, Sub3 may well be the yeast homolog of p34. Suppression by Sub3 likely results from increased transcription of snRNAs (spliceosomal snRNAs except for U6 are polymerase II products) or, alternatively, of genes encoding biogenesis enzymes whose levels are rate-limiting. Once again, one should examine snRNA levels in *brr1-1* in the presence of the suppressor to determine whether suppression arises from restoring the abundance of snRNPs.

MATERIALS AND METHODS

Strains used in this study

YSN183 *MAT***a** *brr1-1 ura3-52 leu2 ade2 his3 lys2*

JO226 *MAT***a**/α *his3/his3 ura3/ura3 lys2/lys2 leu2/leu2 ade2/ade2*

YSN266 *MAT***a**/α *ycl37::LEU2/YCL37 his3/his3 ura3/ura3 lys2/lys2 leu2/leu2 ade2/ade2*

YCL37 gene disruption

A DNA fragment corresponding to *YCL37* was amplified from genomic DNA by a PCR technique, using primers CD7 3' (AAC CGC GGT GCA GTA GTA AGG GAA T) and CD7 5' (TCT GTC TCT AGG AGC TTG A) to generate a 3400 bp PCR fragment. This was digested with *Sac*II and *Kpn*I and subcloned into the Bluescript (-) vector, to create pSN143. (The PCR fragment has at least one mutation in it, as this fragment in a centromere-containing vector will not complement the *YCL37* disruption [S. Noble, unpublished observation]. However, as the purpose of this experiment was to disrupt the *YCL37* gene, such mutations were of no great concern.) Almost the entire *YCL37* coding region was eliminated from pSN143 in a *Bam*HI, *Eco*RV double digest. This fragment was ligated to a blunted *LEU2* gene fragment with *Hin*dIII (blunted with T4 DNA polymerase), *Bam*HI ends, to create pSN147. *Sac*II and *Sph*I were used to liberate the disruption fragment; this was transformed into the wild type diploid strain, JO226. Integrants at the correct chromosomal locus were detected with a PCR procedure used on whole yeast.

SUB1/LHP1 gene disruption

In order to have sufficient flanking DNA sequences to achieve efficient homologous recombination for the gene disruption construct, *SUB1/LHP1* was amplified from genomic DNA using the primers, OS1 5' (*Bam*HI; CCG GAT CCT TCA CCT TAA ACC CTT C) and OS1 3' (*Xho*I; TTC TCG AGG AAC AGC TTA TTG TGA TG). This 1060 base pair fragment was cloned into the Bluescript (-) vector via *Bam*HI and *Xho*I sticky ends. to create plasmid pSN142. All but the first 39 amino acids worth of coding sequence of was removed from the *SUB1/LHP1* gene on pSN142 by digestion with *Eco*RI and *Hpa*I. The resulting linear fragment was ligated to a *hisG-URA3-hisG fragment*, produced by digestion of pAS135 with *Bam*HI (blunted with T4 DNA polymerase) and *Eco*RI. The disruption fragment was released for integration with *Bam*HI and *Xho*I and transformed into ySN266 (*ycl37D::LEU2/YCL37*) in a one-step gene replacement procedure. Uracil prototrophs were screened for integration of the disruption fragment to the correct chromosomal locus by a PCR procedure on whole yeast colonies.

Epitope tags

All epitope-tags introduced via a *Not*I site inserted precisely at the C-terminus of *SUB1*.

pSN173: GFP-tagged *SUB1* on *GAL*-pRS316 (derived the original subclone of *SUB1*, with the *Not*I site of the polylinker destroyed by T4 DNA Polymerase [EagI remains intact]).

pSN181: GFP-tagged *SUB1* (*Eco*RI-*Sac*II fragment) on pRS306.

pSN182: HA3-tagged *SUB1* (*Eco*RI-*Sac*II fragment) on pRS306.

Accession Numbers

SUB3: GenBank Z49219.

