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The stability and fate of a spliced intron from vertebrate cells

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ABSTRACT

Introns constitute most of the length of typical pre-mRNAs in vertebrate cells. Thus, the turnover rate of introns may significantly influence the availability of ribonucleotides and splicing factors for further rounds of transcription and RNA splicing, respectively. Given the importance of intron turnover, it is surprising that there have been no reports on the half-life of introns from higher eukaryotic cells. Here, we determined the stability of IVS1$_{C\beta1}$, the first intron from the constant region of the mouse T-cell receptor-*β* (*TCR-β*) gene. Using a tetracycline (tet)-regulated promoter, we demonstrate that spliced IVS1$_{C\beta1}$ and its pre-mRNA had half-lives of 6.0 ± 1.4 min and 3.7 ± 1.0 min, respectively. We also examined the half-lives of these transcripts by using actinomycin D (Act.D). Act.D significantly stabilized IVS1$_{C\beta1}$ and its pre-mRNA, suggesting that Act.D not only blocks transcription but exerts rapid and direct posttranscriptional effects in the nucleus. We observed that in vivo spliced IVS1$_{C\beta1}$ accumulated predominantly as lariat molecules that use a consensus branchpoint nucleotide. The accumulation of IVS1$_{C\beta1}$ as a lariat did not result from an intrinsic inability to be debranched, as it could be debranched in vitro, albeit somewhat less efficiently than an adenovirus intron. Subcellular-fractionation and sucrose-gradient analyses showed that most spliced IVS1$_{C\beta1}$ lariats cofractionated with pre-mRNA, but not always with mRNA in the nucleus. Some IVS1$_{C\beta1}$ also appeared to be selectively exported to the cytoplasm, whereas TCR-*β* pre-mRNA remained in the nucleus. This study constitutes the first detailed analysis of the stability and fate of a spliced nuclear intron in vivo.

Keywords: actinomycin D; intron half-life; intron lariat; in vivo branchpoint; subcellular fractionation; T-cell receptor; tetracycline-regulated promoter

INTRODUCTION

Nuclear pre-mRNA splicing is an important step in gene expression in which introns and mRNAs are produced in equal molar amounts (Green, 1991; Moore et al., 1993; Sharp, 1994). During this process, the released 5′ end of the intron loops back and covalently links to the branchpoint nucleotide (usually an adenosine) at the 3′ end of the intron to form a lariat molecule that contains a linear tail at its 3′ terminus (Keller, 1984; Padgett et al., 1984; Ruskin et al., 1984). The formation of this lariat structure has been postulated to be a driving force that directs exon ligation to generate mRNA. In addition, the lariat structure itself may play a role in preventing the degradation of the intron during splicing (Keller, 1984). When the lariat introns are removed from primary transcripts, the adjacent exons join and form mature mRNAs, which are exported to the cytoplasm and subjected to mechanisms that regulate their stability.

Although much has been learned about the regulation of mRNA stability, remarkably little is known about the fate or stability of spliced intron lariats. Because eukaryotic cells generate large amounts of spliced introns, their metabolism is probably critical for normal cellular functions. For example, the rate of intron turnover may dictate the recycling rate of splicing factors bound to released introns (Green, 1991). U2, U5, and U6 small nuclear ribonuclear proteins (snRNPs) and Ser-Arg (SR)-related proteins remain bound to released intron lariats in vitro (Wassarman & Steitz, 1992; Blencowe et al., 1995), and thus the metabolism of introns may have a significant influence on the recycling of these splicing factors. Evidence for the importance of efficient intron turnover comes from studies on organisms deficient in debranchase, which specifically cleaves the 2′-5′ phosphodiester bond at the lariat branch site (Ruskin & Green, 1985; Arenas & Hurwitz, 1987; Chapman & Boeke, 1991). *Schizosaccharomyces pombe* deficient in debranchase because of a null

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mutation accumulate high levels of intron lariats and exhibit a severe growth defect (Nam et al., 1997). In contrast, the budding yeast *Saccharomyces cerevisiae*, an organism that contains ~40 times fewer introns than *S. pombe*, grows normally when deficient in debranchase (Chapman & Boeke, 1991).

Because introns comprise a very large fraction of transcribed RNA from higher eukaryotic organisms, efficient intron turnover is probably very important for higher eukaryotic cells. For example, the rate of intron turnover is likely to influence the levels of nucleotides available for further rounds of transcription. In addition, posttranscriptional events may be influenced by the rate of intron turnover. Vertebrate cells execute complex constitutive and alternative-splicing events that depend on precise concentrations of splicing-regulatory factors. The rate of intron turnover may significantly affect the availability of these splicing factors, thereby regulating many cellular processes in multicellular organisms.

Given the importance of intron turnover for normal cellular function, it is surprising that the half-life of a higher eukaryotic pre-mRNA intron has never been reported. In part, this may reflect the fact that very few spliced nuclear pre-mRNA introns have been detected in vertebrate cells. Most of the few excised introns that have been observed in vivo are derived from strongly transcribed genes. For example, excised lariat and linear introns from adenovirus-2 E_2A pre-mRNA were identified in HeLa cells treated with cycloheximide to increase the rate of transcription (Keohavong et al., 1986). β-globin introns were detected in lariat and linear forms from rabbit liver, which is rich in β-globin pre-mRNA (Zeitlin & Esfstratiadas, 1984). In another case, a spliced intron from a strongly transcribed immunoglobulin-κ gene was detected in a stimulated plasmacytoma cell line (Coleclough & Wood, 1984).

The only intron half-lives that have been reported are those from lower organisms. The half-life of a group I intron from the phage T4 *td* gene is 20 to 30 min in *Escherichia coli*, more than 10 times longer than its counterpart mRNA (Chan et al., 1988). The half-life of the actin intron in *S. cerevisiae* is ~5 min (Jacquier & Rosbash, 1986), which is about one third of the average half-life of yeast mRNAs (Brown et al., 1988; Peltz & Jacobson, 1993). In higher eukaryotic cells, some spliced introns have been reported to be "stable" (Michaeli et al., 1988; Farrell et al., 1991; Kopczynski & Muskavitch, 1992), but their half-lives have not been reported. In other studies (e.g., Wijgerde, 1995), the stability of intron-containing RNAs has been assessed, but the half-lives of released introns and that of their respective pre-mRNAs have not been distinguished.

In this report, we present the first stability analysis of a spliced intron from a higher eukaryotic cell. We chose to evaluate the stability of $IVS1_{C\beta1}$, an intron from the constant region of the T-cell receptor-β (*TCR-β*) gene that is detectable by northern blot analysis (Qian et al., 1992). We examined its stability by two independent approaches, including using the tetracycline (tet)-regulated promoter. Because intron half-life is probably dictated by intron lariat debranching (linearization), we identified the branchpoint of $IVS1_{C\beta1}$ used in vivo, and we determined the ability of $IVS1_{C\beta1}$ lariats generated in vivo to be debranched. To examine the fate of $IVS1_{C\beta1}$ after it is excised from pre-mRNA, we performed subcellular-fractionation and sucrose-gradient analyses. These analyses indicated that while some spliced $IVS1_{C\beta1}$ appears to be exported to the cytoplasm, most is in nuclear fractions where it cofractionates with TCR-β pre-mRNA, but not always TCR-β mRNA. This study contributes to our understanding of RNA metabolism, and it provides useful approaches for investigating the regulation and function of spliced introns.

RESULTS

Stability of spliced $IVS1_{C\beta1}$ and its pre-mRNA determined with the tet promoter

To determine the stability of $IVS1_{C\beta1}$ after it is spliced out of pre-mRNA in vivo, we used the tet-repressible promoter system. pTβM, a construct containing $IVS1_{C\beta1}$ and the adjacent exons under the control of the tet promoter, was transfected stably into HeLa cells, along with pTAN, which encodes the transactivator protein necessary for expression from the tet promoter (see Materials and Methods). A stably transfected HeLa cell clone that expressed high levels of transcripts from pTβM was selected for further study. This cell clone expressed four major transcripts that hybridized with an $IVS1_{C\beta1}$ probe: a pre-mRNA (2.1 kb), spliced $IVS1_{C\beta1}$ (0.52 kb), and two other transcripts that we termed bands A and B (Fig. 1A, time = 0). Band A was a 4.1-kb transcript that appeared to be a primary transcript from the tet promoter whose 3′ end is defined by a distal poly(A) site (AAUAAA) ~2 kb downstream of that used by the 2.1-kb pre-mRNA (Gossen & Bujard, 1992). This conclusion was based both on its size and the fact that band A hybridized with a probe downstream of the proximal poly(A) site used by the 2.1-kb pre-mRNA (see Materials and Methods). Band A was apparently unspliceable because no mature mRNA product (which would be 3.6 kb) was detected with an exon probe. Band B (~1 kb) was probably a processing intermediate because it hybridized with both *TCR-β* intron and exon probes, and it was present only in the nucleus and not the cytoplasm.

