Regulation of tissue-specific alternative splicing: exon-specific *cis*-elements govern the splicing of leukocyte common antigen pre-mRNA

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Tissue-specific alternative splicing is an important mechanism for controlling gene expression. Exons 4, 5 and 6 of the human leukocyte common antigen (LCA) gene are included in B cell mRNA but excluded from thymocyte mRNA by differential splicing. In order to study this tissue-specific alternative splicing, we constructed mini-genes that contain only a few of the LCA exons and the SV40 promoter. Mouse B cells and thymocytes were transfected with these mini-gene constructs and the structures of mRNAs were determined by primer extension analysis. The results show that the same primary transcript is spliced alternatively in B cells and thymocytes. This finding suggests that there is a tissue-specific *trans*-acting factor that regulates the alternative splicing of LCA pre-mRNA. By making various deletion mutants, *cis*-elements necessary for tissue-specific splicing were confined within the alternatively spliced exons and their immediate flanking intron sequences. Furthermore, linker scanning analysis shows that there are at least three distinct *cis*-elements within the LCA exon 4 sequence that are required for tissue-specific alternative splicing. Possible mechanisms of LCA alternative splicing are discussed.

Key words: CD45/linker scanning/lymphocyte/T200/thymocyte

Introduction

A detailed knowledge of the mechanism and regulation of splicing is fundamental to our understanding of gene expression. Splicing of conventional pre-mRNA has been shown to depend on the precise identification of splice sites and on the orderly joining of each and every exon (reviewed by Aebi and Weissmann, 1987). In alternative splicing, the precision is maintained, but the pre-mRNA can be spliced in more than one way, thus allowing a gene to encode multiple protein isoforms (reviewed by Leff *et al.*, 1986; Breitbart *et al.*, 1987). Many instances of alternative splicing are stochastic and not obviously regulated. Some other cases of alternative splicing appear to be the consequence of different pre-mRNA structures due either to alternative promoter usage (Nabeshima *et al.*, 1984) or alternative poly(A) site usage (Alt *et al.*, 1980; Early *et al.*, 1980). Regulated alternative splicing has been observed for some virus genes during the course of infection (Ziff, 1980). Splicing pathways of cellular genes can be different in

different sexes (Nagoshi *et al.*, 1988), in different tissues (Laski *et al.*, 1986; Laff *et al.*, 1987), or at different development stages (Breitbart and Nadal-Ginard, 1987). The human leukocyte common antigen (LCA) gene presents an ideal system to study the tissue-specific splicing, mainly due to the very clear difference in splice patterns between two tissue types, B lymphocytes and thymocytes.

The LCAs (CD45, also called T200 or B220) are high mol. wt (180–220 kd) cell surface glycoproteins expressed on essentially all leukocytes and are members of a putative protein tyrosine phosphatase family (Ralph *et al.*, 1987; Streuli *et al.*, 1987a, 1988a; Charbonneau *et al.*, 1988). The extracellular region of LCA molecules show marked polymorphism among different leukocyte lineages, as exemplified by the different mol. wts of LCAs on B cells (220 kd) and thymocytes (180 kd), as well as the expression of different epitopes on these cells. In peripheral blood T cells the pattern is more complex; at least four different mol. wt forms of LCA (180, 190, 205 and 220 kd) can be seen in T cells (Omary *et al.*, 1980; Woollet *et al.*, 1985; Lefrançois *et al.*, 1986; Rudd *et al.*, 1987; Terry *et al.*, 1988). The differential expression of various LCA isoforms have a functional importance to T cells, since human T4⁺ cells can be divided into two functionally distinct subpopulations based on the LCA isoforms expressed (Morimoto *et al.*, 1985a,b; Rudd *et al.*, 1987; Akbar *et al.*, 1988).