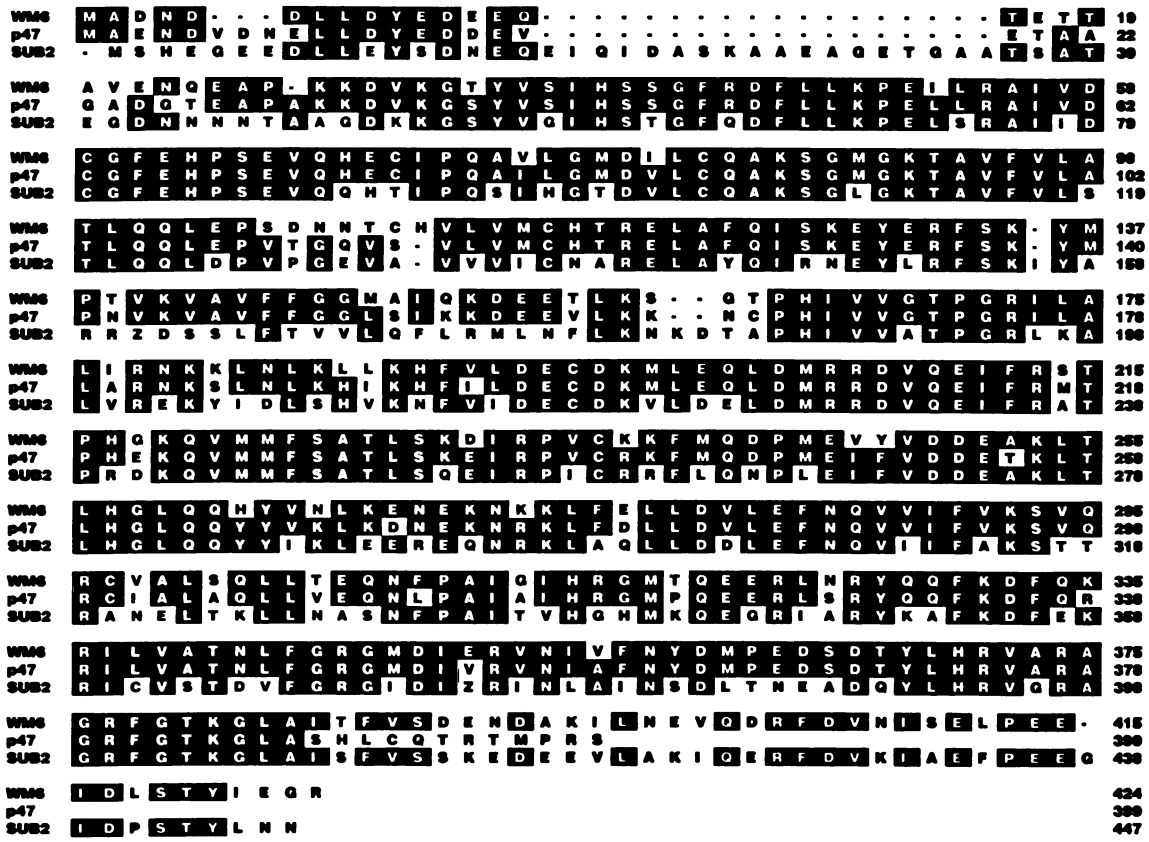


Figure 2. Comparison of conserved helicase motifs.

The conserved helicase motifs described by Gorbalenya et al. (1989) as they occur in Sub2 and in the DEAD, DEAH, and DE(I/V)H subfamilies are shown.

	MOTIF						
SUBFAMILY	I	IA	II	III	IV	V	VI
DECD	AKSGMGKT	HTRELA	VLDECDKML	FSATLS	FKDF	TNLFGRGMDIKR	HRVARAGR
DEAD	AXTGXGKT	PTRELA	VLDEADXML	XSATXP	FXXT	TXVXARGXDXXV	HRIGRXGR
DEAH	GETGSGKT	PRRVAA	MIDEAHERT	TSATMN	FLTG	TSLTIDGIRYVI	QRXGRAGR
DE(I/V)H	AXTSAGKT	PXKALX	IFDE(I/V)HYI	LSATVP	FSXG	TETXAMGVNKPA	QMXGRAGR