To determine the stability of $IVS1_{C\beta1}$ and its pre-mRNA, we performed time-course experiments. We first examined the kinetics of RNA decline in both the nuclear and cytoplasmic fractions to independently determine the fate of the transcripts in these two different compartments. tet decreased the levels of the

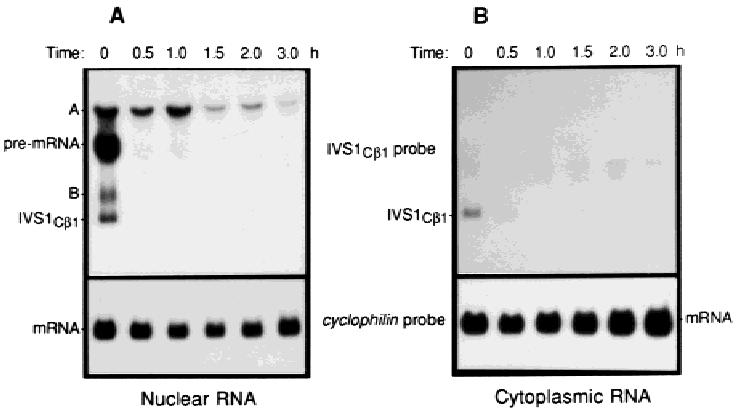

FIGURE 1. Rapid cessation of *TCR-β* minigene transcription in response to tet. The decay of $IVS1_{C\beta1}$ and its pre-mRNA from the pTβM-transfected HeLa cell clone was monitored by Northern blot analysis of nuclear (**A**) and cytoplasmic (**B**) RNA (10 μg) isolated at the times shown after incubation with tet and electrophoresed in a 1.2% agarose gel. The blot was hybridized sequentially with the indicated probes. The levels of cyclophilin mRNA reflect the amount of RNA loaded in each lane.

spliced intron and its pre-mRNA to undetectable levels in the nucleus within 0.5 h of incubation (Fig. 1A). Band B also rapidly disappeared after the cessation of transcription, whereas band A persisted in the nucleus for at least 3 h, consistent with its being an unspliceable primary transcript. In the cytoplasm, the only $IVS1_{C\beta1}$-bearing transcript that was detectable was the spliced intron (Fig. 1B). This suggests the possibility that some spliced $IVS1_{C\beta1}$ is selectively exported to the cytoplasm. It is also possible that the spliced $IVS1_{C\beta1}$ that we observed in the cytoplasmic fraction resulted from leakage from the nucleus during the preparation of cytoplasmic RNA. Northern blot analysis of several independent matched sets of nuclear and cytoplasmic RNA indicated that spliced $IVS1_{C\beta1}$ levels in cytoplasmic RNA were between ~10 to ~30% of that in the nuclear RNA. Given that there is approximately sevenfold more RNA in the cytoplasm than in the nucleus (based on the typical yields that we obtained), this means that spliced $IVS1_{C\beta1}$ is found in similar amounts in the cytoplasm and the nucleus (between 0.7- and 2-fold difference). Because cytoplasmic $IVS1_{C\beta1}$ was not detected after 0.5 h of incubation with tet (Fig. 1B), the low steady-state level of this spliced intron in the cytoplasm may reflect, in part, its rapid degradation in the cytoplasmic compartment.

To determine the half-life of $IVS1_{C\beta1}$ and its pre-mRNA, we performed short time-course experiments. Northern blot analysis revealed that the levels of $IVS1_{C\beta1}$ and its pre-mRNA decreased within 5 min of addition of tet (Fig. 2A). This rapid effect indicated that tet very efficiently blocked transcription. By 10 min, $IVS1_{C\beta1}$ and the pre-mRNA were almost undetectable, whereas the expression of the control housekeeping gene cyclophilin remained unperturbed (Fig. 2A). The half-life of $IVS1_{C\beta1}$ was determined by least-square linear regression analysis assuming that the decay rate of $IVS1_{C\beta1}$ follows first-order kinetics, as has been shown for several mature mRNAs (see Materials and Methods). A representative line-fit plot is shown in Figure 2B, based on the results shown in Figure 2A. The distribution of the experimental data was nearly linear, suggesting that the decay rate of the spliced intron followed first-order kinetics. From analysis of four independent experiments, the half-life value for $IVS1_{C\beta1}$ was determined to be 6.0 $\pm$ 1.4 min. The half-life for the pre-mRNA was 3.7 $\pm$ 1.0 min.

We next determined the relative proportion of spliced $IVS1_{C\beta1}$ molecules that were in the lariat and linear conformations. Polyacrylamide gel electrophoresis was used to separate these two forms; linear molecules are known to migrate according to their molecular weight, whereas lariats migrate more slowly in polyacrylamide gels. Under steady-state conditions, most spliced $IVS1_{C\beta1}$ molecules were lariats, although trace amounts of the linear form were also detected (Fig. 2C; see also Fig. 7 for debranching experiments that further identify these molecules as lariats). After cessation of transcription as a result of tet treatment, there was a rapid decrease in the levels of both lariat and linear $IVS1_{C\beta1}$ molecules. Importantly, the steady-state levels of the linear form did not increase concomitant with a decrease in the lariat form, and degradation intermediates did not appear. This suggests that $IVS1_{C\beta1}$ was degraded very rapidly after being debranched.

To measure the half-life of mature *TCR-β* mRNA, longer time-course experiments were performed. A typical kinetic experiment is shown in Figure 3, in which the kinetic decay of the mRNA in both the cytoplasmic (Fig. 3A) and the nuclear (Fig. 3B) fractions were analyzed. The half-lives of the mRNA were determined by least-square linear regression analysis to be 7.3 h and 5.5 h for the cytoplasmic and nuclear mRNAs, respectively (Figs. 3C and 3D). Because of the inevitable low levels of cytoplasmic contamination in the nuclear RNA, the calculated nuclear half-life may be an overestimate. Nevertheless, the shorter half-life in the nucleus probably reflects, at least in part, transport of *TCR-β* mRNA to the cytoplasm. In *Xenopus laevis* oocytes, it has been shown that in vitro-synthesized mRNAs are transported to the cytoplasm with a $t_{1/2}$ rate of about 30 min to 1 h (Jarmolowski et al., 1994). To our knowl-

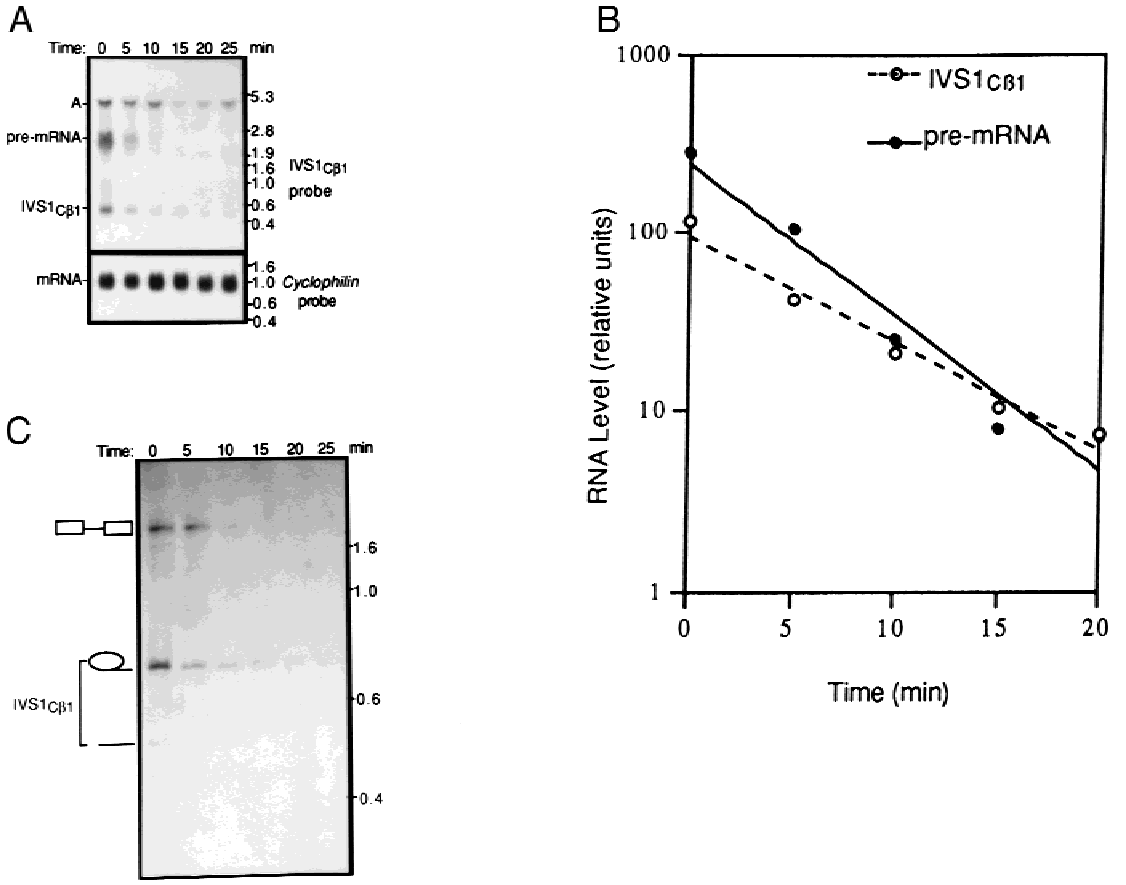

FIGURE 2. Half-life analysis of spliced IVS1$_{C\beta1}$ and its pre-mRNA driven by the tet-repressible promoter. **A**: Northern blot analysis of total cellular RNA (10 μg) electrophoresed in a 1.2% agarose gel. RNA was isolated from the pTβM-transfected HeLa cell clone at the times shown after incubation with tet. The blot was hybridized sequentially with the indicated probes. The levels of cyclophilin mRNA reflect the amount of RNA loaded in each lane. **B**: Line-fit plot of the time-dependent decay of IVS1$_{C\beta1}$ and its pre-mRNA, based on the data from panel A (the values were normalized against cyclophilin mRNA levels). Open circles: IVS1$_{C\beta1}$; closed circles: pre-mRNA. **C:** Northern blot analysis as in panel A, except that the RNA was electrophoresed in a 3.5% denaturing urea-polyacrylamide gel. The blot was hybridized with an IVS1$_{C\beta1}$ probe.

edge, the rate at which mRNAs are transported from the nucleus to the cytoplasm in mammalian cells in vivo has not been reported previously.

