EXPERIMENTAL CELL RESEARCH **229,** 181–188 (1996)
ARTICLE NO. 0357

SPECIAL ARTICLE

Yeast Pre-mRNA Is Composed of Two Populations with Distinct Kinetic Properties

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As an approach to the study of yeast pre-mRNA splicing *in vivo,* we have examined properties of transcripts derived from a gal-UAS intron-containing fusion gene encoding RP51A and a series of its derivatives. RNA half-life measurements were carried out after transcription initiation was blocked by the addition of glucose. Pre-mRNA encoded by GalRP51A decayed with a half-life of ~6 min and was substantially polyadenylated, and transcripts derived from a nonspliced version of the same gene decayed with a similar half-life (~4 min). A comparison of the steady-state levels of these two transcripts suggests that the bulk of GalRP51A pre-mRNA is processed much more rapidly, with an average lifetime of about 2 s. We propose that this inferred population of rapidly processed molecules is spliced cotranscriptionally and that it is the principal precursor to GalRP51A mRNA. Although the pre-mRNA molecules detected are therefore unlikely to be the major splicing precursors, an *in vivo* assay suggests that they are likely to have bound splicing factors. They must then be spliced much more slowly than most primary transcripts, or not spliced at all and then degraded through a different cellular pathway. As a result of its comparatively long lifetime, this minor fraction of the pre-mRNA population makes up the majority of the steady-state level of GalRP51A pre-mRNA.

INTRODUCTION

In eukaryotic cells, newly synthesized transcripts may be directly exported to the cytoplasm, but they often first undergo processing events in the nucleus. These include pre-mRNA splicing, in which introns are removed and exons spliced together to produce functional transcripts.

The nucleus contains an elaborate machinery to carry out splicing, consisting of both RNA and protein components which assemble on the pre-mRNA in a complex called a spliceosome [1–3]. In *in vitro* experiments, spliceosome components from a soluble cell extract assemble stepwise on a synthetic pre-mRNA substrate. Only limited pre-mRNA primary sequence information is essential for spliceosome assembly or for splicing. In yeast (*Saccharomyces cerevisiae*), these are the 5′ splice site, branchpoint (TACTAAC box), and 3′ splice site. Within the mature spliceosome, the 5′ splice site is cleaved and covalently joined to the branchpoint to generate a lariat intermediate and free exon 1. The 3′ splice site is then cleaved, and the two exons are fused to generate mRNA and a free lariat. The RNA products generated *in vivo* from intron-containing genes also reflect the same chemistry. In yeast, for example, precursor (pre-mRNA), lariat intermediate, spliced mRNA, and free lariat can all be detected [1].

To assist in determining the kinetics of *in vivo* splicing, we have measured the half-lives of these various species in living cells. In order to measure transcript turnover times, we used derivatives of the intron-containing RP51A gene [4] under the control of a galactose-inducible promoter. Cells grown under inducing conditions (in medium containing galactose) actively transcribe these genes. This promoter has the additional property of being glucose-repressible; i.e., it can be turned off rapidly by the addition of glucose, which enabled us to measure transcript decay rates in the absence of new RNA synthesis [5]. The results of these experiments suggest the existence of two distinct kinetic classes of RP51A pre-mRNA, one of which is processed extremely rapidly and the other much more slowly. The rapidly processed pre-mRNAs are likely to be the real precursors to RP51A mRNA. The latter class may be true precursors which are processed more slowly, or they may be nonproductive transcripts.

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0014-4827/96 $18.00

MATERIALS AND METHODS

Yeast Strains

RNA half-life measurements. RNA half-life experiments were done in strain MGD353-13D.

Knockout of UPF1. A UPF1 knockout was created in strain MGD353-13D. The *Bam*HI–*Eco*RI fragment of YCpPL63 [6] containing the UPF1 gene and flanking sequences was subcloned into the polylinker of PTZ19R. The internal *Bgl*II fragment containing UPF1 coding sequences was removed from this insert and replaced with a *Bam*HI–*Bgl*II fragment containing hisG/URA3/hisG [7]. This plasmid was used to disrupt the UPF1 gene by transformation after linearization with *Bam*HI and *Eco*RI. URA+ transformants were selected and tested for correct integration of the hisG/URA3/hisG by probing a Southern blot of *Bam*HI–*Bgl*II-digested genomic DNA with the 1.1-kb *Bam*HI–*Bgl*II fragment of YCpPL63 labeled by random priming. Segregants which had lost the URA3 marker by recombination between the hisG repeats were then obtained by growing the cells on YM1 followed by selection on FOA. The resulting cells were transformed with either a centromeric plasmid containing a leu selectable marker (p366) or a derivative of this plasmid containing the *Aat*II–*Bam*HI fragment of YCpPL63 including the UPF1 coding sequence.

