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Cloning of the *RNA8* Gene of *Saccharomyces cerevisiae*, Detection of the RNA8 Protein, and Demonstration that It Is Essential for Nuclear Pre-mRNA Splicing

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Strains of *Saccharomyces cerevisiae* that bear the temperature-sensitive mutation *rna8-1* are defective in nuclear pre-mRNA splicing at the restrictive temperature (36°C), suggesting that the *RNA8* gene encodes a component of the splicing machinery. The *RNA8* gene was cloned by complementation of the temperature-sensitive growth defect of an *rna8-1* mutant strain. Integrative transformation and gene disruption experiments confirmed the identity of the cloned DNA and demonstrated that the *RNA8* gene encodes an essential function. The *RNA8* gene was shown to be represented once per *S. cerevisiae* haploid genome and to encode a low-abundance transcript of approximately 7.4 kilobases. By using antisera raised against β-galactosidase-RNA8 fusion proteins, the *RNA8* gene product was identified in *S. cerevisiae* cell extracts as a low-abundance protein of approximately 260 kilodaltons. Immunodepletion of the RNA8 protein specifically abolished the activity of *S. cerevisiae* in vitro splicing extracts, confirming that RNA8 plays an essential role in splicing.

The removal of introns from nuclear pre-mRNA is an essential step in eucaryotic gene expression and has been implicated as a mechanism for gene regulation (for recent reviews, see references 21, 46, and 51). The biochemical pathway of splicing has recently been elucidated and is similar in all eucaryotes investigated so far, although several important differences in detail exist which may reflect differences in the splicing machinery of various organisms, (1, 22, 27; references therein). Recent studies have indicated that nuclear pre-mRNA splicing takes place within a specific supramolecular assembly, the spliceosome, which has a sedimentation coefficient of approximately 40S to 60S (8, 17, 20). By biochemical analyses and fractionation of in vitro splicing systems, it has been shown that the spliceosome contains several small nuclear RNA molecules, presumably in the form of small nuclear ribonucleoprotein particles (for a review, see reference 39), and a number of other proteins (10, 11, 28).

Another approach to identifying splicing factors has taken advantage of the powerful combination of biochemical, classical genetic, and modern molecular approaches available with the simple eucaryote *Saccharomyces cerevisiae* (57). In particular, much attention has focussed on a set of temperature-sensitive mutations, *rna2* to *rna10/11*. These mutations, which form nine complementation groups, were initially isolated by Hartwell (23) and grouped on the basis of a general defect in RNA metabolism at the nonpermissive temperature (36°C). Although initial work indicated a defect in rRNA metabolism (24, 53, 62), it now appears that the primary defect in strains containing the *rna* mutations is at the level of nuclear pre-mRNA splicing. This defect is demonstrated by the observation that *rna* strains accumulate unspliced transcripts from nuclear-protein-encoding genes at the expense of mature mRNA when incubated at 36°C (18, 31, 42, 48, 59). Indeed, Teem and Rosbash (60) have shown that the mere presence of a yeast intron is sufficient to render a transcript sensitive to the *rna2* mutation. Furthermore, recent studies using extracts prepared from *rna* mutant cells have indicated that the *RNA2*, *3*, *4*, *5*, *7*, *8*, and *11* gene products are required for splicing in vitro (37) and that all but the *RNA2* gene product are required for spliceosome formation (36). It is likely, therefore, that the *RNA* gene products interact with or are part of the yeast splicing machinery.

One approach to investigating the functions of the *RNA* gene products is to clone their genes; this may provide a means by which the *RNA* gene products can be identified and subsequently characterized. To date, the *RNA2*, *3*, *4*, and *11* genes have been cloned (32, 35, 55; D. Shore, personal communication), and sequencing and immunological studies have indicated that each encodes a protein (33, 34; S. Petersen-Bjorn and J. Friesen, personal communication; D. Shore, personal communication). Antisera raised against the RNA2 and RNA3 proteins have allowed the localization of these two proteins to the yeast cell nucleus (33). As yet, however, no specific function has been ascribed to an *RNA* gene product. In this paper, we describe cloning and characterization of the *RNA8* gene, immunological detection of the RNA8 protein, and demonstration that this protein is essential for pre-mRNA splicing in vitro.

MATERIALS AND METHODS

Enzymes. Restriction endonucleases were purchased from Amersham International, Boehringer Mannheim Biochemicals, New England BioLabs, Inc., and Pharmacia. *Escherichia coli* DNA polymerase Klenow fragment and T4 DNA ligase were purchased from Boehringer Mannheim. T7 RNA polymerase was purchased from Pharmacia. All enzymes were used according to the recommendations of the manufacturer.

Strains and microbiological procedures. The following *E. coli* strains were used. HB101 (7) was used for cloning and amplification of plasmid DNAs. BMH71-18 (41) was used for expression of β-galactosidase-RNA8 fusion proteins. The following *S. cerevisiae* strains were used. DBY747 (**a** *his3 leu2-3 leu2-112 trp1-289 ura3-52*) was from D. Botstein, Massachusetts Institute of Technology. BJ2412 (α/**a** *gal2*

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leu2 pep4-3 prb1-1122 prc1-407 trp1 ura3-52) was from E. Jones, Carnegie Mellon. SPJ8.3 (**a** *ade leu2 ura3-52 rna8-1*) was obtained by twice outcrossing *ts*219 (24). SPJO.2831 (α/**a** *ade*/+ *leu2*/*leu2 his3*/+ *ura3-52*/*ura3-52 rna8*/+) was also used.

S. cerevisiae strains were propagated as described by Beggs (3) and Sherman et al. (52). Sporulation and other standard yeast genetic techniques were as described by Mortimer and Hawthorne (44) and Sherman et al. (52). Yeast transformation was by the procedure of Beggs (3) as modified by Lee et al. (35). Temperature-sensitive strains were maintained at 24°C throughout. After being plated (in uracil-deficient medium to select for the *URA3* plasmid marker), transformants of *rna8-1* strains were first incubated at 24°C for 24 h before being transferred to 36°C (to allow expression of plasmid-borne genes before the cells were exposed to the nonpermissive temperature).