Intron and pre-mRNA RNA half-lives determined using transcriptional inhibitors

The half-life that we determined for IVS1$_{C\beta1}$ is comparable to that of short-lived mRNAs such as c-*myc* mRNA (Dani et al., 1984; Hargrove & Schmidt, 1989). Because the half-lives of most mRNAs have been determined using the transcriptional inhibitor actinomycin D (Act.D), we chose to also use this approach to measure the half-life of spliced IVS1$_{C\beta1}$. At a concentration of Act.D that inhibited [^{3}H] uridine incorporation by $>$99% (5 μg/mL), spliced IVS1$_{C\beta1}$ and its pre-mRNA decayed rapidly whereas band A persisted (Fig. 4A), similar to the response to tet (Fig. 2A). However, we found quantitative differences between the results obtained with Act.D and tet: least-square linear regression analysis indicated that the average half-lives of IVS1$_{C\beta1}$ and its pre-mRNA were 6.5 min and 6.6 min, respectively (Fig. 4B). Thus, the half-life of the pre-mRNA in the presence of Act.D was almost double that obtained with the tet system. While the calculated half-life of spliced IVS1$_{C\beta1}$ was only slightly longer in the presence of Act.D than with Tet, there was a significant difference in the kinetics of IVS1$_{C\beta1}$ decline, as was demonstrated in three independent experiments, including one experiment in which Act.D and tet were provided in parallel (data not shown). We therefore conclude that Act.D stabilizes both the spliced intron and its pre-mRNA.

We also examined the effect of Act.D on the stability of other transcripts. Act.D treatment stabilized mature *TCR-β* transcripts, as there was essentially no change in their levels (Fig. 4C) compared with that of the cyclophilin control (Fig. 4D) over the 8-h time course. We

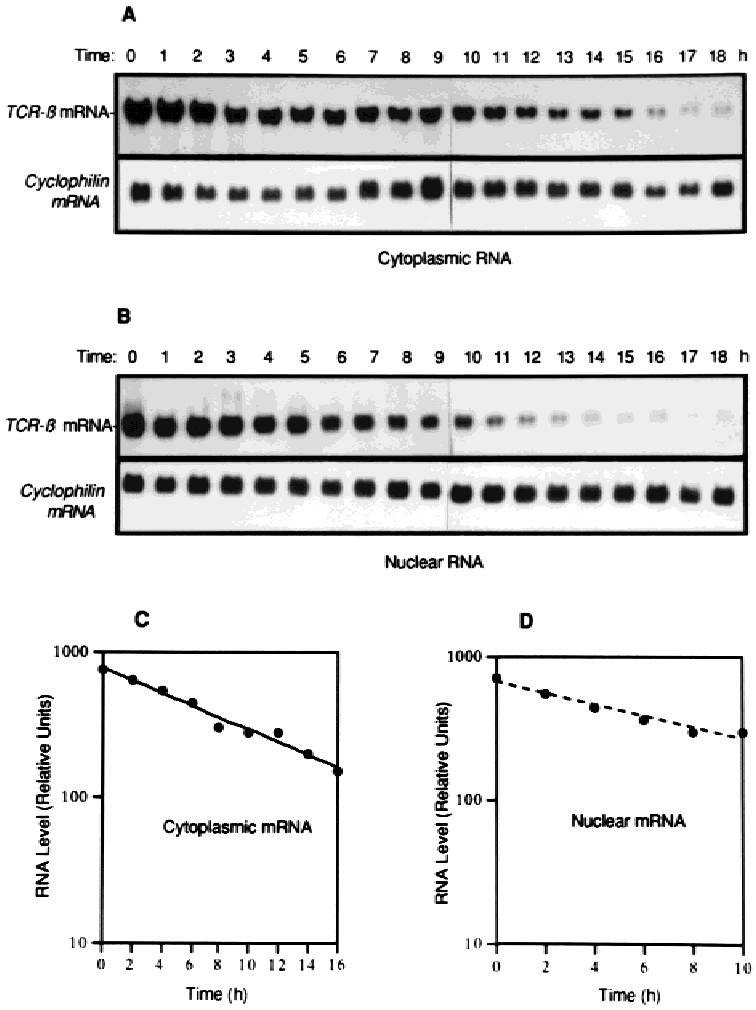

FIGURE 3. Half-life analysis of *TCR-β* mRNA. **A**,**B**: Northern blot analysis of 10 μg of cytoplasmic RNA (**A**) and 10 μg of nuclear RNA (**B**) electrophoresed in a 1.2% agarose gel. The RNA was isolated from the pTβM-transfected HeLa cell clone at the times shown after addition of tet. The blot was hybridized sequentially with the *TCR-β* and cyclophilin probes. The levels of cyclophilin mRNA reflect the amount of RNA loaded in each lane. **C**,**D**: Line-fit plot of *TCR-β* mRNA levels from the data in **A** and **B**, respectively; normalized against cyclophilin mRNA levels.

also examined the stability of the short-lived c-*myc* mRNA (Fig. 4E) to compare its half-life with that of spliced IVS1$_{C\beta1}$ (Fig. 4A). The c-*myc* mRNA half-life was 17.6 min (Fig. 4F), which is similar to that reported by others (~15 min) when Act.D is used to assess its half-life (Dani et al., 1984).

We also examined IVS1$_{C\beta1}$ stability with 5,6-dichloro-1β-D-ribofuranosylbenzimidazole (DRB), which at low concentrations selectively inhibits RNA polymerase II (Tamm & Sehgal, 1978). To our surprise, we found that a concentration of DRB sufficient to block RNA polymerase II (16 μg/mL) had a biphasic effect on the metabolism of *TCR-β* transcripts. The first phase consisted of a rapid decrease in the levels of spliced IVS1$_{C\beta1}$ and its pre-mRNA, and the second phase (beginning 10 min after DRB treatment) was characterized by a gradual increase in the levels of IVS1$_{C\beta1}$-containing transcripts (data not shown). Because of this biphasic effect, we were not able to calculate the half-life of *TCR-β* transcripts in response to DRB. We conclude that DRB is not a useful reagent for measuring the kinetics of nuclear RNA turnover.

Half-lives of spliced IVS1$_{C\beta1}$ and its pre-mRNA from the endogenous *TCR-β* gene

We next tested whether the half-life of IVS1$_{C\beta1}$ is influenced by *TCR-β* gene copy number. Because we had used a stably transfected cell line that probably contains multiple copies of the IVS1$_{C\beta1}$-containing plasmid driven by a highly active promoter, this may have saturated the degradation system responsible for IVS1$_{C\beta1}$ decay. We therefore determined the stability of IVS1$_{C\beta1}$ when it is derived from the endogenous *TCR-β* gene in the murine T-cell lymphoma cell clone SL12.4, which constitutively transcribes a fully rearranged single-copy *TCR-β* gene (Qian et al., 1992, 1993a, 1993b). A typical time-course experiment using Act.D is shown in Figure 5A. The levels of both the intron and pre-mRNA declined rapidly in SL12.4 cells after Act.D. treatment, whereas cyclophilin transcripts did not decline in levels over the short time period examined. The half-life of IVS1$_{C\beta1}$ and its pre-mRNA were determined by least-squares linear regression analysis after normalization against cyclophilin mRNA levels (Fig. 5B). The average