Plasmid Constructions

GalRP51A5′Δss was made by replacing the *Sal*I–*Pvu*II fragments of HZ185′Δss [8] with the *Sal*I–*Pvu*II fragments of GalRP51A containing RP51A. These clones were identified by sequencing.

GalRP51AFS was made by cutting GalRP51A at the *Bam*HI site, filling in the sticky end, and then religating. The clone was identified by its lack of a *Bam*HI site, and this was confirmed by sequencing.

GalRP51AΔIVS was constructed in two steps. First, the *Xho*I–*Hin*dIII fragment, containing RP51A from a PTZ clone containing an intronless RP51A within an *Eco*RI–*Hin*dIII fragment (from F. Stutz), was subcloned into *Xho*I–*Sac*I-digested PTZΔBP (unpublished). This plasmid was then cut with *Eco*RI and *Bam*HI, and the fragment containing the intronless RP51A was subcloned into GalRP51A cut with *Bam*HI and *Pvu*II to generate GalRP51AΔIVS.

RNA Fractionation and Analysis

RNA extractions and primer extension analysis were done as described in [9]. RB1 primer is complementary to exon 2 of RP51A (CGCTTGACGGTCTTGGTTC), RB24 is complementary to exon 2 of the CYH2 gene (CTGTGCTTACCGATACGACC), and DT2133 is complementary to U1 snRNA (GACCAAGGAGTTTGCATCAATG). Gels were quantitated using a phosphorimager. Northern blots were carried out as described in [10]. RNA was fractionated into poly(A)$^+$ and poly(A)$^-$ using a Qiagen oligotex(dT) kit, as described by the manufacturers. Poly(A)$^-$ RNA was prepared by three sequential extractions with oligotex(dT) beads. Poly(A)$^+$ RNA was eluted from the beads after the first incubation. After the second incubation no further poly(A)$^+$ RNA was recovered, demonstrating that poly(A)$^+$ RNA had been quantitatively removed.

RESULTS

Half-Life Measurements Suggest the Existence of Two Classes of RP51A Pre-mRNA with Very Different Kinetic Properties

To investigate the *in vivo* kinetics of splicing, we measured the half-lives of transcripts encoded by GalRP51A. This gene encodes a wild-type RP51A gene downstream of a galactose-inducible promoter. The gene has the same CYC-1 5′ ends as its parent gene, HZ18 [4]. Northern analysis of steady-state RNA from galactose-grown cells indicates that GalRP51A encodes a 0.6-kb transcript as expected (Fig. 1A).