Plasmids. YEp24 (6), YCp50, and YIp5 (58) and the YEp24-based (9) and YCp50-based *Sau*3A libraries of *S. cerevisiae* DNA were provided by D. Botstein. pJDB207 has been described (4). pUR290, pUR291, and pUR292 (50) were provided by D. Lane. pSPT18 and pSPT19 were from Pharmacia. pYA301 was provided by D. Gallwitz and contains the *S. cerevisiae* actin gene subcloned from pYA208 (19).

Nucleic acid procedures. Standard cloning techniques were as described by Maniatis et al. (38). Plasmids in *E. coli* cells were analyzed by using the rapid extraction method of Birnboim and Doly (5). DNA restriction fragments were purified from agarose by the method of Vogelstein and Gillespie (61). ^{32}P-labeled DNA probes were generated by using the random priming procedure of Feinberg and Vogelstein (15, 16). ^{32}P-labeled single-stranded RNA was prepared by transcription of linearized pSPT18- or pSPT19-based plasmid DNAs, with bacteriophage T7 RNA polymerase (13), by using the conditions suggested by Pharmacia. Small quantities of yeast DNA were prepared by the method of Davis et al. (14), and large quantities were prepared as described by Cryer et al. (12). RNA was extracted from yeast cells as described by Hopper et al. (26), and polyadenylated RNA was purified by affinity chromatography on oligo(dT)-cellulose (2). For Northern blot (RNA blot) hybridization analysis, RNA samples (and DNA size markers) were denatured with glyoxal (by the procedure of McMaster and Carmichael [40], except that formamide was substituted for dimethyl sulfoxide) and electrophoresed through agarose in 10 mM sodium phosphate (pH 7.0). RNA was transferred to GeneScreen membranes (New England Nuclear Corp.) by blotting with 1× SSC (1× is 0.15 M NaCl plus 0.015 M sodium citrate). Hybridization and washing procedures were performed as suggested by New England Nuclear. Southern blot-hybridization analysis was carried out as described by Southern (56), with transfer of DNA from agarose gels to GeneScreen Plus membranes (New England Nuclear) in 10× SSC. Hybridization and washing procedures were done as suggested by New England Nuclear.

Protein and immunological procedures. Fractionation of proteins by sodium dodecyl sulfate (SDS)-discontinuous polyacrylamide gel electrophoresis was as described by Laemmli (30). Production of β-galactosidase and β-galactosidase-RNA8 fusion proteins was performed according to the method of Mole and Lane (43) by using *E. coli* BMH71-18. Labeling of yeast proteins with L-[^{35}S]methionine in vivo, extraction of yeast proteins, and immunoprecipitation procedures were as described by Lee et al. (34). For immunization procedures, female rabbits were injected subcutaneously at monthly intervals; each injection contained approximately 75 μg of gel-purified fusion protein as a 1:1 emulsion with Freund complete adjuvant (first injection) or Freund incomplete adjuvant (second and subsequent injections). Antisera for immunoprecipitation experiments were obtained 12 to 14 days after the fourth and subsequent injections.

In vitro splicing reactions. Whole-cell extract of *S. cerevisiae* BJ2412 was prepared as described by Lin et al. (36). ^{32}P-labeled substrate RNA was prepared by in vitro transcription of linearized pSPT19 carrying the 544-base-pair *Alu*I DNA fragment of the yeast actin gene, including the 309-nucleotide intron and flanking sequences from exons 1 and 2. The transcription reaction mixture with T7 RNA polymerase was incubated at 37°C for 15 min, and transcripts were used for in vitro splicing reactions without purification. For immunodepletion experiments, protein A-Sepharose CL-4B (Sigma Chemical Co.) was preswollen in 150 mM NaCl–50 mM Tris hydrochloride (pH7.5)–0.1% (vol/vol) Nonidet P-40 (NTN buffer) and titrated for its immunoglobulin-binding capacity. Protein A-Sepharose (25- to 30-μl packed volume of swollen beads) was mixed with preimmune or immune serum (40 μl) and NTN buffer (final volume, 300 μl) and incubated at 4°C for 12 to 16 h. The Sepharose beads were washed by pelleting and suspension three times in NTN buffer (1 ml) and three times in splicing extract buffer without dithiothreitol (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-K^+ [pH 7.0], 0.2 mM EDTA, 50 mM KCl, 20% [vol/vol] glycerol). Excess liquid was removed from the Sepharose beads, and splicing extract (12 μl) was added and incubated with mixing for 3 to 4 h at 4°C. Immune complexes bound to the protein A-Sepharose beads were sedimented (12,000 × *g* for 2 min), and the supernatant extract was assayed for splicing activity at 25°C for 30 min as described by Lin et al. (36).

RESULTS

Isolation of plasmids that suppress the *rna8-1* mutation. Recombinant plasmids p8500 and p8000 were isolated from YCp50- and YEp24-based yeast DNA libraries, respectively, by the ability to complement the temperature-sensitive growth defect of the *rna8-1* mutant strain SPJ8.3. p8500 and p8000 were subsequently shown to be capable of suppressing the temperature-sensitive growth defects of other *rna8-1* mutant strains but not those of *rna2-1*, *rna3-1*, *rna4-1*, *rna5-1*, *rna6-1*, or *rna11-1* strains (no other *rna* mutants were tested).

Restriction enzyme analysis indicated that p8500 contained a yeast DNA insert of approximately 12 kilobases (kb), which encompassed the approximately 9-kb insert of p8000 (Fig. 1). To more precisely define the limits of the *rna8-1*- complementing sequence, regions of the yeast DNA inserts of p8500 and p8000 were subcloned into yeast replicating plasmids and then tested for the ability to suppress the temperature sensitivity of strain SPJ8.3 (assayed by selection for transformants at 36°C). The results are presented in Fig. 1. The inability of p8133, p8030, and p8530 to suppress the *rna8-1* phenotype showed that the complementing region crossed the *Sal*I site, whereas the inability of p85233, p8022, p8122, and p8520 to suppress temperature sensitivity showed that the complementing region also crossed the right-most *Bam*HI site. Taken together, these data indicated that the minimum size for the complementing sequence was 5.0 kb. The ability of p85888 to suppress the *rna8-1* mutation showed that all sequences necessary for complementation were present within the 7.5-kb insert of this plasmid.