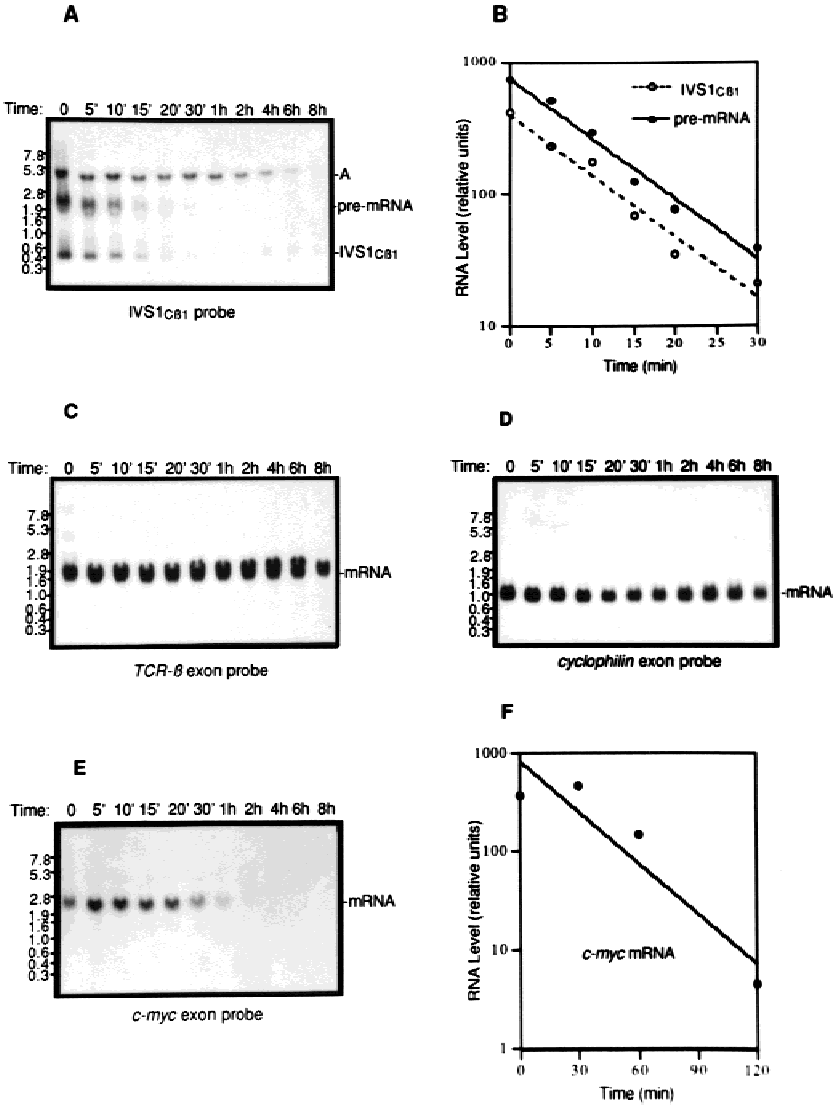

FIGURE 4. Half-life analysis of spliced IVS1$_{C\beta1}$, *TCR-β* pre-mRNA, *TCR-β* mRNA, and c-*myc* mRNA assessed using Act.D. **A**,**C**,**D**,**E**: Northern blot analysis of total cellular RNA (10 μg) electrophoresed in a 1.2% agarose gel. RNA was isolated from the pTβM-transfected HeLa cell clone at the times shown after incubation with Act.D. The blot was hybridized sequentially with the indicated probes. **B**: Line-fit plot of the time-dependent decay of IVS1$_{C\beta1}$ and its pre-mRNA based on the data shown in **A** (the values were normalized against the cyclophilin mRNA levels). Open circles: IVS1$_{C\beta1}$; closed circles: pre-mRNA. **F**: Line-fit plot of the time-dependent decay of cellular c-*myc* mRNA based on the data shown in **E** (the values were normalized against the cyclophilin mRNA levels).

half-life values for spliced IVS1$_{C\beta1}$ and pre-mRNA from four independent time course experiments were 13.4 $\pm$ 3.1 min and 21.0 $\pm$ 7.7 min, respectively, which are longer than that determined from the pTβM-transfected HeLa cell clone (6.5 min and 6.6 min). This strongly suggests that the presence of multiple copies of TCR-β did not inflate the half-life values in stably transfected HeLa cells. Instead, cell type-specific factors may be responsible for the different RNA half-life values in SL12.4 T cells and HeLa epithelial cells.

IVS1$_{C\beta1}$ lariats that accumulate in vivo are debranchable in vitro

IVS1$_{C\beta1}$ may be an unusually stable intron lariat resistant to debranching and therefore not susceptible to degradation by exonucleases. To examine this issue, we first determined the IVS1$_{C\beta1}$ branchpoint sequence. In contrast to most studies that have determined branchpoint usage during in vitro splicing reactions (Reed & Maniatis, 1985), we examined the branchpoint of IVS1$_{C\beta1}$ lariats from an in vivo source. We performed primer extension analysis using poly $(A)^+$ RNA, as it is enriched for IVS1$_{C\beta1}$ lariats because of long internal poly (A) tracts in IVS1$_{C\beta1}$ (Qian et al., 1992). The extension product obtained indicated that the branchpoint was an adenine nucleotide upstream of a homogeneous 35-nt polypyrimidine tract (Fig. 6). Poly$(A)^+$ RNA from control (nontransfected) HeLa cells, which do not express the *TCR-β* gene, did not generate any detectable extension product (data not shown). The IVS1$_{C\beta1}$ branchpoint region was identical to the branchpoint consensus sequence at 5 of 7 nt (Fig. 6); this degree of similarity is typical for mammalian introns. We conclude that IVS1$_{C\beta1}$ does not have an unusual branchpoint sequence and therefore there is no a priori reason that it should not be a good substrate for debranchase.

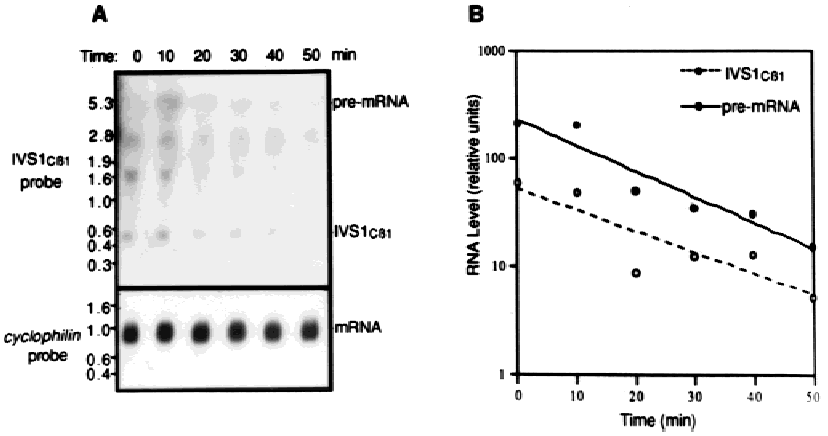

FIGURE 5. Half-life analysis of spliced IVS1$_{C\beta1}$ and its pre-mRNA from the endogenous *TCR-β* gene in the SL12.4 T-cell clone. **A**: Northern blot analysis of total cellular RNA (10 μg) from mouse SL12.4 cells electrophoresed in a 1.2% agarose gel. The time intervals for incubation with Act.D are indicated at the top of the figure. The blot was hybridized sequentially with the indicated probes. **B**: Line-fit plot of the time-dependent decay of spliced IVS1$_{C\beta1}$ and its pre-mRNA based on the data in **A** (the values were normalized against cyclophilin mRNA levels).

We next examined directly whether the IVS1$_{C\beta1}$ lariat can be debranched by performing in vitro debranching experiments. Poly(A)$^+$ RNA from transfected HeLa cells expressing IVS1$_{C\beta1}$ was used as a template and HeLa cell S100 extracts was used as a source of debranchase. IVS1$_{C\beta1}$ intron lariats were debranched completely in the presence of 10% S100 extracts (Fig. 7A). The debranching was time dependent: incubation for 20 min partially debranched IVS1$_{C\beta1}$ (data not shown) and incubation for 50 min led to complete debranching (Fig. 7A). Increasing the concentration of the S100 extract resulted in degradation of the linearized intron, presumably because of exonucleases present in the S100 extract (Fig. 7A). In contrast, cyclophilin mRNA was not noticeably degraded by the S100 extract during the incubation time examined (Fig. 7B). We conclude that IVS1$_{C\beta1}$ is not inherently resistant to debranching and that its linearized product is susceptible to degradation by exonucleases.

To determine the relative debranching efficiency of the IVS1$_{C\beta1}$ lariat, we compared its ability to be debranched with that of an adenovirus intron lariat. The adenovirus lariat was generated by in vitro transcription and splicing (Fig. 7C, lane 2 shows the in vitro transcription product and lane 3 shows the lariat generated by in vitro splicing). Incubation with S100 extract debranched the adenovirus intron lariat to its linear form (Fig. 7C, lane 5; the uppermost and lowermost transcripts are the lariat and linear molecules, respectively). To compare directly the debranching efficiency of the adenovirus intron and IVS1$_{C\beta1}$, the two types of intron lariats were incubated with S100 debranching extract in the same reaction tubes under suboptimal debranching conditions. The debranched products were divided into two equal portions and analyzed in a 12% polyacrylamide gel for the adenovirus intron (Fig. 7C, lane 6) and in a 5% polyacrylamide gel for IVS1$_{C\beta1}$ (Fig. 7D, lane 2). Under conditions that permitted nearly complete debranching of the adenovirus lariat, only a fraction of IVS1$_{C\beta1}$ lariats were debranched. We estimate that the adenovirus intron lariat was debranched 2–3 times more efficiently than IVS1$_{C\beta1}$, based on the percentage of the lariats linearized by the S100 extracts. IVS1$_{C\beta1}$ lariats were also debranched less efficiently than msDNA lariats, which contain a hybrid RNA-DNA branchpoint (Nam et al., 1994; data not shown). We conclude that IVS1$_{C\beta1}$ lariats can be debranched, albeit somewhat less efficiently than at least some other intron lariats.