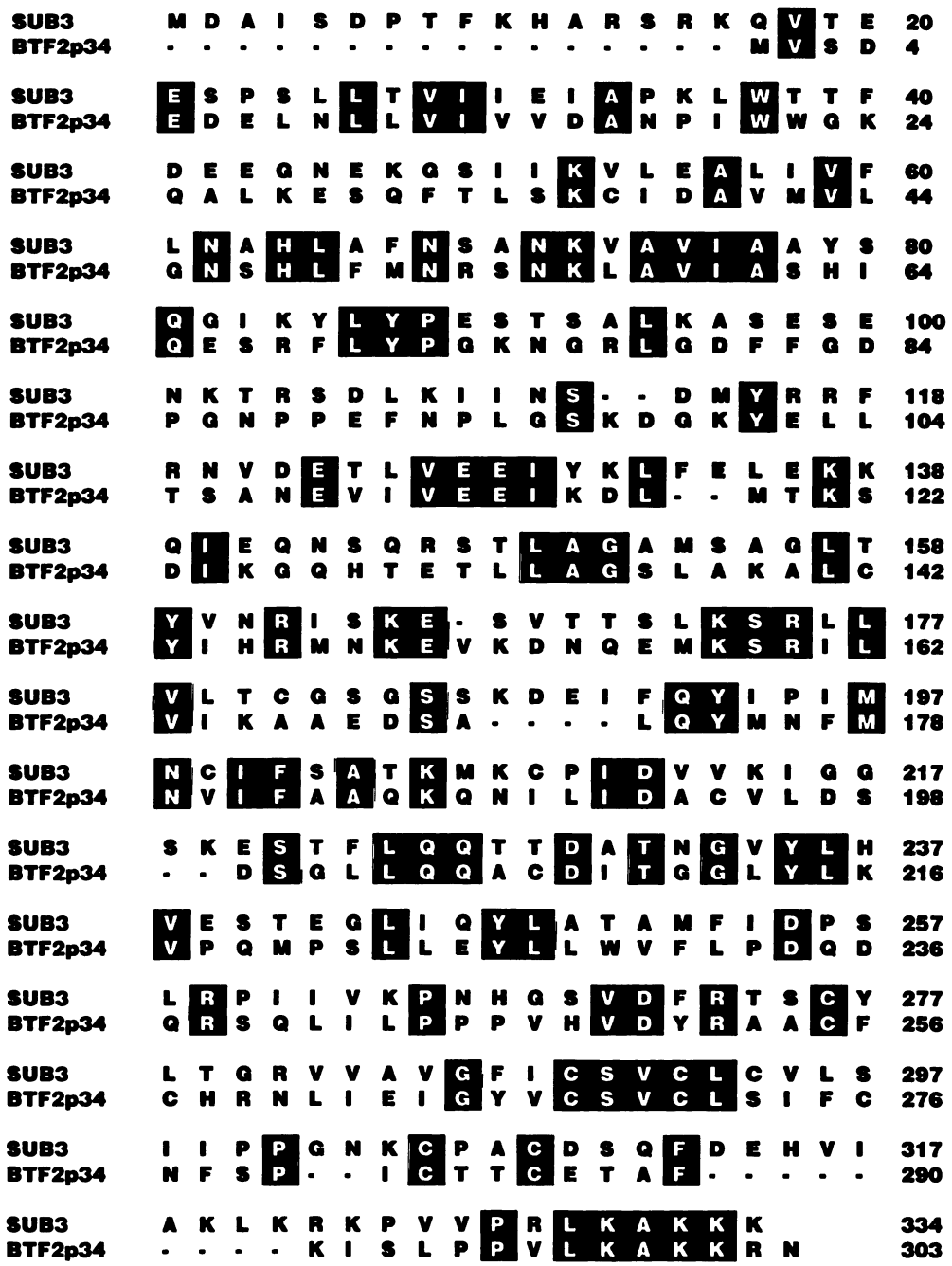

SUB3 M D A I S D P T F K H A R S R K Q V T E 20
BTF2p34 - - - - - - - - - - - - - - - - - M V S D 4
SUB3 E S P S L L T V I I E I A P K L W T T F 40
BTF2p34 E D E L N L L V I V V D A N P I W W Q K 24
SUB3 D E E Q N E K Q S I I K V L E A L I V F 60
BTF2p34 Q A L K E S Q F T L S K C I D A V M V L 44
SUB3 L N A H L A F N S A N K V A V I A A Y S 80
BTF2p34 Q N S H L F M N R S N K L A V I A S H I 64
SUB3 Q Q I K Y L Y P E S T S A L K A S E S E 100
BTF2p34 Q E S R F L Y P Q K N Q R L Q D F F Q D 84
SUB3 N K T R S D L K I I N S - - D M Y R R F 118
BTF2p34 P Q N P P E F N P L Q S K D Q K Y E L L 104
SUB3 R N V D E T L V E E I Y K L F E L E K K 138
BTF2p34 T S A N E V I V E E I K D L - - M T K S 122
SUB3 Q I E Q N S Q R S T L A G A M S A Q L T 158
BTF2p34 D I K Q Q H T E T L L A G S L A K A L C 142
SUB3 Y V N R I S K E - S V T T S L K S R L L 177
BTF2p34 Y I H R M N K E V K D N Q E M K S R I L 162
SUB3 V L T C Q S Q S S K D E I F Q Y I P I M 197
BTF2p34 V I K A A E D S A - - - - L Q Y M N F M 178
SUB3 N C I F S A T K M K C P I D V V K I Q Q 217
BTF2p34 N V I F A A Q K Q N I L I D A C V L D S 198
SUB3 S K E S T F L Q Q T T D A T N G V Y L H 237
BTF2p34 - - D S Q L L Q Q A C D I T G G L Y L K 216
SUB3 V E S T E Q L I Q Y L A T A M F I D P S 257
BTF2p34 V P Q M P S L L E Y L L W V F L P D Q D 236
SUB3 L R P I I V K P N H Q S V D F R T S C Y 277
BTF2p34 Q R S Q L I L P P P V H V D Y R A A C F 256
SUB3 L T Q R V V A V G F I C S V C L C V L S 297
BTF2p34 C H R N L I E I G Y V C S V C L S I F C 276
SUB3 I I P P G N K C P A C D S Q F D E H V I 317
BTF2p34 N F S P - - I C T T C E T A F - - - - - 290
SUB3 A K L K R K P V V P R L K A K K K 334
BTF2p34 - - - - K I S L P P V L K A K K R N 303

Table 1 Suppressors of *brr1-1*

Suppressor	Number of Isolates	Strength of Suppression	Galactose-Dependent Suppression ?	Sequence Homology	Plasmid used for sequencing
SUB1	26	Strong	Yes	Human La	pSN112
SUB2	1	Weak to Moderate	Yes	DECD family of putative RNA helicases	pSN120
SUB3	1	Strong	No	34kD subunit of the BTF2 component of TFIIH	pSN136

REFERENCES

Abovich, N., Liao, X.C. and Rosbash, M. (1994). The yeast MUD2 protein: an interaction with PRP11 defines a bridge between commitment complexes and U2 snRNP addition. Genes Dev *8*, 843-54.

Alani, E., Cao, L. and Kleckner, N. (1987). A method for gene disruption that allows repeated use of URA3 selection in the construction of multiply disrupted yeast strains. Genetics *116*, 541-5.

Bartel, B. and Varshavsky, A. (1988). Hypersensitivity to heavy water: a new conditional phenotype. Cell *52*, 935-41.

Batt, D.B., Rapp, L.M. and Carmichael, G.G. (1994). Splice site selection in polyomavirus late pre-mRNA processing. J Virol *68*, 1797-804.

Bennett, M. and Reed, R. (1993). Correspondence between a mammalian spliceosome component and an essential yeast splicing factor. Science *262*, 105-8.

Blanton, S., Srinivasan, A. and Rymond, B.C. (1992). PRP38 encodes a yeast protein required for pre-mRNA splicing and maintenance of stable U6 small nuclear RNA levels. Mol Cell Biol *12*, 3939-47.

Brosi, R., Gröning, K., Behrens, S.E., Lührmann, R. and Krämer, A. (1993). Interaction of mammalian splicing factor SF3a with U2 snRNP and relation of its 60-kD subunit to yeast PRP9. Science *262*, 102-5.

Butler, J.S. and Platt, T. (1988). RNA processing generates the mature 3' end of yeast CYC1 messenger RNA in vitro. Science *242*, 1270-4.

Butler, J.S., Sadhale, P.P. and Platt, T. (1990). RNA processing in vitro produces mature 3' ends of a variety of Saccharomyces cerevisiae mRNAs. Mol Cell Biol *10*, 2599-605.

Burgess, S., Couto, J.R. and Guthrie, C. (1990). A putative ATP binding protein influences the fidelity of branchpoint recognition in yeast splicing. Cell *60*, 705-17.

Champion-Arnaud, P. and Reed, R. (1994). The prespliceosome components SAP 49 and SAP 145 interact in a complex implicated in tethering U2 snRNP to the branch site. Genes Dev *8*, 1974-83.

Cheng, S.C. and Abelson, J. (1987). Spliceosome assembly in yeast. Genes Dev *1*, 1014-27.

Cheng, Y., Dahlberg, J.E. and Lund, E. (1995). Diverse effects of the guanine nucleotide exchange factor RCC1 on RNA transport. Science *267*, 1807-10.

