Localization and Stability of Introns Spliced from the Pem Homeobox Gene*

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RNA splicing generates two products in equal molar amounts, mature mRNAs and spliced introns. Although the mechanism of RNA splicing and the fate of the spliced mRNA products have been well studied, very little is known about the fate and stability of most spliced introns. Research in this area has been hindered by the widely held view that most vertebrate introns are too unstable to be detectable. Here, we report that we are able to detect all three spliced introns from the coding region of the Pem homeobox gene. By using a tetracycline (tet)-regulated promoter, we found that the half-lives of these Pem introns ranged from 9 to 29 min, comparable with those of short lived mRNAs such as those encoding c-fos and c-myc. The half-lives of the Pem introns correlated with both their length and 5′ to 3′ orientation in the Pem gene. Subcellular fractionation analysis revealed that spliced Pem introns and pre-mRNA accumulated in the nuclear matrix, high salt-soluble, and DNase-sensitive fractions within the nucleus. Surprisingly, we found that all three of the spliced Pem introns were also in the cytoplasmic fraction, whereas Pem pre-mRNAs, U6 small nuclear RNA, and a spliced intron from another gene were virtually excluded from this fraction. This indicates either that spliced Pem introns are uniquely exported to the cytoplasm for degradation or they reside in a unique soluble nuclear fraction. Our study has implications for understanding the regulation of RNA metabolism, as the stability of introns and the location of their degradation may dictate the following: (i) the stability of nearby mRNAs that compete with spliced introns for rate-limiting nucleases, (ii) the rate at which free nucleotides are available for further rounds of transcription, and (iii) the rate at which splicing factors are recycled.

Although spliced introns and mRNAs are spliced from pre-mRNA in equal molar amounts, far less is known about the fate and stability of introns than of mRNAs (1). This may be because most introns have no known function. Apart from a few “specialty introns” that mediate antisense regulation (2), enhance transcription (3), encode small nucleolar RNAs that play a role in rRNA processing (4, 5), or encode proteins that mediate RNA splicing and transposition (6), it is widely assumed that most introns have no specific functional attributes. Instead, evidence suggests that most introns in modern organisms arose serendipitously as a result of the following two processes: the shuffling of small intronless primordial genes to generate large genes with introns (the intron-early theory), and the introduction of introns into intronless genes by transposition-type events (the intron-late theory) (7).

What are the consequences of the fact that modern eukaryotic genomes are saddled with large numbers of introns? First, evidence suggests that introns play a major evolutionary role in shaping protein function by virtue of their ability to promote exon shuffling (8). Second, it is clear that introns are necessary for the efficient expression of most mammalian genes (9–12). Presumably, the presence of introns in pre-mRNA sends the newly transcribed RNA down the appropriate pathway to permit high level accumulation of spliced mRNA.

What is less clear is the importance of introns after they are spliced out of pre-mRNA. Intron sequences exceed the length of exon sequences in most vertebrate genes (13, 14), and thus the major component of spliced transcription units that are ultimately degraded is introns. The rate of this degradation may influence the levels of nucleotides available for further rounds of transcription. In addition, intron turnover rate may influence RNA splicing. U2, U5, and U6 small nuclear ribonucleoproteins and Ser-Arg-containing proteins remain bound to released intron lariats (15), and therefore the metabolism of introns may have a significant influence on the recycling of these splicing factors. 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.

The importance of efficient intron turnover is supported by studies on organisms deficient in debranchase, the enzyme that specifically cleaves the 2′–5′-phosphodiester bond at the branch site of intron lariats (16). Schizosaccharomyces pombe deficient in debranchase because of a null mutation accumulate high levels of intron lariats and exhibit a severe growth defect (17). That this growth defect in fission yeast may stem from a toxic build up of undegraded introns is suggested by a comparison with the budding yeast Sacccharomyces cerevisiae, which contains ~40 times fewer introns than S. pombe and does not display obvious phenotypic defects when rendered debranchase-deficient (18). Vertebrate genomes contain even more introns than S. pombe, and therefore an interference with intron decay is also likely to have serious consequences in vertebrates.

Given the probable importance of intron turnover, it is surprising that very little is known about this topic. In part, this deficiency may reflect the fact that very few spliced nuclear pre-mRNA introns have been detected in vertebrate cells. Most of the spliced introns that have been observed in vivo are

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derived from strongly transcribed genes. For example, excised introns from the adenovirus-2 E2A pre-mRNA were identified in HeLa cells treated with cycloheximide to increase the rate of transcription (19). A β-globin intron was detected from rabbit liver, a source rich in β-globin pre-mRNA (20). A spliced immunoglobulin-k intron was observed in a stimulated plasmacytoma cell line that transcribes high levels of an immunoglobulin-k gene (21).

A widely held belief is that spliced introns accumulate at low levels because they are rapidly degraded (within seconds) at their site of origin in the nucleus (14, 22). However, there is little direct evidence to support this view. The only mammalian intron whose fate and stability have been examined in detail is the IVS1C intron from a mouse T-cell receptor (TCR)β-gene. This intron is easily detectable by the relatively insensitive Northern blot procedure, despite being generated from only a modestly transcribed gene (23). The half-life of IVS1C introns in HeLa cells was determined to be 6 min (24), which is much longer than was originally proposed for introns in general (14, 22). Most spliced IVS1C lariats were found to be in the nuclear compartment, consistent with their origin and degradation in the nucleus (24).