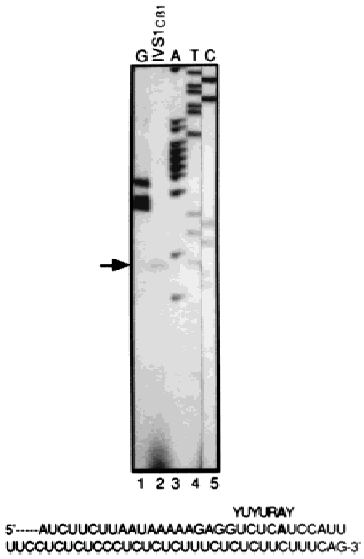

FIGURE 6. Mapping of the IVS1$_{C\beta1}$ branchpoint nucleotide. IVS1$_{C\beta1}$ lariats (enriched in poly(A)$^+$ RNA from *TCR-β*-transfected HeLa cells as a result of the internal polyadenylate tract in IVS1$_{C\beta1}$) were subjected to primer extension analysis with a 20-nt end-labeled synthetic oligonucleotide complementary to the extreme 3′ terminus of IVS1$_{C\beta1}$. Lane 2 contains the primer-extension product (indicated by the arrow). Lanes 1, 3, 4, and 5 are di-deoxy sequencing reaction mixtures performed with the same oligonucleotide primer. As a result of steric hindrance caused by the chemical structure of the branchpoint nucleotide, the extension of the complementary DNA chain terminates one nucleotide downstream from the branchpoint nucleotide (Ruskin et al., 1984). The branchpoint nucleotide (in bold) in the 3′ terminus of IVS1$_{C\beta1}$ is shown at the bottom of the figure, along with the mammalian branchpoint consensus sequence (Y = C,U; R = A,G).

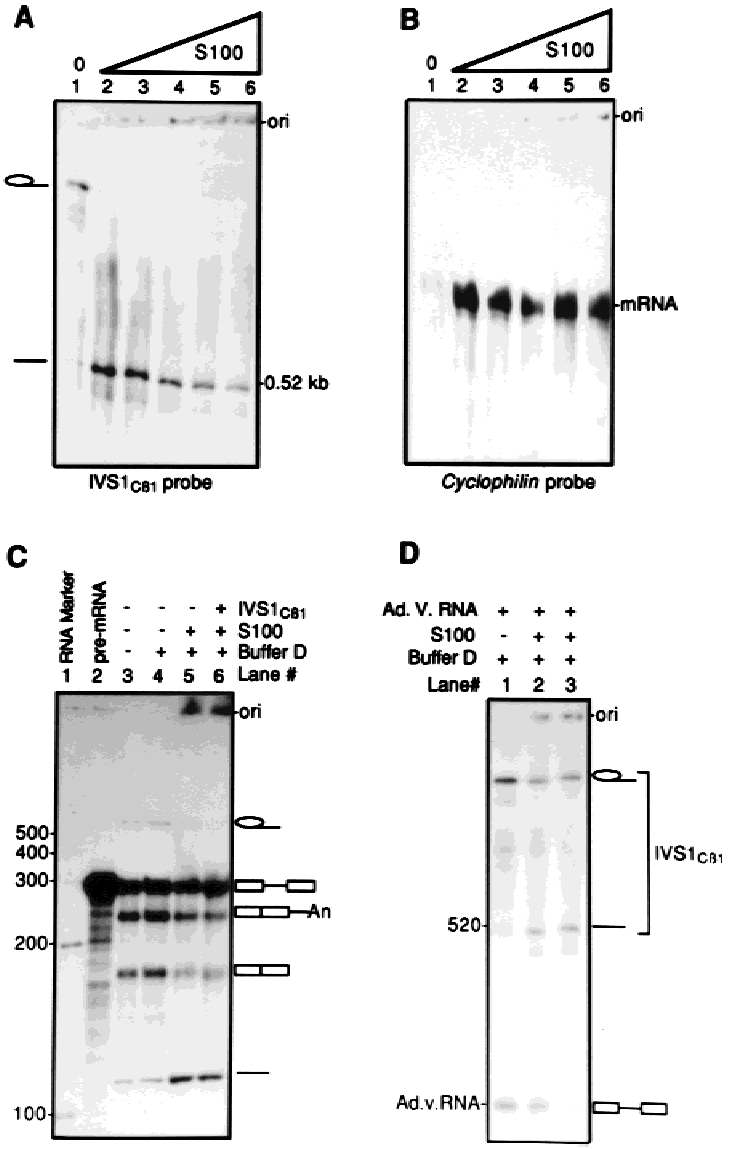

FIGURE 7. Spliced IVS1$_{C\beta1}$ accumulates as lariat molecules in vivo that are debranchable in vitro. **A**,**B**: Northern blot analysis of IVS1$_{C\beta1}$ lariats (enriched in poly(A)$^+$ RNA from *TCR-β*-transfected HeLa cells as a result of the internal polyadenylate tract in IVS1$_{C\beta1}$) subjected to in vitro debranching and electrophoresed in a 5% polyacrylamide gel. Lane 1 contains 1 μg of poly(A)$^+$ RNA incubated without the S100 debranching extract and lanes 2–6 contain 5 μg of poly(A)$^+$ RNA incubated under conditions that permitted complete debranching of IVS1$_{C\beta1}$ (10, 20, 30, 40, and 50% S100 extract in lanes 2–6, respectively, incubated for 50 min at 32 °C). The blot was hybridized sequentially with the indicated probes. The lariat and linear forms of IVS1$_{C\beta1}$ are indicated. **A** and **B** show the same portion of the blot so that the relative positions of the *TCR-β* and cyclophilin transcripts can be seen. ori: origin. **C**: The debranching efficiency of IVS1$_{C\beta1}$ lariats was compared with that of adenovirus intron lariats by comparing their debranchability under suboptimal conditions (as in **B** except incubated for only 20 min with 10% S100 extract). Adenovirus-intron lariats were derived from ^{32}P-labeled adenovirus pre-mRNA (lane 2) by in vitro splicing (lane 3), subjected to in vitro debranching under the same condition as that for IVS1$_{C\beta1}$ lariats (lanes 5 and 6), and analyzed in a 10% denaturing urea-polyacrylamide gel. **D**: IVS1$_{C\beta1}$ lariat debranching performed as in **C** except that the RNA products were electrophoresed in a 5% denaturing urea-polyacrylamide gel, blotted, and hybridized with an IVS1$_{C\beta1}$ probe. Adenovirus-intron lariats were included in all of the reactions (i.e., equivalent to the lane 6 reaction of **C**), so that the debranching efficiency of IVS1$_{C\beta1}$ lariats could be compared directly with that of adenovirus lariats under identical conditions (note that only the ^{32}P-labeled adenovirus pre-mRNA is observable in the 5% gel shown).

Subcellular-fractionation and sucrose-gradient analyses of spliced IVS1$_{C\beta1}$, its pre-mRNA, and *TCR-β* mRNA

To further explore the factors that dictate the fate and stability of IVS1$_{C\beta1}$, we performed subcellular-fractionation analysis. The subcellular-fractionation procedure that we used separates cellular contents into five operationally-defined fractions: cytoplasmic (F1), nuclear-membrane associated (F2), chromatin-associated (F3), high salt-soluble nuclear (F4), and nuclear matrix (F5). The results of a typical subcellular fractionation experiment are shown in Figure 8. TCR-β pre-mRNA was localized exclusively in the nuclear and nuclear-matrix fractions (Fig. 8A, F4 and F5). Bands A and B were also present in these fractions, consistent with their being an unspliced precursor and a splicing intermediate, respectively. Spliced IVS1$_{C\beta1}$ was also located predominantly in these nuclear fractions. Some of this spliced intron was also detected in the cytoplasmic and nuclear membrane fractions (F1 and F2), although this is not visible at the autoradiographic exposure shown in Figure 8A.