Chiara, M.D., Champion-Arnaud, P., Buvoli, M., Nadal-Ginard, B. and Reed, R. (1994). Specific protein-protein interactions between the essential mammalian spliceosome-associated proteins SAP 61 and SAP 114. Proc Natl Acad Sci *91*, 6403-7.

Chiou, H.C., Dabrowski, C. and Alwine, J.C. (1991). Simian virus 40 late mRNA leader sequences involved in augmenting mRNA accumulation via multiple mechanisms, including increased polyadenylation efficiency. J Virol *65*, 6677-85.

Christianson, T.W., Sikorski, R.S., Dante, M., Shero, J.H. and Hieter, P. (1992). Multifunctional yeast high-copy-number shuttle vectors. Gene *110*, 119-22.

Company, M., Arenas, J. and Abelson, J. (1991). Requirement of the RNA helicase-like protein PRP22 for release of messenger RNA from spliceosomes. Nature *349*, 487-93.

Cooper, M., Johnston, L.H. and Beggs, J.D. (1995). Identification and characterization of Uss1p (Sdb23p): a novel U6 snRNA-associated protein with significant similarity to core proteins of small nuclear ribonucleoproteins. EMBO J. *14*, 2066-75.

Couto, J.R., Tamm, J., Parker, R. and Guthrie, C. (1987). A trans-acting suppressor restores splicing of a yeast intron with a branch point mutation. Genes Dev *1*, 445-55.

Cox, J.H. and Strack, H.B. (1971). Cold-sensitive mutants of bacteriophage lambda. Genetics *67*, 5-17.

Dammel, C.S. and Noller, H.F. (1993). A cold-sensitive mutation in 16S rRNA provides evidence for helical switching in ribosome assembly. Genes Dev *7*, 660-70.

Dammel, C.S. and Noller, H.F. (1995). Suppression of a cold-sensitive mutation in 16S rRNA by overexpression of a novel ribosome-binding factor, RbfA. Genes Dev *9*, 626-37.

Dangel, A.W., Shen, L., Mendoza, A.R., Wu, L.C. and Yu, C.Y. (1995). Human helicase gene SKI2W in the HLA class III region exhibits striking structural similarities to the yeast antiviral gene SKI2 and to the human gene KIAA0052: emergence of a new gene family. Nucleic Acids Res *23*, 2120-6.

Decker, C.J. and Parker, R. (1993). A turnover pathway for both stable and unstable mRNAs in yeast: evidence for a requirement for deadenylation. Genes Dev *7*, 1632-43.

Diehl, B.E. and Pringle, J.R. (1991). Molecular analysis of *Saccharomyces cerevisiae* chromosome I: identification of additional transcribed regions and demonstration that some encode essential functions. Genetics *127*, 287-98.

Elledge, S.J. and Davis, R.W. (1988). A family of versatile centromeric vectors designed for use in the sectoring-shuffle mutagenesis assay in *Saccharomyces cerevisiae*. Gene *70*, 303-12.

Forrester, W., Stutz, F., Rosbash, M. and Wickens, M. (1992). Defects in mRNA 3'-end formation, transcription initiation, and mRNA transport associated with the yeast mutation *prp20*: possible coupling of mRNA processing and chromatin structure. Genes Dev *6*, 1914-26.

Frank, D., Patterson, B. and Guthrie, C. (1992). Synthetic lethal mutations suggest interactions between U5 small nuclear RNA and four proteins required for the second step of splicing. Mol Cell Biol *12*, 5197-205.

Furth, P.A., Choe, W.T., Rex, J.H., Byrne, J.C. and Baker, C.C. (1994). Sequences homologous to 5' splice sites are required for the inhibitory activity of papillomavirus late 3' untranslated regions. Mol Cell Biol *14*, 5278-89.

Gallwitz, D. and Sures, I. (1980). Structure of a split yeast gene: complete nucleotide sequence of the actin gene in *Saccharomyces cerevisiae*. Proc Natl Acad Sci *77*, 2546-50.

Gorbalenya, A.E., Koonin, E.V., Donchenko, A.P. and Blinov, V.M. (1989). Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. Nucleic Acids Res *17*, 4713-30.

Gunderson, S.I., Beyer, K., Martin, G., Keller, W., Boelens, W.C. and Mattaj, L.W. (1994). The human U1A snRNP protein regulates polyadenylation via a direct interaction with poly(A) polymerase. Cell *76*, 531-41.

Guthrie, C. (1991). Messenger RNA splicing in yeast: clues to why the spliceosome is a ribonucleoprotein. Science *253*, 157-63.

Guthrie, C. and Fink, G.R. (1991) Guide to yeast genetics and molecular biology. (Academic Press, San Diego).

Guthrie, C. and Patterson, B. (1988). Spliceosomal snRNAs. Annu Rev Genet *22*, 387-419.

Hackl, W., Fischer, U. and Lührmann, R. (1994). A 69-kd protein that associates reversibly with the Sm core domain of several spliceosomal snRNP species. Journal of Cell Biology *124*, 261-72.

Hamm, J., Darzynkiewicz, E., Tahara, S.M. and Mattaj, I.W. (1990). The trimethylguanosine cap structure of U1 snRNA is a component of a bipartite nuclear targeting signal. Cell *62*, 569-77.

Harris, S.D., Cheng, J., Pugh, T.A. and Pringle, J.R. (1992). Molecular analysis of *Saccharomyces cerevisiae* chromosome I. On the number of genes and the identification of essential genes using temperature-sensitive-lethal mutations. J Mol Biol *225*, 53-65.

Harris, S.D. and Pringle, J.R. (1991). Genetic analysis of *Saccharomyces cerevisiae* chromosome I: on the role of mutagen specificity in delimiting the set of genes identifiable using temperature-sensitive-lethal mutations. Genetics *127*, 279-85.