In the present communication, we report on the fate, localization, and stability of spliced introns from the Pem homoeomaine gene, a mammalian gene that contains typical mammalian introns ranging in length from ~0.2 to 2.4 kb (25, 26). Unlike the other genes from which spliced introns have been studied, the Pem gene is not expressed in a cell type-specific manner. Instead, Pem is expressed by different cell types in many different fetal and adult tissues, as well as in tumor cells from several different lineages (25–32). Because the Pem gene is not cell type-specific and its introns appear to be typical, we hypothesized that Pem would be a good candidate to provide information on the metabolism of mammalian introns in general.

We used Northern blot analysis and RPA to detect spliced Pem introns, because these approaches have several advantages, including their ability to provide a quantitative measure of RNA levels and their ability to resolve (by size) spliced introns from intron-containing pre-mRNAs. Other methods used to identify, localize, or measure the stability of intron-bearing RNAs (e.g. pulse-chase analysis, reverse transcription-polymerase chain reaction (PCR) analysis, and in situ hybridization) are either not quantitative or do not distinguish between spliced introns and pre-mRNAs (33–36). We used several independent approaches to verify that we had identified bona fide spliced Pem introns. Our analysis of the three introns in the Pem coding region revealed that they had a range of half-lives that were even longer than that of the other previously analyzed vertebrate intron, IVS1Cβ1. The spliced Pem introns were found in several fractions of the nuclear compartment, consistent with their origin in the nucleus. Surprisingly, the Pem introns were also present in the cytoplasmic fraction of several different cell types. Together, these findings may have important implications for RNA metabolism in general.

**Experimental Procedures**

**Cells**—The cells used for our study were the kind gift of the following investigators: the human HeLa cell line (Susan Berget, Baylor School of Medicine), the rat PS-1 prostate mesenchymal cell line (David Rowley, Baylor School of Medicine), the rat FRTL-5 thyroid cell line (Christine Spatzew, Mayo Clinic), and the rat 208-F, Rat-1/S (src-transformed), and Rat-L/R (ras-transformed) fibroblast cell lines (Bruce Magun, Oregon Health Sciences University). The rat McA-RH8994 liver cell line was obtained from the American Type Culture Collection. The myelin basic protein-reactive rat T-cell line was obtained from Arthur Vandenberg and Halina Offer (Oregon Health Sciences University).

**RNA Isolation and Subcellular Fractionation**—Total cellular RNAs were isolated as described previously (30, 37). For each cell line, the length of time in the lysis buffer (0.6% Nonidet P-40, 0.15 mM NaCl, 10 mM Tris (pH 8), 0.1 mM EDTA) was optimized (between 3 and 10 min) such that >90% of cells exhibited disrupted cell membranes as judged by staining with toluidine blue O. Under the conditions chosen, we found that none of the cell lines displayed nuclear lysis, as judged by microscopic visualization of the released nuclei and by the fact that there was no evidence of viscous nuclear pellets (disrupted nuclei leak DNA, which causes aggregation of nuclei and increased viscosity). Further assurance of purity was demonstrated by methylene blue staining of nylon membranes onto which RNA from the nuclear and cytoplasmic fractions had been transferred (38). We found that cytoplasmic RNA had only mature 18 S and 28 S rRNA, whereas nuclear RNA also had 32 S and 45 S precursor rRNAs, in accordance with its expected properties (38). In cases in which a nuclear wash was performed, the nuclei were resuspended in 0.5% sodium deoxycholate in lysis buffer and immediately centrifuged.

Subcellular fractionation of HeLa cells was performed as described (39, 40). Briefly, trypsinized cells were fractionated into cytoplasmic (after Nonidet P-40 (40), nuclear membrane (after Nonidet P-40 and sodium deoxycholate wash), chromatin-associated (after DNase I digestion), high salt-soluble (after incubation with a high concentration of NaCl), and nuclear matrix (remaining pellet) fractions. The RNA from these five fractions was purified by using method 1 described in Ref. 37.

**Northern Blot and RNase Protection Analysis (RPA)**—For Northern blot analysis, RNA samples were electrophoresed in agarose or polyacrylamide gels, blotted, and hybridized as described (24). Before pre-hybridization, all blots were stained with methylene blue to demonstrate RNA integrity, show equivalent loading and transfer, and judge the purity of nuclear and cytoplasmic RNA (38). Standard RNA molecular weight markers (RNA Molecular Weight marker I (Roche Molecular Biochemicals)) or the 0.16–1.77- and 0.24–9.5-kb RNA ladders (Life Technologies, Inc.) were used to determine the size of RNA transcripts. The DNA probes used for hybridizing the Northern blots were generated by PCR using the DNA oligonucleotides listed in Table I. Table II provides a list of the DNA probes. The names of the probes correspond to the exons (E) or introns (I) in the probes. Most DNA probes were labeled using [32P]dATP and a Roche Molecular Biochemicals random primers labeling kit. To increase the specific activity of the IVS1 probe, both [32P]dATP and [32P]dCTP were used. Band intensities of Northern blots were determined by phosphorimage analysis or densitometry.