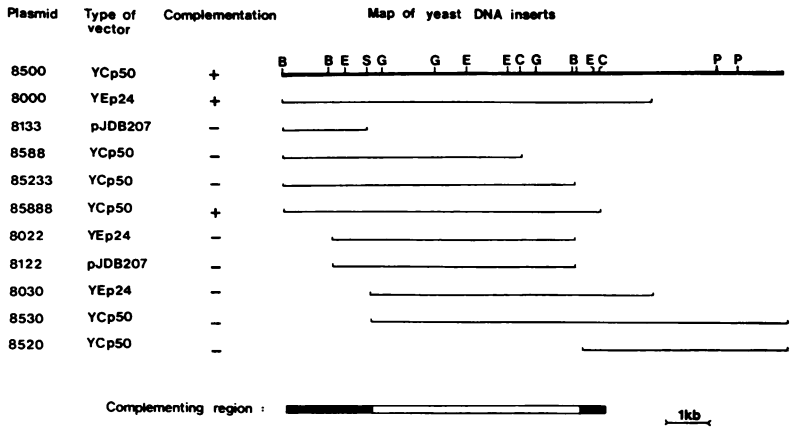

FIG. 1. Subcloning-complementation analysis. Restriction map of the yeast DNA insert of p8500 and complementation data for plasmids that carry portions of this insert. Symbols: + and −, ability and inability, respectively, to suppress the temperature-sensitive growth defect of *rna8-1* strain SPJ8.3 (determined by selection for transformants at 36°C). Restriction enzyme sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; G, *Bgl*II; P, *Pvu*II; S, *Sal*I. The region that confers complementation of the *rna8-1* mutation is indicated. (▬, ends of the complementing region).

Consistent with a defect in pre-mRNA splicing at the nonpermissive temperature, yeast strains that carry the *rna8-1* mutation accumulate unspliced transcripts of nuclear-protein-encoding genes and become depleted in the corresponding mature mRNA (31). The accumulation of unspliced actin pre-mRNA at 36°C in *rna8-1* strain SPJ8.3 (Fig. 2, lane 2) was suppressed by p8500 (lane 4) and p85888 (lane 5) but not by p85233 (lane 6), p8022 (lane 7), or p8530 (lane 8). (The differences in the ratios of precursor to mature mRNA in lanes 2, 6, 7, and 8 probably reflect minor variations in cell growth conditions or RNA extraction procedures or both.) Suppression of the temperature-sensitive growth defect therefore correlated with complementation of the splicing deficiency and strongly suggested that the *rna8-1*-complementing sequence corresponded to the *RNA8* gene.

To determine whether the *rna8-1*-complementing sequence was repeated in the yeast genome, Southern blot hybridization analysis of yeast genomic DNA was performed by using ^{32}P-labeled probes derived from the region of DNA bearing this sequence. These studies (results not shown) showed that the *rna8-1* complementing sequence was unique in the yeast haploid genome; no related sequences were detected, even when low-stringency hybridization and washing procedures were used (hybridization at 40°C; washing in 2× SSC–0.5% [wt/vol] SDS at room temperature).

***rna8-1*-complementing sequence corresponds to the RNA8 gene.** To determine whether the *rna8-1*-complementing sequence represented the *RNA8* gene itself rather than an extragenic suppressor of the *rna8-1* phenotype, one-step gene disruption experiments (49) were performed. The objective was to determine whether the cloned sequence could integrate by homologous recombination at the *RNA8* locus, as it should if it corresponded to the *RNA8* gene.

The plasmid p8244.3 was constructed as described in the legend to Fig. 3 by cloning a region of the *rna8-1*-complementing sequence into the yeast vector YIp5. This plasmid bears the *URA3* gene but no yeast origin of DNA replication and therefore must integrate into the yeast genome to stably transform a Ura3$^-$ yeast cell to uracil prototrophy. p8244.3 was linearized with restriction endonuclease *Bgl*II, which cleaved within the *rna8-1*-complementing sequence, to target integration specifically to a homologous chromosomal locus (45) on transformation of the *rna8-1*/*RNA8* heterozygous diploid strain SPJ0.2831. (This strain is Ura3$^-$ and, due to the recessive nature of the *rna8-1* mutation, is capable of growth at 36°C.) Since p8244.3 carried a region of DNA entirely internal to the *rna8-1*-complementing sequence (see legend to Fig. 3), the integration event would generate two inactive copies of this gene, one truncated at the 5′ end and the other truncated at the 3′ end. Therefore, if the cloned sequence corresponded to the *RNA8* gene, p8244.3 could integrate into either the *RNA8* or the *rna8-1* locus of SPJ0.2831 (Fig. 3A). The two alternative integration events would occur with approximately equal probability, generating phenotypically distinguishable classes of Ura$^+$ transformants: those capable of growth at 36°C (disruption of the *rna8-1* mutant gene and retention of an intact wild-type

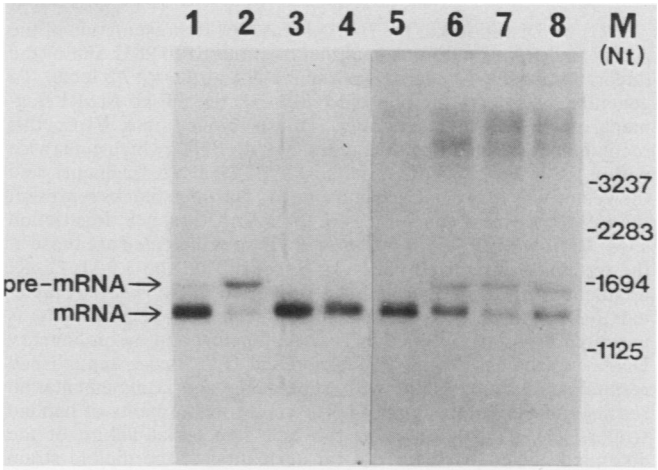

FIG. 2. Suppression of the *rna8-1* splicing defect. RNA samples (12 μg) were denatured with glyoxal, electrophoresed through 1.5% agarose, blotted onto GeneScreen membranes, and hybridized to a ^{32}P-labeled 1-kb *Bam*HI-*Bgl*II restriction fragment of pYA301 (this probe contains the yeast actin sequence). RNA samples were prepared from the following: untransformed *rna*8-1 strain SPJ8.3 (lanes 1 and 2) or SPJ8.3 transformed with p8500 (lanes 3 and 4), with p85888 (lane 5), with p85233 (lane 6), with p8022 (lane 7), or with p8530 (lane 8). Lanes: 1 and 3, RNA extracted from cultures grown at 24°C; 2, 4, 5, 6, 7, and 8, RNA extracted from cultures grown at 24°C and then shifted to 36°C for 1 h; M, DNA markers (sizes in nucleotides [Nt]). Bands corresponding to precursor and mature actin mRNA species are indicated by arrows. Bands seen above the 3,237-nucleotide marker are due to plasmid DNA in the RNA preparations.