Hartwell, L.H., McLaughlin, C.S. and Warner, J.R. (1970). Identification of ten genes that control ribosome formation in yeast. Mol Gen Genet *109*, 42-56.

Hartwell, L.H., Mortimer, R.K., Culotti, J. and Culotti, M. (1973). Genetic control of the cell division cycle in yeast: V. Genetic analysis of the mutants. Genetics *74*, 267-286.

Hermann, H., Fabrizio, P., Raker, V.A., Foulaki, K., Hornig, H., Brahms, H. and Lührmann, R. (1995). snRNP Sm proteins share two evolutionarily conserved sequence motifs which are involved in Sm protein-protein interactions. EMBO J. *14*, 2076-88.

Huang, M.T. and Gorman, C.M. (1990). Intervening sequences increase efficiency of RNA 3' processing and accumulation of cytoplasmic RNA. Nucleic Acids Res *18*, 937-47.

Humbert, S., van Vuuren, H., Lutz, Y., Hoeijmakers, J.H., Egly, J.M. and Moncollin, V. (1994). p44 and p34 subunits of the BTF2/TFIIH transcription factor have homologies with SSL1, a yeast protein involved in DNA repair. EMBO J. *13*, 2393-8.

Izaurralde, E., Lewis, J., Gamberi, C., Jarmolowski, A., McGuigan, C. and Mattaj, I.W. (1995). A cap-binding protein complex mediating U snRNA export. Nature *376*, 709-12.

Jarvik, J. and Botstein, D. (1975). Conditional-lethal mutations that suppress genetic defects in morphogenesis by altering structural proteins. Proc Natl Acad Sci *72*, 2738-42.

Jenny, A., Hauri, H.P. and Keller, W. (1994). Characterization of cleavage and polyadenylation specificity factor and cloning of its 100-kilodalton subunit. Mol Cell Biol *14*, 8183-90.

Johnson, A.W. and Kolodner, R.D. (1995). Synthetic lethality of *sep1* (*xrn1*) *ski2* and *sep1* (*xrn1*) *ski3* mutants of *Saccharomyces cerevisiae* is independent of killer virus and suggests a general role for these genes in translation control. Mol Cell Biol *15*, 2719-27.

Jones, M.H. and Guthrie, C. (1990). Unexpected flexibility in an evolutionarily conserved protein-RNA interaction: genetic analysis of the Sm binding site. EMBO J. *9*, 2555-61.

Kao, H.Y. and Siliciano, P.G. (1992). The yeast homolog of the U1 snRNP protein 70K is encoded by the SNP1 gene. Nucleic Acids Res *20*, 4009-13.

Keller, W. (1995). No end yet to messenger RNA 3' processing! Cell *81*, 829-32.

Keller, W., Bienroth, S., Lang, K.M. and Christofori, G. (1991). Cleavage and polyadenylation factor CPF specifically interacts with the pre-mRNA 3' processing signal AAUAAA. EMBO J. *10*, 4241-9.

Kenan, D.J., Query, C.C. and Keene, J.D. (1991). RNA recognition: towards identifying determinants of specificity. Trends Biochem Sci *16*, 214-20.

Kolodziej, P.A. and Young, R.A. (1991). Epitope Tagging and Protein Surveillance. in Guide to Yeast Genetics and Molecular Biology. Guthrie, C. and Fink, G.R., Eds. (Academic Press, San Diego) pp 508-519.

Konarska, M.M., Grabowski, P.J., Padgett, R.A. and Sharp, P.A. (1985). Characterization of the branch site in lariat RNAs produced by splicing of mRNA precursors. Nature *313*, 552-7.

Konarska, M.M. and Sharp, P.A. (1987). Interactions between small nuclear ribonucleoprotein particles in formation of spliceosomes. Cell *49*, 763-74.

Krämer, A., Legrain, P., Mulhauser, F., Gröning, K., Brosi, R. and Bilbe, G. (1994). Splicing factor SF3a60 is the mammalian homologue of PRP9 of S.cerevisiae: the conserved zinc finger-like motif is functionally exchangeable in vivo. Nucleic Acids Res *22*, 5223-8.

Krämer, A., Mulhauser, F., Wersig, C., Gröning, K. and Bilbe, G. (1995). Mammalian splicing factor SF320 represents a new member of the SURP family of proteins and is homologous to the essential splicing factor PRP21p of *Saccharomyces cerevisiae*. RNA 260-272.

Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol *154*, 367-82.

Lamond, A.I., Konarska, M.M., Grabowski, P.J. and Sharp, P.A. (1988). Spliceosome assembly involves the binding and release of U4 small nuclear ribonucleoprotein. Proc Natl Acad Sci *85*, 411-5.

Larkin, J.C. and Woolford, J.J. (1983). Molecular cloning and analysis of the CRY1 gene: a yeast ribosomal protein gene. Nucleic Acids Res *11*, 403-20.

Last, R.L., Maddock, J.R. and Woolford, J.L., Jr. (1987). Evidence for related functions of the RNA genes of *Saccharomyces cerevisiae*. Genetics *117*, 619-31.

Lehmeier, T., Foulaki, K. and Lührmann, R. (1990). Evidence for three distinct D proteins, which react differentially with anti-Sm autoantibodies, in the cores of the major snRNPs U1, U2, U4/U6 and U5. Nucleic Acids Res *18*, 6475-84.

Lesser, C.F. and Guthrie, C. (1993). Mutational analysis of pre-mRNA splicing in *Saccharomyces cerevisiae* using a sensitive new reporter gene, *CUP1*. Genetics *133*, 851-63.

Li, Z. and Brow, D.A. (1993). A rapid assay for quantitative detection of specific RNAs. Nucleic Acids Res *21*, 4645-6.