We determined the half-lives of the Pem introns by the same procedures we described previously (24). In brief, when the cells reached 70–90% confluence, they were incubated with 1 μg/ml tet (Sigma) for the time intervals indicated in the figures. Total cellular, nuclear, or cytoplasmic RNAs were isolated at various time intervals after the addition of tet. The kinetic loss of RNA was monitored by Northern blot analysis. Levels of Pem RNAs for each time point were normalized against the corresponding levels of cyclophilin mRNA. The RNA half-lives were assessed by least squares linear regression analysis as described previously (24).

RPA was performed essentially as we have done before (25, 30) using riboprobes synthesized with T3 RNA polymerase and labeled with [32P]UTP. The Pem IVS2/exon probe, which contains 234 nt of IVS2 and 70–80% confluence, they were incubated with 1 μg/ml tet (Sigma) for the time intervals indicated in the figures. Total cellular, nuclear, or cytoplasmic RNAs were isolated at various time intervals after the addition of tet. The kinetic loss of RNA was monitored by Northern blot analysis. Levels of Pem RNAs for each time point were normalized against the corresponding levels of cyclophilin mRNA. The RNA half-lives were assessed by least squares linear regression analysis as described previously (24).
Identification. A 0.6-kb transcript was identified as spliced from the tet promoter-driven Pem probes to determine the nature of the transcripts. 

We also detected three other major transcripts, which we call m1, m2, and m3. m2 is mature Pem mRNA, based on its cytoplasmic localization and its hybridization with exon probes (Fig. 2, B and D) but not intron probes (Fig. 2A). Its ∼1.5-kb size indicates that m2 is polyadenylated at the consensus site at the end of the last Pem exon (25). In contrast, the shorter m1 transcript is a mature mRNA whose 3′ terminus is probably generated by use of the polyadenylation signal consensus sequence (AAUAAA) present in the 5′ portion of IVS2 (25). The evidence for this assignment includes the following: its ∼0.9-kb size is consistent with a transcript that polyadenylates in the beginning of IVS2; it hybridized with a 5′ IVS2 probe (Fig. 2A) and an exon 2-IVS2 probe (Fig. 2B) but not with a 3′ IVS2 probe (data not shown); and it was predominantly in the cytoplasm, as expected for a mature mRNA. m3 is probably a 3′-cleavage splicing intermediate because it is a nuclear transcript that hybridized strongly with the exon 1–4 probe (Fig. 2C) and the exon 3/exon 4 probe (Fig. 2D) but not well with an exon 2 probe (Fig. 2B).

We also detected several larger molecular weight transcripts that are probably unspliced and partially spliced mRNAs. The evidence for this assignment is that they are all present exclusively in the nuclear fraction (Fig. 2, A and B), and they hybridized with both intron probes (Fig. 2A and data not shown) but not exon probes (Fig. 2, C and D). Spliced IVS2 was not only present in the nuclear fraction (N), but also in the cytoplasmic fraction (C), a finding that will be addressed below.

We found that spliced IVS2, m1–3, and the precursor RNAs were all derived from the transfected tet-Pem gene, because incubation with the specific transcriptional inhibitor tet strongly reduced the levels of all of these transcripts (Fig. 2, A–D). In contrast, the level of mRNA from the endogenous housekeeping gene cyclophilin was not affected by tet (Fig. 2E).

We conclude that the ptPem construct generates precursor mRNAs, mature mRNAs, and spliced introns that are under stringent tet-regulated control in HeLa cells, thus permitting us to perform further molecular analyses.

Subcellular Fractionation Analysis—The observation that spliced IVS2 was detected in the cytoplasmic fraction (Fig. 2A) was surprising given that introns are known to be spliced out of pre-mRNA in the nucleus and are believed (without direct evidence) to be degraded rapidly within this same compartment (14, 22). To determine whether the presence of Pem IVS2 in the cytoplasmic fraction is a peculiarity of this intron or is...
Instead a property shared by other introns, we also examined the localization of other Pem introns. Because Pem IVS1 is only 167 nt long (Table III), we had difficulty detecting it with a probe generated by the typical random-primed labeling approach. However, when we used a "double labeling" approach to generate an IVS1 probe with higher specific activity (see "Experimental Procedures"), we were able to detect spliced IVS1 by Northern blot hybridization (Fig. 3A). Our analysis showed

**FIG. 1.** Pem homeobox gene transcripts derived from a tet-regulated promoter. A, Pem pre-mRNA transcribed from the ptPem construct. The Pem introns IVS1, IVS2, and IVS3 are indicated (note that additional introns in the 5' untranslated region of Pem pre-mRNA derived from the endogenous Pem distal promoter are not in the ptPem construct). Pem coding sequences are in the 3' portion of exon 1, all of exons 2 and 3, and the 5' portion of exon 4. The location of the DNA probes and oligonucleotides used in the present study are indicated. B, Pem pre-mRNA and mRNA, showing the corresponding DNA probes and oligonucleotides used for analysis.

**FIG. 2.** Identification and characterization of spliced Pem IVS2 and other Pem transcripts in HeLa cells stably transfected with ptPem. A–E, Northern blot analysis of nuclear (N) and cytoplasmic (C) RNA (10 μg) electrophoresed in a 1.2% agarose gel from the ptPem-transfected HeLa cell clone incubated with tet (+) or media alone (−) for 18 h. The blot was hybridized sequentially with the probes indicated (see Table I and Fig. 1 for description of the Pem probes; note that a 5' IVS2 probe is used in A). The Pem transcripts recognized by the probes are spliced IVS2, m1 (mature Pem mRNA polyadenylated in IVS2), m2 (mature Pem mRNA polyadenylated in the last Pem exon), and m3 (probably a 3' cleavage intermediate). The housekeeping gene cyclophilin (E) served as a control to show RNA levels from an endogenous gene not under the control of the tet promoter. This blot and those used for all other figures were stained with methylene blue to demonstrate equivalent loading of all lanes and to show the purity of the nuclear and cytoplasmic RNA (data not shown; see "Experimental Procedures").
that spliced IVS1 was present at higher levels in the cytoplasmic fraction than in the nuclear fraction, supporting the notion that a significant fraction of Pem introns is exported to the cytoplasm.