A single gene spanning >130 kb with 33 exons encodes all the LCA isoforms (Ralph *et al.*, 1987; Streuli *et al.*, 1987a; Hall *et al.*, 1988). Various LCA isoforms are generated by alternative splicing of three exons (exons 4, 5 and 6) that encode peptides near the N-terminus of LCAs (see Figure 1A). The inclusion of all three exons in the LCA mRNA produces the 220-kd isoform, while skipping of all three produces the 180-kd isoform. If these three exons are independently used, there will be potentially eight isoforms. Indeed, human cDNA sequences corresponding to five isoforms have been isolated (Ralph *et al.*, 1987; Streuli *et al.*, 1987a,b, 1988b) and cDNA sequences for a sixth isoform have been isolated from rodent cells (Barclay *et al.*, 1987; Saga *et al.*, 1987). Characterization of the LCA gene and cDNA structures suggest that there is no difference in the mRNA structure 3' of the differentially used exons. Although there are several transcription initiation sites, no correlation was observed between the use of an initiation site and a particular splice pattern (Hall *et al.*, 1988). Thus, with the human LCA gene, the same pre-mRNA appears to be spliced differently in distinct tissues, such as B lymphocytes and thymocytes. Consequently, one must postulate the existence of both *trans*-acting factors and *cis*-acting sequences that regulate the splice pattern of LCA pre-mRNA. Here we show that such *cis*-elements are localized within the differentially spliced exons. The results suggest

Fig. 1. Schematic diagrams of the human LCA gene, alternative mRNA splice pathways and the structures of LCA mini-gene constructs. (A) Exon–intron organization of the human LCA gene (Hall *et al.*, 1988). The top line represents the segment of the human genomic DNA that encodes the human LCA, with the positions and relative sizes of the 33 exons indicated by solid boxes. Exons 4, 5 and 6 are alternatively used to generate different LCA isoforms. Exon 16 encodes the transmembrane (TM) peptide. An enlargement of the 5' portion of the LCA gene (exons 2–9) are indicated by open boxes; not drawn to scale) is shown below the LCA gene along with the splice pathways giving rise to B cell and thymocyte mRNA. B cell mRNA includes exons 4, 5 and 6, while thymocyte mRNA skips exons 4, 5 and 6. (B) Maps of the plasmid vectors used in this work (see Materials and methods for details of the constructions). pSV-SP2 contains the SV40 promoter/enhancer region, the SV40 poly(A) attachment site and pSP65 vector sequences. pSV2neoSP contains the selectable neomycin resistance gene and was used to co-transfect cell lines with LCA mini-gene constructs, which carry no selectable marker. (C) pSV-mini-LCA constructs contain human LCA mini-genes inserted at the *SalI* site in the pSV-SP2 vector (see Materials and methods for details of the constructions). Thin lines, small boxes and large boxes indicate, respectively, segments derived from LCA introns, LCA exons and vector pSV-SP2. Constructs are drawn to scale with the positions of replacements and/or deletions as indicated. Numbers above small boxes indicate LCA exons. Numbers below some constructs indicate the lengths of the intron sequences flanking exon 6 or exon 4.

two alternative mechanisms for the function of the *cis*- and *trans*-factors.

Results

Experimental system

Human LCA gene expression is regulated in different tissues by alternative splicing (Ralph *et al.*, 1987; Streuli *et al.*, 1987a). However, this gene poses several difficulties in terms of the practical analysis of tissue-specific splicing. The dimension of the human LCA gene, i.e. 33 exons spanning > 130 kb (Hall *et al.*, 1988), and encoding a mRNA as large as 5.2 kb (Ralph *et al.*, 1987; Streuli *et al.*, 1987a) is intractable. Transcription of the human LCA gene initiates at two separate major sites as well as at several minor sites (Hall *et al.*, 1988). Furthermore, three exons (exons 4, 5 and 6) are involved in the alternative splicing (Ralph *et al.*, 1987; Streuli *et al.*, 1987a). We have, however, surmounted these difficulties by constructing abbreviated LCA genes (mini-LCA genes) that contain only one of the three alternatively spliced exons flanked by only one or two exons on either side. The mini-LCA genes have been placed in the transcription unit of early SV40 RNA so that the structure of precursor mRNAs produced by this mini-LCA constructs

are well defined at the 5' and 3' ends (Figure 1C and Materials and methods).

To characterize the structures of mRNAs produced from these mini-LCA genes, the murine pre-B cell line 300-19 and murine thymocyte line EL4 were co-transfected with mini-LCA gene plasmids and the selectable pSV2neo-SP plasmid and geneticin-resistant clones were isolated. With the high mass ratio of mini-LCA gene DNA/pSV2neo-SP DNA (10:1), the majority of geneticin-resistant colonies expressed mini-LCA genes. For most of the experiments, we have characterized two independent, stably transfected clones for each combination of plasmid and cell and never observed any discrepancies in splicing patterns between the duplicate samples, although there were noticeable variations in the amounts of mini-LCA mRNA produced. Total cytoplasmic RNAs prepared from the stably transfected cells were hybridized to radioactively labeled oligonucleotide primers complementary to a segment of the individual exons included in the mini-LCA genes and extended using avian reverse transcriptase as described in Materials and methods. The primers were designed so that the endogenous murine LCA mRNA would not cross-hybridize. Moreover, primer extension analysis of RNAs isolated from cell lines transfected only with the selectable pSV2neo-SP plasmid