To examine the distribution of mature mRNA, we hybridized the blot with an exon probe. We observed high levels of mature mRNA (1.6 kb) in the cytoplasmic and nuclear-membrane fractions (F1 and F2), indicating that the mature mRNA was transported to the cytoplasm (Fig. 8B). The distribution of transcripts from the housekeeping gene cyclophilin was also examined (Figs. 8C and 8D). Like *TCR-β* mRNA, cyclophilin mRNA was observed in all nuclear and cytoplasmic fractions. Larger cyclophilin transcripts that were probably cyclophilin pre-mRNAs and splicing intermediates were predominantly in the high salt-soluble and nuclear-matrix fractions (F4 and F5). Note that only a small amount of the chromatin-

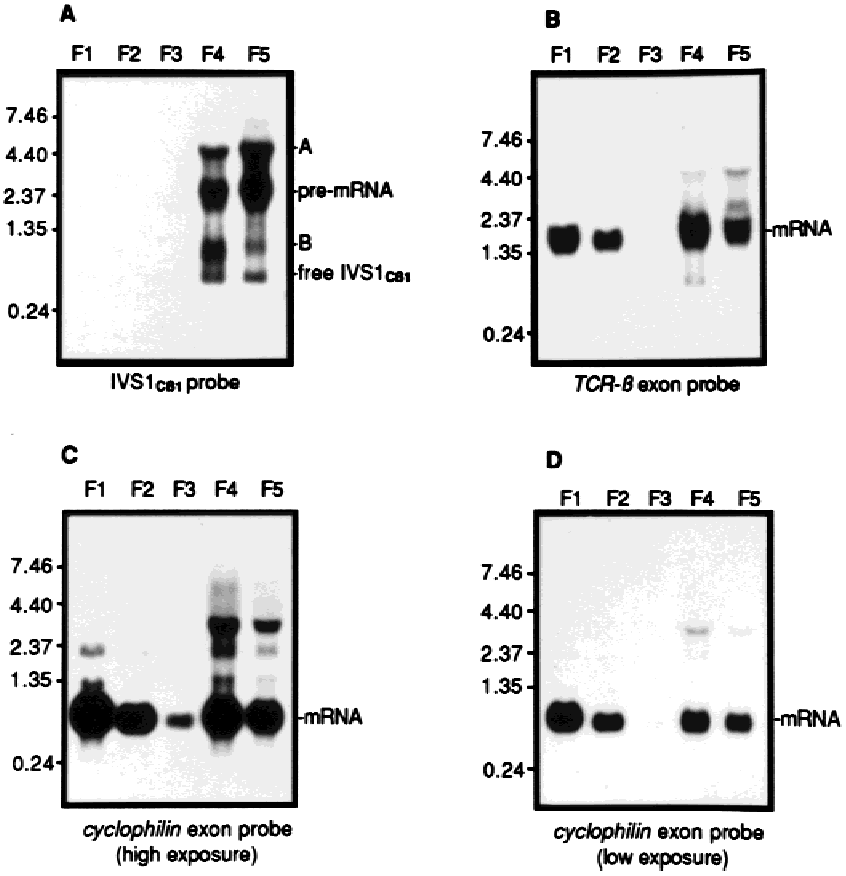

FIGURE 8. Subcellular localization of spliced $IVS1_{C\beta1}$, *TCR-β* pre-mRNA, *TCR-β* mRNA, and cyclophilin mRNA. **A–D**: Northern blot analysis of RNA from five operationally defined fractions (cytoplasmic (F1), nuclear-membrane associated (F2), chromatin-associated (F3), nuclear high-salt soluble (F4), and nuclear-matrix (F5)) obtained from the pTβM-transfected HeLa cell clone. Ten micrograms of RNA were loaded in all lanes, except in F3, in which the entire amount (~1 μg) was loaded. The blot was hybridized sequentially with the indicated probes. The yield of RNA from the operationally defined fractions in the experiment shown was F1: 47%; F2: 37%; F3: ~0.1%; F4: 2%; F5: 14%. Essentially identical results were obtained with subcellular fractions from HeLa cells transfected with the *TCR-β* minigene driven by the β-actin promoter (pAβM) rather than the tet promoter (data not shown).

associated fraction (F3) was loaded on the gel because little was obtained from HeLa cells (see Fig. 8 legend).

Because our subcellular fraction experiments suggested that a fraction of spliced $IVS1_{C\beta1}$ was exported to the cytoplasm, we examined whether $IVS1_{C\beta1}$ associates with mature TCR-β mRNA, which is primarily destined for export to the cytoplasm. We reasoned that if spliced $IVS1_{C\beta1}$ was exported together with mRNA, it might be present in the same nuclear-protein complexes as mRNA. To test this possibility, we performed sucrose-gradient analysis of nuclear lysates. Spliced $IVS1_{C\beta1}$ and its pre-mRNA were found together in the middle fractions of the gradient (Fig. 9A), whereas mature *TCR-β* mRNA was located in the first eight fractions (Fig. 9B). There was very little overlap between the $IVS1_{C\beta1}$- and mRNA-containing fractions. *TCR-β* mRNA was located in fractions typical for mature mRNA, based on our analysis of the distribution of mature mRNA transcribed from the housekeeping genes glyceraldehyde-3-phosphate (*GAPDH*) and β-actin (Fig. 9C and data not shown). These results led us to conclude that spliced $IVS1_{C\beta1}$ cofractionates with its cellular source, pre-mRNA, and not with mature *TCR-β* mRNA.

DISCUSSION

Most vertebrate pre-mRNAs contain more intronic sequences than exonic sequences (Naora & Deacon, 1982; Sharp et al., 1987; Green, 1991). Consequently, the metabolism of introns probably has a major impact on nuclear events. First, intron turnover may provide a dynamic source of ribonucleotides for local *de novo* gene transcription. Thus, the rate of intron decay may dictate the amount of nucleotides that are available for further rounds of RNA synthesis. Second, the degradation of spliced introns may sequester ribonucleases and associated decay-promoting factors away from pre-mRNAs undergoing splicing, as well as fully processed mRNAs undergoing export to the cytoplasm. The greater the proportion of decay-promoting factors occupied degrading spliced introns, the lower the proportion of such factors that can degrade functional mRNAs. Third, intron turnover may release splicing factors that can be used for further rounds of RNA splicing in the nucleus. U2, U5, and U6 snRNPs and some SR-related proteins are known to remain bound to introns after RNA splicing (Wassarman & Steitz, 1992; Blencowe et al., 1995). These known and putative splicing factors may not be released from introns until the introns decay, in which

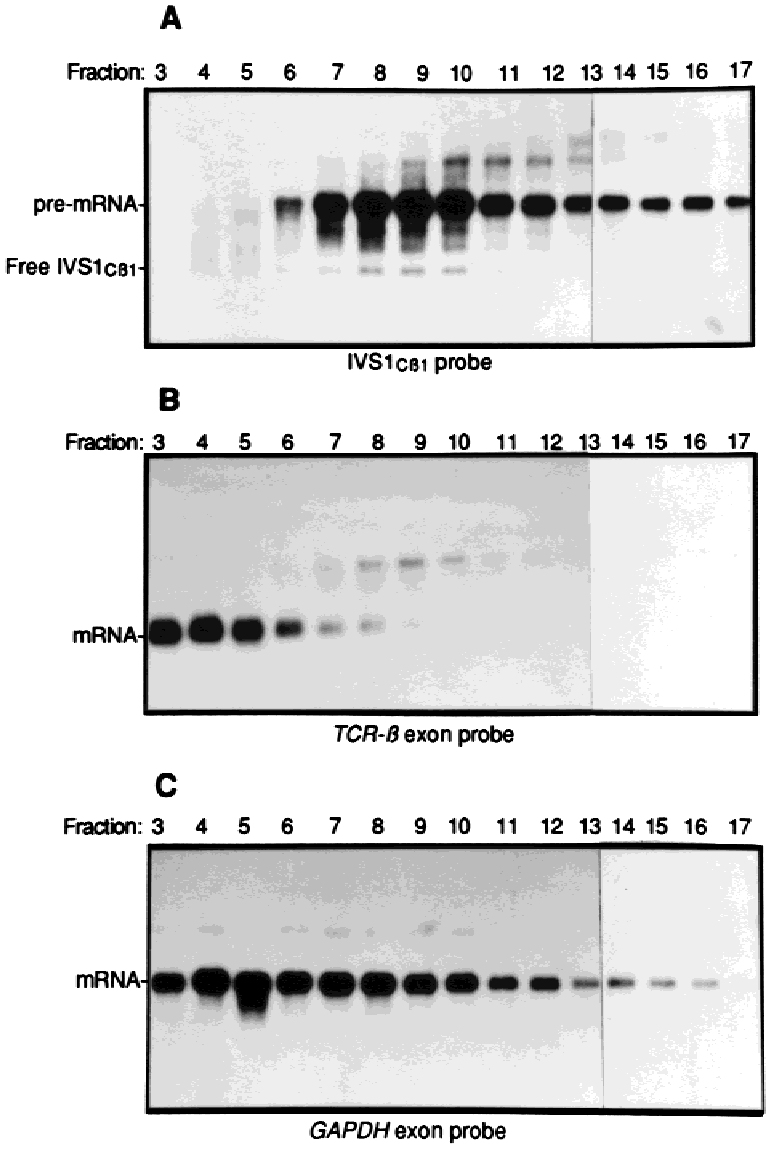

FIGURE 9. Sucrose-gradient analysis of spliced $IVS1_{C\beta1}$, *TCR-β* pre-mRNA, *TCR-β* mRNA, and *GAPDH* mRNA. The nuclear fractions from pAβM-transfected HeLa cells are numbered sequentially from the top to the bottom of the gradient. The Northern blot was hybridized sequentially with the indicated probes. **A**: $IVS1_{C\beta1}$ and its pre-mRNA were found together in the middle fractions of the gradient. **B**: *TCR-β* mRNA was predominantly located in the top eight fractions; there was little overlap with the $IVS1_{C\beta1}$-containing fractions. **C**: The distribution of *GAPDH* mRNA was broader than that for *TCR-β* mRNA.

case the rate of intron turnover would be a limiting event for their release to participate in further rounds of RNA splicing. Thus, intron metabolism is probably critical for regulating RNA splicing, gene transcription, and gene expression in general.