We also considered the possibility that rather than undergoing cytoplasmic export in vivo, the Pem introns instead leaked into the cytoplasmic fraction during our cytoplasmic RNA isolation procedure in vitro. In this case, we predicted that there might be general nuclear leakage into the cytoplasm, and therefore Pem pre-mRNAs would also be in the cytoplasmic fraction. Contrary to this prediction, we found that Pem pre-mRNAs (as detected with either IVS1 or IVS2 probes) remained exclusively in the nuclear compartment (Figs. 2A and 3A). We also considered the possibility that IVS1 and IVS2 preferentially leak out of the nucleus during RNA preparation in vitro because they are smaller than Pem pre-mRNAs. To test this notion, we examined the localization of IVS1 and IVS2 preferentially leak out of the nucleus during RNA preparation in vitro because they are smaller than Pem pre-mRNAs. To test this notion, we examined the localization of Pem IVS3, as it is much larger (2.4 kb) than the other Pem introns (25). We found that IVS3 was present in the cytoplasmic fraction at a level approximately equal to that in the nuclear fraction, when an equal amount of RNA from each fraction was loaded (Fig. 3B). Because ~85% of the RNA in HeLa cells is in the cytoplasmic fraction (based on our typical RNA yields), this indicated that more IVS3 was in the cytoplasmic fraction than in the nuclear fraction on a per cell basis. As a control, we hybridized the same blot with a c-myc probe and found that, as expected, c-myc mRNA was at much higher levels in the cytoplasmic fraction than in the nuclear fraction (Fig. 3C). Given that c-myc mRNA levels were ~5-fold higher in the cytoplasm than in the nucleus and that ~85% of HeLa RNA is cytoplasmic, this meant that ~30-fold more c-myc mRNA was in the cytoplasm than in the nucleus. Calculated in this way, IVS1 and IVS2 exhibited a cytoplasmic-to-nuclear ratio between 3 and 10, whereas IVS3 had a cytoplasmic-to-nuclear ratio between 2 and 5 (some variation was seen from experiment to experiment, presumably because of different degrees of enrichment).

To examine further the intracellular localization of the Pem introns, we performed more extensive subcellular fractionation analysis. The procedure that we used separates cells into five operationally defined fractions: cytoplasmic (F1), nuclear membrane-associated (F2), chromatin-associated (F3), high salt-soluble nuclear (F4), and nuclear matrix (F5) (39).

Analysis with IVS2 and IVS3 probes revealed that both spliced IVS1 and IVS2 were present in most of the nuclear fractions. The chromatin-associated (F3), high salt-soluble nuclear (F4), and nuclear matrix (F5) fractions all contained spliced IVS2 and IVS3 (Fig. 4, A and B). As expected, these same nuclear fractions also contained high levels of unspliced and partially spliced Pem pre-mRNAs. The only nuclear fraction that had few or no spliced introns was the nuclear membrane-associated fraction (F2) (Fig. 4, A and B). This nuclear membrane fraction also lacked precursor mRNA, consistent with the fact that precursor transcripts are known to be localized primarily in the nuclear interior where they undergo splicing.

**TABLE III**

**Pem intron characteristics**

The following abbreviations are used: M is A or C; R is A or G; N is A, C, T, or G; y x is a stretch of mainly pyrimidines (C or T) for at least 10 nt.

| Intron | Length (nt) | Half-life (min) | 5’ Splice site | Score (%) | 3’ Splice site | Score (%) |
|--------|------------|----------------|---------------|-----------|---------------|-----------|
| Consensus | | | MAGgtcagt | 84 | tctttccctctgtgtttcctcttttccag | 92 |
| Pem IVS1 | 167 | 9 | ATGgtcagt | 84 | tctttccctctgtgtttcctcttttccag | 92 |
| Pem IVS2 | 604 | 16 | ATGgtcagt | 86 | ctttttttccttctag | 91 |
| Pem IVS3 | 2438 | 29 | CAgttaggt | 88 | cectttttcctgattaacagt | 88 |

**FIG. 3. Identification and characterization of the spliced Pem introns IVS1 and IVS3.** Northern blot analysis of nuclear (N) and cytoplasmic (C) RNA (10 µg) electrophoresed in a 1.2% agarose gel from the ptPem-transfected HeLa cell clone incubated with tet (+) or media alone (−) for 18 h. The blot was hybridized sequentially with the Pem probes indicated in A and B. The c-myc gene (C) served as a control to show RNA levels from an endogenous gene not under the control of the tet promoter.
Spliced Pem Introns

Fig. 4. Subcellular localization of spliced Pem introns, pre-mRNA, and mRNA. A–E, Northern blot analysis of total cellular RNA (T) and RNA from five operationally defined fractions as follows: cytoplasmic (F1), nuclear-membrane associated (F2), chromatin-associated (F3), high salt-soluble nuclear (F4), and nuclear matrix (F5) obtained from the pt-Pem-transfected HeLa cell clone (10 μg of RNA loaded in all cases). The blot was hybridized sequentially with the indicated probes. F, Northern blot analysis of subcellular fraction RNA from HeLa cells stably transfected with pT/pM, a plasmid containing the TCR-β minigene driven by the tet-regulated promoter (24). The fractionation was performed in parallel with the fractionation of ptPem-transfected cells. The identity of the TCR-β transcripts is described in Ref. 24.