Liao, X.C., Tang, J. and Rosbash, M. (1993). An enhancer screen identifies a gene that encodes the yeast U1 snRNP A protein: implications for snRNP protein function in pre-mRNA splicing. Genes Dev *7*, 419-28.

Liao, X.L., Kretzner, L., Seraphin, B. and Rosbash, M. (1990). Universally conserved and yeast-specific U1 snRNA sequences are important but not essential for U1 snRNP function. Genes Dev *4*, 1766-74.

Lin, R.J., Newman, A.J., Cheng, S.C. and Abelson, J. (1985). Yeast mRNA splicing in vitro. J Biol Chem *260*, 14780-92.

Liu, H., Krizek, J. and Bretscher, A. (1992). Construction of a *GAL1*-regulated yeast cDNA expression library and its application to the identification of genes whose overexpression causes lethality in yeast. Genetics *132*, 665-73.

Liu, X. and Mertz, J.E. (1993). Polyadenylation site selection cannot occur in vivo after excision of the 3'-terminal intron. Nucleic Acids Res *21*, 5256-63.

Lockhart, S.R. and Rymond, B.C. (1994). Commitment of yeast pre-mRNA to the splicing pathway requires a novel U1 small nuclear ribonucleoprotein polypeptide, Prp39p. Mol Cell Biol *14*, 3623-33.

Lührmann, R. (1988). snRNP Proteins. in Structure and Function of the Major and Minor Small Nuclear Ribonucleoproteins. Birnstiel, M., Eds. (Springer-Verlag, Berlin) pp 71-99.

Lührmann, R., Appel, B., Bringmann, P., Rinke, J., Reuter, R., Rothe, S. and Bald, R. (1982). Isolation and characterization of rabbit anti-m3 2,2,7G antibodies. Nucleic Acids Res *10*, 7103-13.

Luo, Y. and Carmichael, G.G. (1991a). Splice site choice in a complex transcription unit containing multiple inefficient polyadenylation signals. Mol Cell Biol *11*, 5291-300.

Luo, Y. and Carmichael, G.G. (1991b). Splice site skipping in polyomavirus late pre-mRNA processing. J Virol *65*, 6637-44.

Lutz, C.S. and Alwine, J.C. (1994). Direct interaction of the U1 snRNP-A protein with the upstream efficiency element of the SV40 late polyadenylation signal. Genes Dev *8*, 576-86.

Madhani, H.D. and Guthrie, C. (1992). A novel base-pairing interaction between U2 and U6 snRNAs suggests a mechanism for the catalytic activation of the spliceosome. Cell *71*, 803-17.

Madhani, H.D. and Guthrie, C. (1994). Dynamic RNA-RNA interactions in the spliceosome. Annu Rev Genet *28*, 1-26.

Madore, S.J., Wieben, E.D. and Pederson, T. (1984). Eukaryotic small ribonucleoproteins. Anti-La human autoantibodies react with U1 RNA-protein complexes. J Biol Chem *259*, 1929-33.

Maraia, R.J., Kenan, D.J. and Keene, J.D. (1994). Eukaryotic transcription termination factor La mediates transcript release and facilitates reinitiation by RNA polymerase III. Mol Cell Biol *14*, 2147-58.

Masison, D.C., Blanc, A., Ribas, J.C., Carroll, K., Sonenberg, N. and Wickner, R.B. (1995). Decoying the cap- mRNA degradation system by a double-stranded RNA virus and poly(A)- mRNA surveillance by a yeast antiviral system. Mol Cell Biol *15*, 2763-71.

Mattaj, I.W. (1988). U snRNP Assembly and Transport. in Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles. Birnstiel, M., Eds. (Springer-Verlag, Berlin) pp 100-114.

Mattaj, I.W., Boelens, W., Izaurralde, E., Jarmolowski, A. and Kambach, C. (1993). Nucleocytoplasmic transport and snRNP assembly. Mol Biol Rep *18*, 79-83.

Mattaj, I.W. and De Robertis, E. (1985). Nuclear segregation of U2 snRNA requires binding of specific snRNP proteins. Cell *40*, 111-8.

Minvielle-Sebastia, L., Preker, P.J. and Keller, W. (1994). RNA14 and RNA15 proteins as components of a yeast pre-mRNA 3'-end processing factor. Science *266*, 1702-5.

Moir, D., Stewart, S.E., Osmond, B.C. and Botstein, D. (1982). Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudoreversion studies. Genetics *100*, 547-63.

Moore, C.L. and Sharp, P.A. (1984). Site-specific polyadenylation in a cell-free reaction. Cell *36*, 581-91.

Moore, M.J., Query, C.C. and Sharp, P.A. (1993). Splicing of Precursors to mRNA by the Spliceosome. in The RNA World. Gesteland, R.F. and Atkins, J.F., Eds. (Cold Spring Harbor Laboratory Press, Plainview, New York) pp 303-358.

Muhlrad, D., Decker, C.J. and Parker, R. (1994). Deadenylation of the unstable mRNA encoded by the yeast MFA2 gene leads to decapping followed by 5'-->3' digestion of the transcript. Genes Dev *8*, 855-66.

Munns, T.W., Liszewski, M.K., Tellam, J.T., Sims, H.F. and Rhoads, R.E. (1982). Antibody-nucleic acid complexes. Immunospecific retention of globin messenger ribonucleic acid with antibodies specific for 7-methylguanosine. Biochemistry *21*, 2922-8.

Murthy, K.G. and Manley, J.L. (1992). Characterization of the multisubunit cleavage-polyadenylation specificity factor from calf thymus. J Biol Chem *267*, 14804-11.

Myslinski, E., Segault, V. and Branlant, C. (1990). An intron in the genes for U3 small nucleolar RNAs of the yeast *Saccharomyces cerevisiae*. Science *247*, 1213-6.

Nair, S., Dey, R., Sanford, J.P. and Doyle, D. (1992). Molecular cloning and analysis of an eIF-4A-related rat liver nuclear protein. J Biol Chem *267*, 12928-35.