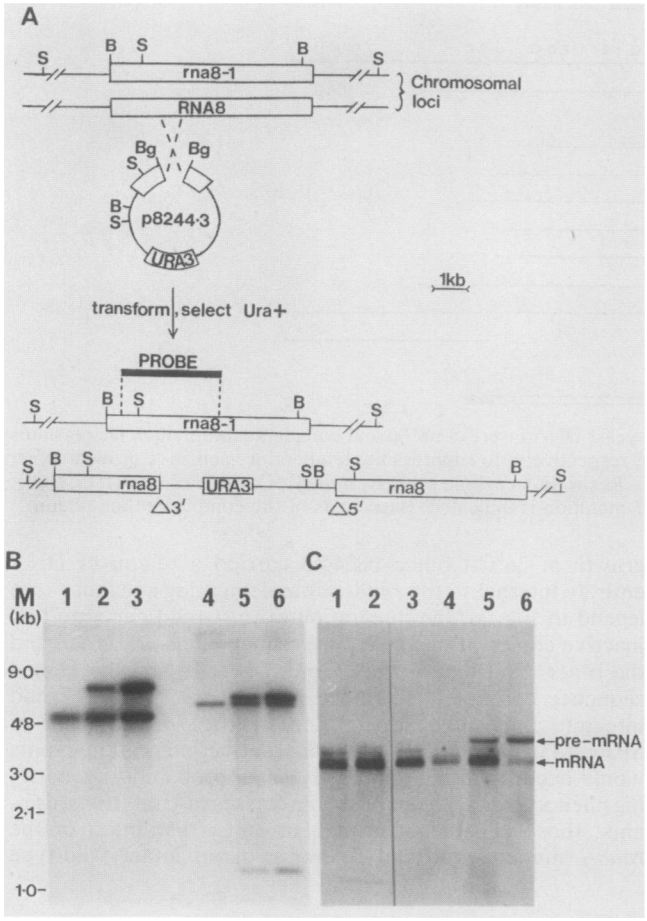

FIG. 3. Disruption of the *RNA8* locus. (A) Representation of the *rna8-1* and *RNA8* chromosomal loci of strain SPJ0.2831 and of the integration of *Bgl*II-linearized p8244.3 DNA at the *RNA8* locus. To generate the integrating plasmid (p8244.3), the 3.0-kb *Eco*RI fragment of the p8500 insert (Fig. 1) was cloned into YIp5; this recombinant plasmid was then digested with *Bgl*II (which cuts twice within the insert sequences, deleting a 1.3-kb DNA fragment), and the vector was recircularized and cloned. The open blocks represent the *RNA8* or *rna8-1* gene or part of the *RNA8* sequence. Restriction sites: B, *Bam*HI; Bg *Bgl*II (the only *Bgl*II sites indicated are those at the termini of linearized p8244.3); S, *Sal*I. That the 3.0-kb *Eco*RI fragment used to generate p8244.3 is internal to the *rna8-1*-complementing sequence (i.e., lacks essential sequences at both ends) is indicated by transcript studies described below and by preliminary DNA sequencing data (S. P. Jackson and J. D. Beggs unpublished results) and is consistent with subcloning and complementation results and the failure of p8244.3 to yield transformants of haploid strains. This is confirmed by the recessive lethal nature of the disrupted alleles produced on transformation of the diploid strain SPJ0.2831. Δ5′ and Δ3′, Truncation of the *RNA8* gene at the 5′ and 3′ ends, respectively. The region of DNA used as the radioactive probe in panel B is indicated. (B) Southern blot hybridization analysis. DNA was prepared from untransformed SPJ0.2831 (lanes 1 and 4), transformant D (lanes 2 and 5), or transformant G (lanes 3 and 6), digested with *Bam*HI (lanes 1 to 3) or *Sal*I (lanes 4 to 6), electrophoresed through 0.9% agarose, blotted onto a GeneScreen Plus membrane, and then hybridized to a ^{32}P-labeled 3.0-kb *Eco*RI fragment of the *rna8-1*-complementing sequence (shown in panel A). Although the 6.0-kb *Bam*HI fragment from the disrupted locus is unresolved from the 5.9-kb *Bam*HI fragment from the intact locus in lanes 2 and 3, it was detected when the blot was rehybridized with a pBR322 probe (results not shown). Lane M, Position of DNA markers (sizes in kilobases). (C) Northern blot hybridization analysis of transformants. RNA was prepared from untransformed SPJ0.2831 (lanes 1 and 2), transformant D (lanes 3 and 4), or transformant G (lanes 5 and 6) which had been grown at 24°C (lanes 1, 3, and 5) or shifted to 36°C for 1 h (lanes 2, 4, and 6). RNA was denatured with glyoxal, electrophoresed through 1.5% agarose, blotted onto a GeneScreen membrane, and hybridized to a ^{32}P-labeled 1-kb *Bam*HI-*Bgl*II fragment of pYA301 (this fragment contains the yeast actin gene). Bands corresponding to precursor (pre-mRNA) and mature actin mRNA are indicated.

RNA8 gene) and those temperature-sensitive for growth at 36°C (disruption of the wild-type gene).