(42–44). This F2 fraction contained intact RNA, as demonstrated by the signals for Pem m1 and m2 mRNA (Fig. 4, D and E) as well as cyclophilin mRNA (Fig. 4C).

Spliced IVS2 and IVS3 were also in the cytoplasmic fraction (F1) (Fig. 4, A and B). This F1 fraction had the characteristics expected of the cytoplasm: (i) higher levels of mature mRNAs, including mature Pem mRNA (m1 and m2) (Figs. 4, D and E) and mature cyclophilin mRNA (Fig. 4C), than did the nuclear fraction, and (ii) no Pem pre-mRNAs, as determined using either IVS2 or IVS3 probes (Fig. 4, A and B). More spliced IVS2 was in the cytoplasmic fraction than in any of the nuclear fractions, when taking into consideration the fact that the cytoplasmic fraction contained ~85% of the total HeLa RNA. Spliced IVS3 displayed a similar distribution pattern as that of spliced IVS2, except that its accumulation in the cytoplasmic fraction was less marked.

The observation that Pem introns accumulated in the cytoplasm differed from what we had observed previously for the intron IVS1C_p1 from the TCR-β gene. Although we had observed trace levels of IVS1C_p1 in the cytoplasm, the vast majority of this intron accumulated in the nucleus (24). We considered the possibility that this difference between Pem introns and IVS1C_p1 resulted from experiment to experiment variation in the subcellular fractionation protocol. To examine this possibility, we prepared fractions from TCR-β- and Pem-expressing cells in parallel. We found that, unlike Pem introns, the TCR-β intron IVS1C_p1 (0.5 kb) accumulated at only trace levels in the cytoplasmic fraction (F1) as compared with the nuclear fractions (F3–F5) (Fig. 4F) (note that the A, B, and pre-mRNA transcripts are described in Clement et al. (24)). We concluded that a significant proportion of Pem spliced introns (IVS1, IVS2, and IVS3) reside in the cytoplasmic fraction, whereas the spliced intron IVS1C_p1, Pem precursor mRNAs, and TCR-β precursor mRNAs accumulate predominantly in nuclear fractions. This indicates that Pem introns are either uniquely exported to the cytoplasm in vivo or they reside in a unique soluble nuclear fraction that leaks out during RNA preparation in vitro.

We considered the possibility that the cytoplasmic accumulation of Pem introns is a peculiarity of the fact that the stably transfected HeLa cell clone used for our analyses expresses high levels of Pem from a heterologous promoter. Although plausible, we considered it unlikely that high expression per se was sufficient for cytoplasmic accumulation, as the TCR-β spliced intron IVS1C_p1 is expressed at high levels from a heterologous promoter, but it does not accumulate in the cytoplasm of HeLa cells (Fig. 4F, Ref. 24). Nevertheless, to examine this issue directly, we elected to examine the localization of Pem introns in cell lines that express only modest levels of Pem. We used two criteria to select such cell lines as follows: 1) they must express lower levels of rat Pem than the stably transfected HeLa cell clone, and 2) they must express the rat Pem gene from its normal endogenous site. After screening several rat cell lines by Northern blot analysis, we identified six cell lines from diverse origins that express modest levels of Pem mature mRNA as follows: PS-1 prostate cells, FRTL-5 thyroid cells, McA-8994 liver cells, and the 208F, Rat-1/S, and Rat-1/R fibroblast cell lines. The levels of Pem mRNA in all of these cell lines were at least 5-fold less than that of the ptPem-HeLa cell clone when levels were normalized against the cyclophilin housekeeping gene (Fig. 5A). The size of Pem mRNA expressed by these cell lines (~1 kb) was the same as expressed in other rodent cell lines and normal tissues (25–27, 29, 30, 32).

To assess the Pem intron levels in these cell lines, we attempted to use Northern blot analysis but were unable to detect a signal, even with previously unhybridized blots. This confirmed that these cell lines expressed only low levels of Pem. To improve sensitivity, we turned to RPA. We generated and tested several different probes for the two larger introns (IVS2 and IVS3) in order to identify those that gave the best signal. We found that the probes that gave unambiguous results were lines containing 234-nt IVS2/106-nt upstream exon and 333-nt IVS3/46-nt upstream exon, respectively. By using these two probes, we examined spliced IVS2 and IVS3 levels in nuclear (N), nuclear wash (NW), and cytoplasmic (C) fractions. The nuclear wash was included to increase the purity of the nuclear fraction; this step involved centrifuging the nuclear pellet in lysis buffer containing the detergent sodium deoxycholate (see “Experimental Procedures”).

