CHARACTERIZATION AND PUTATIVE FUNCTIONAL ROLE OF A STABLE INTRON FROM THE T CELL RECEPTOR GENE

by

Qian Lian

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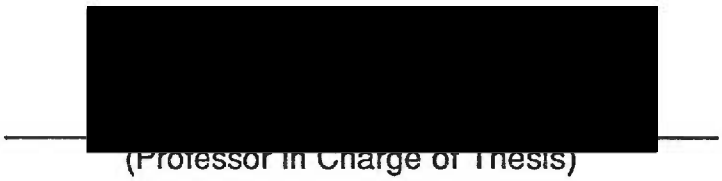

(Professor in Charge of Thesis)

(Chairman, Graduate Council)

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Chapter 1.

ABSTRACT

The majority of higher eukarytic genes are interrupted by non-coding segments termed introns. Introns are excised from pre-mRNAs in the form of lariat structures. It is generally considered that intron lariats are debranched and rapidly degraded following excision from precursor transcripts since most spliced introns are not detectable in vivo.

I have identified an unusual intron that selectively accumulates in the nucleus of cells following its excision from pre-mRNA. This intron, IVS1$_{C\beta1}$, is derived from the constant region of the T cell receptor-β (TCR–β) gene. Its stability is selective since other spliced TCR–β introns do not detectably accumulate in T cells. The property of stability appears to be evolutionarily conserved since the human version of this intron also accumulates in T cells. IVS1$_{C\beta1}$ exists exclusively in the lariat conformation in the nuclei of T cells and transfected non-lymphoid cells. RNase H digestion and ribonuclease protection experiments demonstrate that spliced IVS1$_{C\beta1}$ accumulates as a set of lariat molecules with different length tails. The presence of the tails suggests that this stable intron may be sequestered in a protective nuclear microenvironment, perhaps in the spliceosomal apparatus.

Northern analysis of Hela cells transfected with a variety of TCR-β constructs indicates that the stability of IVS1$_{C\beta1}$ does not require intact secondary structure involving the entire molecule, nor does it require the 5' portion of the molecule. Instead, the 3' terminal region of IVS1$_{C\beta1}$ (minimally defined as 122 nucleotides) engenders the stable phenotype. The most interesting

characteristic of IVS1$_{C\beta1}$ is that its presence is correlated with a retention of fully spliced TCR–β transcripts in the nucleus. Thus, IVS1$_{C\beta1}$ may regulate the nuclear-to-cytoplasmic transport of mature mRNA. The study of this stable intron may lead us to better understand the fate of exons and introns after the process of RNA splicing.

INTRODUCTION

It is a characteristic feature of eukaryotic cells that the functional messenger RNA (mature mRNA) is generated by a variety of post-transcriptional modifications of primary transcripts (pre-mRNAs) derived from genes. One of these modifications is the precise removal of internal sequences by a cleavage-ligation reaction (RNA splicing). Splicing of conventional pre-mRNAs is mediated by spliceosomes. RNA splicing has been shown to depend on the precise recognition of splice sites and the orderly joining of exons. The ability to differentially splice RNA transcripts permits great flexibility in the design and expression of eucaryotic genes. For example, differential splicing enables a series of different but related proteins to be generated, and provides a mechanism for regulating gene expression (see review, Green et al., 1986). The discovery of introns within eucaryotic genes raises many interesting questions regarding their function in the regulation of gene expression. For example, can introns affect the transcriptional activity of genes? Indeed, it has been demonstrated that introns harbor transcriptional enhancer elements (Banerji et al., 1983; Gillies et al., 1983). Do introns also regulate post-transcriptional events? It has been suggested that the mRNA biogenesis machinery might have co-evolved with RNA splicing, so that the splicing of introns might be a necessary step to produce stable mature RNA. For example, some genes have been shown to require the presence of one or more excisable introns for them to efficiently generate normal levels of cytoplamic mRNA [e.g. SV40, rabbit β-globin (Dierks et al., 1981), rat growth hormone and alcohol dehydrogenase-1 genes (see review, Izuarralde et al., 1992)]. In some cases, it has been shown that the stability of the pre-mRNA, as well as the nuclear export

of the mature mRNA is dependent on the presence of these excisable introns (Ryu et al., 1989). However, the requirement for introns to achieve maximal cytoplasmic expression of mature mRNA is apparently not generalizable since some higher eucaryotic genes and most yeast genes lack introns (Izuarralde et al., 1992).

1. Molecular basis for RNA splicing

Major advances have been made in understanding the molecular basis of mRNA splicing since the first discovery of mRNA splicing and introns more than a decade ago (for a review, see Green et al., 1986). There are four major categories of mRNA splicing. These four classes are group I, group II, nuclear mRNA and tRNA splicing. Although the mechanisms of these four classes of RNA splicing have some common features, there are differences in the requirement of particular RNA sequences and the structures of the splicing intermediates (Figure 1, Cech., 1990).

Figure.1.

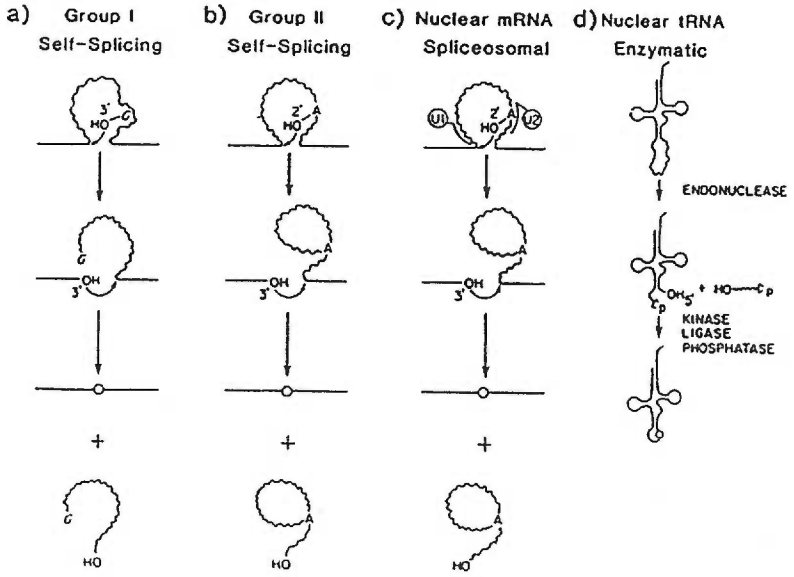
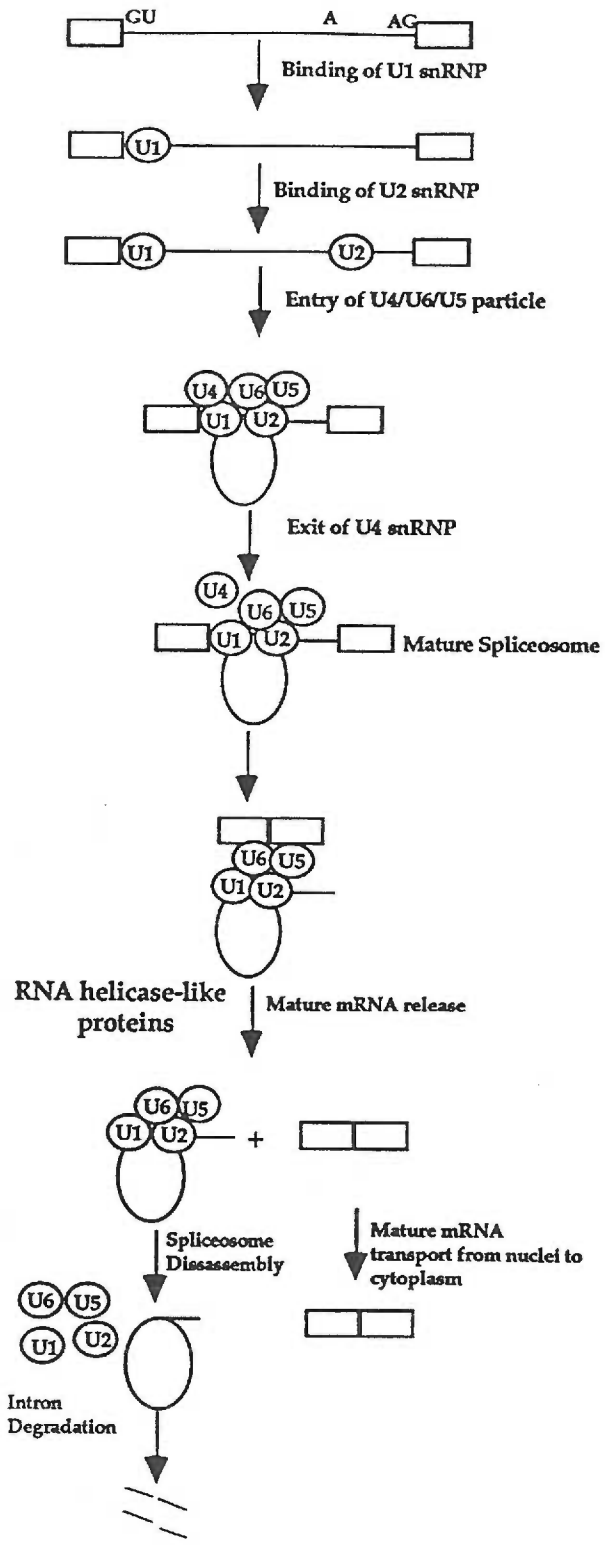


Figure. 2.

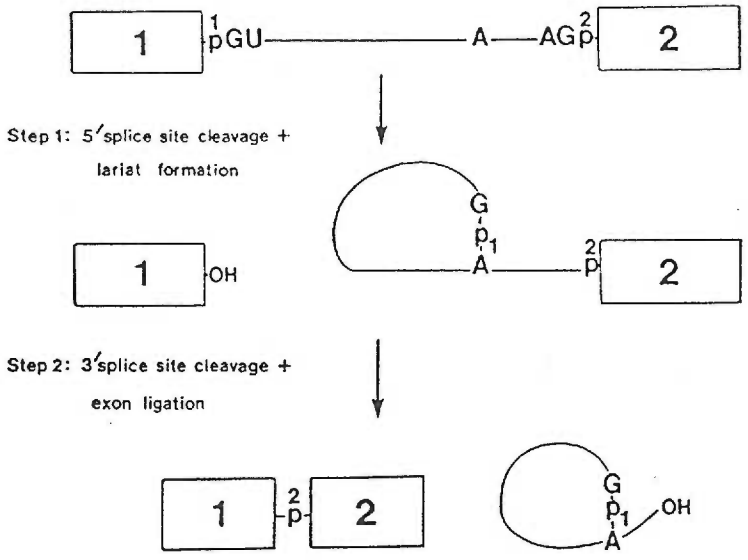

GU
A
AG
Binding of U1 snRNP
U1
Binding of U2 snRNP
U1
U2
Entry of U4/U6/U5 particle
U4
U6
U5
U1
U2
Exit of U4 snRNP
U4
U6
U5
U1
U2
Mature Spliceosome
U6
U5
U1
U2
RNA helicase-like proteins
Mature mRNA release
U6
U5
U1
U2
+
Spliceosome Disassembly
Mature mRNA transport from nuclei to cytoplasm
U6
U5
U1
U2
Intron Degradation

The focus of my literature review is on nuclear mRNA splicing since my project is directed towards understanding the regulation of a particular nuclear mRNA, which displays an unusual pattern of expression in mammalian cells. Nuclear mRNA splicing events take place exclusively in a large multicomponent complex (the spliceosome) in the nucleus. Spliceosomes are composed of small nuclear ribonucleoprotein particles (snRNPs) and other factors. Spliceosomal assembly takes place prior to splicing events and is essential for splicing since it juxtaposes the appropriate splice sites, facilitates RNA branch formation, and catalyses the splicing reaction (Figure.2).

U1 and U2 snRNPs are two important components of the spliceosome. Prior to the first splicing step, the 5' splice site of pre-mRNA is bound by U1 snRNP in the absence of other snRNPs (Seraphin et al., 1989). Then, U2 snRNP binds to the site of the pre-mRNA branchpoint (Green et al., 1986). It has been shown in both yeast (Parker et al., 1987) and mammalian cells (Wu et al., 1989) that the site of U2 snRNP binding determines the branchpoint of the pre-mRNA. Furthermore, binding of U2 snRNP to the branchpoint of the pre-mRNA irreversibly commits the pre-mRNA to the splicing process. It has been shown that binding of U2 snRNP to the branchpoint requires U1 snRNP since U1 appears to align the 5' and 3' splicing sites (Barabino et al., 1990). However, in addition to U1 and U2 snRNP, there are at least three other factors involved in the U2 snRNP/branchpoint interaction in mammalian cells, including U2AF. U2AF is an essential splicing factor for U2 snRNP binding. It binds to the polypyrimidine tract universally present just upstream of mammalian 3' splice sites (Zamore et al., 1991).

Following U1 and U2 snRNP binding to pre-mRNA, a pre-existing particle (consisting of U4/U6/U5 snRNPs) enters the splicing complex (Cheng et al., 1987; Konarska et al., 1987). The U4/U5/U6 particle primarily contacts bound U1 and U2 snRNPs and not the pre-mRNA (Bindereif et al., 1987). The final step in functional spliceosome formation is the dissociation of U4 snRNP from the splicing complex as a result of a conformational change of the splicing complex (Cheng et al., 1987; Konarska et al., 1987). Evidence suggests that the release of U4 snRNP from the spliceosome activates U6 snRNP, the putative catalyst in the splicing reaction (Brow et al., 1989).

Figure.3.



The splicing process, which follows spliceosome assembly, can be divided into a two-step pathway (Fig.3; Green et al., 1986). In the first splicing step, a linear first exon and an intron-second exon lariat are generated after a cleavage at the 5' splice site. This reaction entails joining the 5' end of the intervening sequence, via a 2'-5' phosphodiester bond, to an adenosine residue near the 3'

splice site to produce a lariat (tailed circular molecule) containing an intron-second exon transcript. In the second step of splicing, the lariat intermediate is subsequently resolved by cleavage at the 3' splice site, followed by ligation of the 5' and 3' exons via a 3'-5' phosphodiester bond. Two products are generated: the spliced exons and the excised intervening sequence in the form of a lariat.

It has been shown that the spliced mRNA is released from the spliceosome, while the intron lariat remains bound to some components of the spliceosome following splicing in vitro (Green et al., 1991). RNA helicase-like proteins are required for these processes in yeast (Company et al., 1991). It is generally considered that lariat introns are debranched and rapidly degraded following excision from the precursor transcripts since most spliced introns are not detectable in vivo (Keller et al., 1984). However, it is not known whether the debranching and degradation of spliced lariat introns are required for the dissociation of mature mRNA from the spliceosome in vivo. Furthermore, it is believed that snRNPs recycle, although nothing is known about the mechanisms which dissociate snRNPs from the intron lariat (Figure 2). It has been demonstrated that the presence of excisable intron sequences are required for accumulation of high levels of mature messenger RNAs (Izaurralde et al., 1992), but it is not clear whether this functional effect is conferred by introns present in pre-mRNA or once they are excised. In conclusion, although much is known concerning the mechanism by which introns are removed from pre-mRNAs, little is understood regarding the function or fate of the excised introns.

2. Cis-acting splicing elements

5' splice donor

Virtually, all introns contain the dinucleotide GU at their 5' ends. The 5' splice donor consensus sequence in eukaryotes is AG/GURAGU (/ denotes splice site, R: purine; Green et al., 1986). The actual frequencies of nucleotides at the 5' splice site junction are $A_{64}G_{73}/G_{100}U_{100}$ A_{62} $A_{68}G_{84}U_{63}$; the rest of the sequences beyond this region are relatively random (Mount et al., 1982). The invariant GU dinucleotide is critical for 5' splice site selection; mutations of either the G (Wieringa et al., 1983) or the U (Montell et al., 1982) abolish normal splicing. The other consensus nucleotides at 5' splice site junctions are important in some instances, but not absolutely necessary for splice site selection (Wieringa et al., 1984).

A similarly highly conserved sequence, /GUAYGU (Y: pyrimidine), is found at the 5' splice site of yeast introns. A significant difference between higher eucaryotes and yeast is that mutations of the 5' splice consensus sequence engender different consequences. In yeast, these mutations result in an accumulation of full-length, unspliced pre-mRNA (Gallwitz, D., 1982; Pikielny et al., 1983) or of nonproductive, aberrant 5' splice-site cleavages (Fouser et al, 1986; Parker et al., 1985). In contrast, mutation of 5' splice consensus sequences in higher eucaryotic cells typically result in the activation of "cryptic" splice sites (Montell et al., 1982). The "cryptic" splice sites, which locate in either exons or introns of mammalian genes, typically display a relatively good match with the 5' splice site consensus sequence (Montell et al., 1982; Wieringa et al., 1983). Thus,

there is a much more rigid requirement for the 5' conserved sequence at the 5' splice site in yeast than in mammals.

3' splice acceptor

The consensus sequence at the 3' splice site in yeast is an invariant branchpoint sequence (UACUAAC) followed by an AG dinucleotide at the 3' intron terminus. Since a typical yeast intron is short, these conserved sequences appear to be sufficient to select the splice site (Green et al., 1986). It has been shown in yeast that a cryptic 3' splice site is activated at the first AG dinucleotide downstream of the branchpoint when the normal 3' splice site is deleted and the UACUAAC box (the conserved branchpoint sequence) is intact. However, a normal 3' splice-site cleavage occurs when the AG dinucleotide at the 3' splice site is replaced by a CG dinucleotide (Vijayraghavan et al., 1986).

In mammalian cells, the 3' splice site consensus sequence is $(Y)_nNYAG/G$ (Mount et al.,1982). Unlike yeast, a polypyrimidine tract of variable length [(Y)n] is found upstream of the 3' splice site. Mutagenesis studies have demonstrated that this 3' splice-site consensus sequence is very important for proper RNA splicing in mammalian cells. For example, mutation or deletion of the 3' terminal AG dinucleotide completely abolishes exon ligation and even reduces the efficiency of 5' cleavage. This suggests that the 3' splice site is required for the 5' splice-site cleavage and lariat formation, even though the 3' end is not cleaved until the second step of the splicing (Ruskin et al.,1985; Green et al., 1986). Furthermore, in vivo experiments show that progressive deletion of the polypyrimidine tract can reduce the splicing efficiency and eventually activate a cryptic 3' splice site at another AG dinucleotide (Santen et al., 1985; Wieringa et

al., 1984). In contrast, progressive reduction of the 3' splice-site consensus sequence in yeast does not block 5' cleavage (Langford et al., 1983).

The branchpoint

RNA splicing generates two products: spliced exons and an excised intron in the form of a lariat (Keller et al., 1984). The lariat RNA is a circular molecule produced by the ligation of the 5' end of the intervening sequence to an adenosine residue (the branchpoint) near the 3' splice site. A highly conserved sequence (UACUAAC; A is the branchpoint) functions as the branchpoint sequence in yeast introns. This branchpoint consensus sequence is typically found within 50 bases upstream of the 3' splice site. The UACUAAC element possesses sequences which are complementary with a region of U2 snRNA (Parker et al., 1987). This U2 snRNA–branchpoint base-pairing interaction directs the adenosine in the branchpoint of pre-mRNA to attack the 5' splice junction and form a 2'-5' phosphodiester bond, resulting in a lariat molecule containing an intron-second exon transcript (Green et al., 1986). Deletion or mutation of the authentic yeast branchpoint sequence can block splicing in vivo and in vitro (Newman et al., 1985), which suggests that there is a rigid sequence requirement for a functional branchpoint in yeast.

In contrast, a highly conserved branchpoint element is not found within the introns of mammalian cells; the majority of sequences within many mammalian introns can be deleted without abolishing splicing (Wiringa et al., 1984). The less rigidly defined branchpoint consensus sequence in mammals is YNYURAC (N: any nucleotide). This mammalian sequence resembles the yeast UACUAAC box and is typically located just upstream of the polypyrimidine tract at the 3' end of the intron. The mammalian branchpoint is commonly present 18-40

nucleotides upstream from the 3' splice site, though it is sometimes located more than 150 nucleotides upstream (Helfman et al.; Smith et al., 1989). The highly conserved adenoside nucleotide in the YNYURAC box usually serves as the branchpoint nucleotide (Green et al., 1991). However, even this position is not absolutely conserved; C or U residues can be used as the branchpoints if the regions immediately upstream from polypyrimidine tract is devoid of A residues (Hartmuth et al., 1988). Deletions or mutations of the branchpoint sequence often have no effect on splicing because of the activation of nearby cryptic branchpoints (Padgett et al., 1985; Reed et al., 1985). Some alternatively spliced introns possess multiple branchpoints associated within a single acceptor site (Green et al., 1991). The functional significance of this redundancy, if any, in the regulation of splicing remains unknown.

3. Splice site selection

Despite major advance in our understanding of the splicing mechanism, little is known about the rules governing splice site selection. For example, although splicing consensus sequences are necessary for splicing, they are not sufficient to unambiguously specify a splice site since they are often present *within* exons or introns that are not selected as splice sites. It is therefore a paradox how a multi-intronic pre-mRNA is accurately spliced without exon deletions. A reasonable explanation is that non-consensus sequences flanking splice sites contribute to splice site selection. It has been demonstrated that the accuracy and/or efficiency of splice site selection can be affected by the deletion of exon or intron sequences around the splice sites (Somasekhar et al., 1985; Reed et el., 1986). Furthermore, the activity of inserted splice sites is determined by their

location within pre-mRNA (Nelson et al., 1988). These results suggest that the sequences adjacent to splice sites might form higher order RNA structures which affect the rate of RNA splicing.

4. Lariat RNA:

Two major species of lariat RNAs that result from splicing have been detected in vitro: 1) the intron connected to the downstream exon and 2) the spliced intron alone (see review, Keller et al., 1984). Intron-exon lariats are the intermediates of the splicing reaction which have lost the upstream exon as a result of the first step of splicing. The second splicing product, the spliced intron lariat, is an end product of the splicing reaction. Such spliced intron lariats appear to be rapidly debranched and degraded (see review, Green et al., 1986), although their actual fate has not been rigorously evaluated.

What is the significance of lariat RNA formation during splicing? Several explanations have been suggested. First, lariat formation prevents degradation of the splicing intermediates by exonucleases during the splicing reaction. Second, lariat formation can drive the splicing reaction toward exon ligation. Third, the lariat structure may be a recognition signal for the selection of 3' splice sites since the first AG dinucleotide downstream of the branchpoint usually serves as the 3' splice site. Finally, lariat structures may serve as a mark for the recognition by specific enzymes (such as debranchases) which determine the fate of the spliced intron by, as yet, unknown mechanisms.

It is widely considered that intron lariats are debranched and rapidly degraded following excision from precursor transcripts since most spliced introns are not

detectable in vivo (Keller et al., 1984). The instability of spliced introns appears to be a consequence of debranching. It has been demonstrated that mutant yeast strains that lack debranching activity accumulate high levels of lariat introns (Chapman et al., 1991). Furthermore, mutations within the branchpoint consensus sequence of yeast introns causes inefficient debranching, resulting in an accumulation of the lariat form of these introns (Jacquier et al.,1985). Intron lariats have been observed in higher eukaryotic cells as transient species derived from highly transcribed genes that exist either as RNA processing intermediates generated after 5' cleavage (Rodriguez et al., 1984) or after complete excision from precursor transcripts (Domdey et al., 1984). For example, β-globin introns have been detected in fetal liver nuclei in vivo as lariat and linear molecules (Zeitlin et al., 1984).

Some spliced introns may not exist as transient intermediates, but instead accumulate as stable introns. For example, a spliced SV40 intron accumulates as a lariat structure in the nucleus of injected *Xenopus laevis* oocytes. The function of this SV40 intron is unknown and its physiological relevance is uncertain because it is not stable in transfected mammalian cells (Michaeli et al., 1988). An intriguing example of a "stable intron" is U14 snRNA, a small RNA species involved in RNA processing. Copies of U14 snRNA are present in three of the introns of the cognate hsc70 heat shock gene (Liu et al., 1990). U14 snRNA can be generated by a processing reaction from these introns (Leverette et al., 1992).

5. Nuclear-to-cytoplasmic transport of mature mRNA

It has been shown that mRNA synthesis, processing, and transport might be tightly coupled in time and space (Lawrence et al., 1989). For example, RNAs binds to hnRNP and snRNP particles very rapidly following transcription (see review, Izaurralde et al., 1992). In situ hybridization shows that a cluster of snRNPs and other processing activities are present in a transport track that begins at the site of transcription in nuclei (Lawrence et al., 1989). It has been demonstrated recently that splicing occurs directly within this RNA track (Xing et al., 1993).

Transport of mRNA from the nucleus to the cytoplasm is a process which is not well understood. There are two major questions: why are pre-mRNAs and splicing intermediates retained in the nucleus and what is the signal which permits mature mRNA to be transported quickly to the cytoplasm. It has been shown that RNA transcripts cannot move freely inside the nucleus (Lawrence et al., 1989), perhaps because they are recognized by splicing factors which restrict their migration (Green et al., 1991). Mutations that block spliceosome assembly permit nuclear export of mRNA (Chang et al., 1989; Hamm et al., 1990).

However, the presence of splice sites does not always block pre-mRNAs from leaving the nucleus. For example, mature transcripts generated by alternative splicing contain "unused splice sites" which are functional in other transcripts derived from the same gene; these splice site-containing transcripts are efficiently exported to the cytoplasm (Christopher et al., 1989). It is unknown whether export of these RNAs is permitted as a result of inhibition of the

association between them and splicing factors; or is a result of inefficient recognition of the retained sites by the splicing machinery.

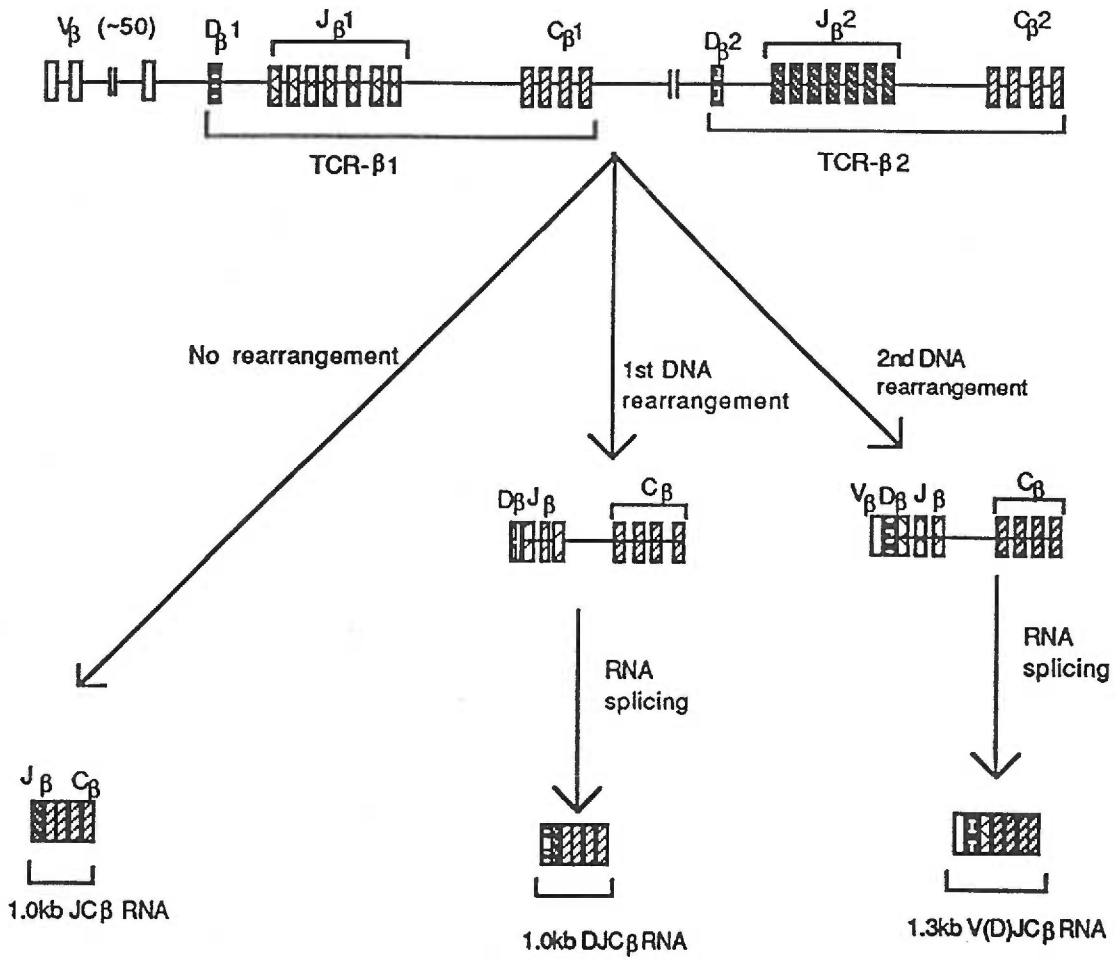
It has been demonstrated that several other cis-acting elements may play a role in nuclear export. For example, a m^7GpppN cap structure forms part of the signal for efficient nuclear export (Hamm et al., 1990). U6 snRNA, which lacks a m7GpppN cap, is not exported from the nucleus (Vankan, et al., 1990). Furthermore, formation of a correct 3' end also appears to be important for export (Eckner et al., 1991). There might be several different nuclear transport mechanisms since rRNAs and tRNAs, which lack a m^7GpppN cap structure, are also exported to the cytoplasm (Green et al., 1991). In addition to cis-acting elements, sequence specific trans-acting factors play an important role in mRNA nuclear export. For example, it has been demonstrated that the HIV-1 rev gene product induces the expression of HIV-1 structural genes by activating the sequence-specific nuclear export of incompletely spliced HIV-1 RNA species (Malim et al., 1989). In the absence of a functional rev gene product, unspliced and partially spliced HIV-RNAs which encode functional viral proteins, are retained in the nucleus and therefore viral replication does not take place. REV protein allows these viral RNAs to be exported to the cytoplasm resulting in the synthesis of viral structural polypeptides and viral replication (Chang et al., 1989; 1990).