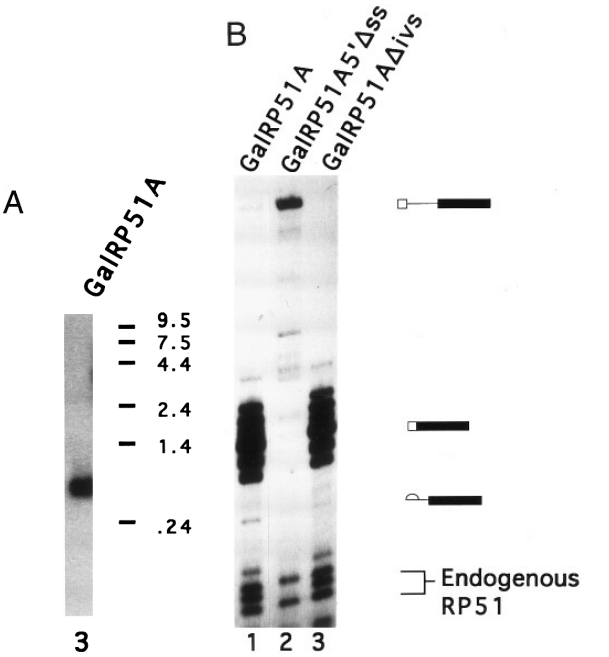

FIG. 1. Analysis of steady-state RNA populations of cells transformed with derivatives of RP51A and grown in media containing galactose. (A) Northern analysis. Ten micrograms of total yeast RNA from GalRP51A-transformed cells was electrophoresed through a 1% formaldehyde–agarose gel alongside an RNA size ladder (BRL) and then transferred onto a Pall Biodyne membrane. This membrane was then probed with the 2-kb *Bam*HI–*Sac*I fragment of HZ18 (which contains both RP51A and LacZ sequences [4, 32]), which was ^{32}P-labeled by random priming (Stratagene). The positions of the size markers are indicated next to the gel. (B) Primer extension analysis. Five micrograms of total yeast RNA from each sample was extended with oligonucleotide primer RB1 and then electrophoresed on a 5% polyacrylamide urea gel.

The GalRP51A transcripts were resolved into mRNA, pre-mRNA, and lariat intermediate by primer extension with RB1 (Fig. 1B; see Materials and Methods). This primer also detects the endogenous (chromosomal) RP51A transcripts, which generate shorter extension products and act as convenient loading controls. GalRP51A mRNA is very stable and decays very slowly following glucose addition (Fig. 2). In contrast, GalRP51A pre-mRNA decays with a half-life of about 6 min (Fig. 2B; the high standard deviation of the data is due to difficulties in reliably quantitating the weak primer extension signal for GalRP51A pre-mRNA). There was no detectable lag in the decay of GalRP51A pre-mRNA.

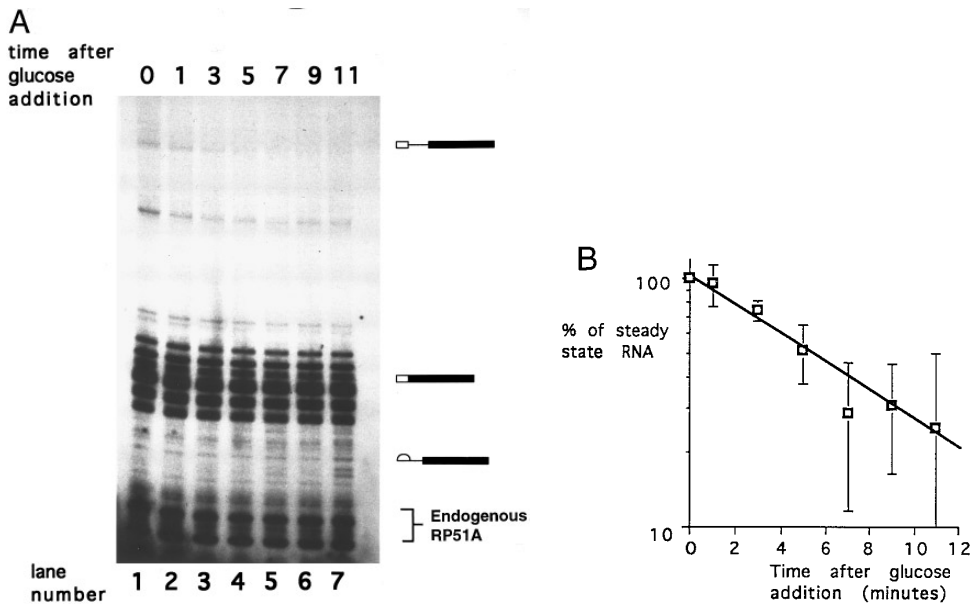

FIG. 2. Decay kinetics of transcripts derived from GalRP51A. GalRP51A-transformed cells were grown in ura$^-$ medium at 30°C to an OD of 0.3 to 0.5. These cells were then spun down, resuspended in a volume of 20 ml of the original medium, and then left in a shaking incubator for 5–10 min. A sample of cells corresponding to steady-state were harvested and frozen on dry ice. Glucose was then added to the remaining cells to a concentration of 2%, and the cells were harvested and frozen on dry ice at the indicated timepoints. Total RNA was then prepared from these cells and analyzed by primer extension. Five micrograms of total RNA was analyzed as described for Fig. 1B. (A) Autoradiograph of primer extension products from each sample of RNA after electrophoresis on a 5% polyacrylamide gel. (B) The data from three independent experiments was quantitated using a Phosphorimager. The major bands corresponding to GalRP51A pre-mRNA were quantitated, and then each was subtracted for the background radiation present in its lane on the gel. These values were expressed as a percentage of the pre-mRNA value in the steady-state RNA, corrected for the amount of RNA loaded in each well (measured by quantitating the upper band of endogenous RP51, which should have been equal in each case). Then the data from the three experiments were averaged and plotted on a logarithmic scale. The standard deviation of the data is shown where this is larger than the point used to plot the graphs.