We found that spliced IVS2 and IVS3 were present in all
The size of the spliced IVS2 and mRNA bands was cellular RNA was hybridized. The upper two panels and the underloaded FRTL-5 lanes (mT) and untransfected HeLa cells (not shown), neither of which protected the IVS2, IVS3, and mRNA bands when 20 μg of rat T-cell line (k rat cell lines indicated and the stably transfected ptPem-HeLa cell clone. The blots in the left and right panels were both hybridized sequentially with the Pem E1–E4 (cDNA) and the cyclophilin housekeeping gene probes. endog = 1.0-kb transcript expressed from the endogenous Pem gene. B, RPA of 5 μg of nuclear (N), 20 μg of nuclear wash (NW), and 20 μg of cytoplasmic (C) RNA from the cell lines shown. The negative controls are a rat T-cell line (T) and untransfected HeLa cells (not shown), neither of which protected the IVS2, IVS3, and mRNA bands when 20 μg of total cellular RNA was hybridized. The upper two panels are different regions (and exposures) of the same lanes hybridized with the IVS2/exon probe. The size of the spliced IVS2 and mRNA bands was ~230 and ~110 nt, respectively. The lower panel is of identical RNA samples as that of the upper panels but hybridized with the IVS3/exon probe and run in different lanes. The size of the spliced IVS3 and mRNA bands was ~330 and ~50 nt, respectively (mature mRNA bands are not shown). All three panels are from the same gel. C, Northern blot analysis of the nuclear, nuclear wash, and cytoplasmic fractions. The three blots were hybridized sequentially with the Pem E1–E4 (cDNA), U6 snRNA, and 18 S rRNA probes, which respectively (mature mRNA bands are not shown).

We first examined the half-life of IVS2, as it was easily further evidence for purity, RPA showed that the levels of IVS2- and IVS3-containing pre-mRNA (~340- and ~380-nt protected bands, respectively) were >10-fold higher in the nuclear fraction than in the cytoplasmic fraction of ptPem-HeLa cells (data not shown). We were unable to assess pre-mRNA levels in the six rodent cell lines, as the signal was insufficiently above background for accurate quantitation, presumably because these cell lines transcribe the Pem gene at a much lower levels than does the ptPem-HeLa cell clone. Table IV shows the ratio of spliced intron-to-mRNA for four of the rat cell lines. The ratio of spliced intron-to-mRNA was between 0.02 and 0.07 (1.50 to 1:15 ratio) in the nuclear fraction. This ratio went down to between 0.001 and 0.01 (1: 1000–1:100 ratio) in both the nuclear wash and cytoplasmic fractions. In part, this decreased ratio is the result of the much higher steady-state level of mature mRNA in the nuclear wash and cytoplasmic fractions than in the nuclear fraction (Fig. 5B).

Table IV

| Cell line | IVS2 | IVS3 |
|-----------|------|------|
|           | N    | NW   | C    | N    | NW   | C    | N    | NW   | C    |
| FRTL-5    | 0.040| 0.002| 0.009| 0.036| 0.001| 0.002|
| McA-RH8994| 0.061| 0.011| 0.009| 0.075| 0.011| 0.005|
| PS-1      | 0.022| 0.001| 0.001| 0.035| 0.001| 0.001|
| 208F      | 0.059| 0.007| 0.001| 0.043| 0.003| 0.001|

The ratio of spliced Pem introns to mRNA in rat cell lines

The ratio of intron to mRNA was calculated by quantifying the IVS2, IVS3, and mRNA-protected bands obtained from RPA (see text). The values were normalized for both length and U content of the protected bands.

Similar results were obtained with the Rat-1/S and Rat-1/R cell lines, but the levels of spliced IVS2 and IVS3 were too low in most fractions to permit accurate quantitation. We conclude that spliced IVS2 and IVS3 are present in both the nuclear and cytoplasmic fractions of cell lines expressing the endogenous rat Pem gene. The modest levels of spliced introns relative to mature mRNA in the cytoplasmic fraction derives, at least in part, from the fact that spliced IVS2 and IVS3 are much less stable than is mature Pem mRNA in this fraction (see below).

Half-life of Spliced Pem Introns in Vivo—We determined the half-life of the Pem introns by performing time course experiments. Pem gene transcription was blocked by incubating the cells with tet; RNA was isolated at different time intervals, and the decrease in RNA levels after transcriptional blockade was analyzed by Northern blot analysis.

We first examined the half-life of IVS2, as it was easily estimated that the half-life of the IVS2 band was between 0.02 and 0.07 (1:15 to 1:50 ratio) in all cell lines tested. This observation was confirmed by performing time course experiments with the Rat-1/S and Rat-1/R cell lines, which were found to have a half-life of IVS2 that was similar to that of the Rat-1/S and Rat-1/R cell lines.
detectable by Northern blot analysis. As Fig. 6A shows, IVS2 levels decreased steadily after addition of tet. A line fit plot of the data revealed that IVS2 decayed with first-order kinetics (Fig. 6B). Least square linear regression analysis of results from three independent experiments showed that the mean half-life for IVS2 was 16.0 ± 4.2 min (Table III). IVS3 had an even longer half-life; stability analysis of four independent experiments showed that spliced IVS3 had an average half-life of 28.7 ± 5.2 min (Fig. 7A and B, and Table III). Stability analysis of IVS1 was more difficult to perform, as spliced IVS1 was only barely detectable by Northern blot analysis, as discussed earlier. However, when we used a double-labeled IVS1 probe (see “Experimental Procedures”), we were able to determine its half-life to be 9.4 min (Fig. 7A and B, and Table III). These intron half-lives were comparable to published half-lives of c-myc and c-fos mRNAs, determined using general transcriptional inhibitors such as actinomycin D (45–47). Our own analysis using actinomycin D showed that c-myc mRNA has a half-life of 18 min in HeLa cells (24).