6. Regulation of T Cell Receptor Gene Expression

T cells recognize antigens through a surface molecule, the T cell receptor (TCR) for antigen. This receptor is a multisubunit receptor composed of two variable chains and several invariant CD3 molecules residing on the cell surface. The TCR-α and β variable chains display binding specificity for antigens in the context of MHC molecules, while the CD3 molecules function in signal transduction events that result from engagement of the TCR (Ashwell et al., 1990). The structure of TCR genes is similar to immunoglobulin genes. The TCR-β gene is composed of variable gene segments ($V\beta$, $D\beta$, and $J\beta$) which are juxtaposed by DNA rearrangement during thymocyte ontogeny (Kronenberg et al., 1986). There are two β-chain constant region genes ($C\beta1$ and $C\beta2$) in all species, which are each associated with a set of distinct $D\beta$ and $J\beta$ elements. The order of rearrangements of the β-chain locus is the same as that of the immunoglobulin heavy chain locus. The first rearrangement event involves D to J joining, with the deletion of the sequences separating these gene elements. This can occur on both chromosomes and for both $D\beta1$ and $D\beta2$ elements sequentially. The second type of rearrangement event directs V to D joining. Collectively, these two rearrangement events combine these elements to produce functional TCR-β genes (Figure. 4).

Figure. 4.

The expression of the TCR- β gene



The expression of TCR–β transcripts during T cell maturation has been well studied. Truncated (1.0kb) TCR–β transcripts are derived from unrearranged or partially rearranged (D-J joining) genes expressed very early in murine ontogeny (at least as early as day 14 of mouse gestation) along with CD3-γ, –δ, –ε and -ζ transcripts (Doskow et al.,1992; Hars et al.,1986; Snodgrass et al., 1985). Full length (1.3kb) TCR–β transcripts derived from a fully rearranged gene are not evident until day 15-16 of mouse gestation. Since this is approximately the same point of gestation when fully rearranged TCR–β genes become evident (Haars et al.,1986; Snodgrass et al., 1985), it is widely assumed that the first expression of functional TCR–β transcripts is limited by gene rearrangement events. As ontogeny proceeds, the level of 1.3kb TCR–β mRNA increases in the thymus. This is, at least in part, due to the fact that increasing numbers of thymocytes undergo rearrangement events in the thymus, but it may also be due to alterations in transcriptional or post-transcriptional regulatory events. On day 17 of murine ontogeny, TCR-α transcripts become apparent in the fetal thymus (Doskow et al.,1992; Snodgrass et al., 1985). This event roughly coincides with the appearance of cell surface TCR-aβ protein on a small proportion of fetal thymocytes (Snodgrass et al., 1985). Thus, the TCR–α chain appears to be the limiting subunit that controls the surface expression of TCR–αβ protein early in ontogeny. TCR–β protein has also been detected on the surface of immature thymocytes or cell lines in the absence of the TCR–α chain (Kishi et al.,1991; Punt et al., 1991), but its function in this context is not yet known.

Although it is clear that rearrangement events are necessary for the functional expression of the TCR–β gene, rearrangement of TCR genes is not sufficient for

expression. Transcriptional and post-transcriptional mechanisms also regulate TCR–β expression. The transcriptional regulation of the TCR–β gene has been studied in cell culture. A tissue specific enhancer element has been defined 3' of the $C_{\beta 2}$ gene segment (Krimpenfort et al.,1988; McDougall et al.,1988). Cis-acting elements important for TCR–β expression have also been characterized in V_β promoters and the J-$C_{\beta 2}$ intron region. DNA binding proteins that possess strong affinity for these regulatory elements have been identified, some of which may be involved in programmed DNA rearrangment events rather than transcriptional regulation *per se* (Hashimoto et al.,1990; Jamieson et al.,1989; Lanier et al.,1991; Royer et al.,1987; Takeda et al.,1990). Negative trans-acting factors have been defined in immature T cell clones that serve to inhibit the transcription of TCR–β genes, as well as other T cell genes that are normally activated during intermediate stages of thymic development (MacLeod et al.,1986; Wilkinson et al., 1991).

The importance of post-transcriptional mechanisms that regulate TCR–β expression or function is not clear. TCR–β transcripts appear to be capable of undergoing alternative splicing events, but the functional significance of the known examples of alternative splicing is not clear. An alternative C_β exon (Cβ) present in the J-Cβ1 intron is utilized in only a minority of mouse TCR–β mature transcripts, and is not present in either rat or human TCR–β genes (Behlke et al.,1986; Dent et al.,1989). Alternative splicing of V_β leader exons has been observed (Chou et al.,1987), but this does not appear to be a general phenomenon.

7. Negative regulation of TCR-β mRNA processing in SL12.4 cells

We have used a set of related murine AKR T-lymphoma cell clones as a model system to study the parameters that regulate TCR–β mRNA levels. These cell clones have been carefully phenotyped and shown to represent distinct stages of thymic development. The SL12.4 cell clone has been the subject of many studies on T cell gene regulation (MacLeod et al.,1986; Wilkinson & MacLeod ., 1988). SL12.4 cells have a surface phenotype ($CD3^-$ $CD4^-$ $CD8^-$ $IL2R\alpha^+$ HSA^+ $CD44^-$ $Thy1^+$) that is characteristic of "intermediate maturity" thymocytes within the double negative subset that have a high potential to differentiate into mature T cells in organ culture or after adoptive intrathymic transfer (Shimonkevitz et al.,1987). SL12.4 cells accumulate CD3–γ, –δ, –ε and -ζ mRNA and possess fully rearranged TCR–α and –β genes, but they do not express the TCR/CD3 complex on their surface due, in part, to low levels of mature TCR–α and -β mRNA in the cytoplasm (MacLeod et al.,1986; Wilkinson & MacLeod.,1988).

The molecular basis for the lack of cytoplasmic TCR–β transcripts in SL12.4 cells has been explored. SL12.4 cells possess a fully rearranged $C\beta1$ gene that is actively transcribed to give rise to large TCR–β transcripts in the nucleus (Wilkinson & MacLeod, 1988). Treatment with the protein synthesis inhibitor cycloheximide (CHX) leads to the disappearance of these larger transcripts, and appearance of fully spliced transcripts in the cytoplasm. Removal of CHX permits reappearance of the larger transcripts in the nucleus and leads to eventual loss of mature transcripts in the cytoplasm. Nuclear run-off analysis shows that the induction of mature TCR-β transcripts by CHX occurs without a significant increase in the rate of transcription. Therefore, the negative

regulation reversed by CHX is exerted at the post-transcriptional level. This negative regulation is specifically directed towards $C\beta1$ transcripts derived from the fully rearranged, not the 1.0 kb $C\beta2$ transcript derived from the unrearranged $C\beta2$ gene in SL12.4 cells.

The underlying mechanism for why CHX induces mature $C\beta1$ transcripts in SL12.4 cells is not known. It may deplete the cells of a labile inhibitor that blocks the processing, transport and/or stability of $C\beta1$ transcripts. Numerous other studies have shown that protein synthesis inhibitors induce transcripts, either by increasing the rate of gene transcription or by increasing the stability of cytoplasmic transcripts (Bindereif, et al., 1987; Brewer, G., 1991) . In most of these cases, it has been hypothesized that an unstable inhibitor protein mediates the negative regulation. However, no study has yet definitively identified the mechanism(s) by which protein synthesis inhibitors augment gene expression.

8. Specific aims of the thesis

T cell receptor (TCR) genes are unusual in that they must undergo rearrangement events which combine V, D, J and C elements to produce functional genes. Although it is clear that rearrangements are necessary for functional expression of TCR–β genes, rearrangement of TCR genes is not sufficient for expression. For example, it has been demonstrated that transcriptional regulation plays an important role in regulating the expression of TCR–β genes (see my literature review). In contrast, little is known about the importance of post-transcriptional mechanisms in regulating TCR–β expression. I have chosen to focus on this topic for my thesis studies.

Aim 1: To analyze unusual T cell receptor–β mRNA splicing intermediates in the immature SL12.4 T cell clone.

It has been shown that SL12.4 cells have a fully rearranged TCR–β1 gene that is actively transcribed and gives rise to large transcripts in the nucleus, yet the cells fail to accumulate mature TCR–β transcripts in the cytoplasm (Wilkinson & MacLeod, 1988). Treatment with the protein synthesis inhibitor CHX leads to an accumulation of fully spliced transcripts in the cytoplasm. The effect of CHX is posttranscriptional since CHX does not significantly increase the rate of TCR–β gene transcription. In this aim, my goal is to characterize the large nuclear TCR–β transcripts, including an analysis of their intron content. The effect of CHX on these nuclear transcripts will be determined. This study provides a first step towards determining the precise post-transcriptional mechanism by which TCR–β transcripts are regulated.

Aim 2: To characterize a novel spliced intron that accumulates in the nucleus.

During the course of analyzing TCR-β pre-mRNA in the SL12.4 cell clone, I identified a spliced intron (IVS1$_{C\beta1}$) which accumulates in the nucleus of SL12.4 cells. This is surprising since it is known that most introns are rapidly debranched and degraded following their excision from pre-mRNA. Discovery of this stable intron raises several interesting questions. For example, is IVS1$_{C\beta1}$ a lariat or linear molecule? Is its stability T cell specific? Is its expression developmentally regulated? In this aim, my goal is to characterize IVS1$_{C\beta1}$ in order to answer these questions. This study provides a first step towards understanding the molecular basis for the stability of IVS1$_{C\beta1}$.

Aim 3: To characterize the cis-acting elements responsible for the stability of IVS1Cβ1 and to determine if this excised intron possesses a function.

It is widely considered that once mammalian introns are spliced out, that they perform no function. The evidence for this notion is that most introns are rapidly degraded following excision from precursor transcripts. The identification of an intron that accumulates following its excision suggests the possibility that this intron possesses a function. In this aim, I evaluate the possibility that IVS1$_{C\beta1}$ performs a functional role following its excision. I also characterized cis-acting elements that are necessary and sufficient for its stability.

9. Historical aspects of the thesis

At the time I initiated my studies on TCR–β regulation, Livia Theodor worked on this project. She was attempting to identify which TCR–β introns display inhibited or inefficient splicing in the SL12.4 cell clone. Her results suggested that all introns except IVS_L were present in TCR–β pre-mRNAs and that no fully sliced TCR–β transcripts were present in SL12.4 cells unless treated with CHX. When I became involved in the project, I used PCR to generate more TCR-β exon and intron probes and used these to analyze the profile of nuclear transcripts present in SL12.4 cells. My results were consistent with Livia's, except that I found that fully spliced Cβ1 transcripts were detectable but at a relatively low level in SL12.4 cells, even before CHX treatment. The fully-spliced Cβ1 transcripts were dramatically increased after treatment with CHX. I also used RT-PCR analysis to show that the ratio of fully-spliced to unspliced TCR–β transcripts increases during normal murine thymic ontogeny (unpublished). Lastly, I sequenced the $IVS2_{C\beta1}$ and $IVS3_{C\beta1}$ in the mouse Cβ1 gene, as a prelude to future work investigating the regulation of TCR–β RNA splicing. This work entitled "T cell receptor–β mRNA splicing: regulation of unusual splicing intermediates" constitutes the first chapter of my thesis and was published in **Molecular and Cellular Biology [13:1686-1696 (1993)].** The contributions of the authors are as follows: L. Qian (PCR analysis, generation and purification of different TCR-β probes, Northern blot analysis, and sequence analysis of the Cβ1 gene); L. Theodor (initiated the project, generated and purified TCR-β probes, and Northern blot analysis); M. Carter (RNase H and Northern analysis); M. Vu (Northern blot analysis); A. Sasaki (sequence analysis of the Vβ5 gene and Northern blot analysis) and M. F. Wilkinson (RNase H and Northern blot analysis, general project development).

While performing Northern blot analysis of SL12.4 cells, I identified an unusual intron from the constant region of the TCR-β gene, IVS1$_{C\beta1}$, which remained relatively stable following its excision from pre-mRNA. To test whether the stability of IVS1$_{C\beta1}$ is unique to T cells, I generated several different constructs and transfected them into Hela, Rat1 and AKR1 cells. I performed the Northern blot analysis showing that its stability is not T cell specific. In order to test whether the stability of IVS1$_{C\beta1}$ is conserved in other animal species, I used PCR to generate several different IVS1 probes from different species for Northern blot analysis. The Northern analysis showed that the property of stability appears to be evolutionarily conserved since the human and rat versions of this intron also accumulate in T cells. I also performed the sequence analysis of the IVS1$_{C\beta1}$ gene. The majority of this work was published in a paper entitled "A Spliced Intron Accumulates as a Lariat in the Nucleus of T Cells" **[Nucleic Acids Research. 20:5345-5350 (1992)]**. The contributions of the authors are as follows: L. Qian (initial observation, IVS1$_{C\beta1}$ sequence analysis, generation of different probes, subcloning different Cβ1 constructs and transfection into different cell lines, Northern blot analysis); M. Vu (purification of RNA and Northern blot analysis); M. Carter (Northern analysis) and M. F. Wilkinson (RNase H digestion analysis, Northern blot analysis, and project development).

In order to further characterize the stable intron and to determine the cis-acting elements which are responsible for its stability, I generated several mutant IVS1$_{C\beta1}$ constructs and transfected them into Hela cells. In addition, I also performed RNase protection analysis to identify the different lengths of the 3' tails of this lariat intron, as well as primer extension analysis to preliminarily locate the branchpoint. The most important observation that I made regarding

this stable intron concerns its possible functional relevance. Northern analysis showed that mature transcripts were retained in the nuclei of Hela cells transfected with chimeric constructs which contained stable forms of $IVS1_{C\beta1}$. This evidence supported the notion that $IVS1_{C\beta1}$ functions to inhibit the export of mature transcripts out of the nucleus. This is a novel regulatory mechanism since it is generally believed that mRNAs are rapidly released from the spliceosome and exported out of the nucleus once their splicing is completed. This work, entitled "Characterization and Putative Functional Role of a Stable Intron from the TCR-β Gene", is described in the last chapter of my thesis. The author contributions are: L. Qian (project development and all experiments) and M. F. Wilkinson (project development).

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Chapter 1

T Cell Receptor–β mRNA Splicing: Regulation of Unusual Splicing Intermediates

(running title: TCR–β RNA splicing)

Lian Qian[1], Livia Theodor[1#], Mark Carter[1], Minh N. Vu[1], Anna W. Sasaki[2] and Miles F. Wilkinson[1*]

Oregon Health Sciences University (O.H.S.U.), Microbiology and Immunology Department, L220, Vollum Institute for Advanced Biomedical Research (V.I.A.B.R.), 3181 S.W. Sam Jackson Park Road, Portland, OR 97201[1], and O.H.S.U., Department of Medicine and V.A. Medical Center, 111AG[2]

[*]To whom correspondence should be addressed. Phone: (503) 494-7578, FAX: (503) 494-6862

[#]Present address: Department of Life Sciences, Bar-Ilan University, Tel Aviv, Israel.

ABSTRACT

The expression of functional T cell receptor–β (TCR–β) transcripts requires the activation of programmed DNA rearrangement events. It is not clear if other mechanisms dictate TCR–β mRNA levels during thymic ontogeny. We examined the potential role of RNA splicing as a regulatory mechanism. As a model system, we utilized an immature T cell clone, SL12.4, that transcribes a fully rearranged TCR–β gene but essentially lacks mature 1.3kb TCR–β transcripts in the cytoplasm. Abundant TCR–β splicing intermediates accumulate in the nucleus of this cell clone. These splicing intermediates result from inefficient or inhibited excision of four of the five TCR–β introns; the only intron that is efficiently spliced is the most 5' intron, IVS_L. The focal point for the regulation appears to be $IVS1_{C\beta1}$ and $IVS2_{C\beta1}$ since unusual splicing intermediates accumulate *in vivo* that have cleaved the 5' splice site, but not the 3' splice site of these two introns. The block in 3' splice site cleavage is of interest since sequence analysis reveals that these two introns possess canonical splice sites. A repressional mechanism involving a labile repressor protein may be responsible for the inhibition of RNA splicing since treatment of SL12.4 cells with the protein synthesis inhibitor cycloheximide (CHX) reversibly induces a rapid and dramatic accumulation of fully spliced TCR–β transcripts in the cytoplasm, concomitant with a decline in TCR–β pre-mRNAs in the nucleus. This inducible system may be useful for future studies analyzing the underlying molecular mechanisms that regulate RNA splicing.

INTRODUCTION

The T cell receptor (TCR) for antigen is a multi-subunit receptor composed of two variable chains that recognize antigen (either the TCR–α and –β, or TCR–γ and -δ subunits) and several CD3 invariant chains that are involved in signal transduction (3). TCR–β chains are of particular interest for several reasons, including the fact that they recognizes so-called superantigens found in the natural environment (22). TCR–β proteins are encoded by gene segments that have undergone DNA rearrangement events. TCR–β genes that have juxtaposed D_β and J_β elements (partial rearrangement) or have remained unrearranged can transcribe a truncated (1.0kb) mRNA molecule of unknown function (28). Fully rearranged TCR–β genes that have joined a V_β gene segment to either $D_\beta J_\beta$ or J_β give rise to full length (1.3kb) mature TCR–β transcripts that can encode a functional TCR–β protein (28).

The expression of TCR–β transcripts during T cell maturation has been well studied. Truncated (1.0kb) TCR–β transcripts are expressed very early in murine ontogeny (at least as early as day 14 of mouse gestation) along with CD3-γ, –δ, –ε and -ζ transcripts (14,19,46). Full length (1.3kb) TCR–β transcripts are not evident until day 15-16 of mouse gestation. Since this is approximately the same point of gestation when fully rearranged TCR–β genes are detectable (19,46), it is widely assumed that the first expression of functional TCR–β transcripts is limited by gene rearrangement events. As ontogeny proceeds, the level of 1.3kb TCR–β mRNA increases in the thymus.

This is, at least in part, due to the fact that increasing numbers of thymocytes undergo rearrangement events in the thymus, but it also may be due to alterations in transcriptional or post-transcriptional regulatory events. On day 17, TCR-α transcripts become apparent in the fetal thymus (14,46). This event roughly coincides with the appearance of cell surface TCR-aβ protein on a small proportion of fetal thymocytes (46). Thus, the TCR–α chain appears to be the limiting subunit that controls the surface expression of TCR–αβ protein early in ontogeny. TCR–β protein has also been detected on the surface of immature thymocytes or cell lines in the absence of the TCR–α chain (25,37), but its function in this context is not yet known.

The transcriptional regulation of the TCR–β gene has been studied in cell culture. A tissue specific enhancer element has been defined 3' of the $C_{\beta 2}$ gene segment (27,33). Cis-acting elements important for TCR–β expression have also been characterized in V_β promoters and the J-$C_{\beta 2}$ intron region. DNA binding proteins that possess strong affinity for these regulatory elements have been identified, some of which may be involved in programmed DNA rearrangment events rather than transcriptional regulation *per se* (21,24,29,41,48). Negative trans-acting factors have been defined in immature T cell clones that serve to inhibit the transcription of TCR–β and other T cell genes that are normally activated during intermediate stages of thymic development (32,55).

The importance of post-transcriptional mechanisms in regulating TCR–β expression or function is not clear. TCR–β transcripts appear to be capable of undergoing alternative splicing events, but the functional significance of the known examples of alternative splicing is not known. An alternative C_β

exon ($C_{\beta 0}$) present in the J-Cβ1 intron is utilized in only a minority of mouse TCR–β mature transcripts, and is not present in either rat or human TCR–β genes (6,13). Alternative splicing of V_β leader exons has been observed (10), but this does not appear to be a general phenomenon.

As a model system to study the parameters that regulate TCR–β mRNA levels, we have used a set of related murine AKR T-lymphoma cell clones that have been carefully phenotyped and shown to represent distinct stages of thymic development. The SL12.4 cell clone has been the subject of many of our studies on T cell gene regulation (32,52,55). SL12.4 cells have a surface phenotype ($CD3^-$ $CD4^-$ $CD8^-$ $IL2R\alpha^+$ HSA^+ $CD44^-$ $Thy1^+$) that is characteristic of "intermediate maturity" thymocytes within the double negative subset that have a high potential to differentiate into mature T cells in organ culture or after adoptive intrathymic transfer (44). SL12.4 cells accumulate CD3—γ, –δ, –ε and -ζ mRNA and possess fully rearranged TCR–α and –β genes, but they do not express the TCR/CD3 complex on their surface (32,52,55). In this report, we show that SL12.4 cells transcribe a fully rearranged TCR–β gene but fail to accumulate mature TCR–β transcripts in the cytoplasm. Instead, conventional and unusual intron-containing TCR–β transcripts accumulate in the nucleus of these cells. The splicing of these transcripts may be inhibited by a labile protein(s) since the protein synthesis inhibitor cycloheximide induces a dramatic accumulation of fully spliced TCR–β transcripts in the cytoplasm of these cells.

MATERIALS AND METHODS

RNA Preparation and Northern Blots.

Total cellular RNA was prepared by lysis in guanidinium isothiocyanate, followed by ultracentrifugation over a 5.7M CsCl cushion, as described (ref. 54; protocol 1). Cytoplasmic and nuclear RNA were prepared as described (ref. 54; protocols 6 & 7). Briefly, the cells were lysed in a tris-saline buffer containing 0.5% Nonidet P-40, 0.25% sodium deoxycholate and 50μg/ml dextran sulphate, followed by immediate centrifugation to generate a nuclear pellet and a cytoplasmic supernatant - these two fractions were completely denatured in guanidinium isothiocyanate buffer and the RNA was prepared as described for the total cellular RNA. Poly(A)$^+$ RNA was prepared directly from cell lysates as described (ref. 54; protocol 9). RNase H mapping was performed as follows: RNA was first incubated with 5μM oligonucleotide in 10mM Tris (pH 7.6), 1mM EDTA in a total volume of 10μl for 30 minutes at 45^{o}C. A RNase H cocktail was then added (15μl) that consisted of 2.5μl 10X RNase H buffer [0.2M Hepes (pH 8.0), 0.5M KCl, 0.1M $MgCl_2$], 2.5μl 10mM DTT, 1 unit of RNase H, and H_2O. Following an incubation for 20 minutes at 37^{o}C in this cocktail, the RNA was extracted and ethanol precipitated.

RNA was electrophoresed in agarose gels in the presence of formaldehyde and MOPS, and capillary blotted onto Nytran membranes (42). RNA was also electrophoresed in denaturing polyacrylamide gels containing 7M Urea

and 1X TBE (42). The RNA from polyacrylamide gels was electroblotted onto Nytran membranes in 10mM Tris (pH 7.8), 5mM sodium acetate, 0.5mM EDTA overnight at 50mA, followed by 1 h at 200mA. All the blots were stained with methylene blue (53) to demonstrate equivalent loading of RNA and to mark the migration of rRNA transcripts and RNA molecular weight ladders (Bethesda Research Laboratories). The blots were hybridized with random oligomer primed ^{32}P-labeled cDNA inserts in the presence of 10% dextran sulphate and 50% formamide for 12-18 h at 42^{o}C (42). Hybridization with single stranded riboprobes was performed by overnight incubation at 60^{o}C in a buffer containing 5X SSPE, 5X Denhardt's, 5mM EDTA, 0.1% sodium pyrophosphate, 0.5% SDS, 50μg/ml sheared salmon sperm DNA, and 50% formamide (see ref. 42 for recipes). All blots were briefly washed at room temperature with 1X SSPE / 0.1% SDS, followed by several longer washes with 0.1X SSPE / 0.1% SDS / 5mM EDTA at 50-60^{o}C. Blots were stripped for sequential hybridization by placing them in boiling 0.1X SSPE / 0.1% SDS, and then gently agitating them while they cooled to room temperature.

Densitometry was performed on XAR-5 film using a Helana Laboratories model "Quick Scan R & D". The densitometer was shown to provide linear values from autoradiographs exposed to a 30 fold range of known amounts of ^{32}P.

Oligonucleotides, DNA Probes, PCR and DNA sequencing.

The oligonucleotides used are described in Table 1. IVS1$_{C\beta1}$ (0.5kb) and IVS2$_{C\beta1}$ (0.13kb) DNA fragments were prepared by the polymerase chain reaction (PCR) with a murine Cβ1 genomic template (17) and the

oligonucleotide pairs I1/I1' and E2'/E3, respectively. The $IVS_{V\beta5.1\text{-}L}$ (0.15kb) fragment was amplified from $V_{\beta5.1}$ genomic DNA (10) using the oligonucleotide pair V/V'. The following probes were generated by restriction endonuclease cleavage and gel purification (38): $IVS3_{C\beta1}$: 0.15kb NcoI/HinfI fragment; $IVS_{JC\beta1}$: 0.75kb EcoRI/KpnI fragment just 5' of Cβ1; Cβ1 exon 4: 0.3kb EcoRI/HindIII fragment from the 3' untranslated region; $C_{\beta2}$ exon 4: 0.2kb NsiI/HpaI fragment from the 3' untranslated region (30); C_{β} (exons 1-4): 0.6kb EcoR1 insert from 86T5 cDNA clone; $J_{\beta1}$: 1.6kb BamHI/SacI fragment encompassing $J_{\beta1.3}$-$J_{\beta1.7}$; $J_{\beta2}$: 1.2kb EcoRI/ClaI fragment encompassing $J_{\beta2.1}$-$J_{\beta2.7}$; $V_{\beta5.1}$ coding exon: 0.2kb EcoRI/RsaI fragment; and $V_{\beta5.1}$ leader and upstream region: 0.8kb SacI/XbaI fragment. Riboprobes were generated according to manufacturer's instructions (Promega Corp.) from the Bluescript vector (Stratagene) containing a 0.9kb XbaI/EcoR1 Cβ1 genomic fragment that includes most of IVS1, all of exon 2, IVS2, exon 3, IVS3, and a small portion of exon 4.

PCR was performed on 1ng of template DNA in a tube containing 2.5µl of 10X PCR buffer [500mM KCl, 100mM Tris (pH 9), 1% Triton X-100], 5µl 1mM dNTPS, 2.5µl 15mM $MgCl_2$, 1µl oligonucleotide #1 (10µM), 1µl oligonucleotide #2 (10µM), 11.8µl H_20, and 0.2µl (1 U) Taq DNA polymerase. The DNA was amplified for 30 cycles under the following conditions for each segment: denaturation, 94°C for 0.8 min; annealing, 54°C for 0.8 min; extension, 72°C for 1.2 min.

The nucleotide sequence of murine IVS2 and $IVS3_{C\beta1}$ was obtained from a 5C.C7 genomic clone (B10.A mouse strain) by dideoxy-sequencing methods with Sequenase reagents (U.S. Biochemical Corp.).

RESULTS

Accumulation of intron-containing TCR–β transcripts in the SL12.4 cell clone.

Our previous studies showed that SL12.4 cells possess a fully rearranged TCR–β gene at the β1 locus (Fig. 1A), but mature β1 transcripts are not detectably expressed in the cytoplasm (52). The basis for the lack of mature β1 transcripts was explored in the present investigation. Northern blots containing SL12.4 poly(A)$^+$ RNA were hybridized with a Cβ1-specific probe. Fig. 1B shows that there was no evidence for mature 1.3kb $V_\beta C\beta 1$ transcripts; instead there was an abundant accumulation of several other Cβ1 transcripts. Our subsequent analysis showed that all of these Cβ1 transcripts contained introns. For example, an $IVS_{JC\beta 1}$ probe hybridized with the two largest TCR–β transcripts of 4.8 and 5.1kb (Fig. 1B), as expected since $IVS_{JC\beta 1}$ is the largest TCR–β intron (Fig. 1A). A detailed analysis of the intron content of each individual Cβ1 pre-mRNA is found in the next section. It should be noted that the terms "pre-mRNA" and "precursor" are used only to designate transcripts that contain introns; these terms are not intended to imply that such transcripts are *necessarily* capable of being spliced to generate mature transcripts. For example, some intron-containing TCR–β transcripts may be irreversibly modified or sequestered in an environment where they are incapable of undergoing splicing.

To determine if all of the Cβ1 transcripts that we detected with double stranded DNA probes are transcribed in the sense orientation, we used sense and anti-sense single stranded riboprobes that contained IVS1, IVS2, IVS3, and the intervening exons. The anti-sense riboprobe hybridized with all major TCR–β transcripts while a sense riboprobe failed to hybridize with any of the transcripts (data not shown).

The pattern of partially spliced transcripts derived from the β1 locus differed dramatically from transcripts derived from the β2 locus. Our previous studies showed that the SL12.4 cell clone accumulates mature 1.0kb transcripts that hybridize with a $J_{\beta 2}$ probe derived from an unrearranged or partially rearranged β2 gene (52). Here, we used a $C_{\beta 2}$-specific probe to confirm that mature 1.0kb $C_{\beta 2}$ transcripts accumulate in SL12.4 cells, and also to demonstrate that no $C_{\beta 2}$ pre-mRNAs are evident, even when poly(A)$^+$ RNA was analyzed on Northern blots (Fig. 1B). Thus, SL12.4 cells are capable of efficiently splicing β2 pre-mRNAs but not β1 pre-mRNAs.

The splicing status of individual TCR–β introns.

By a combination of Northern blot analysis and RNase H mapping we were able to characterize the β1 RNA splicing products that accumulate in the SL12.4 cell clone. The RNase H mapping protocol involves incubation of RNA with specific oligonucleotides, followed by RNase H cleavage of the RNA at the site where the oligonucleotides bind (RNase H specifically cleaves RNA-DNA hybrids). We chose to use the method of RNase H mapping for several reasons: First, this approach complemented Northern blot analysis, since it allowed more detailed analysis of specific transcripts

originally detected by Northern blotting. Second, RNase H mapping permitted us to determine the proportion of transcripts that have spliced out each particular TCR–β intron. Third, RNase H mapping provided information not easily discerned by other methods. It permitted characterization of lariat intermediates. It also allowed characterization of individual transcripts within distinct size classes, while ribonuclease protection analysis is more limited in this regard, particularly if the transcripts are large.

Poly(A)$^+$ RNA from SL12.4 cells was analyzed by RNase H analysis. The expected sizes of the cleavage products were calculated from the known sizes of the TCR–β exons and introns (Fig. 1A). Based on this analysis, we were able to characterize the splicing status of all of the major Cβ1 transcripts in SL12.4 cells. As discussed below, the cleavage products generated were of sizes consistent with normal splicing intermediates. However, due to space limitations we do not discuss the origin of *all* of the major cleavage products generated after RNase H incubation. Figure 2 shows a diagramatic representation of the intron-containing mRNAs mapped by RNase H analysis. The 1.8, 2.0, 2.3, 4.8 and 5.1kb transcripts appear to be conventional splicing intermediates, while the smaller transcripts are unusual products of RNA splicing that will be described in a later section.