	Possible splice pathways	Lengths of primer extension products (nt)				Cells
		E3	E6	E7	E8	
mini-LCA1		E3 227 227	E6 253 -	E7 401 257	E8 474 330	B T, L
mini-LCA2		E3 227 227	E4 255 -	E7 455 257	E8 528 330	B T, L, 3T3, HeLa
mini-LCA4		E3 227 227	E9 260 -	E7 476 257	E8 549 330	B, T, L, 3T3, HeLa
mini-LCA7		E3 227 227	E6 255 -	E8 399 255		B T
mini-LCA11		E3 - -	E7 374 230	E8 447 303		B T
mini-LCA15 mini-LCA16		E8 372 228				B T
mini-LCA17 mini-LCA18 mini-LCA21		E8 426 228				B T
mini-LCA25		E8 331 228				B, T

Fig. 2. Summary of the primer extension analysis of mini-LCA gene alternative splicing. In the left column, the pSV-mini-LCA constructs are schematically drawn. The possible alternative splice pathways for each mini-LCA pre-mRNA are indicated below each construct. In the middle column, the expected nucleotide (nt) lengths of the primer extension products for each exon-specific primer (E3, E4, etc.: see Materials and methods) are given for either of the two possible splice pathways. Only the lengths of the extension products corresponding to the more 5' SV40 promoter start site are given (see Results). A dash (-) signifies that no primer extension product is expected. In the right column, are indicated the cell types that were shown experimentally to splice the mini-LCA pre-mRNAs in one of the two alternative pathways. B, B cell (300-19); T, thymocyte (EL4); L, L cell; 3T3, NIH-3T3 cell; HeLa, HeLa cell.

showed the levels of non-specific primer extension products. By measuring the lengths of the extended fragments, we deduced the usage of individual LCA exons and the structures of the mRNA encoded by the mini-LCA genes. Figure 2 shows schematic structures of mRNA produced from mini-LCA genes and the expected lengths of primer extension products.

A mini-LCA gene faithfully reproduces tissue-specific splicing

To examine the feasibility of using LCA mini-genes to analyze LCA tissue-specific splicing, we transfected 300-19 and EL4 cells with pSV-mini-LCA1 DNA, which contains the human LCA exons 2, 3, 6, 7 and 8 (Figure 1C). Cytoplasmic RNA isolated from transfected cells was hybridized to end-labeled E8 oligonucleotide which is complementary to a segment of exon 8 and then extended by reverse transcriptase. If exon 6 is included in the mature mRNA, the extension fragment will be 474 nt long. On the other hand, if exon 6 is not included in the mature mRNA, the extension fragment will be 330 nt long (Figure 2). There will also be extension fragments that are several nucleotides shorter due to the second initiation site of the SV40 promoter (Benoist and Chambon, 1981). For the sake of simplicity however, we will refer only to the lengths of the longer mRNAs in the following experiments. As is seen in Figure 3A, lanes 3–6, the lengths of the primer extension fragments are consistent with the interpretation that mini-

LCA1 mRNA made in B cells contain exon 6 sequence, whereas mini-LCA1 mRNA made in the thymocytes contains very little or no exon 6 sequence.

To confirm that the other exons are properly spliced in these mRNAs, primer extension analysis were carried out using primers complementary to segments of exons 3, 6 or 7. The results obtained with the exon 3 specific primer E3, demonstrated that in both B cell and thymocyte mRNAs, exon 3 was properly spliced to exon 2. (Figure 3B, lanes 3–6: if the splicing between exons 2 and 3 is proper, a 227-nt extension product is expected. A non-specific extension product of ~550 nt is present in all samples including the control RNA from pSV2neo-SP transfected cells.) These results also confirmed that the SV40 promoter was used to initiate the mini-LCA gene transcription in both cell types. The results with the exon 7 specific primer, E7, were essentially the same as those of the E8 primer experiment, except that the extension fragments were, as expected, shorter by 73 nt in both B cell and thymocyte mRNA (data not shown). Finally, the exon 6 specific primer produced, as expected, a 253-nt extension product from B cell mRNA but essentially no extension product from thymocyte mRNA (data not shown). Thus, the mini-LCA1 construct faithfully reproduced the tissue-specific alternative splicing, in spite of the use of cells from a heterologous species and in spite of the deletion of most of the LCA gene.