Although the Pem introns had half-lives similar to that of c-myc mRNA, we found that they were much less stable than mature Pem mRNA, m1 and m2, the major Pem mRNAs expressed in the pt-Pem-transfected cells, exhibited a slow decrease in levels after cessation of transcription that corresponded to half-lives of greater than 1 h (Fig. 8). m3, which our previous analysis had suggested is a 3’ splicing intermediate (Figs. 2 and 4), had a shorter half-life than that of m1 and m2 mRNAs (Fig. 8).

Branch Point and Debranchability of Pem IVS2—Our analysis of the three introns from the coding region of the Pem gene suggested that these spliced introns had some unexpected characteristics that did not conform to the commonly held view that introns are degraded rapidly in the nucleus of cells (14, 22). Given this, we assessed whether these introns are typical mammalian introns in other respects. Table III shows that all three Pem introns possess 5’ and 3’ splice sites that display a typical degree of identity with the mammalian consensus sequences. The Shapiro and Senpathy rodent scores for the Pem intron splice sites were between 84 and 92%, which is in the normal range, as most mammalian splice sites score >70% (48).

Because intron debranching is considered to be a limiting event that dictates intron stability (18, 24, 49), we next investigated whether Pem introns were unusual in this respect. We restricted our analysis to spliced IVS2 for two reasons. First, we found that only IVS2 accumulated at sufficient levels in vivo for branch point and debranching analyses (the substrates used normally for these assays are abundant introns generated by in vitro splicing (16)). Second, IVS2 is sufficiently small to be analyzed by polyacrylamide gel electrophoresis, which resolves linear and lariat molecules.

The in vivo branch point of IVS2 was determined by primer extension analysis. By using oligonucleotide J (Table I), we found one extension product that corresponded to a branch point at an A nucleotide 55 nt from the 3’ terminus of IVS2 (designated as an “A” in the S100− lane in Fig. 9A). This 55 branch point was located in a region that matched the mammalian branch point consensus 5’-NYNYRAY-3’ (50) at 6 of 7 positions (the branch point nt is in bold). We also observed an extension product that corresponded to a branch point at position −85 (designated as an “a” in the S100− lane in Fig. 9A). This branch point region conformed to the consensus sequence at 5 of 7 positions, which is a typical degree of similarity for mammalian introns (50).

The branch point sequence of introns is known to be a major determinant of their debranchability. Adenylate branch point nucleotides are efficiently debranched (16), whereas nonconsensus branch points are poorly debranched (51, 52). Because our mapping indicated that IVS2 lariats are generated in vivo using consensus adenylate branch point nucleotides, this suggested that these intron lariats are good substrates for the debranchase enzyme. To examine this issue directly, we performed debranching analysis. Polyacrylamide gels were used for Northern blot analysis because they resolve the lariat and linear forms of introns (24). By using this approach, we found that spliced IVS2 accumulated primarily in the lariat conformation in HeLa cells. Two forms of IVS2 lariats were resolved (A and a) that probably differed in their gel migration because their loop sizes differed as a result of usage of the −55 or the −85 branch points, respectively (Fig. 9B). The evidence that these two molecules were both lariats came from the finding that each displayed a different migration in 6% polyacrylamide gels (Fig. 9B) and 4% polyacrylamide gels (data not shown). By contrast, the linear form of IVS2, as well as Pem mRNA (m1), migrated identically with respect to linear molecular weight markers in 4 and 6% polyacrylamide gels.

When incubated with debranchase-containing (S100) extracts, the levels of both IVS2 lariats decreased, whereas the level of the linear form of IVS2 concomitantly increased (Fig. 9B).
This conversion of lariats into linear molecules occurred with two independent S100 preparations (lanes 2 and 3). Further evidence for efficient debranching came from finding that incubation with the S100 debranchase extract prevented the generation of the "α" and "A" primer extension products (S100 lane in Fig. 9A). We conclude that Pem IVS2 is a typical mammalian intron lariat that can be efficiently debranched.