In order to determine what the steady-state level of GalRP51A pre-mRNA would be in the absence of splicing, we used a similar approach to measure the decay kinetics of a derivative of GalRP51A, GalRP51A5′Δss. This gene is almost identical to GalRP51A, with the exception that the 5′ splice site has been deleted and replaced with a site for the restriction endonuclease *Aat*II. GalRP51A5′Δss transcripts are not spliced and accumulate as a species equivalent in size to GalRP51A pre-mRNA. Importantly, the steady-state level of GalRP51A5′Δss transcripts is about 100-fold higher than that of GalRP51A pre-mRNA (Fig. 1B, lanes 1 and 2). Since the 5′Δss and wild-type galactose-induced transcripts should be transcribed at the same rate, the decay (or processing) rate of GalRP51A pre-mRNA is ca. 100-fold faster than that of GalRP51A5′Δss.

Transcripts encoded by GalRP51A5′Δss decayed with a half-life of about 4 min (Fig. 3). A similar half-life was obtained using a second, more 5′-proximal primer for reverse transcription (data not shown). Since the GalRP51A pre-mRNA half-life is comparable (6 min), it is much too long to account for the 100-fold difference in steady-state levels of the two primary transcripts. This indicates the existence of a second population of GalRP51A pre-mRNA molecules, which decays with much faster kinetics and is not detectable using our procedures. Because of the low steady-state level of GalRP51A pre-mRNA, most primary transcripts must traverse this second, rapidly processed population but do not make a significant contribution to the steady-state pool of GalRP51A pre-mRNA.

An in Vivo Assay Suggests that a Class of RP51A Pre-mRNA Molecules Bind Splicing Factors but Turn Over Slowly

The above experiments imply the existence of two populations of RP51A pre-mRNA molecules: one with a very rapid half-life and the other with a much longer half-life. The fast half-life is likely the result of splicing; since the steady-state levels of GalRP51A mRNA are similar to the intronless version of the gene (GalRP51AΔIVS), most primary transcripts must be rapidly spliced (Fig. 1B, compare lanes 1 and 3). In contrast, RP51A5′Δss (which is not an efficient splicing sub-

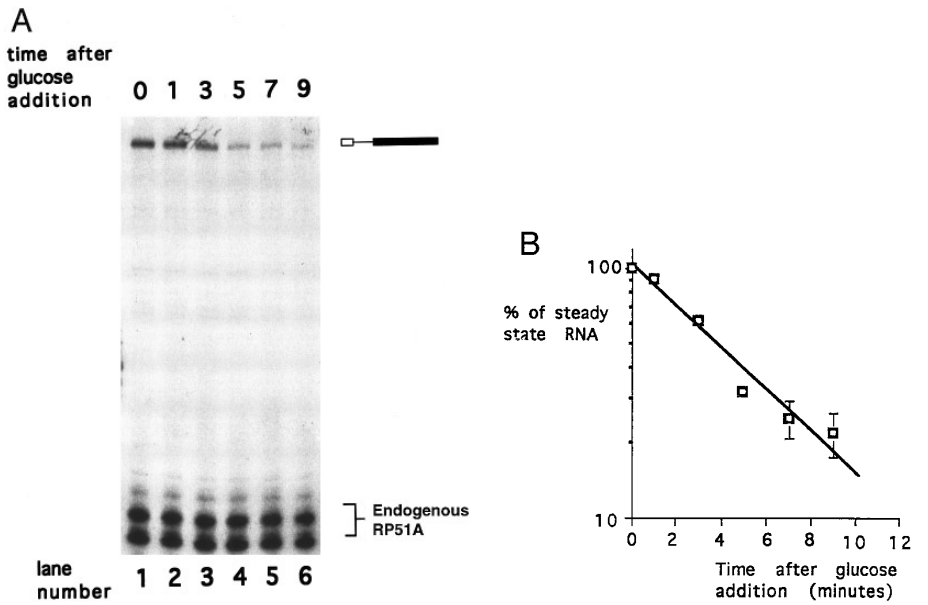

FIG. 3. Decay kinetics of transcripts derived from GalRP51A5′Δss. Total RNA from GalRP51A5′Δss-transformed cells was analyzed as described for Fig. 2. (A) Autoradiograph. (B) Quantitation of the data obtained in two independent experiments, performed as in Fig. 2B.

strate) is composed of a single kinetic class that is processed/degraded by a slow pathway.