Neighboring exons have no effect on LCA exon 4 and 6 alternative splicing

In a broad sense, there are two classes of models that would explain the regulation of alternative splicing. The first class, which might be called the duo-exon models, assumes that the exons flanking the differentially used exons have particular affinity to each other. In the duo-exon models, the alternative splicing is regulated by modulating the affinity of the flanking exons. A similar mechanism has been proposed previously (Leff *et al.*, 1987). The second class of models, which we will call the solo-exon models, assumes that the sequences within and around the differentially spliced exons contain all the information necessary for the regulation of alternative splicing. The prediction of the duo-exon models is that any exon placed in between LCA exons 3 and 7 will be included in B cell mRNA but excluded from thymocyte mRNA, while deletion of exon 3 and/or exon 7 will abolish alternative splicing. On the contrary, the solo-exon models predict the complete opposite: deletion of exon 3 or exon 7 will have no effect on the alternative splicing of exon 6.

In order to examine these two classes of models of tissue-specific alternative splicing, we constructed deletion and substitution mutants of pSV-mini-LCA1 (Figure 1C). pSV-mini-LCA2 has exon 4 (including ~1200 bp and ~600 bp of flanking intron sequences) in place of exon 6 and pSV-mini-LCA4 has exon 9 (with ~700 bp and ~600 bp of flanking intron sequences) in place of exon 6. The human LCA exon 4, like exon 6, is used in a tissue-specific manner, while exon 9 is invariably incorporated into the mature LCA mRNA. pSV-mini-LCA7 and pSV-mini-LCA11 were made from pSV-mini-LCA1 by deleting exon 7 or exon 3 respectively. The primer extension analysis of RNA made from cells transfected with these constructs (Figure 3: see also Figure 2 for the expected fragment lengths) unequivocally demonstrated that exon 6 is alternatively spliced in B cells and in thymocytes even when the neighboring exons are deleted.

Fig. 3. Primer extension analysis of mini-LCA mRNAs. Cytoplasmic RNAs isolated from cells transfected with the pSV-mini-LCA constructs (see Figure 1C) or the control pSV2neo-SP construct alone, were assayed using end-labeled exon-specific primers (E3, E4, E6, E7, E8 and E9: see Materials and methods for sequences) and analyzed by electrophoresis on a 6% denaturing polyacrylamide gel. Primer extension products using the E8 and E3 primers are shown in panels A and B respectively. Each lane is marked at the top with the mini-LCA construct followed by the cell type into which it was transfected. Results with the E4, E6, E7 or E9 primers were all consistent with the interpretation shown in Figure 2, but are not shown because of space limitation, B, B cell (300-19); T, thymocyte (EL-4). Markers in lanes 1 and 2 are *AluI*-digested pSP65 and *DdeI*-digested pSP64 respectively, and their lengths (nt) are shown to the left.

Thus exon 6 was spliced to exons 3 and 8 (mini-LCA7: Figure 3A, lanes 11–14; and 3B, lanes 11–14) or exons 2 and 7 (mini-LCA11: Figure 3A, lanes 15–18; and 3B, lanes 15–18) in B cells, but was skipped in thymocytes. The replacement of exon 6 with exon 4 (pSV-mini-LCA2) demonstrated that the exon 4 sequence was also spliced in a tissue-specific manner, although some mRNA containing exon 4 sequence was detectable even in thymocyte RNA (Figure 3A, lanes 21–24; and 3B, lanes 23–26). A mini-gene construct with exon 5 did not give a clear tissue-specific splicing pattern (data not shown). This result, however, is consistent with the previous finding that the usage of exon 5 in T cell lines is less well regulated than exon 4 or 6 (Streuli *et al.*, 1987a). The replacement of exon 6 with exon 9 (pSV-mini-LCA4) resulted in the inclusion of the exon 9 sequence in both B cell mRNA and thymocyte mRNA (Figure 3A, lanes 7–10; and 3B, lanes 7–10). These results indicate that essential *cis*-information required for LCA tissue-specific splicing is confined to the sequences in and around the alternatively spliced exons themselves and that the flanking exons are not important. Thus, regulation of LCA tissue-specific alternative splicing is consistent with the solo-exon class of models.