IVS2 and IVS3 splicing.

To investigate the splicing status of IVS3, we used the oligonucleotide E3, which binds to a sequence present in exon 3 (Fig. 2). Incubation with this oligonucleotide generated two predominant RNA species of approximately 0.6 and 0.9kb that hybridized with the Cβ1 exon probe (Fig. 3A, upper panel). The larger 0.9kb RNA is derived from TCR–β transcripts that have retained

IVS3 since this cleavage product also hybridized with the IVS3 probe (Fig. 3A, lower panel). The smear of transcripts below the 0.6kb and 0.9kb species is presumably due to variable lengths of poly(A) tails since RNA cleaved with E3 in the presence of oligo(dT) generated discrete sized RNA molecules (Fig. 3A; far right lane). The size of these poly(A)$^-$ cleavage products were about 0.3kb smaller than the predominant poly(A)$^+$ transcripts, indicating that the polyadenylate tail on these TCR–β transcripts is typically 0.3kb in length. The ratio of the 0.35 and 0.65kb cleavage products generated after E3 + dT treatment (determined by densitometic analysis) indicated that approximately 20% of polyadenylated β1 transcripts in SL12.4 cells have retained $IVS3_{C\beta1}$.

IVS2 and IVS3 RNA splicing was analyzed simultaneously with the oligonucleotide E2 which binds to exon 2 (Fig. 2). Cleavage with E2 and dT [oligo(dT) was added to remove polyadenylate tail heterogeneity, as described above] generated 3 different RNA fragments that hybridized with the Cβ1 exon probe: IVS2$^+$IVS3$^+$ (0.75-0.8kb), IVS2$^+$IVS3$^-$ (0.5kb), and IVS2$^-$IVS3$^-$ (0.4kb) cleavage products (Fig. 3B, upper panel). The appropriate products hybridized with IVS2 and IVS3 probes (data not shown). The only potential cleavage product that was *not* present was an IVS2$^-$IVS3$^+$ transcript. These results also revealed the proportion of polyadenylated TCR–β transcripts that have spliced IVS2. Comparison of the abundance ratio of the IVS2-containing transcripts (0.5 and 0.75-0.8kb) to IVS2$^-$ transcripts (0.4kb) indicates that about 50% of the polyadenylated β1 transcripts have retained IVS2.

The splicing status of IVS2 was confirmed by use of the oligonucleotide I2 which binds to the 3' end of IVS2 (Fig. 2). Cleavage with I2 sharply reduced the levels of the 1.8, 2.0, 2.3, 4.8 and 5.1kb β1 transcripts (Fig. 3C, upper panel). Therefore, a proportion of these transcripts must contain this small intron (97 nuc.), as indicated by the diagram in Fig. 2. However, since some uncleaved transcripts persist (even after incubation with 10 fold higher concentration of I2; data not shown) some of these transcripts must have spliced out IVS2.

IVS1 and IVS_{JC} splicing.

The splicing status of IVS1 and IVS_{JC} was analyzed by cleavage with the oligonucleotide E2 and hybridization with a V_β probe to assess the 5' cleavage products that could potentially contain these introns (Fig. 2). The particular V_β element rearranged to the Cβ1 locus in SL12.4 cells was determined by hybridization of ^{32}P-labeled SL12.4 total cDNA to cold V_β probes immobilized on membranes. This analysis revealed that SL12.4 cells expressed a member of the $V_{\beta5}$ family. Sequence analysis (to be described elsewhere) confirmed this result, and in particular, showed that the SL12.4 cell clone transcribed the $V_{\beta5.1}$ gene segment. Fig. 3B (middle panel) shows that a $V_{\beta5}$ probe hybridized with the 1.8, 2.0, 2.3, 4.8 and 5.1kb precursor transcripts, as expected. Incubation with E2 generated three dominant $V_{\beta5}^+$ cleavage products with the expected sizes: 0.8kb (IVS_{JC}^- $IVS1^-$), 1.3kb ($IVS_{JC}^-IVS1^+$) and 4.1kb ($IVS_{JC}^+IVS1^+$). Hybridization with an IVS1 probe confirmed these identities. The proportion of polyadenylated transcripts that excised IVS1 was determined by calculating the ratio of $IVS1^+$ (1.3 and 4.1kb) and $IVS1^-$ (0.8kb) transcripts generated after E2

treatment. This analysis revealed that about 50% of polyadenylated β1 transcripts contained IVS1 sequences.

Further confirmation that many β1 transcripts possess IVS1 was shown by cleavage with oligo(dT). This oligonucleotide cleaves near the 5' end of IVS1 due to the presence of several tracts of adenylate residues that span the region between nucleotides 100 to 155 of $IVS1_{C\beta1}$, including a homogeneous poly(A) tract of 22 nucleotides (39). Incubation with dT cleaved $IVS1^+$ transcripts to the expected sizes, as assessed with the IVS1, $V_{\beta5}$ probe, and Cβ1 exon probes (Figs. 3B & C). Note that the IVS1 probe only recognized the 3' products that result from dT cleavage.

The splicing status of IVS_{JC} was further assessed with oligonucleotide E1 which binds to sequences in exon 1 (Fig. 2). Oligonucleotide E1 generated 0.5kb and 3.4kb cleavage products that hybridized with the $V_{\beta5}$ probe that correspond to IVS_{JC}^- and IVS_{JC}^+ transcripts, respectively (Fig. 4, lane 5). The ratio of these two products shows that only about 10% of polyadenylated β1 transcripts have retained the JC intron.

IVS_L splicing.

The splicing status of the most 5' intron, IVS_L, was also assessed with the oligonucleotide E1. The size of the IVS_{JC}^- cleavage product (0.5kb) generated after E1 cleavage (Fig. 4) is consistent with an RNA that has already spliced out IVS_L. No cleavage product was detected that contains the 135 nuc. IVS_L (this product would be 0.6-0.7kb). Further evidence that IVS_L is spliced out of virtually all β1 transcripts included the following: First, RNase H cleavage in the presence of an oligonucleotide complementary with

the $V_{\beta 5}$ coding segment (olignonucleotide V; see Fig. 2) generated only a 0.1kb (spliced) product recognized by a $V_{\beta 5}$ leader probe (data not shown). Second, an IVS_L probe failed to hybridize detectably with any of the major β1 transcripts (data not shown).

IVS2 5' cleavage intermediate.

RNase H mapping showed that the abundant 0.7kb transcript detected with the Cβ1 and IVS2 probes (Fig. 3C, left lane) is a novel splicing intermediate containing IVS2 at the 5' terminus (Fig. 2). The evidence for this is as follows: Oligo(dT) treatment reduced the size of the 0.7kb transcript to 0.45-0.5kb (Fig. 3C, upper panel) showing that it possessed a 0.45-0.5kb deadenylated body and a 0.2-0.25kb poly(A) tail. This size is consistent with a transcript that has spliced out IVS3 and possesses IVS2 at its 5' termini. This phenotype was confirmed by the following: First, the IVS2 probe hybridized with the 0.45-0.5kb cleavage product generated after dT treatment (Fig. 3C, middle panel). Second, the IVS3 probe did not hybridize with this 0.45-0.5kb cleavage product (Fig. 3C, lower panel). Third, I2 + dT treatment generated a product 0.1kb smaller (approximately 0.4kb) than treatment with dT alone; this cleavage product did not hybridize with the IVS2 probe (Fig. 3C), as expected since I2 incubation should remove IVS2 from the 5' end of this transcript. Fourth, when the 0.7kb transcript was gel purified prior to RNase H analysis, it displayed the appropriate size and hybridization characteristics when cleaved in the presence of the oligonucleotides I2, E3, or E4 (data not shown).

To determine more precisely the 5' terminus of the 0.7kb transcript, poly(A)$^+$ RNA was cleaved with an oligonucleotide complementary to the 3' end of exon 3, E3b, and then subjected to polyacrylamide gel electrophoresis, followed by electroblotting and filter hybridization with an IVS2 probe. The 0.18kb E3b cleavage product derived from the 0.7kb transcript nearly co-migrated with the cleavage product generated after incubation with E3b + E2 (Fig. 5A) indicating that the 0.7kb transcript possesses IVS2 at its 5' terminus (see Fig. 2 and Table 1 for positions of the oligonucleotides). The 5' terminus of the 0.7kb transcript did not include exon 2 since the size of the E3b cleavage product (0.18kb) was clearly smaller than the 0.20kb exon 2-containing cleavage product generated from the conventional TCR–β splicing intermediates after E3b + I1b incubation (Fig. 5A). It is unlikely that IVS2 at the terminus of the 0.7kb transcript is in a lariat conformation since the 0.18kb E3b cleavage product derived from this transcript did not display reduced migration in the polyacrylamide gel, as is typical for intron lariats (36,56), including IVS1$_{C\beta1}$ (39). In fact, the 0.18kb E3b cleavage product derived from the 0.7kb transcript migrated slightly faster than the E3b + E2 cleavage product (Fig. 5A), indicating that it is likely that IVS2 has been debranched and nibbled at the 5' end. We conclude that the abundant 0.7kb β1 transcript is an IVS2$^+$IVS3$^-$ linear RNA molecule that appears to have undergone the first step of IVS2 splicing *in vivo* (5' cleavage), but has not been subjected to the second step (3' cleavage) which would have lead to expulsion of IVS2 and joining of exons 2 and 3.

IVS1 5' cleavage intermediates.

The 1.2 and 1.5kb IVS1$^+$ transcripts appear to be splicing intermediates that contain IVS1 at the 5' terminus (Fig. 2) and differ only in that the former transcript has spliced out IVS3 (Fig. 3A). Evidence for the existence of transcripts that possess IVS1 at the 5' end is as follows: RNase H cleavage in the presence of E3 released a 0.6kb cleavage product that hybridized with IVS1 and IVS2 probes (Fig. 3A, middle panel, and data not shown). The size and hybridization characteristics of this 0.6kb product indicated that it contains IVS2 and that its 5' terminus is the 5' end of IVS1. The intron IVS1 at the 5' termini of the 1.2 and 1.5kb transcripts appears to be in a lariat conformation. Electrophoresis of the RNA in a 6% polyacrylamide gel, followed by electroblotting and filter hybridization with an IVS1 probe did not reveal any transcripts that migrated at 1.2 or 1.5kb, which would be expected if they were linear molecules. Under these conditions, the only major transcripts detected were two RNA species (Fig. 5B, left lane) which we show elsewhere (39) are free (exonless) 0.5kb IVS1 lariats with different length tails (in contrast to their slow migration in polyacrylmaide gels, these free intron lariat molecules migrate as a single 0.5kb transcript in agarose gels, ref. 39).

To provide further evidence that transcripts with IVS1 at the 5' terminus are lariats, SL12.4 poly(A)$^+$ RNA was incubated with the E3 oligonucleotide and RNase H to release the 5' terminal portion of these IVS1$^+$ transcripts. The E3 cleavage product migrated as 0.6kb in agarose gels, as expected (Fig. 3A, middle panel), but clearly no product that migrated at 0.6kb was detectable in a polyacrylamide gel (Fig. 5B). The migration of a linear form of this 0.6kb

RNA was determined by cleavage with E3 and an oligonucleotide to the 5' end of IVS1, I1 (Fig. 5B, last lane). The 0.5kb transcript that was also generated after E3 + I1 treatment (or incubation with I1 alone) resulted from linearization of the free IVS1 lariat (39). It was not feasible to independently analyze whether these splicing intermediates are in a lariat conformation using debranching extracts (HeLa S100 or nuclear extracts) because such extracts only very inefficiently debranch IVS1, perhaps for the same reason that this is intron is not efficiently debranched *in vivo* (39).

Accumulation of TCR-β pre-mRNAs in the nucleus of the SL12.4 cell clone.

One possible mechanism to prevent the splicing of β1 transcripts would be to export partially spliced transcripts to the cytoplasm where RNA splicing does not occur. Retroviruses employ this export mechanism to generate an array of unspliced, partially spliced and fully spliced transcripts in the cytoplasm that give rise to different translation products. We found that such an export mechanism is unlikely to be operating on β1 pre-mRNAs since these transcripts were clearly confined to the nuclear compartment (Fig. 6, first three panels). By comparison, the fully spliced 1.0kb β2 transcript was present in both the nuclear and cytoplasmic compartments (Fig. 6, right panel). Most of the β1 transcripts present in nuclear RNA, including the abundant 0.7kb transcripts possessing IVS2 at the 5' terminus (Fig. 6) were also present in poly(A)$^+$ RNA (Figs. 1B, 3A-C). This shows that the polyadenylation of many β1 transcripts precedes initial RNA splicing events.

A unique nuclear transcript *not* present in poly(A)$^+$ RNA was an abundant 0.8kb $V_{\beta5}^+$ transcript (Fig. 6, left panel). Based on size and hybridization

characteristics, this transcript appears be the 5' portion of precursor transcripts that have undergone 5' cleavage of IVS1 or IVS2, but have failed to undergo 3' cleavage and exon ligation (see earlier). Such a 5' cleavage product would not contain a polyadenylate tail, and thus would not be expected to be in the poly(A)$^+$ fraction.

The protein synthesis inhibitor cycloheximide allows mature TCR–β transcripts to accumulate in the cytoplasm.

The accumulation of partially spliced β1 transcripts in SL12.4 cells shows that the splicing of β1 transcripts is impaired in this cell clone. We previously showed that cycloheximide (CHX) induces the appearance of a fully spliced 1.3kb TCR–β transcript that hybridizes with a $J_{\beta1}$ probe. We concluded that this induction of mature TCR–β mRNA was a post-transcriptional event since it occurred without a significant concomitant increase in the rate of transcription, as judged by nuclear run-off assays (52). It was hypothesized that an unstable inhibitor protein blocks the expression of mature TCR–β transcripts by a post-transcriptional mechanism. If the putative inhibitor acts by inhibiting RNA splicing, it would be anticipated that if the inhibitor was depleted (by addition of CHX) that precursor transcripts would be depressed in levels. Fig. 7A shows that, indeed, CHX treatment lead to the down-regulation of $V_{\beta5}C\beta1$ pre-mRNAs, and the dramatic appearance of a 1.3kb fully spliced $V_{\beta5}C\beta1$ mRNA. A novel 1.7kb $V_{\beta5}C\beta1$ transcript was also induced by CHX - this is a mature cytoplasmic mRNA that is derived from a promoter upstream of the transcription initiation site for 1.3kb transcripts (unpublished observations). Confirmation that CHX induced a fully spliced β1 transcript came from RNase H analysis. IVS2 and IVS3 were fully spliced

following CHX treatment, as evidenced by a single 0.3kb cleavage product recognized by the Cβ1 exon probe after oligonucleotide E2 + dT cleavage (Fig. 7B, right panel).

The mature TCR–β transcripts induced by CHX were exported to the cytoplasm (Fig. 7C, right lane). Virtually no β1 transcripts were in the cytoplasm before treatment (Fig. 7C, left lane). In contrast, 1.0kb β2 transcripts were present in the cytoplasm before and after CHX treatment (Fig. 7C). Removal of CHX led to the rapid reappearance of intron-containing $V_{\beta5}C\beta1$ transcripts and the disappearance of the major mature 1.3kb $V_{\beta5}C\beta1$ transcript (Fig. 7A). The reciprocal expression pattern of intron-containing and mature β1 transcripts in response to CHX suggests that there is a precursor-product relationship between these two classes of transcripts. This notion is substantiated by our observation that treatment of SL12.4 cells with the transcriptional inhibitor actinomycin D does not prevent the induction of mature β1 mRNA in response to CHX (unpublished observations). Taken together, the data indicate that CHX is likely to act, at least in part, by increasing the rate of TCR–β RNA splicing.

Fully spliced TCR–β transcripts accumulate in the nuclei of SL12.4 cells.

If inhibited RNA splicing were the *only* mechanism that prevented TCR–β mRNA accumulation in the cytoplasm of SL12.4 cells, then fully spliced TCR–β transcripts should also be absent in the nucleus. Since we showed that none of the TCR–β introns are completely blocked in splicing, it would not be surprising if some fully spliced TCR–β transcripts *were* generated in SL12.4

cells. Northern blot analysis indicated that fully spliced 1.4-1.5kb TCR–β transcripts with long poly(A) tails that varied somewhat in size were detectable with both $V_{\beta 5}$ and Cβ1 exon probes (Fig. 7A). Cleavage with oligo(dT) and RNase H showed that the deadenylated body of these 1.4-1.5kb transcripts from untreated SL12.4 cells exhibited identical migration (1.2kb) to the deadenylated body of the CHX induced 1.3kb transcript (Fig. 4; note that 5 times more RNA from untreated cells was loaded compared to CHX treated cells). Thus, at least a proportion of the 1.4-1.5kb transcripts in untreated SL12.4 cells are fully spliced TCR–β mRNAs present in the nucleus that have longer poly(A) tails than the 1.3kb mature transcripts induced by CHX to appear in the cytoplasm. This observation is consistent with other studies demonstrating that nuclear mRNAs possess longer poly(A) tails than cytoplasmic mRNAs (8). It should be noted that this analysis provides an overestimate of the relative abundance of fully spliced messages since the analysis was conducted on the poly(A)$^+$ fraction of RNA which lacks many TCR–β pre-mRNAs, particularly those containing $IVS_{JC\beta 1}$ (see earlier discussion). We conclude that although Cβ1 transcripts display inhibited splicing in SL12.4 cells, the presence of *some* fully spliced Cβ1 transcripts in the nucleus implies that other post-transcriptional mechanism(s) must also contribute to the lack of mature transcripts in the cytoplasm.

Sequence of Cβ1 introns.

The sequence of the Cβ1 introns was determined since they displayed unusual splicing behavior. The sequence of $IVS1_{C\beta 1}$ is presented elsewhere (39); our analysis revealed that it possesses canonical splicing signals. The complete sequence of $IVS2_{C\beta 1}$ and $IVS3_{C\beta 1}$ is shown in Fig.

8. Both introns possess the invariant "GU" and "AG" (34) at their 5' and 3' ends, respectively. $IVS2_{C\beta1}$ has many other features expected of "a typical" intron. First, it has a 5' splice junction region that displays perfect complementarity with the portion of U1 known to interact with this region (34). Second, it has a stretch of $^{17}/_{19}$ polypyrimidines (Y_N) followed by the sequence AACAG/C which has some similarity with the known consensus 3' splice junction: $(Y)_N$NYAG/G (34). Third, just upstream of the polypyrmidine tract, it possesses a sequence displaying $^{6}/_{7}$ match with the mammalian branchpoint consensus sequence: YNYURAY (57). Thus, the inhibited splicing of $IVS2_{C\beta1}$ cannot be easily ascribed to inefficient "splicing signals". $IVS3_{C\beta1}$, which was *more* efficiently spliced than IVS2 in SL12.4 cells, has features *less* consistent with a typical intron. Most strikingly, it has a stretch of only $^{9}/_{12}$ polypyrimidine residues upstream of its splice acceptor (Fig. 12). Studies on other introns have indicated that short polypyrimidine tracts typically do not permit efficient splicing (45). However, $IVS3_{C\beta1}$ does have a consensus 3' splice junction [$(Y)_N$GCAG/G] and two potential branchpoint sequences upstream of the short polypyrimidine tract which each display $^{5}/_{7}$ match with the known consensus sequence. The 5' splice site of IVS3 possesses $^{6}/_{9}$ nucleotides in common with the known consensus sequence (34)

DISCUSSION

Inhibitors of RNA splicing have been postulated to play a regulatory role in the expression of several eukaryotic transcripts (7). Most cases of negative splicing regulation involve choices between alternative splice sites (11,15,18,23,31,40,47). Only a few examples of repressional mechanisms are known that inhibit the rate of RNA splicing from a single set of splice acceptors and donors; e.g. "splice-vs.-no splice" decision making. The third intron in the transposase transcript derived from the *Drosophila* P element is prevented from being spliced in somatic tissues. This block in RNA splicing may be controlled by one or more specific RNA binding proteins that have been identified in somatic tissues (50). Autoregulatory control of RNA splicing is exerted by the product of the suppresser of *white apricot* locus of *Drosophila* [*su(W^a)*]. This locus encodes an RNA binding protein that blocks the splicing of the first intron of its own precursor mRNA (7). Similarly, the yeast ribosomal protein L32 blocks the splicing of a single intron in its own pre-mRNA (16). Our own observations suggest that negative splicing regulation also serves to regulate "splice-vs-no splice" decisions in cells of the immune system.

An attractive aspect of the regulation of TCR–β RNA splicing in T cells is that it can be analyzed and manipulated in a cell line. The protein synthesis inhibitor cycloheximide (CHX) has dramatic effects on the accumulation of TCR–β precursor and mature transcripts in the SL12.4 cell clone. CHX treatment triggers a dimunition of precursor transcripts in the nucleus and a

dramatic appearance of spliced transcripts in the cytoplasm. Removal of CHX permits reappearance of precursor transcripts in the nucleus and leads to eventual loss of mature transcripts in the cytoplasm. Taken together, these results suggest that the splicing of TCR–β transcripts is reversibly inducible in SL12.4 cells. The inducibility by CHX suggests that TCR–β RNA splicing is negatively regulated by an unstable protein(s). Other eukaryotic transcripts are also up-regulated in response to protein synthesis inhibitors. Protein synthesis inhibitors potentiate the expression of many cytokine and oncogene transcripts by increasing cytoplasmic RNA half life. The effect is mediated, at least in part, by an AU rich sequence present in the 3' untranslated region (43) which binds to a specific protein that may play a role in the stability of these messages (9). Protein synthesis inhibitors also induce immunoglobulin κ chain gene transcription (51), perhaps as a result of depleting cells of I-κB, an inhibitor of the positive transcription factor NF-κB (4).

Thus, protein synthesis inhibitors have been useful tools to provide evidence for negative regulatory mechanisms that act at the level of gene transcription or cytoplasmic RNA stability. The effect of protein synthesis inhibitors on TCR–β expression is unusual since it does not appreciably affect TCR–β gene transcription (52), but instead it appears to have a profound effect on TCR–β RNA splicing. This provides a unique system to study the mechanism of RNA splicing. It should be stressed, however, that it is not clear that CHX is acting by depleting T cells of a specific inhibitor of RNA splicing. Since the synthesis of most cellular proteins is blocked by CHX, other less specific events may be responsible for its effects on RNA splicing. Nevertheless, the

dramatic effects of CHX on TCR–β transcripts suggests that it will be a useful tool to investigate the regulation of TCR–β RNA splicing.

Several factors must be taken into account when considering the mechanism that controls TCR–β RNA splicing in SL12.4 cells. First, the splicing of several introns is impaired. Second, only the most 5' intron within a V_{β}-containing transcript, IVS_{L}, is efficiently spliced. Third, each individual intron displays different apparent rates of splicing. We assessed the percentage of polyadenylated transcripts that contained each intron as an indirect measure of the splicing rate *in vivo*. $IVS1_{C\beta1}$ and $IVS2_{C\beta1}$ are retained in about 1/2 of β1 transcripts, while $IVS3_{C\beta1}$ is present in only about 1/10th of β1 transcripts. $IVS_{JC\beta1}$ is only present in about 1/10th of polyadenylated β1 transcripts, although it is clearly present in a larger proportion of total nuclear β1 transcripts as detected by Northern blot analysis (see Fig. 6).

One model to explain the pattern of TCR–β RNA splicing that we observe is to hypothesize that unstable regulatory protein(s) independently regulate each of the four introns that display inhibited splicing. Another model is that, instead, only one or a few introns are *directly* inhibited from undergoing splicing by regulatory proteins. The other introns would display inefficient splicing as a consequence of the "regulatory introns". This transfer of inhibitory activity could be mediated by interactions between the introns, as has been noted for other transcripts (35,49). Our data is consistent with a model in which negative regulation is focused on $IVS1_{C\beta1}$ and $IVS2_{C\beta1}$. There are three lines of evidence that support this specific model. First, IVS1 and IVS2 are present in a greater proportion of pre-mRNAs than IVS3 or IVS_{JC}. Second, the particular combination of introns that are present in

TCR–β pre-mRNAs suggests that the splicing of IVS1 and IVS2 is less efficient than the splicing of IVS_{JC} and IVS3, respectively. Third, 3' splice site cleavage is inefficient or repressed for IVS1 and IVS2, but not the other introns. The evidence for this notion is the accumulation of splicing intermediates that have cleaved the 5' splice site but not the 3' splice site of these two introns.

The accumulation of splicing intermediates *in vivo* that have undergone 5' splice cleavage, but not cleavage of the 3' splice site is a novel observation. Other eukaryotic pre-mRNAs have been commonly shown to generate splicing intermediates of this type during *in vitro* splicing reactions (26,36), but such intermediates are rarely observed at detectable levels *in vivo* (56). Presumably, such 5' splicing intermediates accumulate during *in vitro* splicing reactions because splicing is inefficient *in vitro*, while *in vivo* splicing reactions typically display such a rapid kinetic linkage of the first and second steps of splicing that intermediates are not normally detectable. The 5' splice site cleavage intermediates that have been observed *in vitro* and *in vivo* by other workers accumulate as lariat structures (26,36,56). Similarly, TCR–β splicing intermediates which contains $IVS1_{C\beta1}$ at the 5' termini are in a lariat conformation (see Fig. 5B), just as we previously showed for free $IVS1_{C\beta1}$ (39). In contrast, the extremely abundant 0.7kb splicing intermediate which contains $IVS2_{C\beta1}$ at the 5' terminus is likely to be linear, based on migration in polyacrylamide gels. This suggests that the IVS2 lariat structure has been debranched *in vivo*. This debranched transcript may have lost its ability to undergo 3' cleavage and exon ligation. Thus, this unusual transcript may be on a "dead end" pathway. However, we cannot rule out an alternative

possibility that this linear transcript was *not* generated by the classical splicing reaction, but instead by specific endonucleolytic cleavage.

What is the function of this negative post-transcriptional regulation that acts, in part, at the level of RNA splicing? The splicing of (D)JC$_\beta$, CD3-γ, $-\delta$, $-\varepsilon$ and -ζ, CD4 and CD8-α transcripts do not display impaired splicing (ref. 55 and results herein), so the regulation appears to be a selective mechanism that acts on V(D)JC$_\beta$ transcripts. One possibility is that a specific RNA splicing mechanism controls the stage specific accumulation of mature cytoplasmic V(D)JC$_\beta$ transcripts during thymic maturation. Consistent with this hypothesis is our observation that TCR–β splicing intermediates accumulate in normal thymocytes (39). These thymic splicing intermediates correspond in intron content to those which accumulate in the SL12.4 cell clone described in this report (unpublished observations). The rate of RNA splicing may serve to control the expression of TCR–β protein in a developmentally regulated fashion, in combination with other regulated events such as gene rearrangements.

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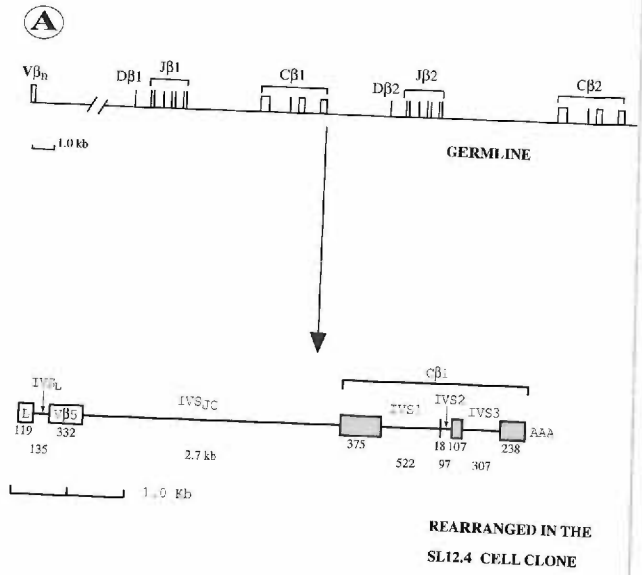
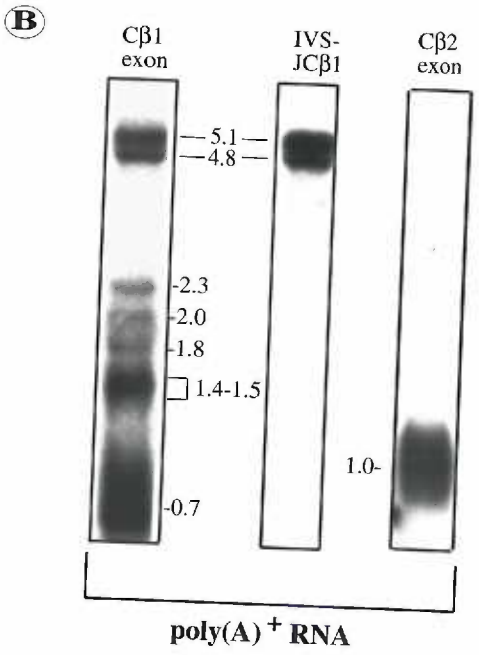
TABLE 1. Oligonucleotides

Name	Orientation	Position[a]	Sequence	Reference
E1	Antisense	Exon 1, 27–49[b]	GAGAGCTCAAACAAGGAGACCTT	14
E2	Antisense	Exon 2, 1–17	TGAGGTAATCCCACAGT	14
E2′	Sense	Exon 2, 1–18	ACTGTGGGATTACCTCAG	14
E3	Antisense	Exon 3, 1–16[c]	CCTTGTTGATAGGATGC	14
E3B	Antisense	Exon 3, 87–107	CATAGCCATCACCACCAGTGT	14
I1	Antisense	IVS1, 1–19	CTCCCCAGGTCCCACTTAC	14
I1B	Antisense	IVS1, 475–491	GGAAAATGGATGAGACC	14
I1′	Sense	IVS1, 17–33	GAGCTGGCAAGAAGAAT	14
V	Antisense	$V_{\beta 5.1}$, 1–14[d]	CAGAATTTGCTGAACCTGGG	8
V′	Sense	L-$V_{\beta 5.1}$, 63–79[e]	CTTTCTCCTGGGAACAAGT	8

[a] Position number is determined from start of exon or intron, unless otherwise noted.
[b] Two-nucleotide mismatch to generate a *Sac*I restriction site.
[c] Includes one nucleotide of IVS2.
[d] Includes five nucleotides of IVS-L.
[e] Includes two nucleotides of IVS-L (position number is determined from the translation start site).