When SPJ0.2831 was transformed with *Bgl*II-linearized p8244.3, 37 of the 82 (45%) Ura$^+$ transformants obtained at 24°C were incapable of growth at 36°C on uracil-deficient media. Furthermore, a randomly chosen temperature-sensitive transformant (G) accumulated unspliced actin pre-mRNA at 36°C (Fig. 3C, lane 6), whereas a randomly chosen transformant capable of growth at 36°C (D) and untransformed SPJ0.2831 showed no such defect (lanes 4 and 2, respectively). To determine whether integration of p8244.3 had occurred through homologous recombination, DNA samples prepared from untransformed SPJ0.2831 and transformants D and G were subjected to Southern blot hybridization by using the 3.0-kb *Eco*RI DNA restriction fragment of the *rna8-1*-complementing sequence as a radioactive probe (Fig. 3A). The results of these analyses are presented in Fig. 3B. As predicted, a 5.9-kb J*Bam*HI fragment (lane 1) and two *Sal*I fragments (6.7 and 3.0 kb; lane 4) were detected in DNA from untransformed SPJ0.2831. (The 3.0-kb *Sal*I fragment gave a weak hybridization signal, since it has only approximately 600 base pairs of homology with the probe.) If the expected integration events had taken place to generate transformants D and G, one locus would remain intact (generating the same fragments as untransformed SPJ0.2831), whereas the other locus (bearing integrated p8244.3) would produce *Bam*HI fragments of 8.2 and 6.0 kb and *Sal*I fragments of 7.0, 6.7, 3.0, and 1.3 kb. All of these fragments were indeed detected (Fig. 3B, lanes 2, 3, 5, and 6). Thus, DNA from the *rna8-1*-complementing sequence could integrate into the yeast genome by homologous recombination, disrupting the resident *RNA8* gene. This demonstrated that the cloned sequence corresponded to the bona fide *RNA8* gene.

Untransformed SPJ0.2831 and temperature-sensitive transformant G were sporulated, and the spores were dispersed and regenerated on complete medium at 24°C. For untransformed SPJ0.2831, all haploid progeny were Ura$^-$ and 20 of 36 (56%) were temperature sensitive. In contrast, 103 of 103 (100%) haploid progeny from transformant G were temperature sensitive, and none of these was Ura$^+$. These results indicated that a disrupted *RNA8* gene is a recessive lethal mutation and that a functional *RNA8* gene is necessary for spore germination or vegetative cell growth or both.

Analysis of the RNA8 Transcript. Northern blot hybridization of yeast RNA was performed by using strand-specific RNA probes homologous to the *RNA8* gene (Fig. 4A). Whereas probe 2 did not detect any *RNA8*-specific transcripts (results not shown), probe 1 detected an approximately 7.4-kb transcript in total yeast RNA (Fig. 4B, lane 1) and in polyadenylated RNA (lane 2). Further evidence that this 7.4-kb RNA corresponded to the *RNA8* transcript was provided by the observations (results not shown) that it was detected by several ^{32}P-labeled DNA restriction fragments from within the *RNA8* gene and that it was overproduced

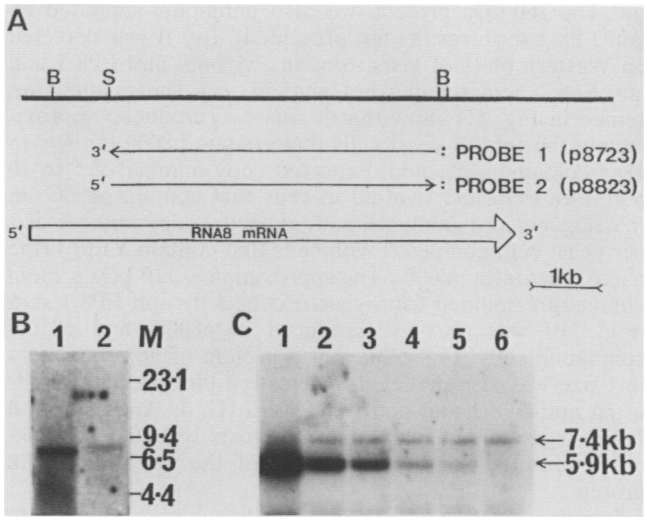

FIG. 4. Analysis of the *RNA8* transcript. A Restriction map of the *RNA8* gene and flanking sequences. Only *Sal*I (S) and *Bam*HI (B) restriction sites are indicated. The 5.0-kb *Sal*I-*Bam*HI restriction fragment of the *RNA8* gene was cloned into pSPT18 and pSPT19 to generate plasmids p8723 and p8823, respectively. These plasmids were linearized with *Sal*I (p8723) or *Bam*HI (p8823) and transcribed in vitro with bacteriophage T7 RNA polymerase to produce ^{32}P-labeled strand-specific RNA probes 1 and 2, respectively. The polarities of these probes and of the *RNA8* transcript are indicated. (B) Northern blot hybridization analysis of the *RNA8* transcript. Samples of yeast RNA were denatured with glyoxal, electrophoresed through 1% agarose, blotted onto a GeneScreen membrane, and hybridized with probe 1 (panel A). Lanes: 1, 7 μg of total yeast RNA; 2, 0.1 μg of polyadenylated yeast RNA; M, DNA markers (sizes in kilobases). (C) Quantification of the *RNA8* transcript. Samples (15 μg) of total yeast RNA (lanes 1 to 6) were mixed with 750 pg (lane 1), 150 pg (lane 2), 75 pg (lane 3), 15 pg (lane 4), 7.5 pg (lane 5), or 1.5 pg (lane 6) of the 5.9-kb *Bam*HI DNA restriction fragment of the *RNA8* gene (see panel A). Mixes were denatured with glyoxal, electrophoresed through 1% agarose, blotted onto a GeneScreen membrane, and hybridized to probe 1 (panel A). Bands corresponding to the 7.4-kb *RNA8* mRNA and the 5.9-kb *Bam*HI fragment are indicated by arrows.

approximately fivefold in cells transformed with p8000 (a plasmid that carries the *RNA8* gene and that is present at 5 to 10 copies per yeast cell). These results therefore indicated that the *RNA8* gene is transcribed in the left-to-right direction of the restriction map (Fig. 4A) to produce a 7.4-kb polyadenylated transcript. The observation that neither the size nor the abundance of this transcript was significantly changed when an *rna4-1* strain was shifted to 36°C for 1 h (results not shown) suggests that the *RNA8* primary transcript is not spliced. However, the possibility that the *RNA8* transcript contains a very small intron or an intron whose removal is not dependent on the *RNA4* gene product cannot yet be excluded. The size of the *RNA8* transcript is therefore approximately equal to the insert size of p85888, the smallest *RNA8* subclone capable of complementing the *rna8-1* mutation (Fig. 1), suggesting that the p85888 insert consists almost entirely of the *RNA8* sequence.