Non-hematopoietic cells splice out exons 4 and 6

Because the same precursor mRNA is spliced in alternative pathways in B cells and thymocytes, one must assume that a *trans*-acting element as well as *cis*-acting elements are

necessary for LCA tissue-specific splicing. The *trans*-acting element may be a positive regulatory factor that allows the usage of otherwise inactive exons, or conversely the *trans*-acting element could be a negative regulatory factor that inhibits the usage of otherwise active exons. Either B cells have a positive regulatory factor, or thymocytes have a negative regulatory factor. Therefore, we examined the splicing pattern of mini-LCA genes in non-hematopoietic cells, namely mouse NIH-3T3, human HeLa and mouse L cells. Given that these cells do not express the endogenous LCA gene, it would seem unlikely that these cells express the *trans*-acting factor that regulates alternative splicing of LCA pre-mRNA. However, of course, it is also possible that the same *trans*-factor regulates splicing of other genes.

The results of the primer extension analysis using non-hematopoietic cells are shown in Figure 4. In the non-hematopoietic cells tested, exon 4 (and exon 6 in one case tested) was excluded from the mini-LCA gene mRNA, although in HeLa cells a small amount of mRNA including exon 4 sequence was observed. Exon 9 was always included in the mRNA. Thus, the three non-hematopoietic cells we tested showed essentially the same phenotype as thymocyte (see Discussion).

Cis-acting elements are located within the alternatively spliced LCA exons and/or their immediate flanking regions

The results from the previous sections indicated that the *cis*-

Fig. 4. Splicing of mini-LCA pre-mRNAs in non-hematopoietic cells. RNA isolated from the mini-LCA gene transfected, non-hematopoietic cell lines, HeLa, NIH-3T3 (3T3) and L cells, and from the hematopoietic cell lines 300-19 (B) and EL4 (T), were assayed by primer extension analysis as in Figure 3 using the exon 8 specific primer, E8.

acting elements are located in or near exons 4 and 6. In order to locate further these *cis*-elements, we constructed a series of mini-LCA genes that contain only exons 2, 4 and 8, or exons 2, 6 and 8. pSV-mini-LCA15 and 16 contain exon 6 with limited lengths of adjoining intron sequences, while pSV-mini-LCA17, 18 and 21 contain exon 4 with limited lengths of adjoining intron sequences (see Figure 1C). pSV-mini-LCA25 is essentially identical to pSV-mini-LCA18, except for a 95-bp deletion located within exon 4. These plasmid DNAs were also used to transfect the B-cell line, 300-19, or the thymocyte line, EL4, and RNA was analyzed by primer extension experiments using the exon 8 specific primers, E8. If mini-LCA mRNAs do not contain exon 4 or 6, the primer extension products will be 228 nt long, whereas if the mRNAs contain either exon 6, intact exon 4 or exon 4 with an internal deletion, the lengths of the extension products will be 372, 426 or 331 nt respectively (see Figure 2).

As is shown in Figure 5, pSV-mini-LCA15, 16, 17, 18 and 21 are still tissue-specifically spliced. mRNA made in B cells invariably contained exon 4 or 6, while the majority of mRNA made in thymocyte skipped exon 4 or 6. As was noted earlier, some mRNA that includes exon 4 or 6 sequence is observed even in thymocytes. From the results of pSV-mini-LCA15 and 16, it can be concluded that essential *cis*-information required for alternative splicing of exon 6 is confined in a 454-bp sequence (140 bp from the 3' end of intron 5 near the 3' splice site, 144 bp of exon 6 and 170 bp from the 5' end of intron 6). Similarly, the results of pSV-mini-LCA17, 18 and 21 confine essential *cis*-

Fig. 5. The effect of intron or exon deletions on mini-LCA pre-mRNA splicing. mRNAs derived from mini-LCA genes (see Figure 1C) with limited exon 4 or 6 flanking intron sequences, or with an internal exon 4 deletion, were assayed by primer extension analysis as in Figure 3 using the exon 8 specific primer, E8.

elements for exon 4 in a 293-bp sequence (75 pb from the 3' end of intron 3, 198 bp of exon 4 and 20 bp from the 5' end of intron 4) (see Streuli *et al.*, 1987a for sequences). The results obtained with pSV-mini-LCA25 is important, because this construct was derived from pSV-mini-LCA18 by deleting sequences within exon 4. In mini-LCA25, nucleotide positions 65–159 of exon 4 are deleted from mini-LCA18 (Figure 1C). mRNA derived from the mini-LCA25 construct included the (partly deleted) exon 4 sequence, whether RNA was derived from B cells or thymocytes (Figure 5, lanes 23–26). Therefore, these results indicate that *cis*-elements necessary for the tissue-specific splicing are confined within exon 4 or 6, and their immediate flanking regions.