Nesic, D., Cheng, J. and Maquat, L.E. (1993). Sequences within the last intron function in RNA 3'-end formation in cultured cells. Mol Cell Biol *13*, 3359-69.

Nesic, D. and Maquat, L.E. (1994). Upstream introns influence the efficiency of final intron removal and RNA 3'-end formation. Genes Dev *8*, 363-75.

Neuman de Vegvar, H. and Dahlberg, J.E. (1990). Nucleocytoplasmic transport and processing of small nuclear RNA precursors. Mol Cell Biol *10*, 3365-75.

Ng, R. and Abelson, J. (1980). Isolation and sequence of the gene for actin in *Saccharomyces cerevisiae*. Proc Natl Acad Sci *77*, 3912-6.

Niwa, M. and Berget, S.M. (1991). Mutation of the AAUAAA polyadenylation signal depresses in vitro splicing of proximal but not distal introns. Genes Dev *5*, 2086-95.

Niwa, M., MacDonald, C.C. and Berget, S.M. (1992). Are vertebrate exons scanned during splice-site selection? Nature *360*, 277-80.

Niwa, M., Rose, S.D. and Berget, S.M. (1990). In vitro polyadenylation is stimulated by the presence of an upstream intron. Genes Dev *4*, 1552-9.

Pandey, N.B., Chodchoy, N., Liu, T.J. and Marzluff, W.F. (1990). Introns in histone genes alter the distribution of 3' ends. Nucleic Acids Res *18*, 3161-70.

Patterson, B. and Guthrie, C. (1987). An essential yeast snRNA with a U5-like domain is required for splicing in vivo. Cell *49*, 613-24.

Patterson, B. and Guthrie, C. (1991). A U-rich tract enhances usage of an alternative 3' splice site in yeast. Cell *64*, 181-7.

Patton, J.G., Porro, E.B., Galceran, J., Tempst, P. and Nadal-Ginard, B. (1993). Cloning and characterization of PSF, a novel pre-mRNA splicing factor. Genes Dev *7*, 393-406.

Pikielny, C.W. and Rosbash, M. (1985). mRNA splicing efficiency in yeast and the contribution of nonconserved sequences. Cell *41*, 119-26.

Ridley, S.P., Sommer, S.S. and Wickner, R.B. (1984). Superkiller mutations in *Saccharomyces cerevisiae* suppress exclusion of M2 double-stranded RNA by L-A-HN and confer cold sensitivity in the presence of M and L-A-HN. Mol Cell Biol *4*, 761-70.

Riles, L. and Olson, M.V. (1988). Nonsense mutations in essential genes of *Saccharomyces cerevisiae*. Genetics *118*, 601-7.

Rosbash, M., Harris, P.K., Woolford, J.J. and Teem, J.L. (1981). The effect of temperature-sensitive RNA mutants on the transcription products from cloned ribosomal protein genes of yeast. Cell *24*, 679-86.

Rose, M.D., Novick, P., Thomas, J.H., Botstein, D. and Fink, G.R. (1987). A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. Gene *60*, 237-43.

Roy, J., Zheng, B., Rymond, B.C. and Woolford, J.J. (1995). Structurally related but functionally distinct yeast Sm D core small nuclear ribonucleoprotein particle proteins. Mol Cell Biol *15*, 445-55.

Rozen, F., Edery, I., Meerovitch, K., Dever, T.E., Merrick, W.C. and Sonenberg, N. (1990). Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F. Mol Cell Biol *10*, 1134-44.

Ruby, S.W., Chang, T.H. and Abelson, J. (1993). Four yeast spliceosomal proteins (PRP5, PRP9, PRP11, and PRP21) interact to promote U2 snRNP binding to pre-mRNA. Genes Dev *7*, 1909-25.

Ruskin, B., Krainer, A.R., Maniatis, T. and Green, M.R. (1984). Excision of an intact intron as a novel lariat structure during pre-mRNA splicing in vitro. Cell *38*, 317-31.

Rymond, B. and Rosbash, M. (1992). Yeast Pre-mRNA Splicing. in The Molecular and Cellular Biology of the Yeast Saccharomyces. Jones, E.W., Pringle, J.R. and Broach, J.R., Eds. (Cold Spring Harbor Laboratory Press, Plainview, New York) pp 143-192.

Rymond, B.C. (1993). Convergent transcripts of the yeast PRP38-SMD1 locus encode two essential splicing factors, including the D1 core polypeptide of small nuclear ribonucleoprotein particles. Proc Natl Acad Sci *90*, 848-52.

Rymond, B.C., Rokeach, L.A. and Hoch, S.O. (1993). Human snRNP polypeptide D1 promotes pre-mRNA splicing in yeast and defines nonessential yeast Smd1p sequences. Nucleic Acids Res *21*, 3501-5.

Sambrook, J., Fritsch, E. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor Laboratory Press, Plainview, N.Y.).

Sauterer, R.A., Feeney, R.J. and Zieve, G.W. (1988). Cytoplasmic assembly of snRNP particles from stored proteins and newly transcribed snRNA's in L929 mouse fibroblasts. Exp Cell Res *176*, 344-59.

Schlenstedt, G., Hurt, E., Doye, V. and Silver, P.A. (1993). Reconstitution of nuclear protein transport with semi-intact yeast cells. J Cell Biol *123*, 785-98.

Schmid, S.R. and Linder, P. (1992). D-E-A-D protein family of putative RNA helicases. Mol Microbiol *6*, 283-91.

Schneider, J.C. and Guarente, L. (1991). Vectors for Expression of Cloned Genes in Yeast: Regulation, Overproduction, and Underproduction. in Guide to Yeast Genetics and Molecular Biology. Guthrie, C. and Fink, G.R., Eds. (Academic Press, San Diego) pp 373-388.