To quantify the *RNA8* transcript, 15-μg samples of yeast RNA were mixed with 1.5 to 1,750 pg of the 5.9-kb *Bam*HI restriction fragment that contains most of the *RNA8* gene sequence, and the samples were analyzed by Northern blot hybridization (Fig. 4C) by using probe 1 (Fig. 4A). Since this probe was equally homologous to the *Bam*HI fragment and the *RNA8* transcript, a comparison of the hybridization intensities produced by these two species allowed approximate quantification of the *RNA8* transcript. Densitometric tracing of the autoradiogram in Fig. 4C revealed that the *RNA8* transcript produced a hybridization signal equivalent to that of 12 pg of the *Bam*HI restriction fragment. Since the transcript was approximately 1.2 times the size of the *Bam*HI fragment, it was concluded that it represented approximately 14.5 pg of the 15 μg of total yeast RNA. Finally, assuming that 2% of yeast RNA is mRNA under conditions of exponential growth (25), that the average length of yeast mRNA is 1,500 nucleotides, and that a yeast cell contains 5.3×10^{10} daltons of mRNA (54), we calculated that the *RNA8* transcript constitutes approximately 0.005% (by mass) or 0.001% (by moles) of yeast mRNA and is present at approximately one copy per yeast cell.

Raising antisera against the RNA8 protein. The observation that the *RNA8* gene is transcribed to yield a large polyadenylated transcript suggested that it encoded a protein. To detect the putative RNA8 protein, antisera were raised against β-galactosidase-RNA8 fusion proteins. These proteins were generated by cloning the internal 1.3- and 2.4-kb *Bgl*II restriction fragments of the *RNA8* gene (Fig. 5A) into the 3′ end of the *lacZ* gene of expression vectors pUR290, pUR291, and pUR292 (50) and then expressing the recombinant genes in *E. coli* cells (see legend to Fig. 5A). The 1.3- and 2.4-kb *Bgl*II fragments cloned in pUR290 gave rise to fusion proteins FP8.1 and FP8.2, respectively. These fusion proteins were estimated by SDS-polyacrylamide gel electrophoresis to be 170 and 200 kilodaltons (kDa), respectively, consistent with the *RNA8* open reading frame spanning the entire length of these fragments. FP8.1 and FP8.2 were purified from SDS-polyacylamide gels and then used to immunize rabbits as described in Materials and Methods.

Immunological detection of the RNA8 protein. Figure 5B shows L-[^{35}S]methionine-labeled proteins immunoprecipitated from extracts of yeast strain BJ2412 by preimmune or immune sera from two rabbits (rabbits 35 and 36) immunized with FP8.1. Whereas preimmune sera failed to specifically immunoprecipitate any labeled proteins, the immune sera immunoprecipitated three proteins in each case, two of which (M_w, approximately 110 and 260 kDa) were recognized by both antisera. Since laboratory yeast strains do not contain proteins with immunological cross-reactivity to β-galactosidase, these two proteins are good candidates for the *RNA8* gene product. Although the antisera were raised against the same antigen preparation, each individually precipitated a different low-molecular-weight polypeptide. These polypeptides are either RNA8 degradation products produced during immunoprecipitation or immune artifacts, as they are not detected on Western blots (immunoblots) with affinity-purified antibodies (G. J. Anderson and J. D. Beggs, unpublished results).

There are several lines of evidence which indicate that the approximately 260-kDa yeast protein immunoprecipitated by anti-FP8.1 sera corresponds to the RNA8 protein. (i) Its large size is consistent with the previously determined sizes of the *RNA8* gene and the *RNA8* transcript. (ii) Immunoprecipitation of this protein (indicated by an arrow in Fig. 5C, lanes 1 and 9) by anti-FP8.1 serum was completely blocked by the addition of partially purified FP8.1 (lanes 2 to 4) but not by β-galactosidase (lanes 5 to 8), indicating that the antibodies responsible for immunoprecipitation were specific for the RNA8 moiety of FP8.1. The high level of background proteins observed in this experiment was the result of very abundant yeast proteins sticking nonspecifically to the immunoprecipitated FP8.1 or to β-galactosidase.