FIG. 1 TCR–β gene rearrangments and expression in the SL12.4 cell clone. (A) Upper portion shows TCR–β genomic structure (28) and the lower portion provides information on the rearranged β1 gene expressed in SL12.4 cells. The length of the exons were derived either from published work (1,10,17) or from our sequence analysis of the expressed $V_{\beta 5.1}$ element in SL12.4 cells. The lengths of IVS1, IVS2 and IVS3 were also derived by our sequence analysis (Fig. 12 and ref. 39). (B) Northern blot analysis of SL12.4 poly(A)$^+$ RNA (1μg) electrophoresed in a 1% agarose gel. The blot was sequentially hybridized with the probes shown.


A
Vβn
Dβ1
Jβ1
Cβ1
Dβ2
Jβ2
Cβ2
1.0 kb
GERMLINE
Cβ1
IVS1
IVS2
IVS3
AAA
119
332
135
2.7 kb
375
522
18
107
97
307
238
1.0 Kb
REARRANGED IN THE
SL12.4 CELL CLONE


B
Cβ1
exon
IVS-
JCβ1
Cβ2
exon
5.1
4.8
2.3
2.0
1.8
1.4-1.5
0.7
1.0
poly(A) + RNA

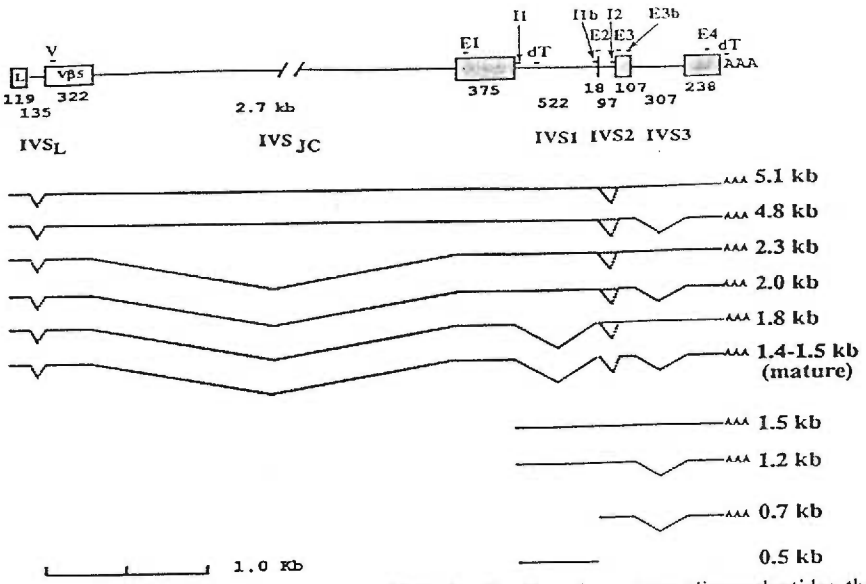
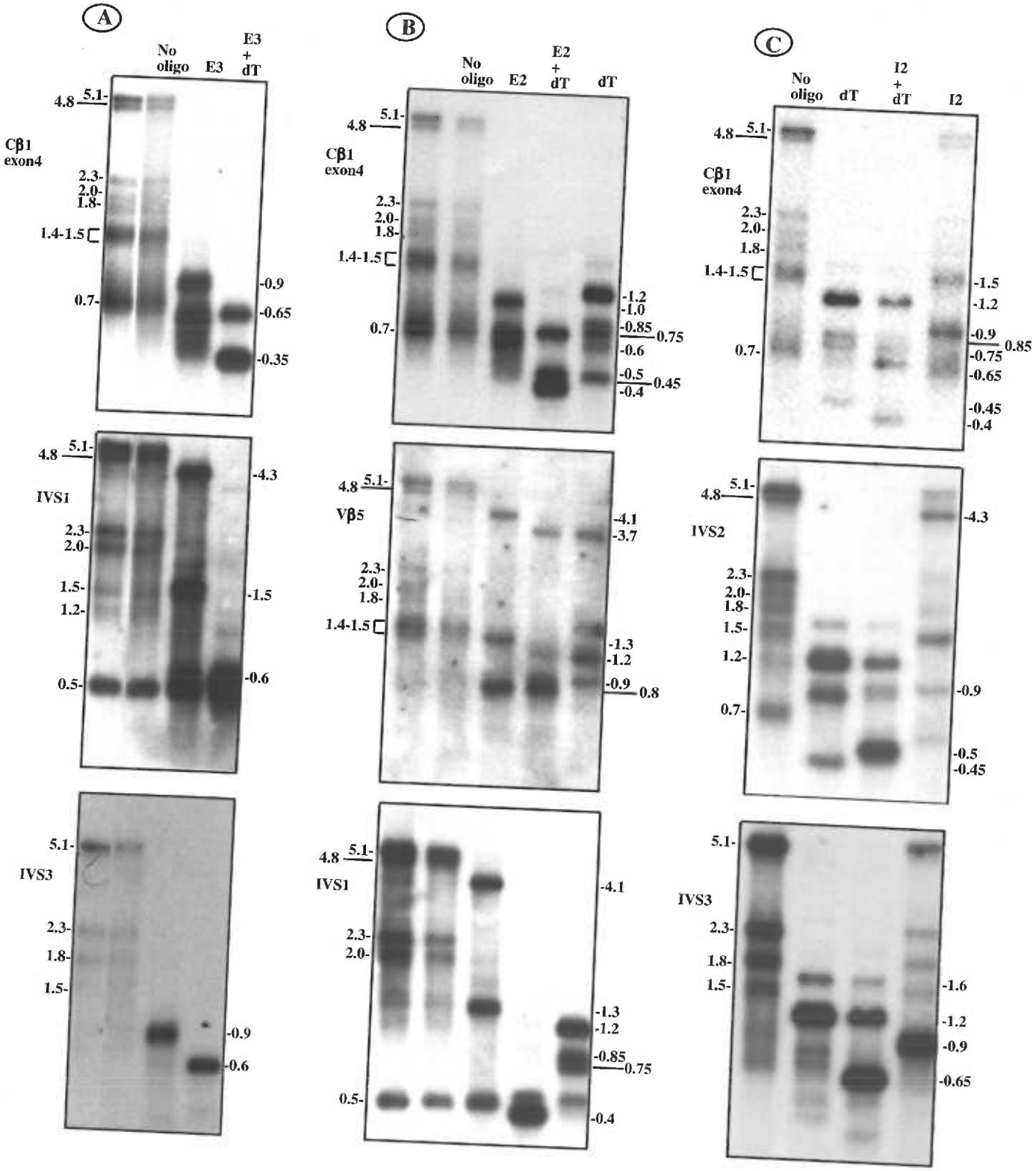


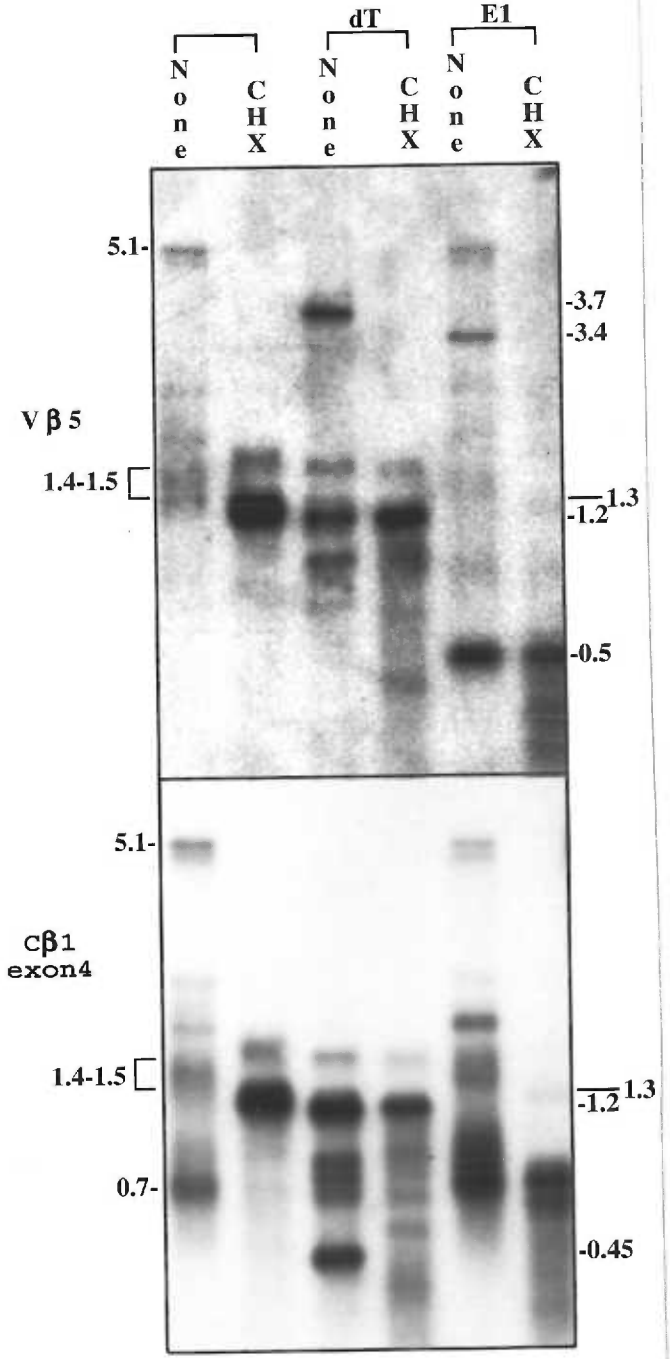
FIG. 2. Schematic diagram of $C_{\beta 1}$ transcripts expressed in SL12.4 cells. Complementary oligonucleotides that bind to SL12.4 TCR-β pre-mRNA at the locations shown were designed (see Table 1 for more information on the oligonucleotides). The splicing pattern and approximate lengths of mapped transcripts were determined by the analysis shown in Fig. 3 and 4. IVS2 was variably present in some transcripts, as indicated by the dotted lines.

FIG. 3 RNase H analysis of Cβ1 transcripts in SL12.4 polyadenylated RNA. SL12.4 poly(A)$^+$ RNA (1μg) was subjected to RNase H analysis using the oligonucleotides shown, followed by electrophoresis in 1% agarose gels and Northern blot hybridization. Each panel (A,B and C) shows the results with a single blot sequentially hybridized with the probes shown. Lanes marked "no oligo" refers to RNA subjected to the RNase H procedure in the absence of a specific oligonucleotide, while unmarked lanes show untreated RNAs, as a control.


A
No oligo
E3
E3 + dT
Cβ1 exon4
4.8
5.1-
2.3-
2.0-
1.8-
1.4-1.5
0.7-
-0.9
-0.65
-0.35
IVS1
-4.3
-1.5
-0.6
0.5-
IVS3
-0.9
-0.6
B
No oligo
E2
E2 + dT
dT
-1.2
-1.0
-0.85
0.75
-0.6
-0.5
0.45
-0.4
Vβ5
-4.1
-3.7
-1.3
-1.2
-0.9
0.8
IVS1
-4.1
-1.3
-1.2
-0.85
0.75
-0.4
C
No oligo
dT
I2 + dT
I2
-1.5
-1.2
-0.9
0.85
-0.75
-0.65
-0.45
-0.4
IVS2
-4.3
-0.9
-0.5
-0.45
IVS3
-1.6
-1.2
-0.9
-0.65

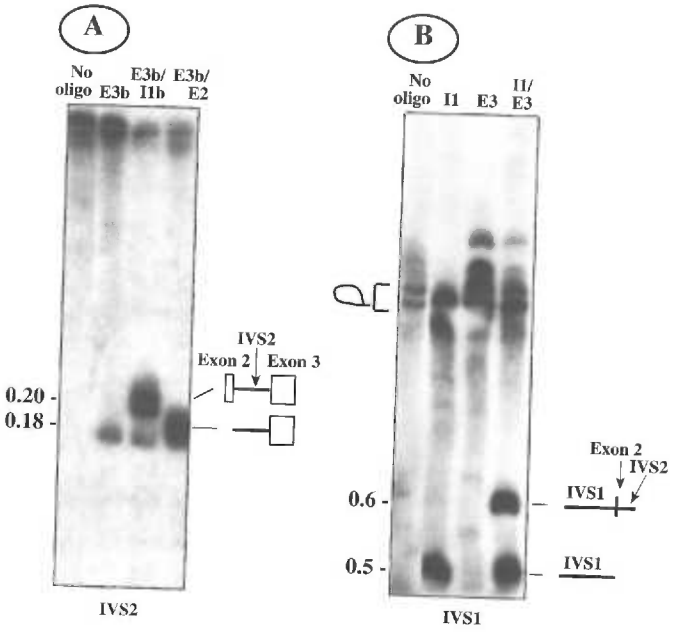
F3

FIG. 4 RNase H analysis: effect of cycloheximide on SL12.4 Cβ1 transcripts. SL12.4 poly(A)$^+$ RNA [0.2μg RNA from CHX treated cells (10μg/ml for 6hr) and 1μg RNA from control cells (labeled "none")] was subjected to the analysis described in the legend from Fig. 3. The first two lanes show RNA incubated under the same conditions as the others, but without a specific oligonucleotide.


dT
E1
None
CHX
None
CHX
None
CHX
5.1-
-3.7
-3.4
Vβ5
1.4-1.5
1.3
-1.2
-0.5
5.1-
Cβ1
exon4
1.4-1.5
1.3
-1.2
0.7-
-0.45

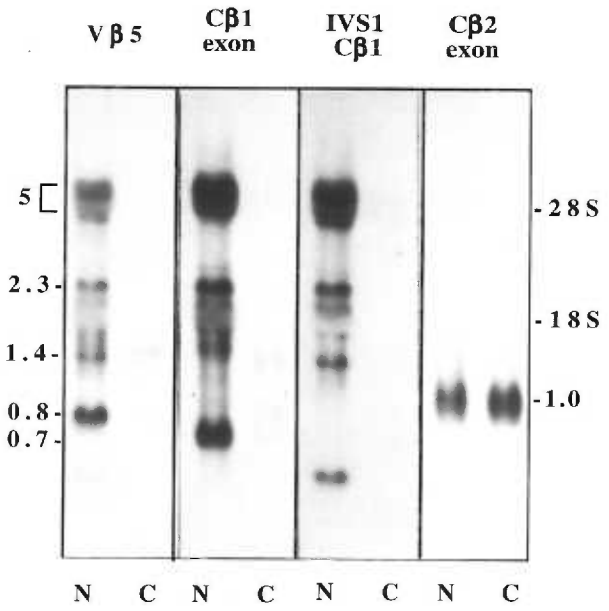
F4

FIG. 5 Characterization of Cβ1 transcripts that possess introns at the 5' end. (A) SL12.4 poly(A)$^+$ RNA (1μg) was subjected to RNase H analysis using the oligonucleotides shown, followed by electrophoresis in a 7% denaturing polyacrylamide gel and Northern blot hybridization with a $IVS2_{C\beta1}$ probe. The lane marked "no oligo" refers to RNA subjected to the RNase H procedure in the absence of a specific oligonucleotide. (B) The same same conditions as described for panel A, except that a 5% polyacrylamide gel is used, and the hybridization probe is $IVS1_{C\beta1}$.


A
No oligo
E3b
E3b/ I1b
E3b/ E2
0.20 -
0.18 -
IVS2
Exon 2
Exon 3
IVS2
B
No oligo
I1
E3
I1/ E3
0.6 -
0.5 -
Exon 2
IVS2
IVS1
IVS1
IVS1

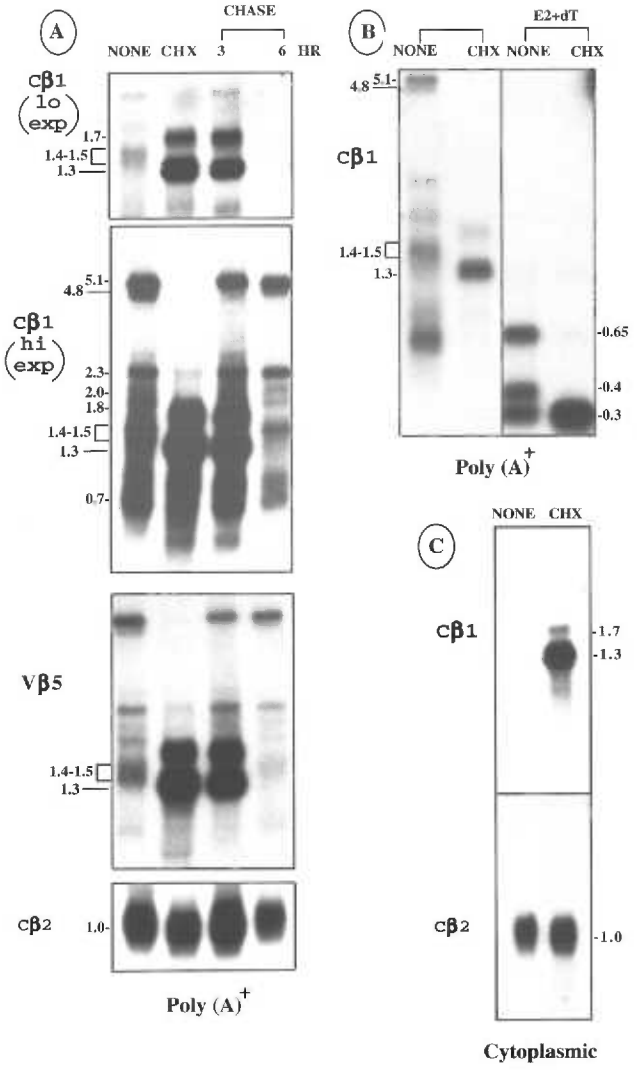
E5

FIG. 6 Localization of SL12.4 Cβ1 transcripts to the nuclear compartment. Northern blot analysis of nuclear (N) and cytoplasmic (C) RNA (10μg) from SL12.4 cells electrophoresed in a 1% agarose gel. The blot was sequentially hybridized with the probes shown.



F6

FIG. 7 Reversible effects of cycloheximide on TCR–β mRNA accumulation. (A) Northern blot analysis of SL12.4 poly(A)$^+$ RNA (1μg) from control cells (none); cells incubated for 12hr with 10μg/ml CHX (CHX); or cells treated the same except they were washed and re-cultured for 3hr or 6hr (chase). The RNA was electrophoresed in a 1% agarose gel, blotted, and sequentially hybridized with the probes shown. Equivalent amounts of RNA were loaded in each lane as assessed by methylene blue staining (53) except the last lane, which had approximately 1/2 as much RNA as the other lanes. (B) SL12.4 poly(A)$^+$ RNA [0.5μg RNA from CHX treated cells (10μg/ml for 6hr) and 1μg RNA from control cells (labeled "none")] was subjected to RNase H analysis in the absence of oligonucleotides (first two lanes) or the presence of the oligonucleotides E2 and oligo(dT) (last two lanes). The RNA was electrophoresed in 1% agarose gels, blotted and hybridized with the probe shown. (C) Northern blot analysis of cytoplasmic RNA (10μg) from SL12.4 cells. Performed as described for panel A.


A
CHASE
NONE CHX 3 6 HR
cβ1 (lo exp)
1.7-
1.4-1.5
1.3
4.8 5.1-
cβ1 (hi exp)
2.3-
2.0-
1.8-
1.4-1.5
1.3
0.7-
Vβ5
1.4-1.5
1.3
cβ2 1.0-
Poly (A)+
B
E2+dT
NONE CHX NONE CHX
4.8 5.1-
cβ1
1.4-1.5
1.3-
-0.65
-0.4
-0.3
Poly (A)+
C
NONE CHX
cβ1
-1.7
-1.3
cβ2
-1.0
Cytoplasmic

$IVS2_{C\beta1}$

```
1   CAG/GTAAGT GAGTTGACTC TCTCTTCCCC TCTCATGATT ATGACTGTAG
    CAG/GTAAGT
51  AGCTAGCTAG CTGTCCAAAG GTCCTTACCT GCTCTCCTTT TCTGTCAACAG/C
                          YNYURAY                       NYAG/G
```

$IVS3_{C\beta1}$

```
1   TAT/GGTAAG GAACAGGCAG ATGGAGCTTA TGGGGAGGTG ACACATGCAA
    CAG/GTAAGT
51  CCCAGGCATT TACAACCAGT GGCTTAGGCC TCTTCCAGAA GTAGAGGGGA

101 GCAGTAGAGA GAGGTCTGCT TCCATGGTGT AAAAGACAAG AAGAAACTTC

151 AAAGAACTGT AGTGGATAGC TATAGAGGAT AAGCTGTGAT AACTCAAAAG

201 CCCAAGGATG TTTTAGTAGC TCTATCGTTT GTCTGAACAA ATGGGCCTTT

251 GTATATTCTC TCAAGCCACA CTATGCAAGG CCATTGGTAA ACCTAAAATG
                                    YNYUR AY
                                          YNYURAY

301 ATTCTCATCT GCAG/G
               NYAG/G
```

FIG. 8. Nucleotide sequences of $IVS2_{C\beta1}$ and $IVS3_{C\beta1}$. The sequences shown in bold below the $C_{\beta1}$ intron sequence represent the consensus sequences for mammalian branchpoint regions (53) and 5′/3′ splice junctions (30). The sequence shown is in agreement with a partial sequence of murine $IVS2_{C\beta1}$ and $IVS3_{C\beta1}$ reported previously (14).

Chapter 2

A spliced intron accumulates as a lariat in the nucleus of T cells

running title: TCR–β intron lariats

Lian Qian, Minh N. Vu, Mark Carter and Miles F. Wilkinson*

Microbiology & Immunology Department, Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University, L220, 3181 S.W. Sam Jackson Park Rd., Portland, Oregon 97201, USA

*To whom correspondence should be addressed. Phone: (503) 494-7578, FAX: 494-6862

ABSTRACT

The vast majority of mammalian genes are interrupted by non-coding segments of DNA termed introns. Introns are spliced out of RNA transcripts as lariat structures, and then are typically debranched and rapidly degraded. Here, we describe an unusual spliced intron from the constant region of the T cell receptor–β (TCR-ß) locus that is relatively stable in mammalian cells. This intron, $IVS1_{C\beta1}$, accumulates as a set of lariat RNA structures with different length tails in the nucleus of T cells. The accumulation of this spliced intron is developmentally regulated during murine thymocyte ontogeny. The property of stability appears to be evolutionarily conserved since the human version of this intron also accumulates in T cells. The stability is selective since other spliced TCR–β introns do not detectably accumulate in T cells. The unusual stability of this intron does not depend on T cell specific factors since non-T cells transfected with TCR–β gene constructs also accumulate spliced $IVS1_{C\beta1}$. The discovery of a mammalian intron that accumulates as a lariat *in vivo* provides an opportunity to elucidate mechanisms that regulate intron debranching, stability, and nuclear localization.

INTRODUCTION

RNA splicing is a process whereby exons are joined and intervening sequences (introns) are removed in the form of lariat structures (1). Following excision, intron lariats are typically debranched and rapidly degraded (1). Thus, spliced introns are generally not sufficiently stable to be detected *in vivo*. However, yeast intron lariats have been observed as transient species that exist either as RNA processing intermediates generated after 5' cleavage (2) or after complete excision from precursor transcripts (3,4). For example, a spliced intron derived from a yeast nuclear actin transcript can be detected as various forms along a degradative pathway, including a lariat form that possesses a tail 3' of the branchpoint, a tail*less* lariat form, and a debranched linear molecule (3). The intron excised from the self-splicing yeast mitochondrial *oxi3* transcript exists transiently as either a tailed or a tail*less* lariat structure (4).

The instability of yeast introns appears to be a consequence of the debranching mechanism. When the conserved branchpoint sequence of a yeast intron was mutated so that the intron was only inefficiently debranched, the intron accumulated as a stable lariat structure (5). Furthermore, mutant yeast strains that lack debranching activity accumulate high levels of lariat introns (6). Presumably, introns that are maintained in a lariat conformation are relatively stable due to the low levels of endonucleases in the nuclear

environment. However, once introns are debranched these linear molecules become susceptible to exonuclease attack.

Spliced introns have also been detected in higher eukaryotic cells. A spliced SV40 intron accumulates as a lariat structure in the nucleus of injected *Xenopus laevis* oocytes (7). Herpes simplex virus (HSV type I) generates a latency associated transcript (LAT) that has recently been defined as a stable intron (8). The basis for the stability of LAT is not known. Furthermore, it has not been determined if LAT is a linear or a lariat molecule. A portion of this stable intron is complementary with an immediate early gene involved in trans-activation, implicating LAT as a possible regulator of viral latency. Evidence that LAT inhibits viral trans-activation has been obtained in transient transfection assays (8). In contrast to these examples of stable viral introns, there is little evidence that introns derived from cellular transcripts accumulate in higher eukaryotic cells. The spliced introns that have been observed are derived from highly transcribed genes, perhaps because only under this circumstance can their transient existence be detected. For example, ß-globin introns have been observed in fetal liver nuclei *in vivo* as lariat and linear molecules (9). The J-C intron from the immunoglobulin-κ (Ig-κ) locus has been detected as a discrete species in the nuclei of B cells (10). The conformation of the spliced Ig-κ J-C intron is not known. The heat shock gene hsc70 possesses three copies of the U14 snRNA gene within its intron sequences (11). U14 snRNA may be a "stable intron" generated by RNA splicing, or alternatively, U14 snRNA may be derived from a transcriptional promoter located within the hsc70 intron. An acetylcholine receptor (AChR) intron has been localized to the nuclear membrane of myotubes by *in situ* hybridization (12). It has not been determined if this AChR intron is a transient

intermediate, or instead selectively accumulates in the nucleus of these cells. In the present communication, we provide evidence for a mammalian intron that selectively accumulates in the nucleus of cells. It exists exclusively as a set of lariat structures that possess different length tails. This characteristic may have relevance to the stability and possible function of this unique intron.

MATERIALS AND METHODS

Cell culture and transfection

All cells were cultured as described (13). Transfection of DNA into HeLa and rat1 cells was performed by calcium phosphate precipitation (14). HeLa–β and rat1–β cells were generated by stable transfection of the following constructs: a 12kb HindIII $V_{\beta 3}C\beta 1$ genomic fragment (5C.C7) that possesses a functional V_β promoter was inserted into the pcDNA-neo vector (Invitrogen Corp.) and transfected into HeLa cells; a 1.0kb StuI Cβ1 fragment (15), that includes IVS1, was inserted in pcDNA-neo so that it is under the control of the CMV immediate early promoter, and transfected into rat-1 cells.

RNA preparation and northern blots

Total cellular RNA was prepared by lysis in guanidinium isothiocyanate, followed by ultracentrifugation over a 5.7M CsCl cushion, as described (ref. 16; protocol 1). Cytoplasmic and nuclear RNA was prepared as described (ref. 16; protocols 6 & 7). Briefly, the cells were lysed in a tris-saline buffer containing 0.5% Nonidet P-40, 0.25% sodium deoxycholate and 50μg/ml dextran sulphate, followed by immediate centrifugation to generate a nuclear pellet and a cytoplasmic supernatant - these two fractions were completely denatured in guanidinium isothiocyanate buffer and the RNA was prepared as described for the total cellular RNA. Poly(A)$^+$ RNA was prepared directly from cell lysates as described (ref. 16; protocol 9). The RNA was

electrophoresed in 1-1.5% agarose gels in the presence of formaldehyde, and capillary blotted onto Nytran membranes (14). RNA was also electrophoresed in denaturing polyacrylamide gels containing 7M Urea and 1X TBE (14). The RNA from polyacrylamide gels was electroblotted onto Nytran membranes in 10mM Tris (pH 7.8), 5mM sodium acetate, 0.5mM EDTA overnight at 50mA, followed by 1 h at 200mA. All the blots were stained with methylene blue (17) to demonstrate equivalent loading of RNA and to mark the migration of 18S rRNA, 28S rRNA, and RNA molecular weight ladders (Bethesda Research Laboratories, Bethesda, MD). The blots were hybridized with random oligomer primed ^{32}P-labeled cDNA inserts in the presence of 10% dextran sulphate and 50% formamide for 12-18 h at 42^{o}C, followed by washing with 0.1X SSPE / 0.1% SDS at 50-60^{o}C (14). Blots were stripped for sequential hybridization by placing them in boiling 0.1X SSPE / 0.1% SDS, and then gently agitating them while they cooled to room temperature.

RNA manipulation

The 0.5kb RNA fraction was prepared from gel slices derived from 1% agarose gels containing formaldehyde (14) by the method originally designed for DNA purification (18), except that the gel slices were melted in gel running buffer instead of TE, and extracted with phenol/chloroform prior to isopropanol precipitation. RNase H mapping was performed as follows: RNA was first incubated with 5μM oligonucleotide in 10mM Tris (pH 7.6), 1mM EDTA in a total volume of 10μl for 30 minutes at 45^{o}C. A RNase H cocktail was then added (15μl) that consisted of 2.5μl 10X RNase H buffer [0.2M Hepes (pH 8.0), 0.5M KCl, 0.1M $MgCl_2$], 2.5μl 10mM DTT, 1 unit of RNase H,

and H_2O. Following an incubation of 30 minutes at 37°C in this cocktail, the RNA was extracted and ethanol precipitated.

DNA probes and DNA sequence analysis

The murine $IVS1_{C\beta1}$ 0.5kb DNA fragment used as a probe for blot analysis was prepared by the polymerase chain reaction (PCR) with a murine Cβ1 genomic template, an oligonucleotide that corresponds to nucleotides 17-33, and oligonucleotide C (see Fig. 2). 5' and 3' specific $IVS1_{C\beta1}$ probes were prepared by cleaving the full length fragment with HpaII. The Cβ1 exon probe is a 0.3kb EcoR1/HindIII fragment from the 3' untranslated region of murine Cβ1 genomic DNA (15). The $C_{\beta2}$ exon probe is a 0.2kb NsiI/HpaI fragment from the 3' untranslated region of murine $C_{\beta2}$ genomic DNA (15,19). The human $IVS1_{C\beta1}$ fragment was prepared by PCR amplification from a human Cβ1 genomic template (20,21) with an oligonucleotide that corresponds to sequences starting 22 nuc from the exon1/IVS1 border (5'-GCCTGGAGGAGATTAG-3') and another oligonucleotide complementary to the 3' terminus of IVS1 (5'-CTGAAAGAAAGCAGGG-3'). This 0.4kb fragment was then cleaved with PvuII to generate 5' and 3' specific probes.