DISCUSSION

Spliced introns constitute a major portion of the RNA metabolized in vertebrate cells, yet little is known about their fate or stability. In this report, we determined the stability and localization of the three introns that are spliced from the coding region of the Pem homeobox gene. We measured the half-lives of these introns using a tet-regulated promoter (Figs. 6 and 7 and Table III). This tet promoter approach has the advantage that only the transcription of the test gene is shut off for half-life analysis, thus minimizing the chance of artifacts. In contrast, most studies investigating RNA half-life have used general transcriptional inhibitors, such as actinomycin D or DRB, which not only block the synthesis of the transcript under scrutiny but also all other mRNAs, including those encoding putative regulators of RNA half-life (53, 54). By using this tet promoter approach, we found that the Pem introns IVS1, IVS2, and IVS3 had half-lives of 9, 16, and 29 min, respectively (Figs. 6 and 7 and Table III). This was surprising given that the prevailing view (without direct evidence) that introns have half-lives on the order of only seconds (14, 22). All of the Pem introns had half-lives longer than that for the TCRβ intron IVS1C (6 min), the only other vertebrate intron whose half-life has been determined (24). The Pem intron half-lives were comparable to those of unstable mammalian mRNAs, such as those encoding c-myc and c-fos (24, 45, 46). Little is known about the stability of introns from other organisms. The S. cerevisiae actin intron has a half-life of ~5 min, which is about one-third the average half-life of yeast mature mRNA (49). Studies in yeast suggest that lariat debranching is the rate-
limiting step in intron degradation. Mutation of the branch point sequence in an actin intron inhibited its debranching and dramatically increased its stability in S. cerevisiae (49). Mutant S. cerevisiae lacking debranchase activity accumulated high levels of circular introns missing the lariat tail (18). Collectively, these data indicate that the circular portion of an intron lariat is relatively impervious to degradation, but when the circle is opened by debranching, this permits rapid exonuclease digestion of the intron. Consistent with this model, we found that most of Pem IVS2 that accumulated in vivo was in the lariat conformation (Fig. 9B). If debranching is indeed rate-limiting for intron degradation, then introns may differ in stability because of differences in their intrinsic debranchability. We observed a wide range of different intron half lives, as brief as 6 min for TCRβ IVS1 Cβ (24) and as long as 29 min for Pem IVS3 (Fig. 7). We were not able to determine if the differences in the stability of Pem introns were due to differences in their debranchability, as only Pem IVS2 was amenable to in situ debranching analysis (IVS1 levels were too low and IVS3 was too large for polyacrylamide gel analysis).

Factors other than debranchability may also regulate intron stability, including intron length and their relative 5' to 3' position, as we found that both of these factors correlated with intron half-life. Another factor that may control intron stability is the ability to be released from the spliceosome. Mutant S. cerevisiae have been isolated that accumulate higher than normal levels of introns, including mutants for PRP22, which accumulate intron lariats in the spliceosome (55). If Pem introns vary in their ability to be released from the spliceosome complex, this could be a factor that dictates their half-lives.

Our subcellular fractionation analysis revealed that spliced Pem introns are present in three of the four nuclear fractions that we purified: the nuclear matrix, high salt-soluble, and chromatin-associated fractions (Fig. 4). These same fractions were also the ones containing Pem pre-mRNA, consistent with their being the site of origin of the spliced Pem introns. Consistent with our results, other investigators (56) have demonstrated that transcription and RNA processing occur in the same nuclear compartments, including the nuclear matrix. We observed few or no spliced Pem introns or pre-mRNA in the nuclear membrane-associated fraction. This is consistent with the available evidence indicating that RNA splicing occurs in the nucleoplasm rather than at the nuclear membrane (42–44). Because all three Pem introns and the TCRβ IVS1 Cβ intron exhibited a similar relative distribution in the four nuclear fractions, it appears that this nuclear distribution pattern is a general one for spliced vertebrate introns.

A surprise was our observation that all three spliced Pem introns were in the cytoplasmic fraction of HeLa and rat cell lines. For some of the Pem introns, we found more in the cytoplasmic fraction than in the nuclear fractions. Several lines of evidence suggested that this reflected their cytoplasmic localization in vivo rather than leakage of nuclear RNA into the cytoplasmic fraction during RNA isolation in vitro. First, we performed cellular lysis under conditions that did not cause noticeable nuclear disruption (see “Experimental Procedures”). Second, we found that U6 snRNA and precuror mRNAs (both from Pem and TCR-β), which would be expected to be primarily in the nucleus, were indeed found in the nuclear fractions and were virtually excluded from the cytoplasmic fraction. Third, even the largest Pem intron (2.4 kb) was in the cytoplasmic fraction, indicating that preferential leakage of small RNAs was not responsible for what we observed. Fourth, we found that, unlike the Pem introns, the TCRβ IVS1 Cβ intron was predominantly in the nuclear fraction, indicating that the cytoplasmic localization of the Pem introns was gene-specific.

An apparent contradiction with the possibility that Pem introns accumulate in the cytoplasm are the data from in situ hybridization studies using intron probes. These in situ studies have not detected intron-containing transcripts in the cytoplasm; instead such transcripts were only detected near the site of gene transcription in the nucleus, sometimes in the form of “tracks” (35, 42, 45). Although this appears to differ with our own observation, there are several explanations for this apparent discrepancy. First, as described above, some introns may not share with Pem introns the ability to accumulate at high levels in the cytoplasm. Second, in situ hybridization analysis does not distinguish between spliced introns and pre-mRNAs;
mRNA did not cotranslate when HeLa nuclear extracts were separated on sucrose gradients (24). However, a caveat to this interpretation is that since the data from both the sucrose gradient nuclear fractions and subcellular fractions reflect steady-state concentrations of RNA, we cannot definitely distinguish between sites of transport and sites of degradation.

What are the functional consequences of the accumulation and degradation of spliced introns? We predict that if there were degradation of introns in the cytoplasm, then this would have an impact on the cytoplasmic decay of mature mRNAs. Cytoplasmic introns undergoing degradation would sequester ribonucleases and associated decay-promoting factors away from mRNAs, thus permitting cytoplasmic mRNAs to accumulate to higher steady-state levels. Similarly, the decay of introns in the nucleus would play a protective role for nuclear pre-mRNAs and mRNAs. The rate of nuclear intron decay may also dictate the amount of ribonucleotides and splicing factors available for further rounds of transcription and RNA splicing. Herein we conclude that intron metabolism has the potential to influence gene expression at several different levels in higher eukaryotic cells.

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