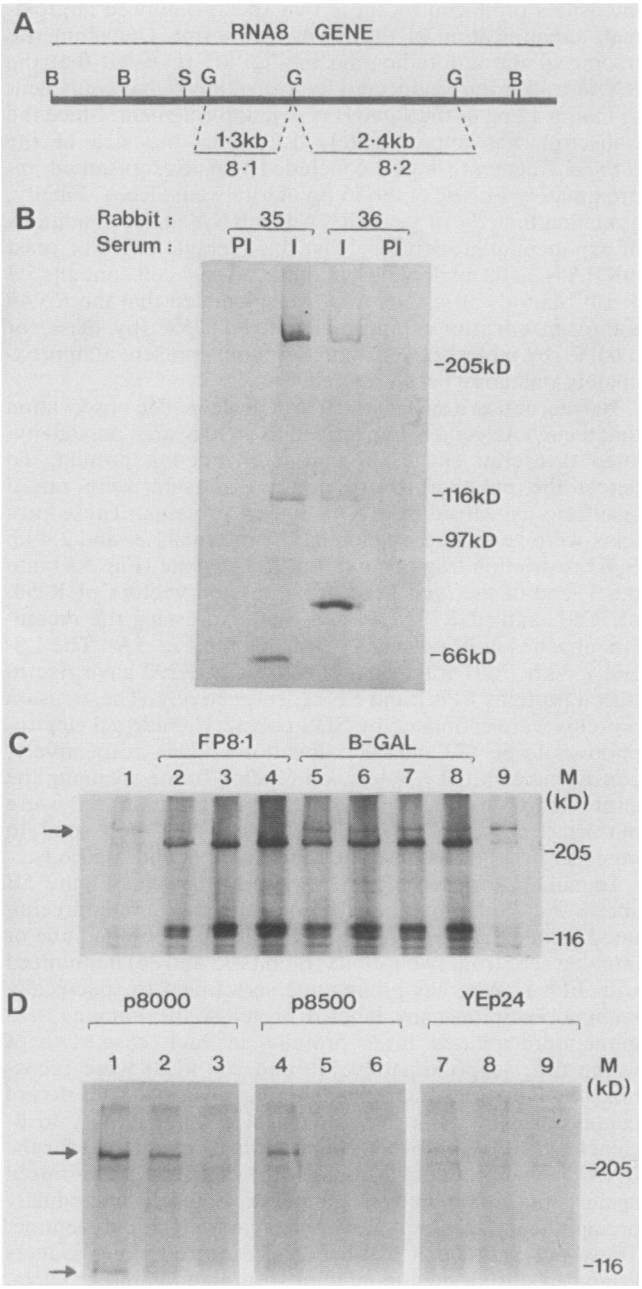

FIG. 5. Immunological detection of the RNA8 protein. (A) Representation of the 1.3- and 2.4-kb *Bgl*II fragments of the *RNA8* gene that were cloned into the *Bam*HI site in the *lacZ* gene of pUR290, pUR291, and pUR292. B, *Bam*HI; S, *Sal*I; G, *Bgl*II. In each case, only pUR290-based recombinant plasmids produced fusion proteins significantly larger than β-galactosidase. Subsequently, DNA sequencing confirmed that only the pUR290 recombinants had the *RNA8* sequences fused in the appropriate frame of *lacZ* to make fusion proteins. (B) Immunoprecipitation of proteins from L-[^{35}S]methionine-labeled extracts of yeast strain BJ2412 with 10 μl of preimmune serum (PI) or 10 μl of immune serum (I) raised against FP8.1 in rabbits 35 or 36. Immunoprecipitates were fractionated on an 8.5% SDS-polyacrylamide gel. The positions of marker proteins are indicated (sizes in kilodaltons [kD]). (C) Effect of adding purified FP8.1 or β-galactosidase (B-GAL) on immunoprecipitation by anti-FP8.1 antiserum of proteins from L-[^{35}S]methionine-labeled extracts of yeast strain DBY747. FP8.1 and β-galactosidase were purified by electroelution from SDS-polyacrylamide gel slices and concentrated to 0.25 mg/ml. Purified FP8.1 or β-galactosidase (lanes 1 and 9, none added; lanes 2 and 5, 1 μg; lanes 3 and 6, 2.5 μg; lanes 4 and 7, 6 μg; lane 8, 12 μg) were mixed with [^{35}S]methionine-labeled yeast extract (0.8 ml) just before the addition of antiserum. Immunoprecipitates were fractionated on an 8.5% SDS-polyacrylamide gel. The positions of marker proteins (M) are indicated (sizes in kilodaltons [kD]). The high level of background proteins is caused by certain abundant yeast proteins sticking nonspecifically to the immunoprecipitated FP8.1 or β-galactosidase. (D) Immunoprecipitation of protein from L-[^{35}S]methionine labeled extracts from yeast strain SPJ8.3 that contains p8000, p8500, or YEp24 (as indicated). Immunoprecipitations were from undiluted extracts (lanes 1, 4, and 7) or from extracts that had been diluted 1:1 (lanes 2, 5, and 8) or 1:3 (lanes 3, 6, and 9) with immunoprecipitation buffer. Anti-FP8.1 antiserum (10 μl) was used in each case. Immunoprecipitates were fractionated on an 8.5% SDS-polyacrylamide gel. The approximately 260- and 110-kDa proteins are indicated by arrows. The positions of marker proteins (M) are indicated (sizes in kilodaltons [kD]).

(iii) The 260-kDa protein was also immunoprecipitated by anti-FP8.2 sera (results not presented). (iv) It was detected on Western blots of yeast proteins by both anti-FP8.1 and anti-FP8.2 sera (results not shown). (v) The results presented in Fig. 5D show that it was overproduced approximately fourfold in yeast cells that contain p8000 (an *RNA8* gene-containing plasmid; expected copy number of 5 to 10 per yeast cell) and twofold in cells that contain p8500 (an *RNA8* gene-containing plasmid; expected copy number of 1 per yeast cell) compared with cells that contain YEp24 (the parent vector for p8000). The approximately 110-kDa protein immunoprecipitated from yeast extracts by anti-FP8.1 sera (Fig. 5B) was also overproduced in p8000- and p8500-containing cells (Fig. 5D); that a protein of approximately this size was also detected by Western blotting procedures using anti-FP8.1 and anti-FP8.2 sera (G. J. Anderson and J. D. Beggs, unpublished data) suggests that it is a breakdown product or processed form of the 260-kDa RNA8 protein.

Immunodepletion of RNA8 protein abolishes splicing in vitro. Yeast whole-cell splicing extract was incubated with FP8.1-specific antibodies bound to protein A-Sepharose according to the method of Kramer et al. (29) to deplete RNA8 protein. After the removal of immune complexes, the depleted extract was assayed for splicing activity. Incubation of splicing extract with protein A-Sepharose in the absence of antiserum or with preimmune serum had very little effect on splicing activity (Fig. 6A and B, compare lanes 2, 3, and 4). Similarly, incubation of splicing extract with antibodies raised against another β-galactosidase fusion protein which had no reaction with RNA8 protein (Fig. 6B lanes 5 and 6) did not significantly affect splicing activity. On the other hand, incubation of splicing extract with antibodies raised against FP8.1 reproducibly resulted in a significant loss of splicing activity (Fig. 6A, lane 5 and 6B, lanes 7 and 8), such that the production of both intermediates (Fig. 6, IVS+E2 and E1) and products (Fig. 6, IVS and E1+E2) of the reaction was prevented. These results suggest that the use of RNA8-specific antibodies to remove RNA8 protein from splicing extracts depletes the activity of the extract, preventing the conversion of pre-mRNA either to intermediates or to fully spliced mRNA, and support the conclusion of Lustig et al. (37) that functional *RNA8* gene product is required for pre-mRNA splicing.