The nucleotide sequence of murine $IVS1_{C\beta1}$ was obtained from a 5C.C7 genomic clone (B10.A mouse strain; ref. 22) by dideoxy-sequencing methods with Sequenase reagents (U.S. Biochemical Corp., Cleveland, OH).

RESULTS AND DISCUSSION

Identification of a spliced TCR–β intron that accumulates in the nucleus of T cells

The murine genome contains two TCR–β constant genes (Cβ1 and $C_{\beta 2}$) which are similar in genomic structure and encode similar proteins (23). Each C_β gene contains three introns: IVS1, IVS2 and IVS3 (23). These introns are present in TCR–β pre-mRNAs that accumulate in the nucleus of T cells (13; Qian *et al.*, submitted). During the course of characterizing these splicing intermediates in the murine T-lymphoma cell clone, SL12.4, we noted that in addition to large pre-mRNAs, that a small 0.5kb RNA species hybridized with an $IVS1_{C\beta 1}$ probe on Northern blots (Fig. 1A). The size of this small RNA transcript was identical to the size of $IVS1_{C\beta 1}$ alone. Both 5' and 3' specific $IVS1_{C\beta 1}$ probes (which do not contain sequences in common) hybridized with the 0.5kb RNA species. This transcript was enriched in poly(A)$^+$ RNA (Fig. 1A), as expected, since two poly(A) tracts are present in the 5' portion of the intron (Fig. 2). Taken together, these results suggests that $IVS1_{C\beta 1}$ accumulates as a free intron following excision from TCR–β pre-mRNA.

The 0.5kb $IVS1_{C\beta 1}$ transcript was present in the nucleus of SL12.4 cells; little or none accumulated in the cytoplasm (Fig. 1A). Large $IVS1_{C\beta 1}$-containing pre-mRNAs were also confined to the nuclear compartment. In contrast, $JC_{\beta 2}$ (1.0kb) transcripts, which are fully spliced in SL12.4 cells (13), were present in both the nuclear and cytoplasmic compartments, as expected (Fig. 1A).

It is possible that the accumulation of free $IVS1_{C\beta1}$ RNA in SL12.4 T-lymphoma cells is an oddity of this cell line, rather than a general property of this intron. We tested normal thymocytes and found that they also accumulated 0.5kb $IVS1_{C\beta1}$. Fig. 1B shows expression of $IVS1_{C\beta1}$-containing transcripts during fetal thymocyte ontogeny. Upper panel displays different autoradiographic exposures for each developmental stage to allow visualization of the 0.5kb $IVS1_{C\beta1}$ transcript at each stage, whilst the second panel displays a single autoradiographic exposure to show relative levels of expression at different stages of thymic development. The results indicate that the spliced $IVS1_{C\beta1}$ intron exhibits a pattern of expression during fetal ontogeny that is different than other TCR–β transcripts. Pre-mRNAs (1.5-2.5kb and 5-7kb transcripts migrating similar to 18S and 28S rRNA transcripts, respectively) and mature mRNAs (1.0 and 1.3kb) increased in levels 15-20 fold between day 16 of fetal ontogeny and the adult stage, whilst 0.5kb $IVS1_{C\beta1}$ transcripts increased >50 fold over the same time interval (Fig. 1B). This difference suggests that the level of the free intron which accumulates is not entirely dependent on the rate of transcription, but may also reflect alterations in the rate of RNA splicing or RNA degradation.

We tested other murine TCR–β introns to determine whether they also accumulate in T cells. Northern blot analysis indicated that a probe to the first intron of the $C_{\beta2}$ gene ($IVS1_{C\beta2}$) also hybridized with an abundant 0.5kb transcript present in SL12.4 nuclear RNA (data not shown). In contrast, murine probes specific for $IVS2_{C\beta1}$, $IVS2_{C\beta2}$, $IVS3_{C\beta1}$, $IVS3_{C\beta2}$ and $IVS_{JC\beta1}$ did not detect transcripts of a size consistent with free introns in either nuclear SL12.4 RNA or total adult thymus RNA. These intron probes

did hybridize with TCR–β pre-mRNAs, showing their efficacy as hybridization probes (data not shown). Thus, IVS1 from both the Cβ1 and $C_{\beta 2}$ loci display a unique stability that is not reflected in other TCR–β introns. If stability is an important characteristic of IVS1, one may expect this feature to be conserved in animal species besides mice. Hybridization analysis of total cellular RNA from human Jurkat T-leukaemia cells showed the presence of an $IVS1_{C\beta 1}$ transcript (Fig. 1C) of the size predicted for the free intron (0.4kb) based on sequence analysis (20,21). This 0.4kb transcript was present in the nucleus of Jurkat cells (data not shown). Since human $IVS1_{C\beta 1}$ does not contain internal polyadenylate tracts (20,21), it was not detectable in poly(A)$^+$ RNA, even when the autoradiogram was sufficiently exposed to observe the larger $IVS1_{C\beta 1}$-containing pre-mRNAs (Fig. 1C).

IVS1 exists in the form of multiple lariat molecules

Stable introns accumulate in the form of lariats in yeast mutant strains that lack intron debranching activity (6). Hence, we considered the possibility that $IVS1_{C\beta 1}$ may remain in a lariat conformation, permitting it to be resistant to exonuclease attack. Lariats are known to migrate slower than linear molecules of equivalent size in acrylamide gels (2-4,6,7,9). To test whether $IVS1_{C\beta 1}$ is a lariat molecule, total cellular RNA from adult thymus was electrophoresed in a denaturing polyacrylamide gel, electroblotted, and hybridized with the $IVS1_{C\beta 1}$ probe. This probe failed to detect any transcripts migrating at 0.5kb (Fig. 3A, lane 2), indicating that the free $IVS1_{C\beta 1}$ intron is not a linear molecule. Instead, the probe hybridized with three discrete transcripts that migrated in a 5% polyacrylamide gel to a linear size equivalent of 0.9-1.2kb (Fig. 3A, lane 2), well above the linear $IVS1_{C\beta 1}$ DNA

standard that hybridized with the probe (Fig. 3A, lane 1). The thymic transcripts larger than 1.3kb detected by the $IVS1_{C\beta1}$ probe (Fig. 3A, lane 2) are $IVS1_{C\beta1}$-containing pre-mRNAs, based on their ability to hybridize with the Cβ1 exon probe (data not shown).

Spliced IVS1 from the $C_{\beta2}$ gene ($IVS1_{C\beta2}$) was also a non-linear molecule since it migrated as a single dominant transcript to a linear size equivalent of over 1kb (Fig. 3A, lane 4), even though its actual length is 0.5kb (19). The $IVS1_{C\beta2}$ probe did not cross-hybridize with $IVS1_{C\beta1}$ sequences, as judged by the distinct pattern of RNA transcripts which hybridize to the probe, and because this probe failed to detectably hybridize with $IVS1_{C\beta1}$ DNA (Fig. 3A, lane 5). Thus, neither $IVS1_{C\beta1}$ nor $IVS1_{C\beta2}$ transcripts appear to exist as linear molecules in the thymus.

We also tested whether $IVS1_{C\beta1}$ is a non-linear molecule in the SL12.4 T cell line. To conclusively demonstrate that the free 0.5kb intron rather than the larger intron-containing-pre-mRNAs is responsible for the hybridization signal, the 0.5kb fraction of SL12.4 poly $(A)^+$ RNA was purified in an agarose gel, and then run on polyacrylamide gels for blot analysis. On a 5% polyacrylamide gel, purified free $IVS1_{C\beta1}$ migrated as three discrete species (Fig. 3B, left panel) to positions equivalent to that observed for the thymus RNA sample (Fig. 3A, lane 2). The 0.5kb $IVS1_{C\beta1}$ transcripts migrated even slower in a 7% polyacrylamide gel (Fig. 3B, right panel), as expected for non-linear lariat molecules (9). We conclude that free $IVS1_{C\beta1}$ is not a linear molecule, but instead is likely to be a set of lariat molecules.

We further characterized the multiple species of spliced $IVS1_{C\beta1}$ molecules by incubation with oligonucleotides (oligos) complementary to different

regions of this intron (see Figs. 2 & 3C), followed by RNase H digestion, and electrophoretic analysis. The RNA used for the analysis was free 0.5kb $IVS1_{C\beta1}$ RNA purified in an agarose gel (Fig. 3C, lane labeled "none"; note that the two major $IVS1_{C\beta1}$ transcripts appear as a single band in the 6% polyacrylamide gel shown). Oligo D, an oligonucleotide complementary with the 3' terminus of $IVS1_{C\beta1}$, was used to determine if some of the putative lariat molecules still possess a 3' tail. Oligo D treatment increased the migration of the two largest $IVS1_{C\beta1}$ transcripts. This result indicates that both of these larger transcripts contain a 3' tail, and that they may differ in migration due to different tail lengths. Incubation with an oligonucleotide complementary with the 5' terminus of $IVS1_{C\beta1}$, oligo A, generated 0.5kb transcripts (Fig. 3C). This is consistent with the conversion of the lariat molecules to linear molecules. We presume that the inefficient cleavage mediated by oligo A is due to steric hindrance from the branchpoint region of the lariat. Incubation with both oligos A and D generated a single 0.5kb linear transcript with slightly lower migration than that generated by oligo A treatment alone, as expected if cleavage occurred at both ends of the intron. Oligo B released two intermediate size transcripts; this is consistent with the generation of two Y-shaped molecules (9) from the two major transcripts that possess different length tails. The third minor band of 0.5kb resulting from oligo B treatment is likely to be generated from the low abundance $IVS1_{C\beta1}$ lariat molecule. The migration of this smaller cleavage product implies that its parental lariat molecule has little or no 3' tail.

We also used RNase H analysis to investigate the approximate position of the branchpoint in this intron. Intron branchpoints typically occur at an

adenylate residue just upstream of the polypyrimidine tract present at the 3' termini of introns (1). Treatment with oligo C, which is complementary to such a region in $IVS1_{C\beta1}$, generated a 0.5kb linear molecule, implying that this region does indeed contain a branchpoint. In order to precisely determine the branchpoint sequence(s), we have attempted primer extension experiments using primer D and the gel purified $IVS1_{C\beta1}$ lariat. These experiments have not yielded specific sized products, perhaps because of the close proximity of the 3' end of the intron to the putative branchpoint.

Taken together, the results indicate that $IVS1_{C\beta1}$ exists as three discrete lariat molecules that possess different length tails. The heterogeneity of tail length may be due to exonuclease cleavage of the tail *in vivo* to preferred sites, or alternatively, the use of more than one branchpoint site. A tail has also been detected on a ß-globin intron lariat *in vivo*, but this tailed lariat may simply be a short-lived intermediate since the debranched linear form of the intron is also observed (9). In yeast, tailed intron lariats have been detected in wild type cells, but in mutants where debranching is inhibited, only tail*less* lariats accumulate, presumably due to exonuclease degradation (5,6). Since it appears that $IVS1_{C\beta1}$ may be blocked in debranching, it is curious that it exists primarily in a tailed lariat form. Perhaps, $IVS1_{C\beta1}$ is sequestered in a niche in the nuclear environment that protects its tail from degradation.

The accumulation of $IVS1_{C\beta1}$ in transfected non-lymphoid cells

We sought to determine if accumulation of $IVS1_{C\beta1}$ lariat structures is a peculiarity of T cells, or if this intron is also stable in non-lymphoid cells. HeLa cells (of epithelial origin) stably transfected with a construct containing $IVS1_{C\beta1}$ (including donor and acceptor splice junctions) accumulated free

$IVS1_{C\beta1}$ (0.5kb) which co-migrated with the endogenous $IVS1_{C\beta1}$ transcript from SL12.4 T-lymphoma cells on agarose gels (Fig. 3D). As with T cells, transfected HeLa cells accumulated the free intron in the nuclear compartment (Fig. 3D). Similarly, transfected rat1 cells (of mesenchymal origin) accumulated the 0.5kb $IVS1_{C\beta1}$ transcript (Fig. 3D). The hybridization of the $IVS1_{C\beta1}$ probe to the 0.5kb transcript in transfected cells was specific since RNA from control cells (that were not transfected) failed to give rise to a detectable hybridization signal (Fig. 3D and data not shown). To determine if free $IVS1_{C\beta1}$ exists in lariat form in transfected HeLa cells, the free intron (0.5kb fraction) was purified on an agarose gel, followed by blot analysis on a polyacrylamide gel. Transfected HeLa cells accumulated three lariat species with identical migration and relative abundance as the endogenous transcripts in the SL12.4 T cell clone (Fig. 3E).

Thus, $IVS1_{C\beta1}$ can accumulate as nuclear lariat molecules in cell types derived from multiple lineages. This implies that the stability of $IVS1_{C\beta1}$ may be an intrinsic property that is independent of cell type. $IVS1_{C\beta1}$ may adopt a secondary or tertiary structure that is inherently resistant to debranching. Alternatively, the stability of $IVS1_{C\beta1}$ may be mediated by a general factor(s) that is present in many different cell lineages. Since stable $IVS1_{C\beta1}$ exists in lariat form, one could envisage that a stabilizing protein might act by preventing debranching. One hypothesis is that $IVS1_{C\beta1}$ remains associated with splicesomes following its excision from pre-mRNAs. However, $IVS1_{C\beta1}$ does not have an intrinsic affinity for snRNPs since antibodies against these molecules failed to co-immunoprecipitate free $IVS1_{C\beta1}$ (M.W.; unpublished observations).

The cis-acting sequences that are important in dictating the stability of $IVS1_{C\beta1}$ are not known. Examination of the $IVS1_{C\beta1}$ sequence (Fig. 2) and the adjacent exon sequences (15) indicates that it possesses canonical RNA splicing motifs. The 5' splice junction displays perfect complementarity with the portion of U1 snRNA known to base pair with this region (24). Just upstream of the "AG" at the 3' splice junction, it has a long tract of polypyrimidine residues (a homogeneous stretch of 35 polypyrimidines), as is typical of mammalian introns (24). Two potential branchpoint adenylate residues are found immediately upstream of the polypyrimidine stretch; the sequences surrounding these residues both display a 5/7 match with the mammalian branchpoint consensus sequence: YNYURAY (25). Since the debranching of $IVS1_{C\beta1}$ appears to be inefficient, it may be that RNA sequences nearby the branchpoint mediate this effect, either because the sequences generate a conformation that is inherently resistant to debranching, or because the sequences bind to a regulatory protein that inhibits the debranching reaction. Sequence comparison of the putative branchpoint region of murine $IVS1_{C\beta1}$ with the corresponding region of the two other stable introns that we identified (murine $IVS1_{C\beta2}$ and human $IVS1_{C\beta1}$) reveals the existence of a precisely conserved 6 nucleotide motif, AUUUUC (Fig. 2), that is not present in murine TCR–β introns that do not display stability (15,19). This sequence may be important in preventing debranching, as well as promoting stability and nuclear retention. In addition, this sequence motif could be responsible for the inefficient splicing of $IVS1_{C\beta1}$ from TCR–β pre-mRNAs. However, it should be noted that $IVS2_{C\beta1}$ and $IVS3_{C\beta1}$ lack this sequence motif but are also inefficiently spliced in T cells (Qian *et al.*, submitted).

Concluding remarks

A few other mammalian introns have been observed following their excision from precursor transcripts, including ß-globin (9) and immunoglobulin-κ introns (10). It is not clear if these spliced introns are selectively stable or, instead, if they are transient species which were detected simply because they are derived from highly abundant transcripts. In this report, we demonstrate that the $IVS1_{C\beta1}$ and $IVS1_{C\beta2}$ lariats are selectively stable; that other C_β introns do not detectably accumulate. The evidence that spliced $IVS1_{C\beta1}$ molecules exist exclusively as a set of lariat molecules *in vivo* is based on RNase H mapping analysis. Independent evidence that spliced $IVS1_{C\beta1}$ transcripts in SL12.4 cells are lariats comes from experiments with HeLa debranching extracts (nuclear or S100) where we have reproducibly shown that $IVS1_{C\beta1}$ lariat transcripts are converted to a linear sized transcripts (M.W.; unpublished observations).

The observation that the property of stability has been retained in the human and mouse versions of this intron suggests that $IVS1_{C\beta1}$ may perform a function. Since this intron is confined to the nucleus, an attractive possibility is that $IVS1_{C\beta1}$ participates in the regulation of splicing. Portions of murine $IVS1_{C\beta1}$ are self-complementary (based on computer analysis; L.Q., unpublished observations), suggesting that the free intron may hybridize with $IVS1_{C\beta1}$-containing pre-mRNAs *in vivo* and thereby play an autoregulatory role. Alternatively, the free intron may compete with pre-RNAs for binding to proteins that specifically regulate TCR–β RNA splicing. It is possible that an

unstable protein(s) regulates the splicing of TCR–β transcripts since protein synthesis inhibitors appear to dramatically augment the splicing of Cβ1 pre-mRNAs in SL12.4 cells (13; Qian *et al.*, submitted). The stable intron and cell lines described in this report should provide a system suitable for an analysis of factors that are important in the regulation of RNA splicing, intron debranching, and RNA stability.

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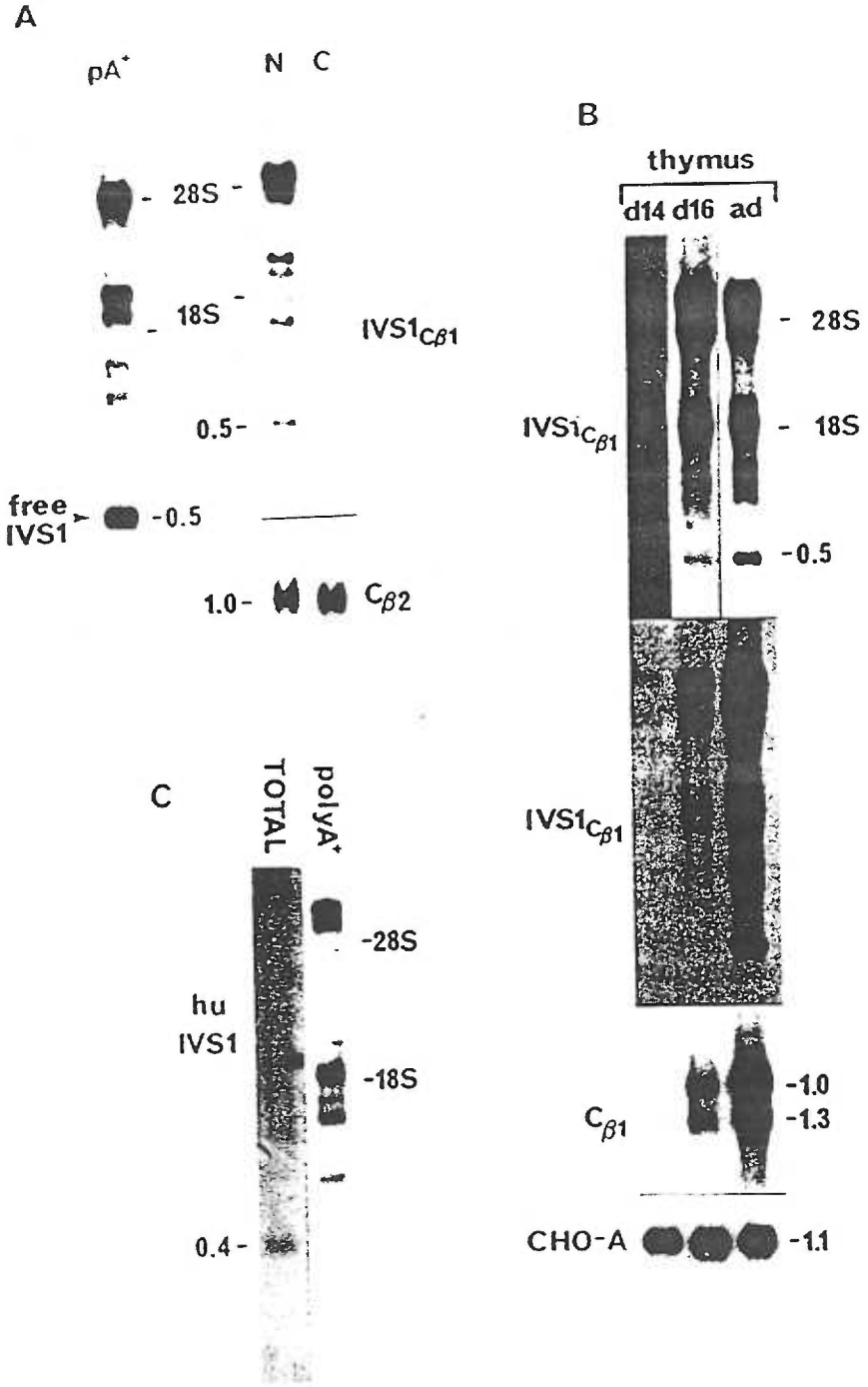
Figure 1. Northern blot analysis of $IVS1_{C\beta1}$ in murine and human T cells. All panels show RNA electrophoresed in agarose gels, blotted, and hybridized with the probes indicated. **A.** Left panel: blot containing 2μg SL12.4 poly(A)$^+$ (pA$^+$) RNA hybridized with the murine $IVS1_{C\beta1}$ probe; right panel: blot containing 10μg nuclear (N) and cytoplasmic (C) RNA from SL12.4 cells sequentially hybridized with murine $C_{\beta2}$ exon and $IVS1_{C\beta1}$ probes. The blot shown provides results with a full length $IVS1_{C\beta1}$ probe; the same hybridization pattern was observed with either 5' or 3' specific probes (see materials and methods). **B.** Blot containing total cellular RNA (5μg) from thymus derived from fetal mice (days of ontogeny are shown) or a 6 week old adult (ad). The upper panel shows different autoradiographic exposures of the individual lanes. The same blot was sequentially hybridized with murine $IVS1_{C\beta1}$, Cβ1 exon, and CHO-A cDNA probes. The CHO-A probe detects a ubiquitously expressed housekeeping gene (13,26) which serves as a control for RNA loading. **C.** Blot of total cellular (10μg) or poly(A)$^+$ (2μg) RNA from human Jurkat cells hybridized with a human $IVS1_{C\beta1}$ probe. The blot shown provides results with the 3' probe; the 5' probe also hybridized with the 0.4kb transcript in Jurkat cells (see materials and methods).

Figure. 1.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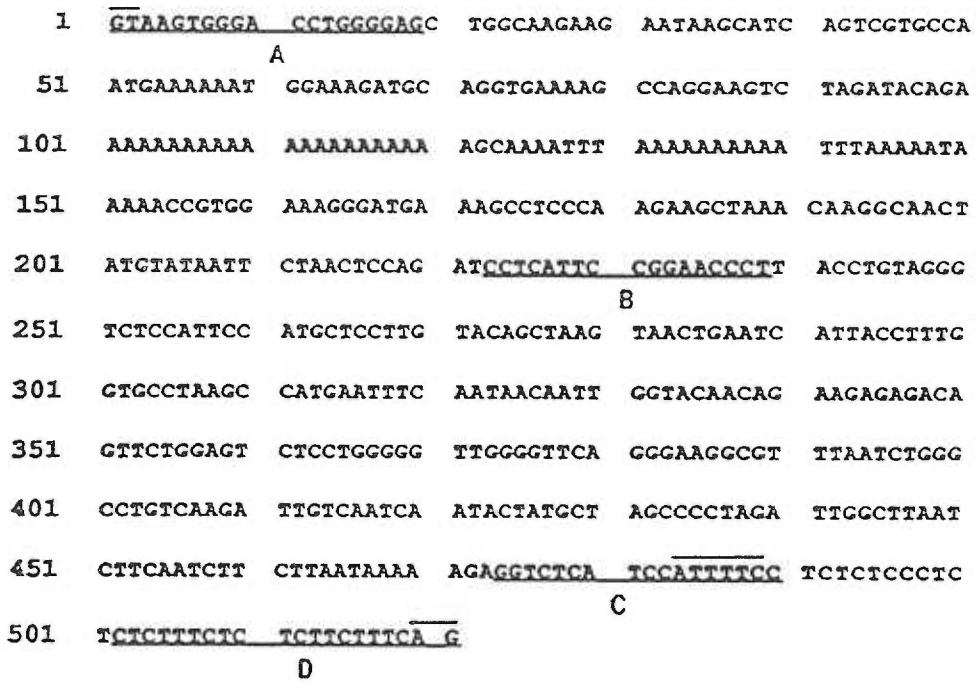
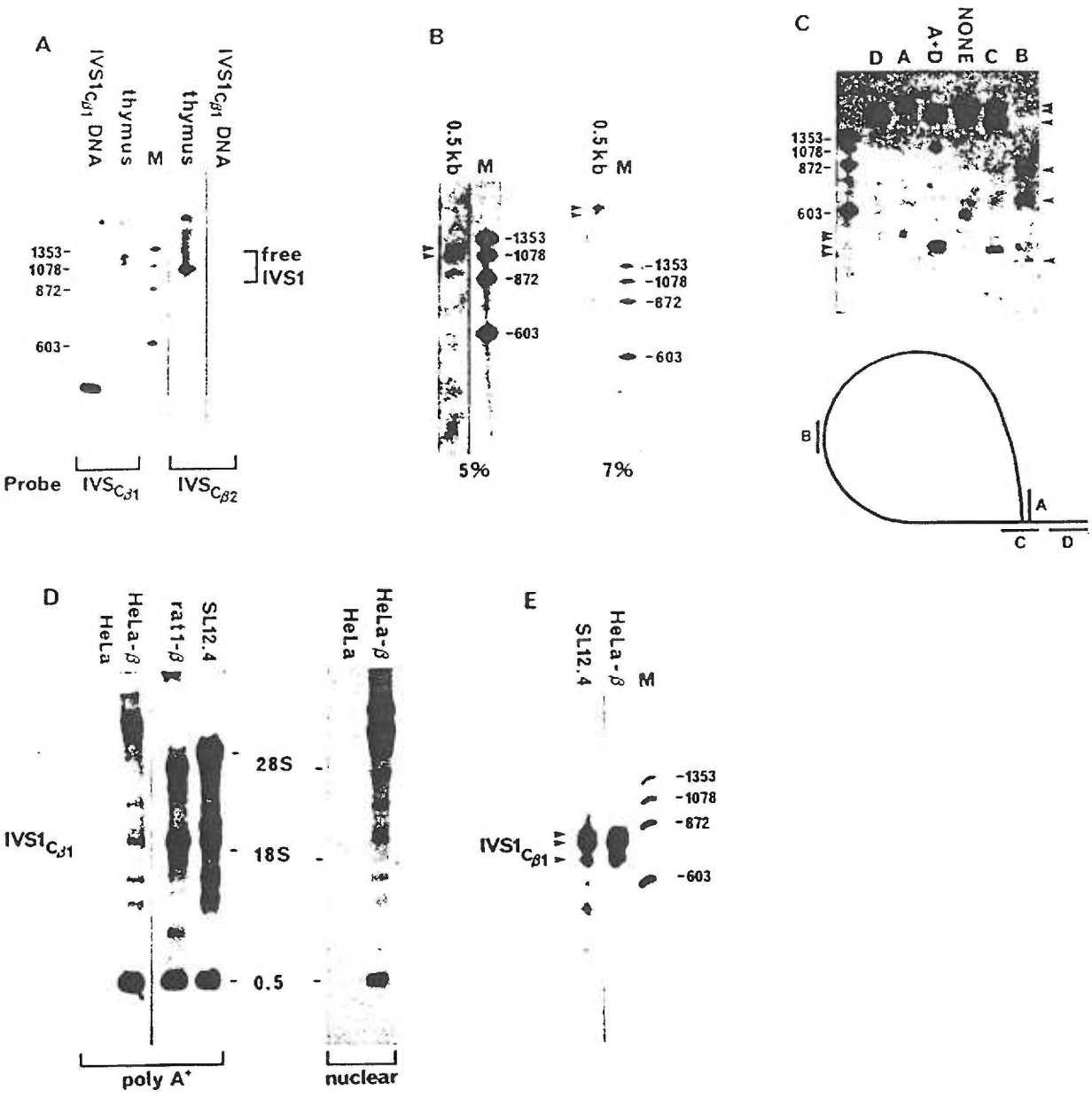


Figure 2. Nucleotide sequence of murine $IVS1_{C\beta1}$. Underlined sequences indicate the position of complementary oligonucleotides used for RNase H analysis and PCR amplification. Lines drawn above the sequences indicate the conserved dinucleotides at the termini of the intron, and a conserved motif also present in mouse $IVS1_{C\beta2}$ (15,19) and human $IVS1_{C\beta1}$ (20,21). The partial sequence analysis of murine $IVS1_{C\beta1}$ which was previously published (15) is in agreement with the full sequence reported here except that these authors show a 'T' at position 12.

Figure 3. Northern blot analysis of $IVS1_{C\beta1}$ and $IVS1_{C\beta2}$ lariat structures which accumulate *in vivo*. The panels show RNA electrophoresed in either polyacrylamide or agarose gels, blotted, and hybridized with the probes indicated. **A.** Electroblot of a 5% polyacrylamide gel loaded with adult mouse thymus RNA (10μg), a 0.5kb $IVS1_{C\beta1}$ DNA fragment generated by PCR amplification (10pg), and an end-labeled HaeIII digested OX174 DNA marker (M). RNA molecular weight ladders displayed a similar migration pattern as the DNA marker (not shown). The blot was sequentially hybridized with the murine probes $IVS1_{C\beta1}$ and $IVS1_{C\beta2}$, as shown. **B.** Polyacrylamide gels (% polyacrylamide is indicated) were loaded with the 0.5kb fraction of SL12.4 $polyA^+$ RNA. The gels were electroblotted and the blots were hybridized with the murine $IVS1_{C\beta1}$ probe. **C.** The 0.5kb fraction of SL12.4 $poly(A)^+$ RNA was incubated with the oligonucleotides shown (see Fig. 2), followed by treatment with RNase H. The treated samples were then electrophoresed in a 6% polyacrylamide gel, the gel was electroblotted, and the blot hybridized with the $IVS1_{C\beta1}$ probe. **D.** Blots of 1.5% agarose gels loaded with $poly(A)^+$ RNA (2μg) or nuclear RNA (10μg). **E.** Electroblot of a 4.5% polyacrylamide gel loaded with the 0.5kb RNA fraction of $poly(A)^+$ RNA from the cell lines indicated.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thymus
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1078-
872-
603-
free IVS1
Probe
IVSCβ1
IVSCβ2
B
0.5kb
M
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-1078
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1078-
872-
603-
B
A
C
D
D
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HeLa-β
rat1-β
SL12.4
HeLa
HeLa-β
IVS1Cβ1
28S
18S
0.5
poly A+
nuclear
E
SL12.4
HeLa-β
M
IVS1Cβ1
-1353
-1078
-872
-603

Figure.3.