The class of GalRP51A pre-mRNA that slowly turns over may escape commitment to the splicing pathway altogether, or it may bind splicing factors but then fail to splice (or splice much more slowly than most of the population). In order to distinguish between these possibilities, we used an *in vivo* assay to monitor the relationship of these transcripts to the translation machinery. The principle requires pre-mRNA association with the translation machinery as an indication of a cytoplasmic location and a failure to commit stably to the splicing pathway [11]. The current assay is based on a decay pathway which degrades out of frame transcripts that are undergoing translation. This cytoplasmic pathway is nonessential and can be disabled by the deletion of the UPF1 gene, which encodes one of its key components [6]. This viable deletion strain shows increased levels of frame-shifted, cytoplasmic transcripts. Most yeast pre-mRNAs have an initiation codon followed by a short open reading frame which terminates within the intron; hence, they are effectively frame-shifted transcripts. Experiments using this UPF1 assay to monitor the cellular location of yeast pre-mRNAs showed that a number of inefficiently spliced pre-mRNAs are stabilized in the deletion strain. The interpretation is that they leak into the cytoplasm at some frequency where they associate with the translation machinery [12].

We deleted the gene encoding UPF1 from the same strain used for the half-life measurements and then either rescued this deletion with UPF1 on a centromeric plasmid (strain Y59 UPF1$^+$) or pseudorescued with the vector alone (strain Y59 UPF1$^-$). The pair of isogenic strains was then transformed with GalRP51A and the level of transcripts monitored by primer extension. Experiments using a primer complementary to RP51A showed that GalRP51A pre-mRNA was not stabilized in the strain deleted for UPF1, and so most of it is not normally degraded by the UPF1 pathway (Fig. 4, lanes 7 and 8). We carried out three controls. First, cells were transformed with a version of GalRP51A in which a stop codon had been introduced near the 5′ end of the mRNA (GalRP51AFS). This mRNA is present at much lower steady-state levels in a UPF1$^+$ background, but at near wild-type levels in a UPF1$^-$ background, indicating that this frame-shifted GalRP51A mRNA is a substrate for UPF1-mediated degradation as expected (Fig. 4, lanes 5 and 6); the spliced mRNA is presumably exported to the cytoplasm and associates with the translation machinery. Since pre-mRNA levels from this construct are still unaffected by deleting UPF1, the RP51A intron presumably prevents association of the pre-mRNA with the translation machinery. Second, we assayed these same RNA preparations for the levels of CYH2 transcripts. CYH2 pre-mRNA is inefficiently spliced and at least some of it is degraded by the UPF1 decay pathway [12]. Consistent with this report, the level of CYH2 pre-mRNA was increased in the UPF1$^-$ background, while the level of CYH2 mRNA was unaffected (Fig. 4, lanes 1–4). Third, transcripts encoded by GalRP51A5′Δss are UPF1-sensitive (data

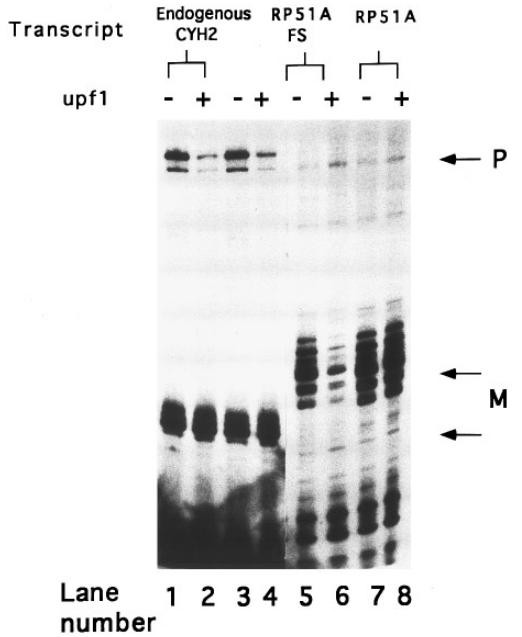

FIG. 4. Deletion of UPF1 does not stabilize GalRP51A pre-mRNA. RNA was prepared from GalRP51A-transformed cells and analyzed by primer extension, using oligonucleotides complementary to RP51 (RB1) and CYH2 (RB24), followed by electrophoresis on a 5% polyacrylamide gel. Lanes 1–4 correspond to the same RNA samples as lanes 5–8.

not shown; [8]). The results suggest that even the GalRP51A pre-mRNA molecules that slowly turn over bind splicing factors *in vivo*, making them relatively inaccessible to the UPF1-mediated decay machinery.