One barrier for the analysis of intron and pre-mRNA stability has been the difficulty in detecting spliced introns and splicing intermediates in vivo. Reverse transcriptase (RT)-polymerase chain reaction (PCR) has typically been used for such analyses because of its high sensitivity. However, RT-PCR is only semiquantitative and thus is not appropriate for precisely determining RNA half-lives. In our study, we chose to use northern blot analysis, as it is a quantitative method for assaying RNA levels. To further ensure accurate determination of RNA half-life, we adopted the tet-regulated promoter system. We found that the tet promoter was ideal for the kinetic analysis of intron and pre-mRNA half-life, because transcriptional repression in response to tet was robust ($>$10-fold) and very rapid ($<$5 min; Figs. 1 and 2).

Using this system, we report the first calculated half-life for an intron from a higher eukaryotic cell. Our analysis was conducted on mouse $IVS1_{C\beta1}$, which is a typical vertebrate intron that is 521 nt long, has a consensus 5′ splice site (CAG/GTAAGT), and an uninterrupted 35-nt polypyrimidine tract upstream of the 3′ splice site (Qian et al., 1992). We determined that the half-life of $IVS1_{C\beta1}$ was 6.0 ± 1.4 min (Fig. 2B). Surprisingly, this intron half-life was comparable to that for short-lived mammalian mRNAs, including c-*myc* and c-*fos* mRNAs, which have half-lives of 10 to 30 min (Fig. 4E; Dani et al., 1984; Hargrove & Schmidt, 1989). The stability of $IVS1_{C\beta1}$ was similar to that of a typical *S. cerevisiae* mRNA (15 min) (Brown et al., 1988; Peltz & Jacobson, 1993). Spliced $IVS1_{C\beta1}$ was significantly more stable than most bac-

terial mRNAs, which typically possess half-lives between 2 and 4 min (Belasco, 1993). To our knowledge, the only other eukaryotic intron whose stability has been reported is the *S. cerevisiae* actin intron, which has a half-life of ~5 min (Jacquier & Rosbash, 1986).

We determined that the half-life of the pre-mRNA that gave rise to $IVS1_{C\beta1}$ was 3.7 ± 1.0 min. This value is similar to that for 15S β-globin pre-mRNA, which is 2.5 min, based on Act.D transcriptional blockade experiments (Curtis et al., 1977). The measured half-life value of the *TCR-β* pre-mRNA is about half of that for the free $IVS1_{C\beta1}$ intron. These half-life values reflect different processes, as the pre-mRNA levels are reduced as a result of both RNA splicing and degradation, whereas the only fate of the spliced intron is degradation. In addition to the normal 2.1-kb pre-mRNA, we detected a longer (4.1 kb) $IVS1_{C\beta1}$-containing RNA, band A, that was polyadenylated at a downstream site (Figs. 1A, 2A, and 4A). Interestingly, this larger $IVS1_{C\beta1}$-containing transcript was not spliceable in vivo. This suggests that either the site of polyadenylation or the strength of the polyadenylation signal in band A is not sufficient for RNA splicing. Our results are in agreement with several studies demonstrating that polyadenylation strongly influences the splicing of the immediately upstream intron in vitro and in vivo (Niwa et al., 1990; Niwa & Berget, 1991; Nesic et al., 1993; Nesic & Maquat, 1994; Cooke & Alwine, 1996). Band A persisted in the nucleus in an unspliced form for several hours after cessation of transcription (Figs. 1 and 4). This is of interest given the widely held notion that RNAs that are not allowed exit to the cytoplasm are rapidly turned over in the nucleus. The nuclear environment may be more permissive for RNA stability than previously supposed, or band A may have unique properties that permit its stability in the nucleus.

The transcription inhibitor Act.D has been widely used for RNA half-life analysis. However, because it blocks transcription globally, it renders the results subject to various artifacts. For example, Act.D blocks not only the synthesis of the transcript under scrutiny but also transcripts that encode putative regulators of RNA half-life. A reduction in the concentration of RNA decay-promoting factors would explain why Act.D stabilizes many cytoplasmic mRNAs (Müllner & Kühn, 1988; Belasco & Brawerman, 1993; Wang & Hawley, 1993; Caruccio & Ross, 1994; Dubois et al., 1994; Ross, 1995; Seiser et al., 1995). We predicted that if Act.D stabilized transcripts by this mechanism *only*, then rapidly turned-over messages (such as most nuclear transcripts) would be amenable to accurate half-life analysis using Act.D, because insufficient time would accrue over the short time course for RNA decay-promoting factors to decay significantly. We found this not to be the case. Act.D *did* significantly stabilize the normally short-lived *TCR-β* pre-mRNA and its spliced intron (Fig. 4A,B). Therefore, we believe that Act.D can directly regulate RNA metabolism. Further evidence for a direct effect of Act.D is that it causes rapid restructuring of nuclear morphology, including redistribution of chromatin and collapse and aggregation of the nuclear matrix, without new protein synthesis (Nickerson et al., 1989). How Act.D rapidly stabilizes RNA transcripts is not known, but it may confer this effect by intercalating between the bases of RNA, as it is known to do in DNA.

The branchpoint sequence is a critical *cis* element that dictates the stability of spliced introns in vivo. Two lines of evidence support this notion. First, mutation of the branchpoint sequence in the *S. cerevisiae* actin intron inhibits debranching and dramatically increases its half-life (Jacquier & Rosbash, 1986). Second, mutant *S. cerevisiae* that lack debranchase activity accumulate high levels of circular introns lacking the lariat tail (Chapman & Boeke, 1991). These data from yeast support the notion that debranching is the rate-limiting event for intron degradation, presumably because debranching must occur before the circular portion of introns can be degraded by exonucleases. Consistent with this notion, we found that most $IVS1_{C\beta1}$ that accumulates in vertebrate cells was in the lariat conformation. Cessation of transcription rapidly decreased the levels of these lariats without increasing the levels of the linearized form of $IVS1_{C\beta1}$ (Fig. 2C). This indicates either that the $IVS1_{C\beta1}$ lariat is degraded without conversion to the linear form or that after debranching, the lariat is degraded so rapidly that the linear form does not accumulate. We believe the latter is more likely because $IVS1_{C\beta1}$ can be debranched in vitro (Fig. 7A). Furthermore, we determined that $IVS1_{C\beta1}$ used a consensus adenosine branchpoint in vivo (Fig. 6), which is consistent with its being amenable to debranching (Ruskin et al., 1984; Ruskin & Green, 1985; Hartmuth & Barta, 1988; Zhuang et al., 1989). In contrast, nonconsensus C,U, and G branchpoints are less efficiently debranched (Horning et al., 1986; Jacquier & Rosbash, 1986; Adema et al., 1988; Hartmuth & Barta, 1988).

We found that $IVS1_{C\beta1}$ was debranched less efficiently than an adenovirus intron in vitro (Fig. 7C,D), perhaps because the $IVS1_{C\beta1}$ branchpoint region matched the branchpoint consensus sequence at only five of seven residues. Although many introns have this degree of divergence from the branchpoint consensus sequence (Padgett et al., 1985; Moore et al., 1993; Sharp, 1994), it may cause $IVS1_{C\beta1}$ to be debranched inefficiently in vivo and thereby be a contributing factor to its in vivo half-life. Debranching may be a limiting factor for the decay of other introns. An intron from the leader region of late 16S SV40 RNA accumulates exclusively as a lariat in *X. laevis* oocytes, suggesting that it may also be somewhat resistant to debranching in vivo (Michaeli et al., 1988).

Our subcellular-fractionation analysis demonstrated that TCR-β primary transcription products, splicing in-

termediates, and spliced IVS1$_{C\beta1}$ were all predominantly in the nuclear matrix and salt-soluble nuclear fractions of HeLa cells (Fig. 8). That most of these transcripts were in the nuclear matrix fraction is consistent with *in situ* hybridization and labeling studies showing that the nuclear matrix is where both gene transcription and RNA splicing take place (Nickerson et al., 1989; Berezney & Jeon, 1995). The close association of spliced IVS1$_{C\beta1}$ with its pre-mRNA was also demonstrated by sucrose-gradient analysis (Fig. 9A). This suggested the possibility that these transcripts are components of the same macromolecular complex (e.g., the spliceosome), a likely possibility given the precursor-product relationship of IVS1$_{C\beta1}$ and its pre-mRNA. What remains for future studies is to determine where IVS1$_{C\beta1}$ molecules are degraded. If most IVS1$_{C\beta1}$ lariats decayed in the same nuclear subcompartment where they are spliced out of pre-mRNA, this would be optimal for the recycling of ribonucleotides for further rounds of transcription and the release of splicing factors to assemble new spliceosomes on nascent transcripts.