Chapter 3

Characterization and Potential Function of a Stable Intron

Lian Qian and Miles F. Wilkinson*

Microbiology & Immunology Department, Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University, L220, 3181 S.W. Sam Jackson Park Rd., Portland, Oregon 97201, USA

*To whom correspondence should be addressed. Phone: (503) 494-7578, FAX: 494-6862

ABSTRACT

We have identified an unusual spliced intron from the T cell receptor β gene, IVS1$_{C\beta1}$, that accumulates in the nucleus following its excision from pre-mRNA in mammalian cells. To further characterize this stable intron and study its potential function, we have generated a series of TCR-β constructs and analyzed their expression in stably transfected Hela cells. Northern analysis indicates that the stability of IVS1$_{C\beta1}$ does not require intact secondary structure involving the entire molecule, nor does it require sequences present in the 5' portion of the molecule. Instead, sequences present in the 3' terminal region of IVS1$_{C\beta1}$ (minimally defined as 122 nucleotides) engender the stable phenotype. Ribonuclease protection experiments demonstrated that spliced IVS1$_{C\beta1}$ accumulates as a set of lariat molecules with different length tails. The presence of the tails suggests that this stable intron may be sequestered in a protective nuclear microenvironment, perhaps in the spliceosomal apparatus. A functional role for this intron is suggested by a series of experiments demonstrating that the presence of IVS1$_{C\beta1}$ is correlated with a retention of fully spliced TCR–β transcripts in the nucleus.

INTRODUCTION

Introns are excised from pre-mRNAs in the form of lariat structures (Green et al., 1991). It is generally considered that intron lariats are debranched and rapidly degraded following excision from precursor transcripts since most spliced introns are not detectable in vivo (Keller et al., 1984). However, intron lariats have been observed as transient species that exist either as RNA processing intermediates generated after 5' cleavage (Rodriguez et al., 1984) or after complete excision from precursor transcripts (Domdey et al., 1984). For example, β-globin introns have been detected in fetal liver nuclei in vivo as lariat and linear molecules (Zeitlin et al., 1984). Such spliced introns are unlikely to be stable - instead they probably have been detected because they are derived from highly transcribed genes.

Some spliced introns may not exist as transient intermediates, but instead accumulate as stable introns. For example, a spliced SV40 intron accumulates as a lariat structure in the nucleus of injected *Xenopus laevis* oocytes. The function of this SV40 intron is unknown and its physiological relevance is uncertain because it is not stable in transfected mammalian cells (Michaeli et al., 1988). An intriguing example of a "stable intron" is U14 snRNA, a small RNA species involved in RNA processing. Copies of U14 snRNA are present in three of the introns of the cognate hsc70 heat shock gene (Liu et al., 1990). U14 snRNA can be generated by a processing reaction from such hsc70 introns (Leverette et al., 1992). Herpes simplex virus (HSV type I) generates a latency associated transcript (LAT) that may be a stable intron (Farrell et al., 1991) but

this has not been rigorously tested. For example, the precise termini of LAT RNA has not been determined, nor is it known if LAT is a linear or a lariat molecule.

We have identified an unusual intron that selectively accumulates in the nucleus of cells following its excision from pre-mRNA (Qian et al., 1992). This intron, IVS1$_{C\beta1}$, is derived from the constant region of the T cell receptor-β (TCR–β) gene. Its stability is selective since other spliced TCR–β introns do not detectably accumulate in T cells. The property of stability appears to be evolutionarily conserved since the human version of this intron also accumulates in T cells. IVS1$_{C\beta1}$ exists exclusively in the lariat conformation in the nuclei of T cells and transfected non-lymphoid cells. RNase H digestion studies indicated that some species of this intron lariat still possess tails, suggesting that this spliced intron resides in a protective nuclear microenviroment. In present study, we sought to further characterize this unusual spliced intron and analyze its potential functional role. Ribonuclease protection experiments confirmed that IVS1$_{C\beta1}$ molecules possess tails and allowed accurate determination of their length. A functional role for this intron was suggested by transfection studies which showed that pre-mRNAs that contains IVS1$_{C\beta1}$ give rise to mature transcripts that are retained in the nucleus. Thus, IVS1$_{C\beta1}$ may regulate the nuclear-to-cytoplasmic transport of mature mRNA.

MATERIALS AND METHODS

Enzymes and reagents

Restriction endonucleases were purchased from New England Biolabs, Inc. (Beverlt, Mass.), Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) and Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Taq DNA polymerase was purchased from Promega (Madison, Wisconsin). DNA polymerase and T4 DNA ligase was purchased from New England Biolabs, Inc. α-^{32}P dATP and α-^{32}P UTP were from Dupont, NEN Research Products (Boston, Mass.). Ribonuclease A, ribonuclease T1 and RNA molecular weight ladders were purchased from Bethesda Research Laboratories Inc.

Construction of Vectors

StuI-Cβ1 construct : A 1.0 kb Cβ1 StuI fragment containing IVS1$_{C\beta1}$ was subcloned into the PvuII site of pSP73 vector (Promega).

HaeIII-Cβ1 construct (Fig.5): A 0.46 kb HaeIII fragment that contains a portion of IVS1$_{C\beta1}$ was sub-cloned into the EcoRV site of the pSP72 vector (Promega).

IVS1$_{C\beta1}$ (nuc. 403-490) construct: a 474 bp IVS1$_{C\beta1}$ fragment was generated by the polymerase chain reaction (PCR, Qian et al., 1992) using the StuI-Cβ1 genomic construct as a template, and the following oligonucleotides: one corresponds to nuc.16-33 of IVS1$_{C\beta1}$ (GAGCTGGCAAGAAGAAT) and another is complementary with nuc. 473-490 of IVS1$_{C\beta1}$ (GGAAAATGGATGAGACC). A 87

bp IVS1$_{C\beta1}$ fragment was released from the PCR product with HaeIII, and subcloned into the EcoRV site of Bluescript (Stratagene)

IVS1$_{C\beta1}$ construct (Fig.1A): A 0.7 kb Cβ1 fragment was generated by the polymerase chain reaction (PCR, Qian et al., 1992) using the StuI-Cβ1 genomic construct as a template, and the following oligonucleotides: one corresponds to the T7 promoter (AATACGACTCACTATAGGGAG) and another is complementary with exon2$_{C\beta1}$ and also includes a BglII restriction site (GCGAGATCTGAGGTAATCCCACA). The 0.7 kb PCR product was digested with XbaI and BglII and substituted for the 3' Cβ1 sequences in the StuI-Cβ1 construct at the XbaI and BglII sites. The insert was then released by digestion with XhoI and BglII and subcloned into the SalI and XbaI site of the pHβ–APr1-neo vector such that it was fused with the 5' portion of the actin gene (Gunning et al., 1987): 78 bp of the 5' untranslated region and all of IVS1$_{actin}$ (832 bp) and a portion of the downstream exon (32 bp). The Cβ1 sequences in this construct include 195 bp of exon1$_{C\beta1}$, all of IVS1$_{C\beta1}$ and all of exon2$_{C\beta1}$.

Cβ1 construct (Fig. 8): A 1.95 kb Cβ1 KpnI/HindIII fragment was filled in with DNA polymerase and subcloned into the EcoRV site of Bluescript (Stratagene; La Jolla, CA). This insert was then released with SalI and BamHI, and sub-cloned into the SalI and BamHI site of the pHβ–APr1-neo vector (Gunning et al., 1987). The Cβ1 construct contains 230 bp of intron$_{JC\beta1}$ and all of Cβ1.

Cβ1–Gin construct (Fig. 2B). A 0.8 kb EcoR1 fragment of the 3' globin untranslated region (Lawn et al., 1980) was filled in with DNA polymerase and inserted into the XbaI site of the Cβ1 construct in a 3'-5' orientation.

Cβ1–2Gin construct (Fig. 2C). This construct was generated in the same manner as the Cβ1–Gin construct

IVS1$_{C\beta1}$Δ95-231 construct (Fig.3): A 1.4 kb Cβ1 HpaII fragment was subcloned into the ClaI site of the pSP72 vector (Promega). This insert was then released from pSP72 by digestion with XbaI and BglII and substituted for the 3' Cβ1 sequences in the Cβ1 construct (Fig.8) at the XbaI and BamHI sites. This construct contains is identical to the Cβ1 construct except that a 136 bp internal region of IVS1$_{C\beta1}$ was deleted.

IVS1$_{C\beta1}$ Δ95-401 construct (Fig.3): A 0.46 kb fragment was released from the HaeIII-Cβ1 construct by digestion with XbaI and BglII and substituted for the 3' Cβ1 sequences in the Cβ1 construct (Fig.8) at the XbaI and BamHI sites.

G-Cβ1 construct (Fig.4A): A 1.1kb β–globin HindIII/DraI fragment (Lawn et al., 1980) was sub-cloned into the EcoRV site of the Bluescript vector (Stratagene). This insert was then released with SalI and XbaI and substituted for the 5' Cβ1 sequences in the Cβ1 construct (Fig.8) at the SalI and XbaI sites.

Cβ1–G construct (Fig.4B): A 0.67 kb β–globin DraI fragment (Lawn et al., 1980) was sub-cloned into the EcoRV site of the Bluescript vector (Stratagene). This insert was removed by digestion with ClaI and BamHI and sub-cloned into the pSP73 vector (Promega). This insert was then released from pSP73 by digestion with XbaI and BglII and substituted for the 3' Cβ1 sequences in the Cβ1 (Fig.8) construct at the SalI and XbaI sites.

Globin construct (Fig.6): A 2.1 kb β–globin HindIII/BglII fragment (Lawn et al., 1980) was subcloned into the HindIII and BamHI sites of the pHβ–APr1-neo vector (Gunning et al., 1987).

ΔIVS1$C\beta1$ construct (Fig.7B): A 0.7 kb EcoR1 fragment of TCR-β cDNA (86T5, Hedrick et al., 1984) was sub-cloned into the EcoR1 site of the Bluescript vector (Stratagene). A 0.6 kb fragment was generated by the polymerase chain reaction (PCR) with the Bluescript -TCR-β cDNA template and 2 oligonucleotides: one oligonucleotide corresponds to the T7 promoter (AATACGACTCACTATAGGGAG) and the other is complementary to exon2$C\beta1$ and also includes a BglII restriction site (GCGAGATCTGAGGTAATCCCACA). A 0.1 kb TCR-β cDNA fragment was released from the PCR product with NcoI and BglII, and substituted for the 3' Cβ1 sequences in the StuI-Cβ1 construct at the NcoI and BglII sites. A 0.2kb insert was then released from pSP73 by digestion with XhoI and BglII and subcloned into the SalI and BglII site of the pHb-APr1-neo vector (Gunning et al., 1987). The ΔIVS1$C\beta1$ construct contains 195 bp of exon1$C\beta1$ and 18 bp of exon2$C\beta1$.

IVS3$C\beta1$ construct (Fig7C): A 0.44 kb HincII /EcoRI Cβ1 fragment that contains IVS3$C\beta1$ was subcloned into the pSP73 vector (Promega). The insert was then released from pSP73 by digestion with XhoI and BglII and subcloned into the SalI and XbaI sites of the pHβ–APr1-neo vector (Gunning et al., 1987). The IVS3Cβ1 construct contains 135 bp of exon3$C\beta1$, all of IVS3$C\beta1$ and 17 bp of exon4$C\beta1$.

RNA preparation and Northern blot analysis

All cells were cultured as described (Wilkinson and MacLeod., 1988). Transfection of DNA into HeLa cells was performed by calcium phosphate precipitation (Sambrook et al., 1989).

Total cellular RNA was prepared as described (Wilkinson, 1991, protocol 1). Cytoplasmic and nuclear RNA of Hela cells was prepared as described (Wilkinson, 1991, protocols 6 & 7) with some modifications. Hela cells were lysed for 4 min at 4^{o}C in a lysis buffer [final concentration: 0.6% Nonidet P-40, 0.15 M NaCl, 10 mM Tris (pH7.9), 1mM EDTA], followed by immediate centrifugation at 3,000 rpm/min for 30 seconds to generate a nuclear pellet and a cytoplasmic supernatant. The cytoplasmic supernatant was then centrifuged at 15,000 cpm/min for 30 seconds. The cytoplasmic supernatant was collected and phenol:choloform extracted in the presence of 1% SDS and additional salt (final NaCL concentration was 0.3 M), followed by ethanol precipitation. The nuclear pellet was resuspended in lysis buffer and incubated for 4 min at 4^{o}C followed by immediate centrifugation at 3,000 rpm/min for 30 seconds to obtain a more purified nuclear pellet. The supernatant was discarded and the nuclear pellet was incubated in lysis buffer for a second time, followed by centrifugation. The final nuclear pellet was then completely denatured in guanidinium isothiocyanate buffer followed by ultracentrifugation over a 5.7M CsCl cushion, as described (Wilkinson, 1991 protocol 6). Poly(A)$^{+}$ RNA was prepared directly from cell lysates as described (Wilkinson, 1991; protocol 9).

RNA was electrophoresed in 1-1.5% agarose gels in the presence of formaldehyde, and capillary blotted onto Nytran membranes (Sambrook et al., 1989). All the blots were stained with methylene blue (Wilkinson et al., 1990) to demonstrate equivalent loading of RNA and to mark the migration of 18S rRNA, 28S rRNA, and RNA molecular weight ladders. The blots were hybridized with random oligomer primed ^{32}P-labeled cDNA inserts in the presence of 10% dextran sulphate and 50% formamide for 12-18 h at 42^{o}C, followed by washing with 0.1X SSPE / 0.1% SDS at 50-60^{o}C. Blots were stripped for sequential

hybridization by placing them in boiling 0.1X SSPE / 0.1% SDS, followed by gentle agitation, while they cooled to room temperature.

Ribonuclease Protection Assays

Riboprobes (Figure 5) were prepared as follows:

Probe A was derived from the HaeIII-Cβ1 construct (122 bp of the 3' terminal region of $IVS1_{C\beta1}$, plus downstream exon and intron sequences). The plasmid was linearized with EcoR1 and transcribed with T7 RNA polymerase to yield a 0.4kb riboprobe.

Probe B was derived from the $IVS1_{C\beta1}$ (nuc. 403-490) construct (87 bp of a 3' portion of $IVS1_{C\beta1}$ and lacks that 3' terminal 32 nucleotides) The plasmid was linearized with XhoI and transcribed with T7 RNA polymerase to yield a 0.1kb riboprobe.

Riboprobe transcription was carried out as described (Kreig and Melton, 1987) with ^{32}P-UTP (800 ci/mmole). Nuclear RNA (10 µg) was denatured at 85°C for 10 min and hybridized for >12 hr at 45°C with $2x10^5$ cpm of RNA probe in 30 µl of 80% formamide, 40 mM PIPS (pH 6.4), 0.4 m NaCl, 1mM EDTA and then digested for 30 min at 37°C with RNAase A and T1 (final concentration of 40 µg/ul and 100U/ml, respectively) in 350 µl of 10 mM Tris (pH7.5), 5mM EDTA, and 0.3M NaCl. After proteinase K treatment and phenol-chloroform extraction, samples were ethanol precipitated and electrophoresed on 6% polyacrylamide, 8 M urea sequencing gels. Gels were dried and analyzed by autoradiography with X-omat™ AR film (Kodak) for 1 to 4 days at -70°C with a Cronex Lightning-Plus intensifying screen.

DNA probes

$IVS1_{C\beta1}$ probe: a 0.5kb DNA fragment was prepared by the polymerase chain reaction (PCR) with a murine Cβ1 genomic template as described (Qian et al., 1992).

Cβ1 exon probe: a 0.3kb EcoR1/HindIII fragment which contains the 3' untranslated region of murine Cβ1 genomic DNA (Qian et al., 1993).

TCR-β exon probe: a 0.6 kb EcoRI fragment of the 86T5 gene TCR-β cDNA clone that contains all Cβ1 exon sequences (Hedrick et al., 1984).

3' globin probe: a 0.67 kb globin DraI fragment which contains 0.35 kb of $IVS2_{globin}$ and 0.32 kb of downstream $exon3_{globin}$ plus 3' untranslated sequences (Lawn et al., 1980).

β–actin probe: a 0.9 kb XhoI/SalI fragment which contains 78 bp of 5' untranslated region, 832 bp of IVS1 and a small proportion of the downstream exon (32 bp) (Gunning et al., 1987).

RESULTS

Accumulation of unusual splicing intermediates in the nucleus of Hela cells tranfected with the IVS1$_{C\beta1}$ construct

In a previous study we identified an unusual intron from the constant region of the TCR–β gene, IVS1$_{C\beta1}$, which accumulates following excision from pre-mRNA (Qian et al., 1992). To further characterize this intron, we chose to study it in transfected Hela cells for the following reasons: First, Hela cells do not transcribe endogenous TCR-β genes, and therefore, the analysis of the expression of the transfected genes will not be obscured by endogenous products. Second, Hela cells are widely used for analysis of RNA splicing, thus allowing the splicing of TCR-β transcripts to be compared with the splicing of other transcripts. Finally, we have been shown that IVS1$_{C\beta1}$ is properly spliced and accumulates in the nucleus of stably transfected Hela cells. Furthermore, stably transfected Hela cells accumulated the same three lariat species of IVS1$_{C\beta1}$ in the same molar ratio as in T cells (Qian et al., 1992).

We first analyzed the expression of a simple IVS1$_{C\beta1}$ mini-gene construct in stably transfected Hela cells. This construct consists of a β-actin promoter, the first actin exon and intron, and a portion of the second actin exon fused to a mini Cβ1 gene followed by a SV40 polyadenylation signal (Fig. 1A). Nuclear and cytoplasmic RNA from stably transfected Hela cells were analyzed with different probes. Nuclear RNA contained spliced IVS1$_{C\beta1}$ (species VI) as expected (Fig. 1B). In addition, nuclear RNA from these cells contained an array of splicing intermediates of sizes predicted from the known lengths of the exons and introns

in the IVS1$_{C\beta1}$ construct. This was not unexpected since we previously showed that TCR-β splicing intermediates normally accumulate in T cells (Qian et al., 1993). Species I in transfected Hela cells is the unspliced precursor since it hybridized with IVS1$_{C\beta1}$, IVS1$_{actin}$ and exon$_{C\beta1}$ probes (Fig.1B, lanes 1, 2 and 3). Species II and III are splicing intermediates which have spliced out IVS1$_{C\beta1}$ and IVS1$_{actin}$, respectively (Fig. 1B). Species V is fully spliced mRNA; as expected it is the only species which is detected in the cytoplasm of the transfected cells (Fig.1B, lane 2; and Fig. 1C). Species IV and VII appear to be splicing intermediates that result from inefficient 3' cleavage of IVS1$_{C\beta1}$. Although we have not determined that species IV is a lariat molecule as depicted in Fig. 1B, we previous showed that the SL12.4 T cell clone accumulates IVS1$_{C\beta1}$-5' terminal transcripts that migrate in polyacrylamide gels as lariats (Qian et al., 1993).

We conclude that this mini-gene construct gives rise to correctly spliced TCR-β1 transcripts in Hela cells, including spliced IVS1$_{C\beta1}$, thus it is suitable for our study. The nuclear accumulation of splicing intermediates is a reflection of the inefficient splicing of IVS1$_{C\beta1}$ and its ability to inhibit the splicing of neighboring introns (Qian et al., manuscript in preparation).

The stability of IVS1$_{C\beta1}$ is not abolished by internal sequence insertions or deletions

It has been reported that the stability of the Herpes LAT transcript (reported to be a spliced intron) is dependent on precise secondary structure since its stability is abolished by an insertion of a 0.44 kb fragment (Block et al., 1990). We considered the possibility that the stability of IVS1$_{C\beta1}$ may also depend on

particular sequences or intact secondary structure. To determine this, we generated several mutant IVS1$_{C\beta1}$ constructs and transfected them into Hela cells. First, an EcoR1 fragment of the β-globin 3' untranslated region (0.8 kb) was inserted into an internal site of IVS1$_{C\beta1}$ (Fig. 2B, upper panel). We introduced a construct containing this form of IVS1$_{C\beta1}$ into cultured Hela cells, selected stably transfected cell clones and examined expression by Northern analysis. The addition of the insert did not abolish the stability of IVS1$_{C\beta1}$ since a RNA species of the appropriate size (1.3 kb) accumulated in the nucleus of transfected cells (Fig.2B, lower panel). This 1.3 kb species hybridized with IVS1$_{C\beta1}$ (Fig.2B, lower panel) and 3' globin probes (data not shown). The intron was appropriately spliced since mature Cβ1 transcripts of the appropriate size were detected in both the nuclear and cytoplasmic compartments (Fig.7D).

To further confirm that the stability of IVS1$_{C\beta1}$ does not depend on integrity of its entire structure, we inserted two fragments the β-globin 3' untranslated region into IVS1$_{C\beta1}$ (Fig.2C, upper panel). Northern analysis showed the accumulation of a 2.1 kb transcript that hybridized with IVS1$_{C\beta1}$ (Fig.2C, lower panel) and 3' globin probes (data not shown).

To examine whether internal sequences of IVS1$_{C\beta1}$ are necessary for its stability, we generated two constructs in which middle portions of IVS1$_{C\beta1}$ (IVS1$_{C\beta1}$ Δnuc.95-231 and Δnuc. 95-401) were deleted (Fig.3, upper panel). Hela cells stably transfected with a construct containing the 136 bp internal deletion in IVS1$_{C\beta1}$ (nuc.95-231) accumulated spliced IVS1$_{C\beta1}$ transcripts of the appropriate size (0.3 kb) transcripts in the nucleus (Fig.3, lane 1). Likewise, the Δnuc.95-401 form of IVS1$_{C\beta1}$ (0.2 kb) transcript was detected in the nuclei of Hela cells (Fig.3, lane 2). The IVS1$_{C\beta1}$Δ95-401 transcript accumulated at

relatively low levels, perhaps because it was inefficiently spliced; the level of fully spliced mRNA was relatively low in both nuclear and cytoplasmic compartments (data not shown).

Collectively, these results support the notion that the intact secondary structure of $IVS1_{C\beta1}$ is not necessary for its stability and further indicate that a major portion of its internal sequence (306 nuc.) can be removed without abolishing its stable phenotype. Deletions that extend further towards the 5' and 3' terminus of $IVS1_{C\beta1}$ were not tested because it is likely that they would inhibit the ability of this intron to be spliced out.

The stability of $IVS1_{C\beta1}$ is dependent on the 3' portion of its sequence

We sought to determine whether the stability of $IVS1_{C\beta1}$ depends on 5' or 3' terminal sequences, or both. Two chimeric minigene constructs were generated to test this. One of the chimeric constructs (G–$C\beta1$) contains globin exon 2 and the 5' portion of $IVS2_{globin}$ ligated to the 3' portion of $IVS1_{C\beta1}$, followed by the adjacent $exon_{C\beta1}$ (Fig.4A, upper panel). The other construct ($C\beta1$–G) was generated in the reciprocal manner: it contains $IVS1_{C\beta1}$ 5' sequences, followed by $IVS2_{globin}$ 3' sequences. (Fig.4B, upper panel). To determine whether the 3' end of $IVS1_{C\beta1}$ confers stability, the G–Cβ1 construct was tested. Northern analysis shows that a 0.9 kb species that hybridized with both $IVS1_{C\beta1}$(Fig.4A, lower panel, left lane) and globin probes (data not shown) is present in the nucleus. The size and hybridization characteristics of this transcript are consistent with those expected of the chimeric intron. This intron efficiently spliced out as indicated by the accumulation of the fully spliced (1.0 kb) G–Cβ1

transcript in both the nucleus (Fig.4A, lower panel, middle lane) and cytoplasm (Fig. 4A, lower panel, right lane).

To determine whether the 5' end of IVS1$_{C\beta1}$ can confer its stability, the Cβ1-G construct was transfected into Hela cells. In contrast to the G–Cβ1 intron, the Cβ1-G intron was not detectable in the nucleus of Hela cells transfected with the Cβ1-G construct (Fig.4B, lower panel, left and middle lane). This intron was presumably spliced efficiently since mature Cβ1-G transcripts was present in the nucleus (Fig.4B, lower panel, left lane) and cytoplasm (Fig. 4B, lower panel, right lane). Collectively, these results indicate that sequences in the 3' portion and not the 5' portion of IVS1$_{C\beta1}$ are necessary and sufficient for its stability.

IVS1$_{C\beta1}$ accumulates as a set of lariat molecules with different length tails

Our previous data demonstrated that spliced IVS1$_{C\beta1}$ migrates as three discrete species in a 5% polyacrylamide gel. RNase H digestion in the presence of an oligonucleotide complementary with the 3' terminus of IVS1$_{C\beta1}$ decreased the size of the largest species, suggesting that these species possessed lariat tails (Qian et al., 1992). Here, we investigated the length of the lariat tails by ribonuclease protection analysis. One riboprobe (riboprobe A) used for the analysis is complementary with the 3' terminal region of IVS1$_{C\beta1}$ and the downstream exon (Fig.5, upper panel). We used the probe to analyze nuclear RNA isolated from Hela cells transfected with the IVS1$_{C\beta1}$ construct (Fig.5, upper panel). Four major sizes of RNA species were protected by this probe: the largest one was the unspliced pre-mRNA, while the three small RNAs corresponded to the stable IVS1$_{C\beta1}$ lariat with different length tails (Fig.5, lower

left panel, lane 3). These RNA species were only observed in RNA from Hela cells transfected with IVS1$_{C\beta1}$, not the parental Hela cells (Fig.5, lower left panel, lane 2). In order to test the unlikely possibility that these protected RNA species are due to different 5' ends rather than different 3' ends, another riboprobe (riboprobe B) was used in the analysis. Riboprobe B has a 5' end which is identical to riboprobe A but is shorter in length at the 3' end; it lacks 32 nucleotides at the terminus of IVS1$_{C\beta1}$ (Fig.5, upper panel). A single protected band was evident with this probe, as expected if the three species detected by riboprobe A differ in length at the 3' end, not the 5' end (Fig.5, lower right panel, lane 3). The sizes of the three species of IVS1$_{C\beta1}$ detected by riboprobe A were determined by comparing their migration to an RNA ladder and a DNA sequencing reaction. Species VIa has a full length tail, while species VIb and species VIc have lost approximately 16 and 37 nucleotides of their tails, respectively.

Spliced IVS1$_{C\beta1}$ may regulate the nuclear-to-cytoplasmic transport of mature TCR–β mRNA

During the course of evaluating the pattern of TCR-β expression in transfected Hela cells, we noted that constructs which possessed the IVS1$_{C\beta1}$ gene gave rise to mature transcripts which accumulated in the nucleus of T cells. This was a surprising observation since fully spliced transcripts typically rapidly exit to the cytoplasm (see review, Izaurralde et al., 1992). To test the notion that IVS1$_{C\beta1}$ regulate the nuclear-to-cytoplasmic transport of fully spliced transcripts, five different constructs were evaluated by transfection (Fig. 6A). Highly purified nuclear RNA (see materials and methods) from the stably transfected cells was subjected to Northern analysis. Constructs that contained stable forms of

IVS1$_{C\beta1}$ gave rise to mature transcripts that accumulated in the nucleus at levels similar to or greater than pre-mRNA (Fig. 6B). This included the wild type form of IVS1$_{C\beta1}$ (construct Cβ1) as well as IVS1$_{C\beta1}$ containing an internal insert (Cβ1–Gin) or β–globin 5' sequences (G-Cβ1). In contrast, constructs that do not encode a stable intron gave rise to mature transcripts that are barely detectable in the nucleus (Fig.6C). Collectively, these results suggested that the presence of IVS1$_{C\beta1}$ might inhibit or delay the nuclear export of mature mRNA.

We considered the possibility that the nuclear retention of mature Cβ1 transcripts might be due to sequences that neighbor IVS1$_{C\beta1}$, rather than IVS1$_{C\beta1}$ itself. In order to definitively determine which sequences are responsible for the nuclear retention of mature Cβ1 transcripts, we examined the expression profile of other constructs. First, a mini-gene construct that contains only IVS1$_{C\beta1}$ and the directly adjacent exon sequences was stably transfected into Hela cells. Mature transcripts derived from this construct displayed the nuclear retention phenotype (Fig.7A). To determine if the exons which neighbor IVS1$_{C\beta1}$ (exons 1 and 2) engender nuclear retention rather than IVS1$_{C\beta1}$ itself, we tested an exon-only construct (ΔIVS1$_{C\beta1}$) that lacks IVS1$_{C\beta1}$ (Fig.7B). Mature mRNA derived from this construct was detected exclusively in the cytoplasm, not the nucleus (Fig.7B). Thus, exons 1 and 2 do not confer the nuclear retention phenotype.

To further test the specificity of the effect of IVS1$_{C\beta1}$, we examined the expression characteristics of a mini-gene construct which contains another TCR-β intron, IVS3$_{C\beta1}$ (Fig.7C), which is not stable following excision (Qian et al., 1992). Unlike the IVS1$_{C\beta1}$ construct, the IVS3$_{C\beta1}$ construct gave rise to mature transcripts that accumulated exclusively in the cytoplasm, not the nucleus (Fig.7C). Thus, IVS3$_{C\beta1}$ does not confer the nuclear retention phenotype.

Lastly, we examined the expression pattern of mutant IVS1$_{C\beta1}$ constructs that encode stable forms of IVS1$_{C\beta1}$ to determine if they also retained the ability to engender the nuclear retention phenotype. Constructs with insertions (Fig.7D and 7E) or deletions (Δ95-231 and Δ95-401, data not shown) in IVS1$_{C\beta1}$ gave rise to mature transcripts that accumulated in the nuclear compartment. Thus, these alternations have no effect on either the stable phenotype of IVS1$_{C\beta1}$ or its apparent ability to cause nuclear retention of mature transcripts.