GalRP51A Pre-mRNA Is Substantially Polyadenylated

The 6 minute half-life of GalRP51A pre-mRNA suggests that these molecules are not nascent transcripts. Since polyadenylation and 3′ end formation are concomitant events, this suggests further that much of the GalRP51A pre-mRNA transcripts should be polyadenylated. To test this prediction, steady-state RNA from cells transformed with GalRP51A was fractionated into poly(A)$^+$ and poly(A)$^-$ populations. To obtain equivalent loadings, equal amounts of total RNA and poly(A)$^-$ RNA, as well as poly(A)$^+$ RNA corresponding to 2 and 5% of this amount, were assayed by primer extension (Fig. 5). In each case the sum of the poly(A)$^-$ fraction plus the 5% poly(A)$^+$ fraction was approximately equal to the signal from total RNA, consistent with the reasonable expectation that ~5% of total yeast RNA in cells grown under these conditions is polyadenylated.

GalRP51A mRNA and endogenous RP51 mRNAs were found mainly in the poly(A)$^+$ fraction. More than 90% of the U1 snRNA was in the poly(A)$^-$ fraction as expected. A significant fraction of the recovered RP51A pre-mRNA and lariat intermediate was polyadenylated, suggesting that it is full length (i.e., nonnascent). Like total GalRP51A pre-mRNA (see above), even the poly(A)$^-$ population of GalRP51A pre-mRNA decayed with a ca. 6-min half-life (data not shown), suggesting that these molecules compose the same slowly decaying population despite losing most of their poly(A) tail, as a result of either cleavage or terminal deadenylation.

DISCUSSION

We have examined the *in vivo* decay characteristics of a collection of transcripts that contain the RP51A intron. The data suggest the existence of two distinct populations of pre-mRNA, which are likely in different states if not compartments within the cell. We have direct experimental evidence for the existence of a population with a long half-life which either does not splice or splices very slowly (Fig. 6). It is possible that a small fraction of RP51A pre-mRNA molecules may be in an inappropriate molecular configuration to support rapid splicing. Nonetheless, the UPF1 data suggest that these pre-mRNAs still bind splicing factors. This suggests that splicing catalysis may be restricted to a particular time frame (or cellular location) of mRNA biogenesis; if molecules fail to splice at this time or place, they will no longer be spliced but will be degraded by a different pathway. The frequency of these abortive splicing events may be much higher for less efficiently spliced transcripts like CYH2, in which case a significant fraction of pre-mRNA might also escape the stable binding of splicing factors and, as a consequence, nuclear retention. The binding of splicing factors may anchor pre-mRNA in the nucleus or otherwise prevent transport to the cytoplasm. Alternatively, and as a result of the bound splicing factors, pre-mRNA may be exported in a translationally incompetent form, and so would not access the upf1 decay pathway (Fig. 6).

We infer the existence of a second class that rapidly turns over from the difference in abundance of the steady-state populations of GalRP51A pre-mRNA and that of a cognate nonspliced version of this transcript, GalRP51A5′Δss. The relative levels suggest that GalRP51A pre-mRNA should have a dramatically shorter half-life than GalRP51A5′Δss, but the opposite is actually the case. The predicted lifetime of this second, invisible class of pre-mRNA transcripts must be at least 100-fold shorter than that of GalRP51A5′Δss, or about 2 s (Fig. 6). If the lifetime is considerably longer than 2 s, then we should have seen by primer extension a population of poly(A)$^+$ pre-mRNA that decayed rapidly after glucose addition.