DISCUSSION

In this paper, we describe the cloning of a DNA sequence which suppresses the temperature-sensitive growth and pre-

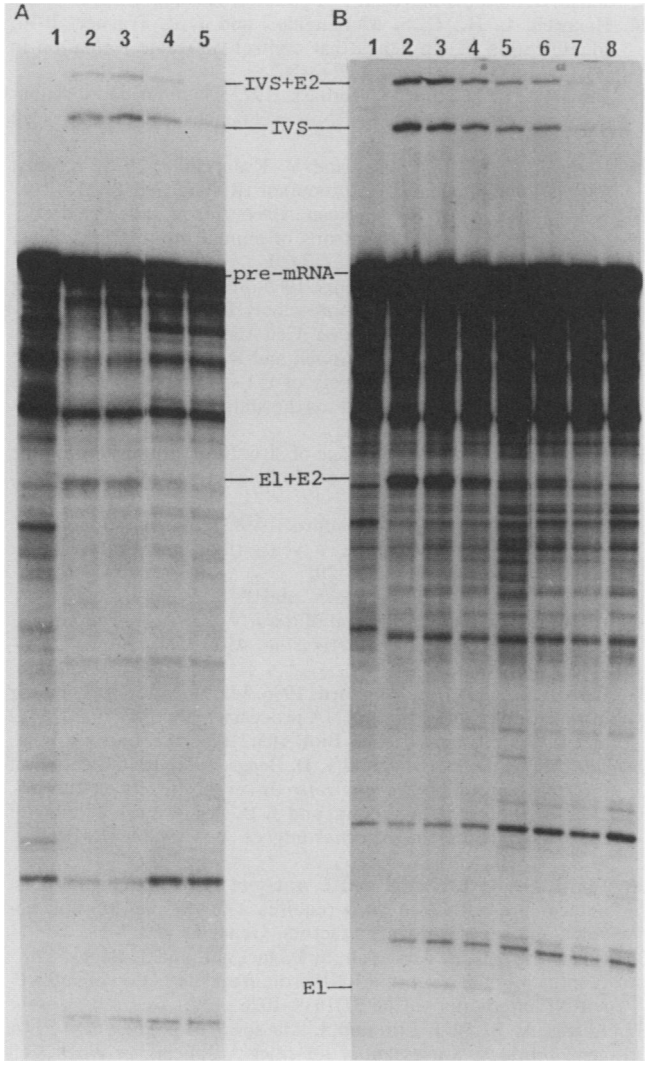

FIG. 6. Immunodepletion of in vitro splicing activity. Yeast whole-cell splicing extract was treated with antibodies bound to protein A-Sepharose as described in Materials and Methods and assayed in vitro for splicing activity as described by Lin et al. (36) by using a 544-nucleotide ^{32}P-labeled synthetic actin transcript as the substrate. The reaction products were fractionated on a 7% polyacrylamide–8 M urea gel, followed by autoradiography. (A) Splicing reaction with no splicing extract (lane 1), untreated splicing extract (lane 2), splicing extract incubated with protein A-Sepharose alone (lane 3), splicing extract incubated with protein A-Sepharose-bound preimmune antibodies (lane 4; from the same rabbit used to raise the anti-FP8.1 response), and splicing extract incubated with protein A-Sepharose-bound antibodies raised against FP8.1 (lane 5). (B) Splicing reaction (lanes 1 and 2 are as in panel A) with extract preincubated at 4°C with a volume of splicing buffer equivalent to the void volume of the protein A-Sepharose beads (lane 3), extract treated with protein A-Sepharose alone (lane 4), extract treated with protein A-Sepharose-bound antibodies raised against a β-galactosidase fusion protein having no cross-reaction with RNA8 protein (lanes 5 and 6), or extract treated with protein A-Sepharose-bound antibodies raised against FP8.1 (lanes 7 and 8). For both panels, the positions of the substrate (pre-mRNA), splicing intermediates (intron-exon 2 [IVS+E2] and exon 1 intermediate [E1]), and splicing products (lariat intron [IVS] and mRNA product [E1+E2]) are indicated. These species were identified by their migration properties and by the fact that their production is ATP-dependent and splicing extract-dependent; they are also precipitable with anti-trimethylguanosine antibodies which precipitate spliceosomes.

mRNA splicing defects of an *rna8-1* yeast strain, and we demonstrate through gene disruption studies that this sequence corresponds to the *RNA8* gene. The cloned gene was characterized by restriction mapping and subcloning and shown to be unique in the yeast haploid genome and to encode a low-abundance polyadenylated transcript of approximately 7.4 kb. We raised antisera against β-galactosidase-RNA8 fusion proteins, and we show that these antisera recognize a low-abundance yeast protein of approximately 260 kDa. Evidence that this protein is the *RNA8* gene product includes its detection by antisera raised against two different fusion proteins containing nonoverlapping regions of the RNA8 protein, its overproduction in yeast cells containing multiple copies of the *RNA8* gene, and the demonstration that the antibodies which recognized this protein are specific for the RNA8 moiety of a β-galactosidase-RNA8 fusion protein. Thus, the *RNA8* gene encodes a protein, as does each of the other *RNA* genes characterized to date. At approximately 260 kDa, however, the RNA8 protein is much larger than any of the other RNA proteins (the RNA2, 3, 4, and 11 proteins have estimated sizes of 100, 55, 52, and 30 kDa, respectively [32, 35; S. Petersen-Bjorn and J. Friesen, personal communication; D. Shore, personal communication]). Indeed, to our knowledge, it is the largest protein identified in *S. cerevisiae* to date. Anti-RNA8 sera also recognized an approximately 110-kDa yeast protein which may represent a degradation product or processed form of the 260-kDa RNA8 protein. Finally, we used anti-RNA8 sera to immunodeplete the RNA8 protein from yeast in vitro splicing extracts. The observation that these depleted extracts no longer possessed significant splicing activity provides strong support for a requirement for the *RNA8* gene product in pre-mRNA splicing. Although other possibilities cannot yet be excluded, the fact that the *rna8-1* mutation leads to a defect in RNA splicing in vivo (31) and in vitro (37) and our observation that depletion of the *RNA8* protein from an in vitro splicing extract inhibited RNA splicing suggest a direct role for the RNA8 protein in the splicing reaction. One obvious possibility, therefore, is that the RNA8 protein interacts with or is an integral part of the yeast spliceosome (8). More specifically, within this structure, the RNA8 protein might associate with products of the other *RNA* genes, with pre-mRNA, or with the yeast small nuclear RNA species that have been identified as spliceosomal components (47). Indirect evidence for association of the RNA8 protein with the spliceosome is provided by the observation that heat-inactivated extracts from *rna8-1* cells are unable to form spliceosome complexes (36); however, these studies were unable to distinguish whether the RNA8 protein is actually part of the spliceosome or is merely required for spliceosome assembly. Using anti-RNA8 antibodies, we have recently shown that the RNA8 protein is a component of small nuclear ribonucleoprotein particles containing small nuclear RNAs which are known to be present in spliceosomal complexes (36a). With further immunological approaches it should be possible to demonstrate the presence of RNA8 protein in spliceosomes and gain a better understanding of the function of this protein in nuclear pre-mRNA splicing.

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