The fate of IVS1$_{C\beta1}$ and mature TCR-β transcripts after cessation of transcription

If IVS1$_{C\beta1}$ blocks the transport of mature TCR-β transcripts out of the nucleus, why do some fully spliced transcripts manage to accumulate in the cytoplasm? One possible explanation is that spliced IVS1$_{C\beta1}$ has a defined half-life which control mature transcript level. According to this hypothesis, the inhibition on the export of mature TCR-β transcripts will be released when IVS1$_{C\beta1}$ is degraded. To test this, we examined the kinetics of IVS1$_{C\beta1}$ and mature TCR-β transcript levels following treatment of cells with the transcriptional inhibitor actinomycin D (AMD). Hela cells stably transfected with a full length Cβ1 construct were treated with AMD (Fig.8A, upper panel). After 1 hr AMD treatment, the level of spliced IVS1$_{C\beta1}$ remained relatively constant (Fig.8B, upper panel). This is likely to be due both to its relative stability and the generation of more spliced IVS1$_{C\beta1}$ from pre-mRNA that were present before AMD treatment (Fig.8B). After 3 hr AMD treatment, the level of spliced IVS1$_{C\beta1}$ declined precipitously (Fig.8B, upper panel), indicating that its half life must <3hr. Similarly, fully spliced transcripts in

the nucleus decreased after 1hr AMD treatment and disappeared after 3hr AMD treatment (Fig.8B, lower panel). Thus, the kinetics of mature transcript reduction in the nucleus following AMD treatment paralleled that for spliced IVS1$_{C\beta1}$. This data is consistent with the hypothesis that IVS1$_{C\beta1}$ regulates the transport of mature transcripts, but it clearly does not prove a causal relationship. In contrast to spliced IVS1$_{C\beta1}$, the expression pattern of IVS1$_{C\beta1}$-containing pre-mRNAs did not correlate with mature transcripts; these pre-mRNAs dramatically declined in level after 1 hr AMD treatment (Fig.8B).

Functional role of IVS1$_{C\beta1}$ in T cells

In a previous report, we showed that TCR-β1 splicing intermediates and mature mRNA accumulate in the nucleus of a T cell clone, SL12.4, which transcribes a fully rearranged TCR-β1 gene but essentially lacks mature 1.3 kb TCR-β1 transcripts in the cytoplasm (Qian et al., 1993). Protein synthesis inhibitors (e.g. cycloheximide) induce the appearance of fully spliced 1.3 kb TCR-β transcripts in the cytoplasm of these T cells. This induction event is primarily posttranscriptional since CHX does not significantly increase the rate of transcription, as measured by the nuclear run-on assay (Wilkinson & MacLeod., 1988). Results from the present investigation suggested the possibility that spliced IVS1$_{C\beta1}$ may play a role in this unusual nuclear regulation. We tested the hypothesis that IVS1$_{C\beta1}$ prevents the cytoplasmic accumulation of mature TCR-β1 transcripts and that protein synthesis inhibitors reverse the regulatory effect of IVS1$_{C\beta1}$. To examine this possibility, we treated SL12.4 cells with CHX and compared the expression of spliced IVS1$_{C\beta1}$ (0.5kb) and the fully spliced TCR-β transcripts (1.3 kb). Consistent with the hypothesis, we observed that CHX induced mature 1.3 kb TCR-β transcripts in SL12.4 cells concomitant with a

dramatic decline in spliced IVS1$_{C\beta1}$ (Fig.9). In addition, a 1.7 kb mature TCR-β1 transcript derived from a distal promoter rather than the "classical" proximal promoter (Wilkinson et al., unpublished data) was also induced by CHX treatment (Fig.9, lower panel). By comparison, no obvious change was observed in the expression of the housekeeping gene CHO-A before and after CHX treatment (Fig.9, middle panel).

The reduction of spliced IVS1$_{C\beta1}$ and induction of the fully spliced 1.3 kb TCR-β transcripts was reversible. The level of spliced IVS1$_{C\beta1}$ returned to normal level by 3 hrs after removal of CHX (Fig.9, upper panel). In contrast, fully spliced 1.3 kb and 1.7 kb TCR-β transcripts decreased after removal of CHX (Fig.9, lower panel). The lag before mature transcripts decline (between 3-6 hr post-CHX treatment) may be due to their relatively long intrinsic half-life in the cytoplasm (see Fig.8C).

DISCUSSION

The 3' terminal portion of IVS1Cβ1 is necessary and sufficient for its stability

In order to understand the molecular basis for the stability of IVS1$_{C\beta1}$, we performed a preliminary investigation to study the cis-acting elements which are responsible for the stability of IVS1$_{C\beta1}$. It has been reported that the secondary structure of RNA plays an important role in maintaining the stability of RNA. For instance, the stability of LAT is presumably dependent on unique secondary structure since a 440 nucleotide insertion destabilizes this transcript (Farrell et al., 1991; Block et al., 1990). The unusual stability of the group I td intron is due to a complex secondary structure (Shub et al., 1988). Therefore, if the stability of IVS1$_{C\beta1}$ depends on sequences or precise secondary structure in the central portion of this intron, deletions or insertions within this intron will abolish its stability. However, our analysis indicates that this not the case since large insertions in IVS1$_{C\beta1}$ did not destabilize the intron (Fig. 2B &C). Furthermore, IVS1$_{C\beta1}$ remains stable when large portions of its internal sequence were deleted (Fig.3). Hence, we conclude the stability of IVS1$_{C\beta1}$ does not depend on internal sequences or intact secondary structure involving the entire molecule.

To determine the role of the 5' and 3' termini of IVS1$_{C\beta1}$ in its stability, we tested chimeric TCR-β/β–globin constructs which contained either the 5' or 3' portion of IVS1$_{C\beta1}$. A construct in which a 5' portion of IVS1Cβ1 was replaced by a 5' portion of IVS2$_{globin}$ gave rise to an intron which accumulated in transfected Hela cells (Fig. 4A. left lane). In contrast, the substitution of a 3' portion of

IVS1Cβ1 with 3' portion of IVS2$_{globin}$ gave rise to an unstable intron (Fig. 4B). Therefore, sequences present in the 3' terminal region of IVS1Cβ1 (minimally defined as 122 nucleotides) are necessary and sufficient for its stability, while 5' sequence of IVS1Cβ1 do not confer stability.

We have not yet determined which particular 3' sequences in IVS1Cβ1 confer the stable phenotype. The 3' spliced site and polypyrimidine tract [(Y)$_{34}$CAG/A] of IVS1Cβ1 is an exceedingly good match to the consensus sequence: (Y)$_{>12}$ NYAG/G (Green et al., 1986). Thus, the unusual stability of IVS1Cβ1 is unlikely to be due to its generic 3' polypyrimidine tract / 3' splice site. Our preliminary primer extension experiments suggest that IVS1Cβ1 possesses multiple branchpoints. It is possible that these branchpoints are resistant to debranching, thus conferring a stable phenotype to IVS1Cβ1 by virtue of resistance to exonuclease attack. However, this mechanism fails to explain why IVS1Cβ1 lariats maintain their tails. A more likely explanation is that IVS1Cβ1 possesses a unique sequence that binds to one or more spliceosomal components and block spliceosomal disassembly (see later). Mutagenesis studies will be performed to test this hypothesis.

IVS1Cβ1 consists of a set of lariat molecule possessing different length tails

What is the mechanism which permits IVS1Cβ1 to remain stable? One possibility is that it is unable to undergo debranching and is therefore resistant to exonuclease attack. It has been reported that stable introns accumulate in yeast as a result of defects in debranching (Jaquier et al., 1986; Chapman et al., 1991). These yeast introns accumulate as circular molecules that presumably have lost

their tails as a result of exonucleolytic digestion. Thus, if a block in debranching was the only mechanism that permits the accumulation of IVS1$_{C\beta1}$, it would be predicted that the circular portion of IVS1$_{C\beta1}$ would remain intact, but that the lariat tail would be completely degraded by exonucleases. However, our evidence obtained from RNase H mapping experiments indicates that IVS1$_{C\beta1}$ lariats do possess tails (Qian et al., 1992). This unusual feature suggests that spliced IVS1$_{C\beta1}$ lariats are not only prevented from undergoing debranching, but are also able to escape exonuclease attack of their tails. In the present study, we used ribonuclease protection analysis to determine the length of the tails. This analysis revealed that many spliced IVS1$_{C\beta1}$ transcripts have full-length tails. Two other discrete IVS1$_{C\beta1}$ species also exist that have lost approximately 16 and 37 nucleotides of their tails, respectively (Fig. 5). Collectively, these results suggest that spliced IVS1$_{C\beta1}$ lariats are present in a protective microenvironment in the nucleus. An attractive, but as yet untested notion, is that IVS1$_{C\beta1}$ lariats remain associated with spliceosomes following splicing (see later discussion).

The potential function of IVS1$_{C\beta1}$

What is the function of IVS1$_{C\beta1}$? One possibility is that it encodes a protein. This is unlikely for at least two reasons: 1) spliced IVS1$_{C\beta1}$ transcripts can only be detected in the nucleus, not in the cytoplasm. 2) Long open reading frames are not present in its sequence. However, we cannot exclude the possibility that non-proteinacious factors might be encoded by the IVS1$_{C\beta1}$ sequence. For example, it has been shown that U14 snRNA is encoded in introns of the heat shock hsc70 gene (Liu et al., 1990).

Our results suggest that IVS1$_{C\beta1}$ might inhibit the exit of mature transcripts from the nucleus since the presence of nuclear IVS1$_{C\beta1}$ lariats is correlated with an accumulation of mature transcripts in the nuclear compartment of transfected cells (Fig. 6B).

This effect on nuclear-to-cytoplasmic transport was revealed while studying the expression of a construct which contains IVS1$_{C\beta1}$ and the adjacent exon sequences driven by a β-actin promoter. A high level of mature transcripts was detected in the nucleus of cells transfected with this IVS1$_{C\beta1}$ construct (Fig. 1B and Fig.7A). In contrast, little or no mature transcripts was observed in the nucleus of cells transfected with a gene containing an unstable intron (Fig.7C). To test if the nuclear retention of mature transcripts is due to adjacent exons next to IVS1$_{C\beta1}$ rather than spliced IVS1$_{C\beta1}$ itself, we transfected Hela cells with a construct which *only* contains the adjacent exons and lacks IVS1$_{C\beta1}$ (ΔIVS1$_{C\beta1}$). We predicted that mature RNA will accumulate in the nucleus if the adjacent exons are responsible for the block in nuclear-to-cytoplasmic transport. Alternatively, if IVS1$_{C\beta1}$ itself is responsible, then, mature transcripts should not accumulate in the nucleus. Our results are agreement with the latter hypothesis (Fig.7B), thus further supporting the notion that it is spliced IVS1$_{C\beta1}$ itself which possesses this function. Further evidence for this hypothesis was the observation that mutations which failed to abolish the stability of IVS1$_{C\beta1}$ also did not perturb the nuclear retention phenotype (Fig. 7D and 7E).

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Figure 1. Northern blot analysis of Hela cells transfected with the $IVS1_{C\beta1}$ construct. All panels show 10 µg of RNA electrophoresed in agarose gels, blotted, and hybridized with the probes indicated. (A) $IVS1_{C\beta1}$ construct. Bars represent exons and lines represent introns. (B) A blot containing nuclear RNA from stably transfected Hela cells. The blot was sequentially hybridized with murine $IVS1_{C\beta1}$, $Exon_{C\beta1}$ and $IVS1_{actin}$ probes. The lengths of $IVS1_{C\beta1}$, splicing intermediates and mature transcripts are derived from published work (Gunning et al., 1987) and our sequence analysis (Qian et al., 1992). Note that species IV did not hybridize with the $exon_{C\beta}$ probe because exon2 is only 18 nuc. in length. Note also that since the $IVS1_{actin}$ probe also contains 78 nuc. of $exon1_{actin}$ (see materials and methods), that it cross-hybridizes with species III and V. (C) A blot containing cytoplasmic RNA from stably transfected Hela cells hybridized with the Cβ exon probe.

Fig.1.

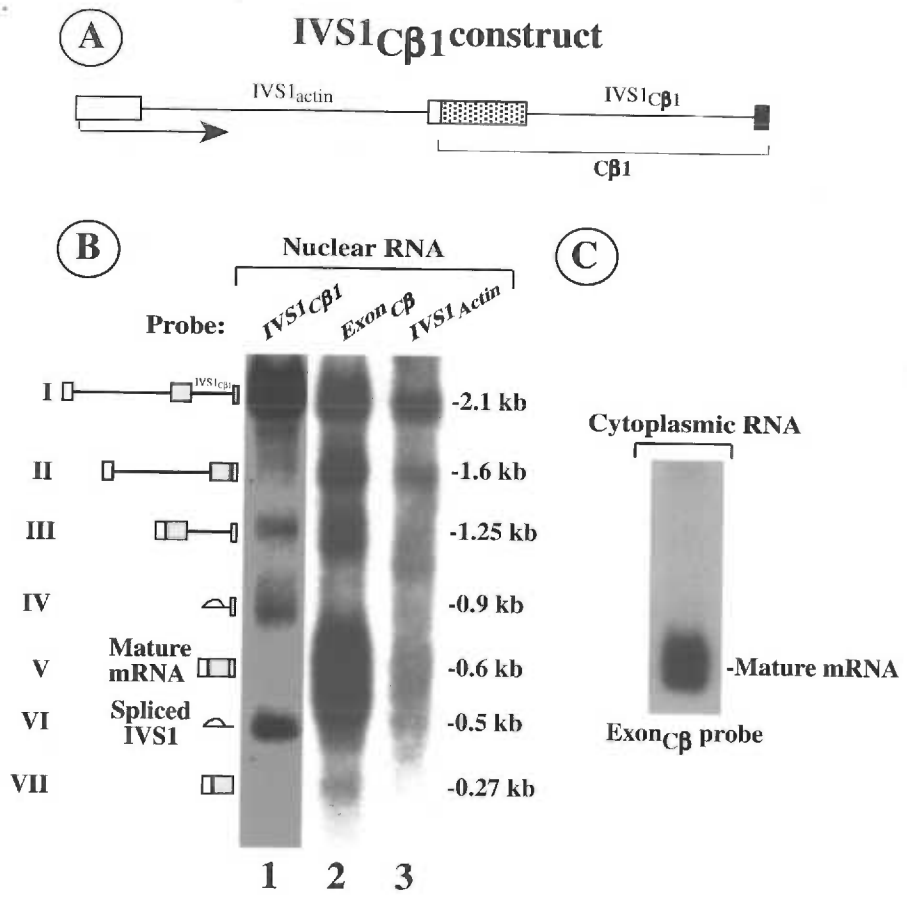

A
IVS1Cβ1 construct
IVS1actin
IVS1Cβ1
Cβ1
B
Nuclear RNA
Probe:
IVS1Cβ1
Exon Cβ
IVS1Actin
I
II
III
IV
V
VI
VII
Mature mRNA
Spliced IVS1
-2.1 kb
-1.6 kb
-1.25 kb
-0.9 kb
-0.6 kb
-0.5 kb
-0.27 kb
1 2 3
C
Cytoplasmic RNA
-Mature mRNA
Exon Cβ probe

Figure 2. Northern blot analysis of Hela cells transfected with the constructs containing insertions within IVS1$_{C\beta 1}$. All panels show 10 μg of nuclear RNA electrophoresed in agarose gels, blotted, and hybridized with the IVS1$_{C\beta 1}$ probe. (A), (B),(C) The upper panel shows the construct transfected. The lower panel shows Northern analysis of nuclear RNA from Hela cells stably transfected with these constructs. The lengths of mutant IVS1$_{C\beta 1}$ sequences are derived from published work (Lawn et al., 1980) and our sequence analysis (Qian et al., 1992).

Fig.2.

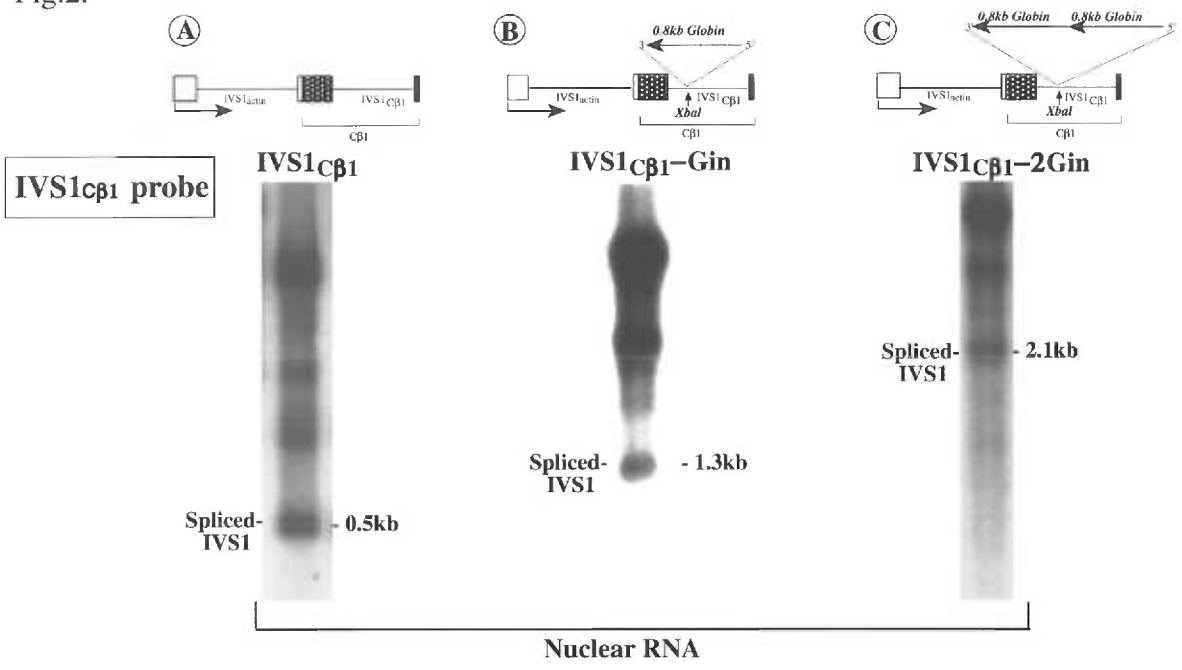

A
B
C
0.8kb Globin
0.8kb Globin
0.8kb Globin
IVS1actin
IVS1Cβ1
Cβ1
XbaI
IVS1Cβ1
IVS1Cβ1–Gin
IVS1Cβ1–2Gin
IVS1Cβ1 probe
Spliced-IVS1
- 0.5kb
Spliced-IVS1
- 1.3kb
Spliced-IVS1
- 2.1kb
Nuclear RNA

Figure 3. Northern blot analysis of Hela cells transfected with the constructs containing deletions within IVS1$_{C\beta1}$. All panels show 10 μg of nuclear RNA electrophoresed in agarose gels, blotted, and hybridized with the IVS1$_{C\beta1}$ probe. The upper panel shows the construct transfected. The lower panel shows Northern analysis of nuclear RNA from Hela cells stably transfected with these constructs. The lengths of mutant IVS1$_{C\beta1}$ sequences are derived from our sequence analysis (Qian et al., 1992).

Fig.3.

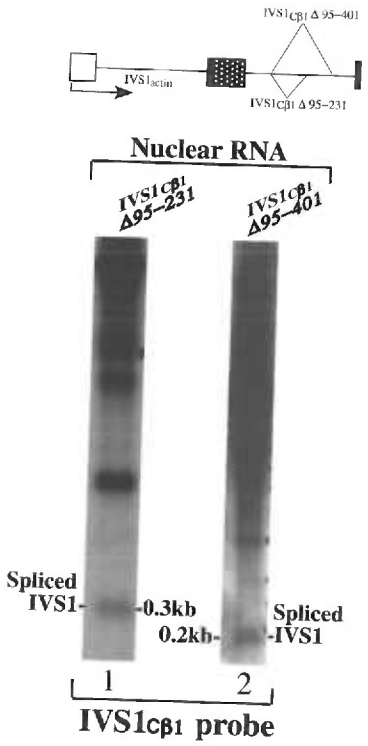

IVS1cβ1 Δ 95–401
IVS1actin
IVS1cβ1 Δ 95–231
Nuclear RNA
IVS1cβ1 Δ95–231
IVS1cβ1 Δ95–401
Spliced IVS1
0.3kb
0.2kb
Spliced IVS1
1
2
IVS1cβ1 probe

Figure 4. Northern blot analysis of Hela cells transfected with TCR-β/β-globin chimeric constructs. All panels show 10 μg of RNA electrophoresed in agarose gels, blotted, and hybridized with the probes indicated. (A) Upper panel: the G-Cβ1 construct that was stably transfected into Hela cells. Lower panel: the left two lanes shows a blot containing nuclear RNA from stably transfected Hela cells sequentially hybridized with murine $IVS1_{C\beta1}$ and $Exon_{C\beta1}$ probes. The lengths of the chimeric IVS and mature transcripts are derived from published work (Lawn et al., 1980) and our sequence analysis (Qian et al., 1992 and see materials and methods). The right lane shows the blot containing cytoplasmic RNA from stably transfected Hela cells hybridized with the $exon_{C\beta1}$ probe. (B) Upper panel: the Cβ1–G construct that was stably transfected into Hela cells. Lower panel: The left two lanes show a blot containing nuclear RNA from stably transfected Hela cells sequentially hybridized with the 3' globin (see materials and methods) and murine $IVS1_{C\beta1}$ probes. Note that the 3' globin probe can hybridize with both the chimeric intron and mature Cβ1–G transcripts since it contains 0.35 kb of IVS2 and 0.32 kb of downstream $exon3_{globin}$ plus 3' untranslated sequences. The lengths of the chimeric mature transcripts are derived from published work (Lawn et al., 1980) and our sequence analysis (Qian et al., 1992 and see materials and methods). The right lane shows the blot containing cytoplasmic RNA from stably transfected Hela cells hybridized with the 3' globin probe.

Fig.4.

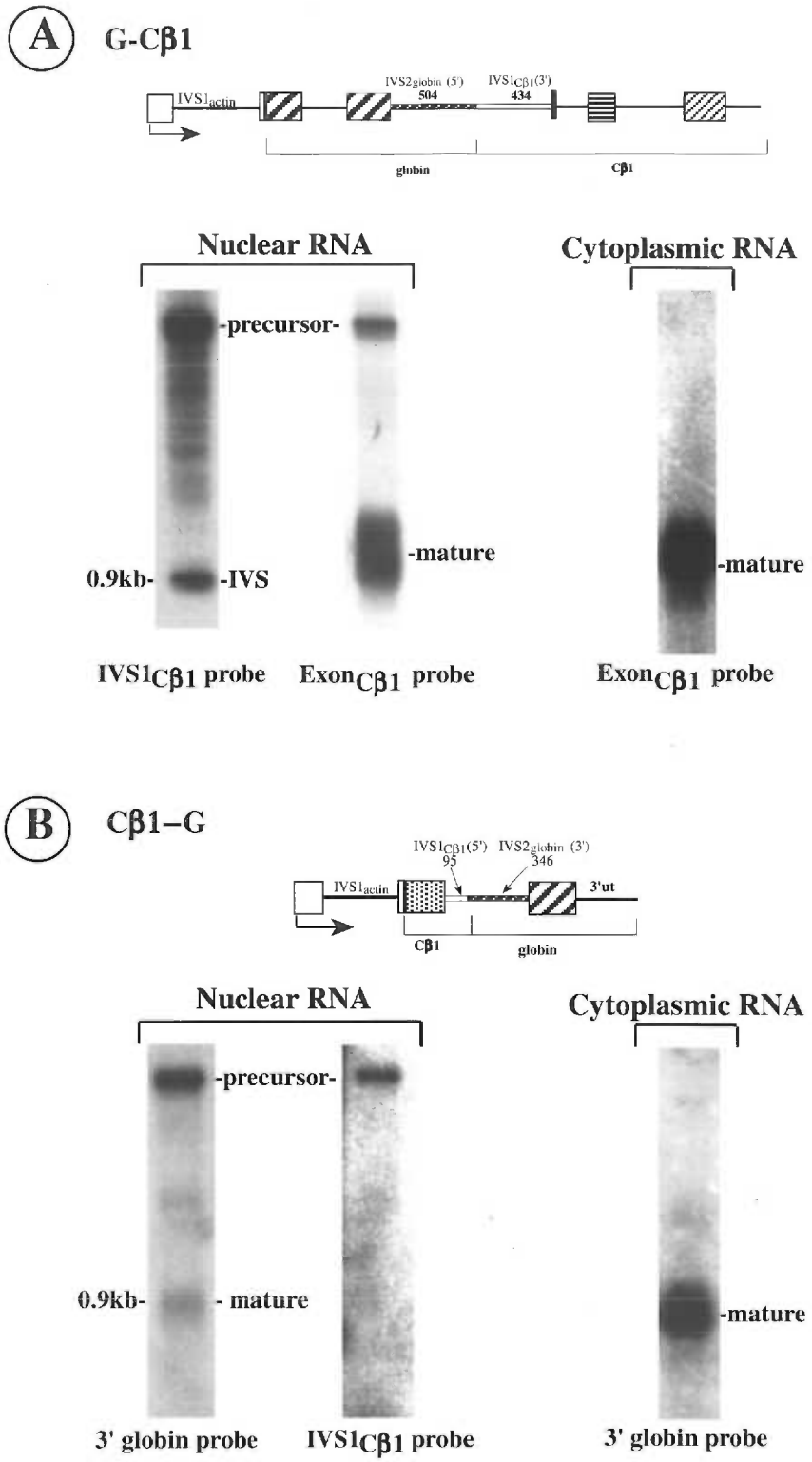

A
G-Cβ1
IVS1actin
IVS2globin (5')
504
IVS1Cβ1(3')
434
globin
Cβ1
Nuclear RNA
Cytoplasmic RNA
-precursor-
-mature
0.9kb-
-IVS
-mature
IVS1Cβ1 probe
ExonCβ1 probe
ExonCβ1 probe
B
Cβ1–G
IVS1Cβ1(5')
95
IVS2globin (3')
346
IVS1actin
3'ut
Cβ1
globin
Nuclear RNA
Cytoplasmic RNA
-precursor-
0.9kb-
- mature
-mature
3' globin probe
IVS1Cβ1 probe
3' globin probe

Figure 5. Ribonuclease protection analysis $IVS1_{C\beta1}$ tail length

10 μg of nuclear RNA was assayed in each lane except lane 1 which is the undigested probe. The RNA from parental Hela cells alone or Hela cells stably transfected with the $IVS1_{C\beta1}$ construct is indicated. Upper panel: diagrammatic representation of the regions recognized by the riboprobes A and B (see materials and methods). Lower panel: ribonuclease protection results using the riboprobes indicated. The diagrams on the right side of the autoradiographs depict the RNA structures defected by the analysis.

Fig.5.

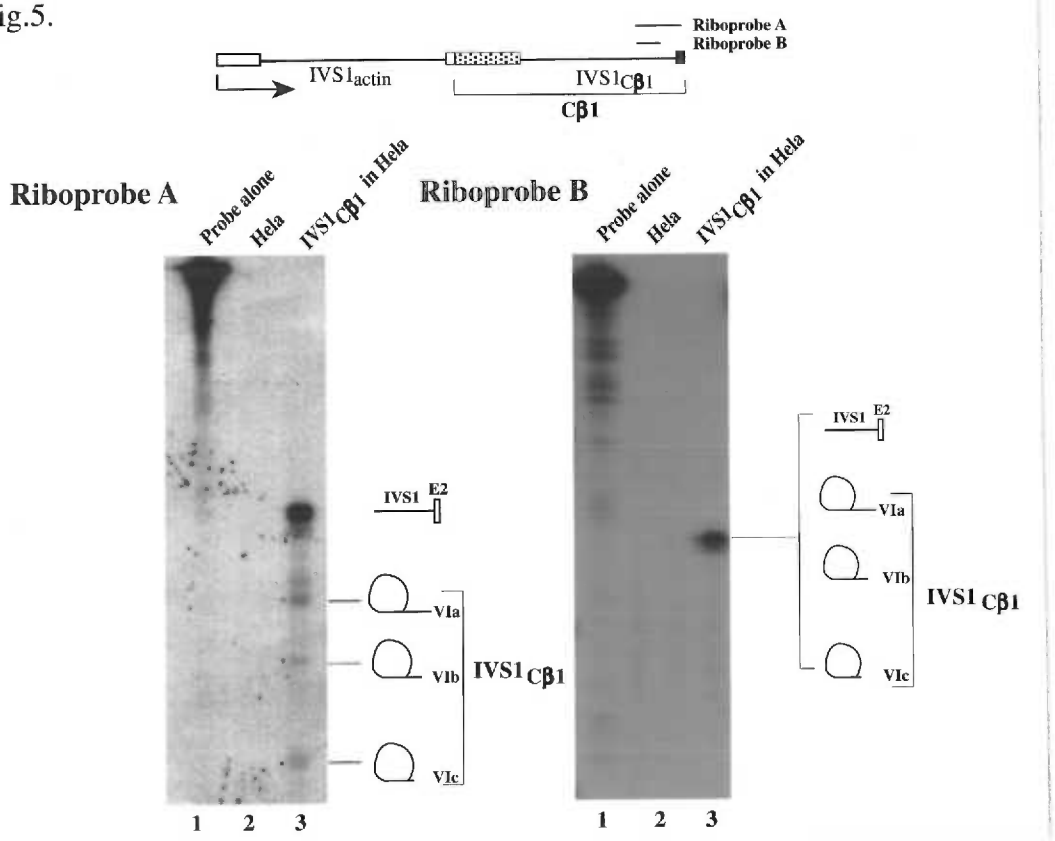

Riboprobe A
Riboprobe B
IVS1actin
IVS1Cβ1
Cβ1
Riboprobe A
Probe alone
Hela
IVS1Cβ1 in Hela
Riboprobe B
Probe alone
Hela
IVS1Cβ1 in Hela
IVS1
E2
VIa
VIb
VIc
IVS1 Cβ1
1 2 3
IVS1
E2
VIa
VIb
VIc
IVS1 Cβ1
1 2 3

Figure 6. Mature transcripts are retained in the nucleus when spliced IVS1$C\beta 1$ is present. All panels show nuclear RNA (10 μg) electrophoresed in agarose gels, blotted, and hybridized with the probes indicated. (A) Structure of the constructs stably transfected into Hela cells. (B) and (C) Northern analysis of nuclear RNA from Hela cells stably transfected with the constructs indicated in the upper panel. The lengths of mature RNA are derived from published work (Lawn et al., 1980; Gascoigne et al., 1984) and our sequence analysis (Qian et al., 1992).