Schwer, B. and Guthrie, C. (1991). PRP16 is an RNA-dependent ATPase that interacts transiently with the spliceosome. Nature *349*, 494-9.

Scotti, P.D. (1968). A new class of temperature conditional lethal mutants of bacteriophage T4D. Mutat Res *6*, 1-14.

Ségault, V., Will, C.L., Sproad, B.S. and Lührmann, R. (1995). In vitro reconstitution of mammalian U2 and U5 snRNPs active in splicing: Sm proteins are functionally interchangeable and are essential for the formation of functional U2 and U5 snRNPs. EMBO J. *14*, 4010-4021.

Séraphin, B. (1995). Sm and Sm-like proteins belong to a large family: identification of proteins of the U6 as well as the U1, U2, U4 and U5 snRNPs. EMBO J. *14*, 2089-98.

Seroz, T., Hwang, J.R., Moncollin, V. and Egly, J.M. (1995). TFIIH: a link between transcription, DNA repair and cell cycle regulation. Curr Opin Genet Dev *5*, 217-21.

Shannon, K.W. and Guthrie, C. (1991). Suppressors of a U4 snRNA mutation define a novel U6 snRNP protein with RNA-binding motifs. Genes Dev *5*, 773-85.

Sheets, M.D., Ogg, S.C. and Wickens, M.P. (1990). Point mutations in AAUAAA and the poly (A) addition site: effects on the accuracy and efficiency of cleavage and polyadenylation in vitro. Nucleic Acids Res *18*, 5799-805.

Shuster, E.O. and Guthrie, C. (1988). Two conserved domains of yeast U2 snRNA are separated by 945 nonessential nucleotides. Cell *55*, 41-8.

Sikorski, R.S. and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics *122*, 19-27.

Smith, V. and Barrell, B.G. (1991). Cloning of a yeast U1 snRNP 70K protein homologue: functional conservation of an RNA-binding domain between humans and yeast. EMBO J. *10*, 2627-34.

Strauss, E.J. and Guthrie, C. (1991). A cold-sensitive mRNA splicing mutant is a member of the RNA helicase gene family. Genes Dev *5*, 629-41.

Svitkin, Y.V., Meerovitch, K., Lee, H.S., Dholakia, J.N., Kenan, D.J., Agol, V.I. and Sonenberg, N. (1994a). Internal translation initiation on poliovirus RNA: further characterization of La function in poliovirus translation in vitro. J Virol *68*, 1544-50.

Svitkin, Y.V., Pause, A. and Sonenberg, N. (1994b). La autoantigen alleviates translational repression by the 5' leader sequence of the human immunodeficiency virus type 1 mRNA. J Virol *68*, 7001-7.

Tai, P.C., Kessler, D.P. and Ingraham, J. (1969). Cold-sensitive mutations in *Salmonella typhimurium* which affect ribosome synthesis. J Bacteriol *97*, 1298-304.

Takagaki, Y. and Manley, J.L. (1992). A human polyadenylation factor is a G protein beta-subunit homologue. J Biol Chem *267*, 23471-4.

Takagaki, Y. and Manley, J.L. (1994). A polyadenylation factor subunit is the human homologue of the Drosophila suppressor of forked protein. Nature *372*, 471-4.

Teem, J.L. and Rosbash, M. (1983). Expression of a beta-galactosidase gene containing the ribosomal protein 51 intron is sensitive to the *rna2* mutation of yeast. Proc Natl Acad Sci *80*, 4403-7.

Vijayraghavan, U., Company, M. and Abelson, J. (1989). Isolation and characterization of pre-mRNA splicing mutants of *Saccharomyces cerevisiae*. Genes Dev *3*, 1206-16.

Villarreal, L.P. and White, R.T. (1983). A splice junction deletion deficient in the transport of RNA does not polyadenylate nuclear RNA. Mol Cell Biol *3*, 1381-8.

Warbrick, E. and Glover, D. (1994). A *Drosophila* gene encoding a DEAD box RNA helicase can suppress loss of wee1/mik1 function in Schizosaccharomyces pombe. Mol Gen Genet *245*, 654-7.

Wassarman, K.M. and Steitz, J.A. (1993). Association with terminal exons in pre-mRNAs: a new role for the U1 snRNP? Genes Dev *7*, 647-59.

Widner, W.R. and Wickner, R.B. (1993). Evidence that the SKI antiviral system of *Saccharomyces cerevisiae* acts by blocking expression of viral mRNA. Mol Cell Biol *13*, 4331-41.

Wise, J.A., Tollervey, D., Maloney, D., Swerdlow, H., Dunn, E.J. and Guthrie, C. (1983). Yeast contains small nuclear RNAs encoded by single copy genes. Cell *35*, 743-51.

Yang, H., Moss, M.L., Lund, E. and Dahlberg, J.E. (1992). Nuclear processing of the 3'-terminal nucleotides of pre-U1 RNA in Xenopus laevis oocytes. Mol Cell Biol *12*, 1553-60.

Yoo, C.J. and Wolin, S.L. (1994). La proteins from Drosophila melanogaster and *Saccharomyces cerevisiae*: a yeast homolog of the La autoantigen is dispensable for growth. Mol Cell Biol *14*, 5412-24.

Zavanelli, M.I. and Ares, M.J. (1991). Efficient association of U2 snRNPs with pre-mRNA requires an essential U2 RNA structural element. Genes Dev *5*, 2521-33.

Zavanelli, M.I., Britton, J.S., Igel, A.H. and Ares, M.J. (1994). Mutations in an essential U2 small nuclear RNA structure cause cold-sensitive U2 small nuclear ribonucleoprotein function by favoring competing alternative U2 RNA structures. Mol Cell Biol *14*, 1689-97.

Zieve, G.W. and Sauterer, R.A. (1990). Cell biology of the snRNP particles. Crit Rev Biochem Mol Biol *25*, 1-46.