The relative rates of splicing and transcription will determine whether a pre-mRNA will be spliced while it

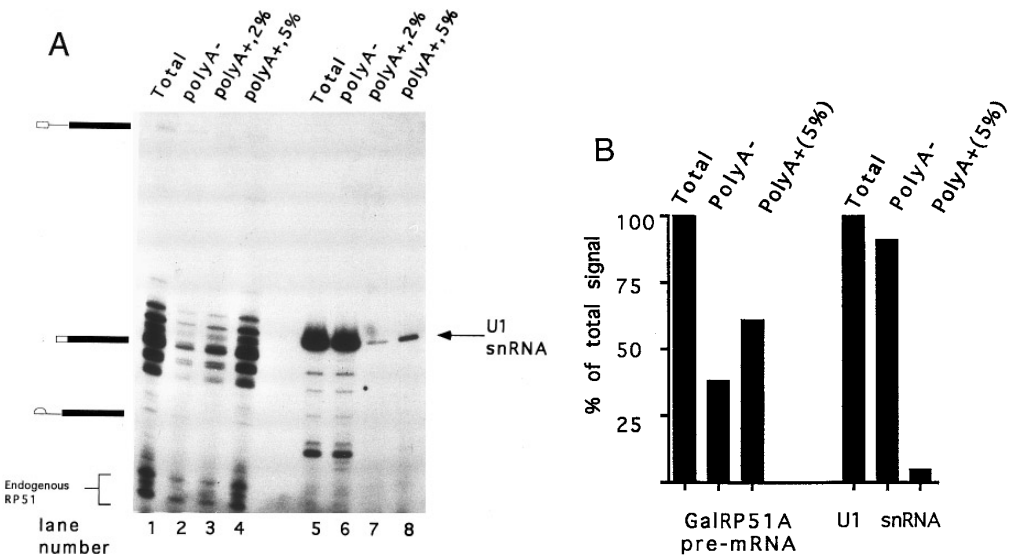

FIG. 5. Poly(A) fractionation of steady-state RNA obtained from GalRP51A-transformed cells grown in galactose-containing media. One hundred micrograms of total RNA was fractionated into poly(A)$^+$ and poly(A)$^-$ populations as described under Materials and Methods. Four micrograms of total RNA and poly(A)$^-$ RNA and 0.08 μg (corresponding to 2% of total RNA) and 0.2 μg (corresponding to 5% of total RNA) of poly(A)$^+$ RNA were analyzed by primer extension from oligonucleotides RB1 (complementary to RP51) and 2133 (complementary to U1 snRNA). (A) The primer extension products were analyzed on a 5% polyacrylamide urea gel. (B) The gel shown in (A) was then quantitated using a Phosphorimager. The values obtained for the poly(A)$^-$ and poly(A)$^+$ fractions of the primer extension products corresponding to RP51A pre-mRNA and U1 snRNA were expressed as a percentage of the values obtained for total RNA. Each sample was corrected for background radiation present in its lane.

is still being transcribed (nascent splicing) or afterward (posttranscriptional splicing). While there is no published elongation rate for yeast RNA polymerase II, several estimates for *Drosophila* are between 1 and 1.5 kb/min [13–16]. There are reasons to believe that yeast elongation rates are not much different [16, 17]. If such figures also apply to yeast, a 2-s lifetime means that splicing occurs shortly after transcription of the 3′ splice site. The reverse transcriptase primer used in this study begins only a few nucleotides after the 3′ splice site and so should detect nascent pre-mRNA even if splicing occurs rapidly after transcription of the 3′ splice site. While our biochemical procedures might have failed to extract nascent RNA, a 2-s half-life suggests that the true pre-mRNA population is undetectable because it is present at very low concentration and/or has been spliced or degraded during the rapid harvesting and freezing of the cells.

Experiments to measure the kinetic properties of pre-mRNA in organisms, and how this relates to where and when splicing takes place, do not provide a uniform picture. Nascent splicing has been inferred from morphological examination of Miller spreads, in which chromatin with attached nascent transcripts is visualized under the electron microscope [18]. Interpretation of these spreads suggested that factors bound rapidly to the 5′ splice site, followed by transcription through the rest of the intron, spliceosome assembly, and splicing, all on nascent transcripts. In *Drosophila*, nascent splicing of upstream exons before the transcription of downstream RNA has been demonstrated biochemically in the case of extremely long, developmentally regulated transcription units [19]. More recently, nascent splicing has been demonstrated for transcripts encoded by *Chironomus* balbiani rings, by microdissection of nascent complexes [20]. Furthermore, trans-splicing has been shown to occur rapidly and on nascent RNA in trypanosomes [21].