Fig.6.

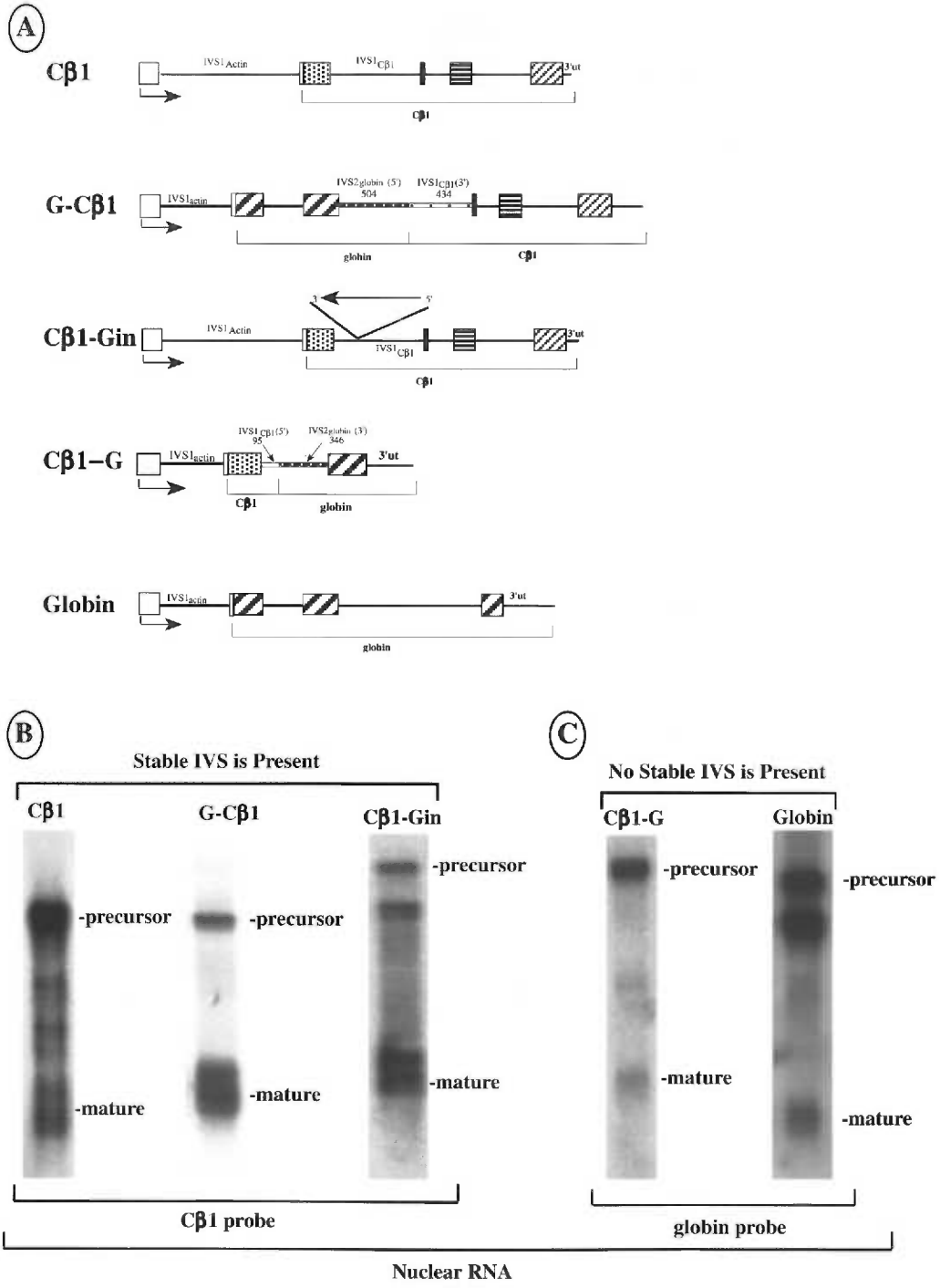

A
Cβ1
IVS1 Actin
IVS1 Cβ1
3'ut
Cβ1
G-Cβ1
IVS1actin
IVS2globin (5')
504
IVS1Cβ1(3')
434
globin
Cβ1
Cβ1-Gin
IVS1 Actin
IVS1 Cβ1
3'ut
Cβ1
Cβ1–G
IVS1Cβ1(5')
95
IVS2globin (3')
346
IVS1actin
3'ut
Cβ1
globin
Globin
IVS1actin
3'ut
globin
B
Stable IVS is Present
Cβ1
G-Cβ1
Cβ1-Gin
-precursor
-mature
-precursor
-mature
-precursor
-mature
Cβ1 probe
C
No Stable IVS is Present
Cβ1-G
Globin
-precursor
-mature
-precursor
-mature
globin probe
Nuclear RNA

Figure 7. IVS1$_{C\beta1}$ may retain mature transcripts in the nucleus. Each panel contains 10 μg of nuclear (N) or cytoplasmic (C) RNA from Hela cells stably transfected with the construct shown. RNA was electrophoresed in agarose gels, blotted, and hybridized with the Exon$_{C\beta1}$ probe (see materials and methods).

Fig.7.

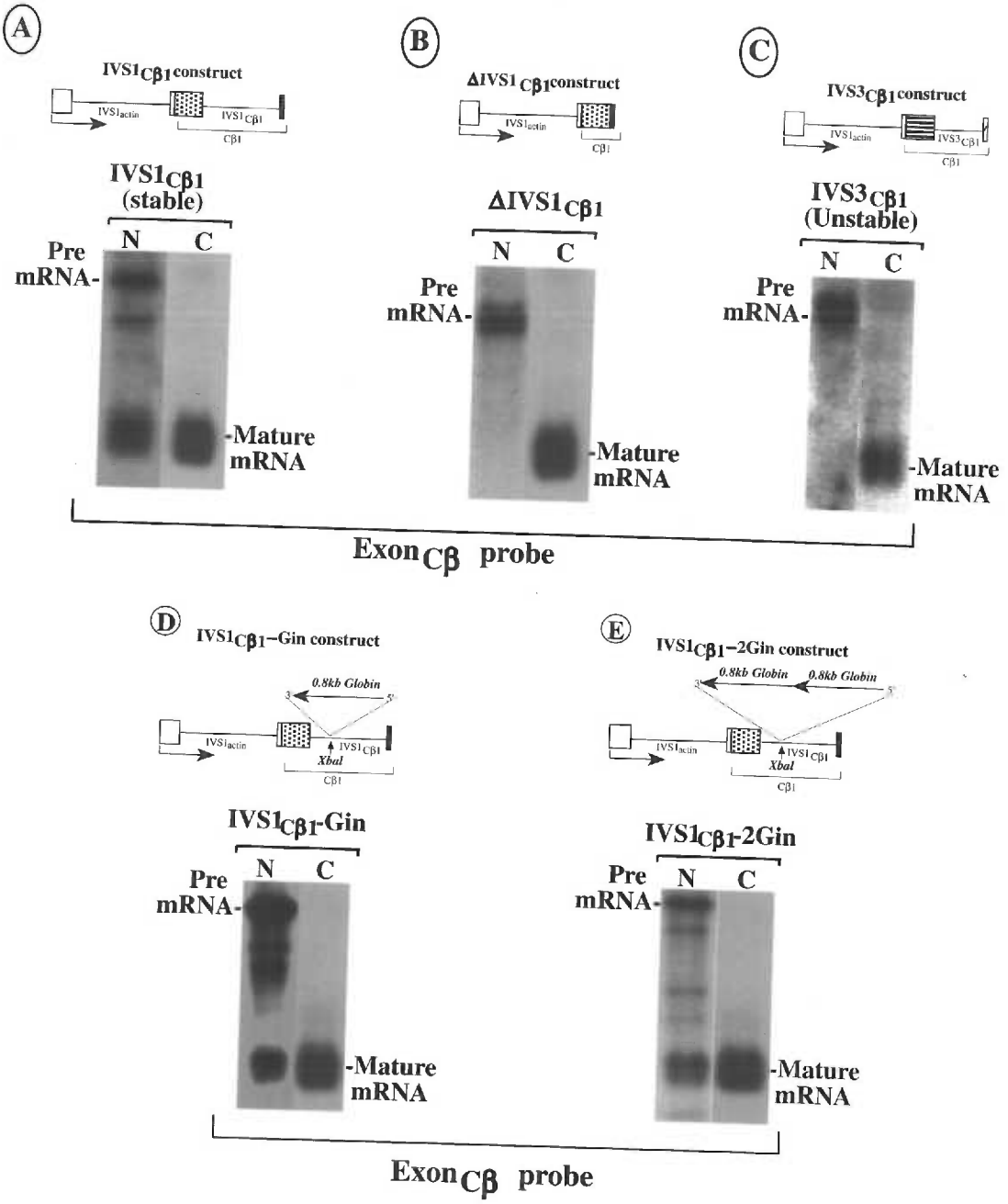

A
IVS1Cβ1 construct
IVS1actin
IVS1Cβ1
Cβ1
IVS1Cβ1 (stable)
N
C
Pre mRNA-
-Mature mRNA
B
ΔIVS1 Cβ1construct
IVS1actin
Cβ1
ΔIVS1Cβ1
N
C
Pre mRNA-
-Mature mRNA
C
IVS3Cβ1 construct
IVS1actin
IVS3Cβ1
Cβ1
IVS3Cβ1 (Unstable)
N
C
Pre mRNA-
-Mature mRNA
Exon Cβ probe
D
IVS1Cβ1−Gin construct
0.8kb Globin
IVS1actin
IVS1Cβ1
XbaI
Cβ1
IVS1Cβ1-Gin
N
C
Pre mRNA-
-Mature mRNA
E
IVS1Cβ1−2Gin construct
0.8kb Globin
0.8kb Globin
IVS1actin
IVS1Cβ1
XbaI
Cβ1
IVS1Cβ1-2Gin
N
C
Pre mRNA-
-Mature mRNA
Exon Cβ probe

Figure 8. Effect of cessation of transcription on spliced $IVS1_{C\beta1}$ and mature TCR-β transcripts. (A) The Cβ1 construct was used to stably transfected Hela cells. (B) Northern blot analysis of transfected Hela cells (10 μg of nuclear RNA). RNA was electrophoresed in agarose gels, blotted, and hybridized with $IVS1_{C\beta1}$ and $Exon_{C\beta1}$ probes as indicated (see materials and methods). RNA was obtained from untreated cells or cells treated with 5 μg/ml AMD for the number of hours shown. Equivalent amounts of RNA were loaded in each lane as assessed by methylene blue staining (Wilkinson et al., 1990). Note that the upper and lower panels are different blots but they contain the same RNA electrophoresed in different agarose gels. Also note that the Cβ1 construct contains 230 bp of the 3' portion of $IVS_{JC\beta1}$; this alternative acceptor can be used instead of the acceptor of $IVS1_{actin}$, as a consequence, two species of mature transcripts are generated (C) Northern analysis of cytoplasmic RNA from stably transfected Hela cells treated with AMD.

Fig. 8.

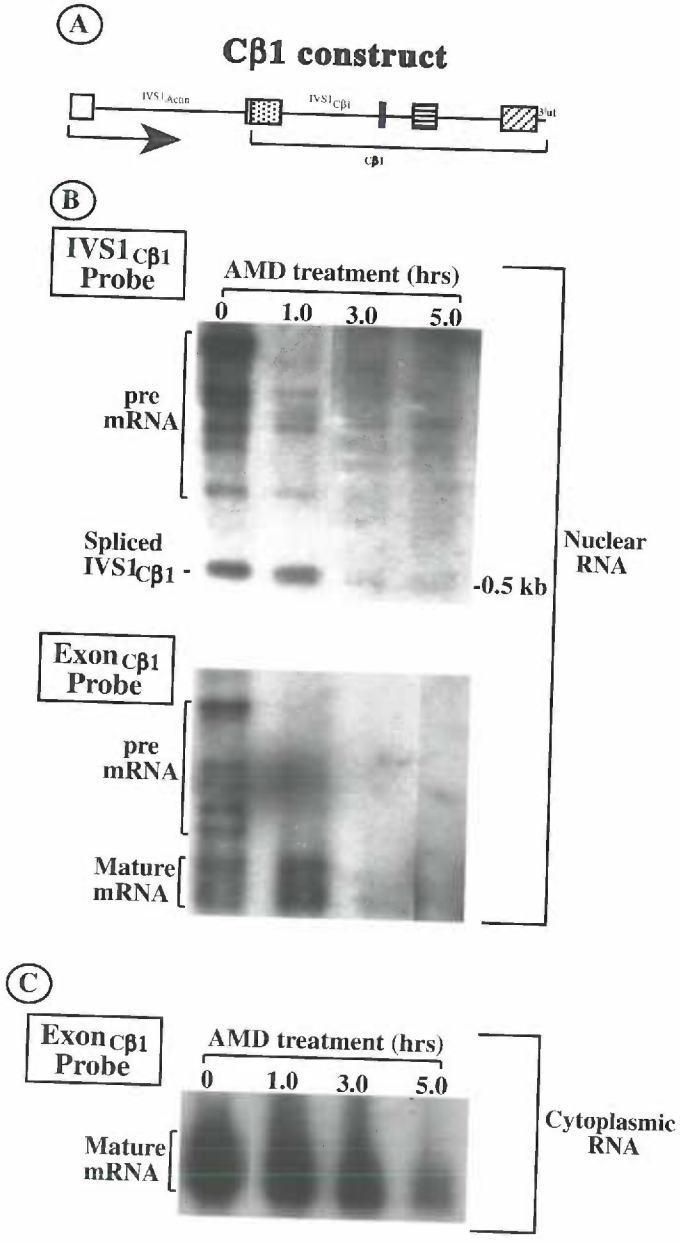

A
Cβ1 construct
IVS1Actin
IVS1Cβ1
3'ut
Cβ1
B
IVS1Cβ1 Probe
AMD treatment (hrs)
0
1.0
3.0
5.0
pre mRNA
Spliced IVS1Cβ1 -
-0.5 kb
Nuclear RNA
ExonCβ1 Probe
pre mRNA
Mature mRNA
C
ExonCβ1 Probe
AMD treatment (hrs)
0
1.0
3.0
5.0
Mature mRNA
Cytoplasmic RNA

FIG. 9 Reversible effects of cycloheximide on the accumulation of spliced IVS1$_{C\beta1}$ and mature TCR–β transcripts. Northern blot analysis of SL12.4 poly(A)$^+$ RNA (1μg) from untreated cells (0 hr) or cells incubated for 12hr with 10μg/ml CHX (CHX); or cells treated the same except they were washed and re-cultured for 3hr or 6hr. The RNA was electrophoresed in a 1% agarose gel, blotted, and hybridized with the probes shown. Equivalent amounts of RNA were loaded in each lane as assessed by methylene blue staining (Wilkinson et al., 1990) except that the last lane of the lower panel had approximately 3/4 as much RNA as the other lanes. Note that the upper and lower panel show the same RNA preparation, but contained in different blots. Middle panel: the same blot as the upper panel, but hybridized with the CHO-A probe.

Fig.9.

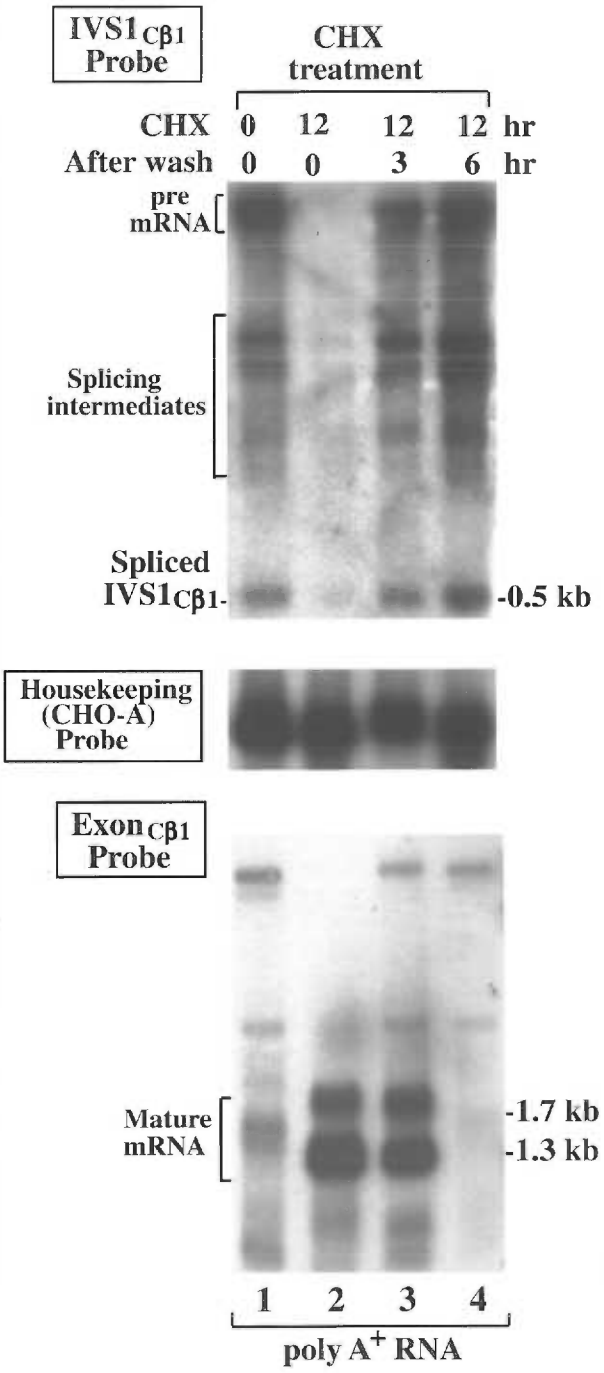
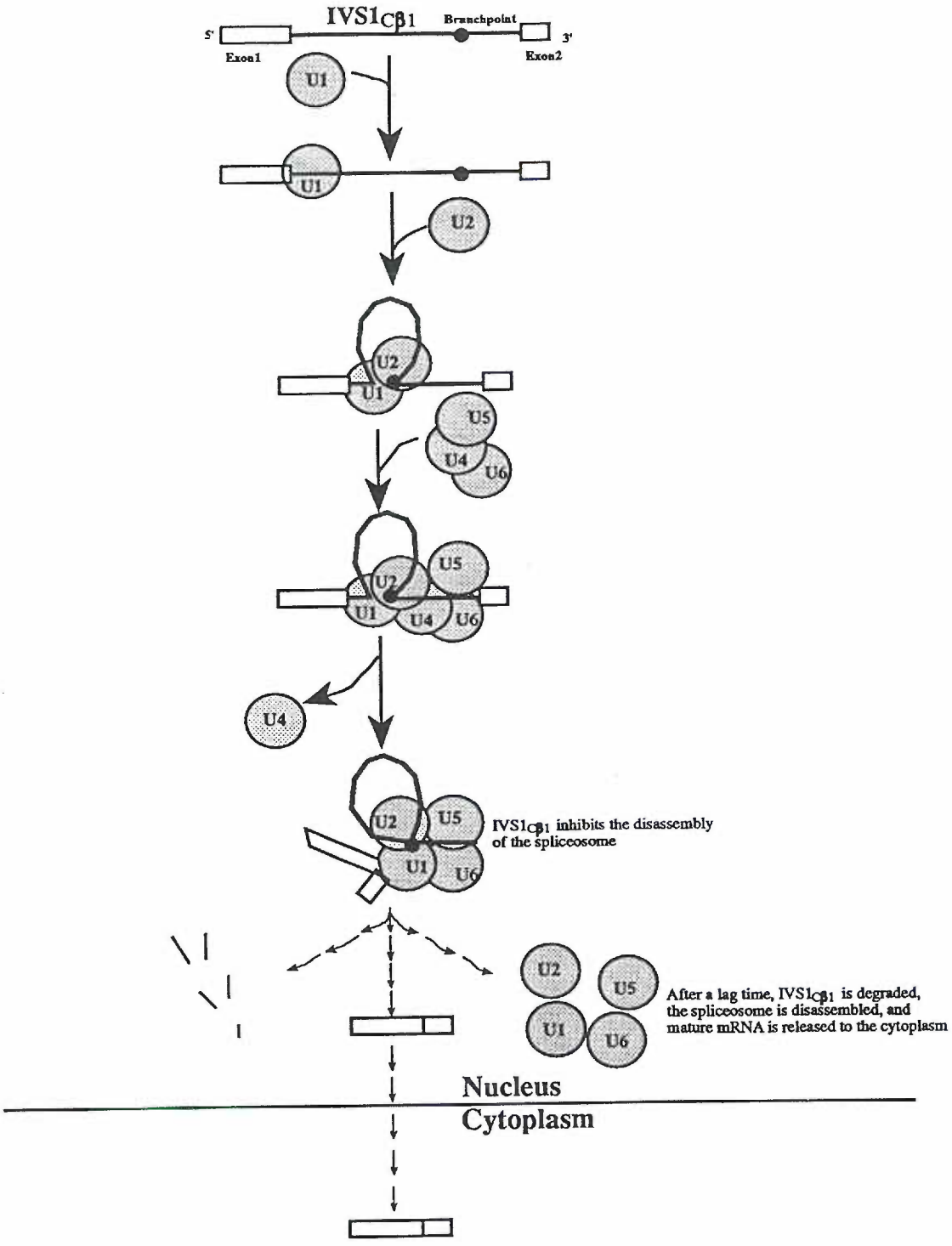

IVS1 Cβ1 Probe
CHX treatment
CHX 0 12 12 12 hr
After wash 0 0 3 6 hr
pre mRNA
Splicing intermediates
Spliced IVS1 Cβ1-
-0.5 kb
Housekeeping (CHO-A) Probe
Exon Cβ1 Probe
Mature mRNA
-1.7 kb
-1.3 kb
1 2 3 4
poly A+ RNA

Fig.10 A Model of IVS1$_{C\beta1}$ Function



CONCLUSION AND DISCUSSION

Characteristics of $IVS1_{C\beta1}$.

During the course of analyzing TCR-β pre-mRNA in the SL12.4 cell clone, I identified an unusual spliced intron (Qian et al., 1992). This intron, $IVS1_{C\beta1}$, is derived from the constant region of the T cell receptor-β (TCR–β) gene. Its stability is selective since other spliced TCR–β introns do not detectably accumulate in T cells. The property of stability appears to be evolutionarily conserved since the human version of this intron also accumulates in T cells. $IVS1_{C\beta1}$ exists exclusively in the lariat conformation in the nuclei of T cells and transfected non-lymphoid cells. RNase H digestion and ribonuclease protection experiments confirmed that $IVS1_{C\beta1}$ molecules still possess tails, suggesting that this spliced intron resides in a protective nuclear microenviroment.

$IVS1_{C\beta1}$ has several unique characteristics different from "stable" introns which have been reported before. First, the stability of $IVS1_{C\beta1}$ does not rely on intact secondary structure involving the entire molecule since its stability is not abolished by internal sequence insertions or deletions of $IVS1_{C\beta1}$. In contrast,. the "stability" of the LAT intron transcript may be dependent on unique secondary structure since its stability is compromised by the insertion of a fragment into its sequence (Block et al., 1990). Second, the stability of $IVS1_{C\beta1}$ is independent of cell type since it is not only stable in T cells but also in epithelial cells and fibroblasts. In contrast, the SV40 "stable intron" accumulates in *Xenopus* oocyte, but not in transfected monkey cells (Michaeli et al., 1988).

Models of the molecular mechanism of IVS1$C\beta1$ function

A novel function attributes that IVS1$C\beta1$ appears to possess is an ability to regulate the export of mature mRNA out of the nucleus.

Several models could be proposed to explain why IVS1$C\beta1$ inhibits with the nuclear export of mature transcripts. One model is that IVS1$C\beta1$ exerts its regulatory function while present in pre-mRNA. For example, it is possible that an irreversible conformational change in the spliceosome is elicited when it binds to IVS1$C\beta1$–containing pre-mRNA. This conformational change could result in a block in spliceosomal disassembly, thus inhibiting the release of spliced IVS1$C\beta1$ and mature transcripts following splicing. As a consequence, spliced IVS1$C\beta1$ and mature transcripts would accumulate in the nucleus. A second model is that free IVS1$C\beta1$, which has been *spliced out* of pre-mRNA, is the agent which blocks the release of the mature transcripts. Evidence that supports this second model is that the level of mature transcripts in the nucleus of transfected cells correlates with the level of spliced IVS1$C\beta1$, not the level of TCR-β pre-mRNA (Fig.7A, D&E).

If model 2 is correct, how does spliced IVS1$C\beta1$ mediate its effect on nuclear-to-cytoplasmic transport? One possibility is that spliced IVS1$C\beta1$ hybridizes with mature transcripts, thereby inhibiting their release to the cytoplasm. This is unlikely since computer sequence searches did not show any obvious complementary between IVS1$C\beta1$ and Cβ1 exons. Another possibility is that IVS1$C\beta1$ directs spliceosomes to a nuclear compartment that does not efficiently export mature transcripts. Presumably, this hypothetical nuclear compartment would provide a protective environment which would allow IVS1$C\beta1$ to escape

immediate degradation. A simple model which I favor is that spliced IVS1Cβ1 interacts with some component of the spliceosome which prevents spliceosomal disassembly following splicing. As a consequence, mature mRNA would be prevented from leaving the nucleus and IVS1Cβ1 would remain in the protective microenvironment of the spliceosome (Fig.10). A prediction of this model which remains to be tested is whether spliced IVS1Cβ1 actually remains bound to spliceosomes in vivo.

The experiments performed with the protein synthesis inhibitor CHX suggest that a labile protein may be involved in the stability of IVS1Cβ1 and the nuclear retention of mature transcripts. For instance, an unstable regulatory protein may bind to spliced IVS1Cβ1 and block spliceosomal disassembly. The evidence that a labile protein is involved comes from the following experiment: incubation with CHX causes a marked reduction in the nuclear levels of spliced IVS1Cβ1 and a simultaneous augmentation in the cytoplasmic levels of mature transcripts in SL12.4 T cells (Fig.9). This effect is reversible. Clearly, other explanations besides the presence of an unstable inhibitor protein could be involved to explain this effect of CHX since it is a general inhibitor of protein synthesis. Nevertheless, this provides a good model system to reversibly modulate the levels of spliced IVS1Cβ1 and mature TCR-β transcripts in order to study the molecular basis for their co-regulation.

Although IVS1Cβ1 appears to inhibit the release of mature transcripts from the nucleus, its effect is not complete; some mature transcripts accumulate in the cytoplasm. It is not clear why this is so. One possibility is that IVS1Cβ1 is not absolutely efficient in its capacity to prevent the release of mature mRNA to the cytoplasm. Another explanation is that after a lag time, mature transcripts are

permitted exit to the cytoplasm. What is the trigger that allows mature transcripts to exit? One possibility is that the presence of spliced IVS1$_{C\beta1}$ is absolutely necessary for the nuclear retention of mature transcripts; once IVS1$_{C\beta1}$ is degraded, mature mRNA is permitted release from the nucleus (the half-life of IVS1$_{C\beta1}$ is <3hr; Fig.8B). This hypothesis is consistent with time course studies which show that the presence of IVS1$_{C\beta1}$ is correlated with the retention of mature transcripts in the nucleus of transfected Hela cells (Fig.8B). If this hypothesis is correct, we suggest that the debranching and degradation of spliced introns, in general, might be a prerequisite for the nuclear export of mature transcripts. A defect in this step may result in a defective spliceosome which is incapable of releasing the products of the splicing reaction.

What is the physiological relevance for the retention of mature TCR-β transcripts in the nucleus?

What is the biological function of retaining mature TCR-β mRNAs in the nucleus? One possibility is that this allows defective TCR-β transcripts to be identified and down-regulated by a nuclear mechanism which our evidence suggests operates in T cells. Defective TCR-β transcripts are generated as a result of the normal process of DNA rearrangement during thymocyte ontogeny. Such rearrangement events combine Vβ, Dβ and Jβ elements to produce functional genes. However, since nucleotides are added and excised from the junctions of the rearranged gene segments in a random fashion, TCR-β genes are also commonly non-productively rearranged such that they are out-of-frame. Evidence indicates that defective transcripts derived from such out-of-frame genes are dramatically down-regulated (L.Q., M. C., and M. W.; unpublished observations). Evidence for this novel nuclear mechanism is as follows: First, the endogenous TCR-β1 gene in

SL12.4 cells is out-of-frame (M. W.; unpublished observations) and gives rise to precursor and mature transcripts that accumulate in the nucleus, not in the cytoplasm, unless treated with CHX (Qian et al., 1993). Second, this type of regulation is also exerted on transcripts derived from transfected out-of-frame TCR-β genes (M. W.; unpublished observations). In contrast, transfected *in-frame* constructs give rise to abundant mature TCR-β transcripts in the cytoplasm, regardless of the presence of CHX.

This nuclear down-regulatory mechanism may require a lag time to scan and inhibit the expression of defective (out-of-frame) TCR-β transcripts. Thus, if IVS1$_{C\beta1}$ delays the exit of TCR-β transcripts from the nucleus, this may allow the scanning mechanism to operate at maximum efficiency. In accordance with this model, I observed that removal of spliced IVS1$_{C\beta1}$, as a result of incubation with the metabolic inhibitor CHX, permitted defective mature TCR-β transcripts in SL12.4 cells to enter the cytoplasm (Fig.9. lower panel). It remains for future investigations to definitively determine if the presence of IVS1$_{C\beta1}$ plays a role in the scanning mechanism that recognizes defective TCR-β transcripts. The discovery of this unusual mammalian intron provides an opportunity to elucidate mechanisms that regulate intron debranching, stability, spliceosomeal disassembly and the regulation of the TCR-β gene expression .

Reference:

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Leverette, R.D., Andrews, N.T.and Maxwell, E.S.(1992) Mouse U14 snRNA is a processed intron of the cognate hsc 70 heat shock pre-messenger RNA Cell.71:1215-1221

Michaeli, T., Pan, Z. and Prives, C. (1988) Genes and Develop. 2:1012-1020.

Qian, L., Vu, M. N., Carter, M. and Wilkinson, M. (1992) A spliced intron which accumulates as a lariat in the nucleus of T cells Nucleic. Acids Res.20:5345-5350