An unexpected observation was that some spliced IVS1$_{C\beta1}$ was in the cytoplasmic fraction of HeLa cells (Fig. 1B). This may have been due to either transport out of the nucleus in vivo or nuclear leakage during the isolation of cytoplasmic RNA. That nuclear-to-cytoplasmic intron transport may be a bona fide in vivo event was supported by finding only spliced IVS1$_{C\beta1}$, not *TCR-β* pre-mRNA, in the cytoplasm. Because IVS1$_{C\beta1}$ is much smaller than *TCR-β* pre-mRNA (0.5 kb versus 2.1 kb) it could be argued that it preferentially leaked out of the nucleus during the isolation of cytoplasmic RNA. Yet, we found that three spliced introns from the *Pem* homeobox gene (including a large 2.4-kb intron) are found at even higher levels in the cytoplasm (relative to the nucleus) than is spliced IVS1$_{C\beta1}$ (J.Q. Clement & M.F. Wilkinson, unpubl. observations). Our findings are consistent with earlier work showing that some spliced introns are found at low levels (10–20%) in the cytoplasm of mammalian cells (Coleclough & Wood, 1984; Keohavong et al., 1986). A putative spliced intron from herpes simplex virus type-1 (Farrell et al., 1991) is found at even higher levels in the cytoplasm than the nucleus (Nicosia et al., 1994). *In situ* hybridization studies have shown that transcripts hybridizing with intron probes are located near the nuclear membrane (where they could exit to the cytoplasm), but it is not known if these RNAs are spliced introns, pre-mRNAs, or both (Berman et al., 1990; Kopczynski & Muskavitch, 1992). Recently, several transport pathways that translocate different classes of RNA between the nucleus and the cytoplasm have been identified (Jarmolowski et al., 1994; Nigg, 1997; Izaurralde & Adam, 1998). Our observation that a large proportion of spliced IVS1$_{C\beta1}$ was not associated with mature *TCR-β* mRNA in nuclear fractions of HeLa cells (Fig. 9) suggests the possibility that its pathway of export differs from that of mRNA. However, this difference may reflect differences in nuclear accumulation rather than differences in transport. Future studies are required to determine (a) whether IVS1$_{C\beta1}$ and other spliced introns are actually transported to the cytoplasm in vivo, (b) by what mechanism this occurs, and (c) what role, if any, these spliced introns have in the cytoplasmic compartment.

MATERIALS AND METHODS

Cell culture and transfection

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells cultured to ~50% confluence were cotransfected with 15 μg of pTβM or pAβM and 5 μg of pTAN by the calcium phosphate precipitation method (Kriegler, 1990). Cell clones that stably integrated the transfected plasmids were selected with 1,000 μg/mL G418. Positive clones that expressed either pTβM or pAβM were identified by Northern blot analysis and were maintained without antibiotic.

Plasmid construction and DNA probes

pTβM, the *TCR-β* mini-gene driven by the tet-responsive promoter, was created by replacing the coding region of the luciferase gene (between the *Sal*I and *Eco*RV sites) in the reporter construct pUHC 13-3 (Gossen & Bujard, 1992) with a *Stu*I*Bgl*II 0.74-kb fragment containing IVS1$_{C\beta1}$ and its adjacent exons (Qian et al., 1992). pTAN, the transactivator construct, was made by inserting the transactivator protein gene fragment from pUHD 15-1 (Gossen & Bujard, 1992) into pNEO (Carter et al., 1995).

pAβM, the *TCR-β* mini-gene driven by the human β-actin promoter, was created by inserting the *Stu*I*Bgl*II 0.74-kb fragment containing IVS1$_{C\beta1}$ and its adjacent exons into a β-actin expression vector (Qian et al., 1992).

The 476-bp IVS1$_{C\beta1}$ probe used for Northern blot analysis was generated by PCR amplification with primers I1′ and I1B (Qian et al., 1993a) and pTβM as the template. The *TCR-β* exon probe was a 0.6-kb *Eco*RI fragment from the 86T5 *TCR-β* cDNA clone (Hedrick et al., 1984). The 324-bp vector probe used to distinguish polyadenylation sites in pre-mRNAs was generated by PCR with primers 5′-CTCAATGCTCAC GCTGTAGG-3′ (sense) and 5′-CCGGATCAAGAGCTACCA AC-3′ (antisense) that are located at positions 3517–3538 and 3821–3840, respectively, from the vector pUHC 13-3 (Gossen & Bujard, 1992). The cyclophilin cDNA housekeeping-gene probe used as an internal loading control was described by Carter et al. (1995).

RNA isolation and northern blot analysis

Total cellular RNA was isolated by lysing cells in guanidinium isothiocyanate buffer and ultracentrifugation, as described previously (Wilkinson, 1991). Nuclear and cytoplasmic RNA were prepared as described previously (Carter et al., 1996). Poly(A)$^+$ RNA was isolated from total RNA with an oligo-dT column (Wilkinson, 1991). The isolated poly(A)$^+$ RNA was used as an enriched source of IVS1$_{C\beta1}$ RNA by virtue of the

long internal poly(A) tracts present in $IVS1_{C\beta1}$ (Qian et al., 1992). The RNA was electrophoresed in agarose and polyacrylamide gels, blotted, and hybridized as described previously (Qian et al., 1992). Standard RNA molecular weight markers (RNA I; Boehringer Mannheim) were used to mark the migration of RNA samples for all the figures shown in the this paper.

For RNA half-life analysis, cells cultured to ~50 to ~80% confluence were treated with 1 μg/mL tetracycline, 5 μg/mL Act.D, or 16 μg/mL DRB (Sigma Chemical Co.). Quantification of the results from Northern blot analysis was assessed by densitometry and phosphoimager scanning. The levels of *TCR-β* spliced $IVS1_{C\beta1}$, pre-mRNA, and mRNA were normalized against the levels of cyclophilin mRNA at each time point of the time-course experiments. The RNA half-lives were assessed by least-squares linear regression analysis, as described previously (Belasco & Brawerman, 1993).

Primer extension

The primer extension experiment was performed as described previously (Ruskin et al., 1984; Zeitlin & Efstratiadis, 1984; Grabowski, 1994). In brief, we annealed a ^{32}P-labeled oligonucleotide complementary with the 3′ lariat tail region of $IVS1_{C\beta1}$ (5′-CTGAAAGAAGAGAGAAAGAG-3′) with poly(A)$^+$ RNA from pAβM-transfected HeLa cells, followed by cDNA synthesis using reverse transcriptase. The $IVS1_{C\beta1}$ sequencing ladder was generated as previously described using the same 3′ $IVS1_{C\beta1}$ oligonucleotide (Qian et al., 1992).

In vitro splicing and debranching of lariat introns

The control intron lariat RNA used in the in vitro debranching experiments was generated by in vitro splicing of an adenovirus pre-mRNA. The adenovirus pre-mRNA was synthesized by in vitro transcription using pIVPX linearized with *Bgl*II as a template (Zillman et al., 1988). The capped and labeled pre-mRNA was gel purified and spliced in vitro as described by Eperon & Krainer (1994). The incubation time of 20 min was chosen because we found that a maximum yield of intron lariats was produced within that time period. The template construct and nuclear extracts used in the in vitro splicing reactions were kindly provided by Dr. Susan Berget (Baylor College of Medicine, Houston, Texas).

Intron lariats were debranched by incubating poly(A)$^+$ RNA from transfected HeLa cells enriched for $IVS1_{C\beta1}$ at 32 °C for 50 min in a 50-μL volume containing 1× buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 Moler (M) KCl, 0.5 mM dithiothreitol, and 0.5 mM phenyl-methyl-sulfonyl fluoride), S100 extract, 50 mM EDTA, and 0.5 mM fresh dithiothreitol. The S100 extract used for in vitro debranching analysis was isolated from HeLa cells as described by Dignam et al. (1983), except that the cells were cultured as adherent monolayers instead of nonadherent cells. The debranching reaction conditions used were based on the results of extensive pilot experiments to optimize the debranching activities of the S100 extract while limiting the amount of nonspecific degradation, following the guidelines described by Ruskin & Green (1985).

Subcellular-fractionation and sucrose-gradient analysis

For subcellular RNA fractionation experiments, trypsinized cells were fractionated into cytoplasmic (after NP-40 lysis), nuclear-membrane (after NP-40 and sodium deoxycholate wash), chromatin-associated (after DNase I digestion), high salt-soluble (after incubation with high concentrations of NaCl), and nuclear matrix (remaining pellet) fractions according to the protocol in Leppard & Shenk (1989).

The procedure for sucrose-gradient analysis of nuclei was adapted from Sperling et al. (1985) with some modifications. In brief, trypsinized pAβM-expressing HeLa cells grown to 50–80% confluency were washed twice with cold Tris saline and resuspended in lysis buffer (0.6% NP-40, 0.15 M NaCl, 10 mM Tris [pH 8.0], and 1 mM EDTA), and incubated on ice for 4 min. The cell suspension was centrifuged at 5,000 × *g* for 1 min to collect the pellet, which was then resuspended in the lysis buffer and incubated 30 s on ice. The centrifugation was repeated and the pellet was resuspended in ST2M buffer (10 mM Tris [pH 8.0], 100 mM NaCl, 2 mM $MgCl_2$, 0.15 mM spermine, and 0.05 mM spermidine). The resulting total nuclear lysate was loaded onto a 15–45% sucrose gradient and ultracentrifuged at 40,000 × *g* at 0 °C for 1.5 h. The fractions were recovered in 0.5-mL aliquots, starting from the top of the gradient. Each sample was incubated with proteinase K at 37 °C for 15 min, extracted with phenol and chloroform, and stored in ethanol at −70 °C before Northern blot analysis. One third of each fraction was used for Northern analysis.

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