Other evidence, however, suggests splicing may be posttranscriptional. These experiments reported that the pre-mRNAs of ovalbumin, ovomucoid, immunoglobulin, and late adenoviral genes are polyadenylated, and pulse-chase experiments suggested they were genuine precursors [22–24]. However, there is biochemical evidence for cotranscriptional splicing at some frequency from late adenoviral and immunoglobulin genes [25–27]. These conflicting data on the pathways of pre-mRNA maturation can be reconciled if we generalize our result with yeast RP51A, predicting that many intron-containing genes encode two or more pre-mRNA classes with different kinetic properties. Although some genes may splice by entirely different pathways, most transcripts of most genes are spliced rapidly on nascent RNA, with a minor proportion occurring more slowly and posttranscriptionally. Hence, experiments that indicate posttranscriptional splicing may have

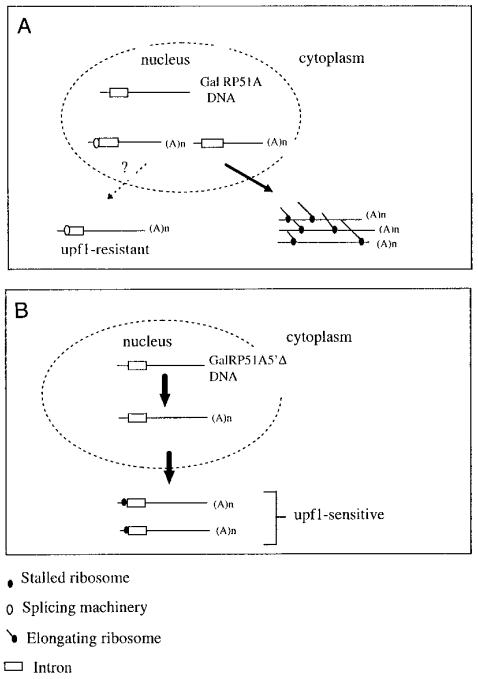

FIG. 6. Model of pre-mRNA metabolism in yeast. (A) GalRP51A is transcribed into two populations of pre-mRNA. A large population is rapidly spliced (perhaps cotranscriptionally), polyadenylated, and exported to the cytoplasm, where it is translated. A second smaller population binds splicing factors but fails to splice or splices more slowly. This second class does not access the translation machinery as a result of splicing factors sequestering translational initiation signals and so is not degraded by the upf1 decay system. As a result of its slow decay, this second class makes a large contribution to the steady-state level of gal RP51A pre-mRNA. (B) Gal RP51A 5′Δss is transcribed into a single population of molecules which cannot be spliced and is polyadenylated and exported into the cytoplasm where it is degraded by the upf1 decay system.

been assaying molecules with a substantially longer half-life than the principal precursor population. In support of this generalization, a similar phenomenon has also been reported based on microscopic observation of the transcripts encoded by the *Chironomus* balbiani ring. In this case, a fraction (2–5%) of mRNP molecules in the nucleoplasm still contain an intron, which is usually spliced cotranscriptionally. Despite their much slower turnover, the minor fraction still bound splicing factors [20]. This is consistent with the upf1 insensitivity of the slowly processed GalRP51A pre-mRNA molecules. Similarly, examination of the structure of pre-mRNAs in rat liver nuclei provides evidence for both nascent spliced pre-mRNA and soluble polyadenylated pre-mRNA [27].

Because of their much longer half-lives, the slowly or poorly spliced fraction may make a substantial or even dominant contribution to steady-state pre-mRNA levels. Indeed, in mammalian cells a significant fraction of nuclear poly(A)$^+$ RNA does not decay rapidly after transcriptional inhibition [28]. This view has the potential to explain different *in situ* hybridization observations of different pre-mRNAs within mammalian nuclei [29–31]; for genes with a greater fraction of slowly spliced primary transcripts, the location of their steady-state pre-mRNA population may be different from that of primary transcripts which are rapidly spliced cotranscriptionally. The biochemical characteristics of the slowly spliced population may resemble those reported here for yeast GalRP51A pre-mRNA transcripts.

We thank our colleagues, especially F. Stutz and M. Moore, for helpful comments during the course of these experiments. The work was supported in part by the National Institutes of Health to M.R. (GM 23549).

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Received September 20, 1996