Multiple distinct sequences within LCA exon 4 are necessary for tissue-specific splicing

The results in the preceding section suggested that the sequences within exon 4 are important for the tissue-specific splicing of the LCA pre-mRNA. However, since pSV-mini-LCA25 has a large deletion within exon 4 (nearly half of exon 4 is deleted) the effect could be either due to a removal of specific sequences or due to non-specific shortening of the exon size. To distinguish between these possibilities, and to define further the sequences in exon 4 that are essential for tissue-specific splicing, we dissected the exon 4 sequence by the linker scanning method (McKnight and Kingsbury, 1982). In each linker scanning construct (LS1–LS18), a 10-bp *Clal* linker sequence (CCATCGATGG) substituted 9–17 bp within various segments of the exon 4 sequence (Figure 6: see Materials and methods for the details of the

constructions). Therefore, the changes in the size of exon 4 were kept minimal. The linker scanning plasmid DNAs were used to transfect pre-B cells (300-19) and thymocytes (EL4). For each combination of cell and plasmid DNA, six independent, geneticin-resistant colonies were pooled and cytoplasmic RNA was extracted. The structures of the mini-LCA mRNAs were examined by primer extension analysis using the exon 8 specific primer, E8. The results of this analysis (Figure 7: summarized in Table I) demonstrated that any linker substitution mutation within exon 4 did not affect the ability of exon 4 to be included in the mRNA made by B cells. However, several linker scanning mutants lost their ability to be alternatively spliced. For example, LS1 pre-mRNA (substitution between nucleotide positions 8 and 17 of exon 4) has lost its ability to be alternatively spliced. On the contrary, LS2 pre-mRNA, which has substitutions between nucleotide positions 11 and 24, still retained its full activity to be alternatively spliced. Thus, nucleotide positions 8–10 of exon 4 are part of a *cis*-acting element that affects the tissue-specific splicing of the human LCA gene. In a similar fashion, two other segments of exon 4, namely nucleotide positions 40–91 and 127–137, are essential for the tissue-specific splicing. It should be noted that some of the linker scanning mutants, which have no gross change in the length of exon 4 (LS5 and LS6), nevertheless lost their tissue specificity in splicing. Therefore, specific sequences within exon 4, rather than the length of the exon, is the

Fig. 6. Exon 4 linker scanning mutants. (A) The pSV-mini-LCA18 construct is schematically shown at the top with the exon 4 segment enlarged underneath. The lengths and positions of the nucleotides replaced in mutants are represented by solid bars. Numbers in parentheses to the left of each mutant indicate the 5' and 3' endpoints (inclusive) of the deletions that were substituted with the 10-bp *Clal* linker (CCATCGATGG). The position of the exon 4 deletion in pSV-mini-LCA25 is also indicated by a solid bar. The segments in 4 that were shown to be essential for the alternative splicing are indicated by shading. (B) The nucleotide sequence of LCA exon 4 (upper case letters) and flanking introns (lower case), which was shown to contain essential information for tissue-specific alternative splicing.

Fig. 7. The effect of exon 4 linker scanning mutations on tissue-specific splicing. (A) mRNA isolated from cells transfected with the exon 4 linker scanning mutant constructs LS1–LS9, and (B) from the LS10–LS18 constructs (Figure 6), as well as the mini-LCA18 and control pSV2neo-SP constructs, were assayed by primer extension analysis as in Figure 3 using the exon 8 specific primer, E8. LS1–LS18 constructs were derived from the mini-LCA18 construct as described in Materials and methods. The extension products corresponding to the mini-LCA mRNA without exon 4 sequences appear as a doublet at 228 nt, whereas extension products corresponding to the mini-LCA mRNA with exon 4 sequences appear at ~426 nt (slightly variable because of the size differences of exon 4 in linker scanning mutants). B, B cell (300-19); T, thymocyte (EL4).

crucial factor determining tissue-specific splicing of LCA pre-mRNA.

Discussion

Cis-acting elements

We demonstrated that essential *cis*-acting elements regulating tissue-specific alternative splicing of the human LCA pre-mRNA are located within the differentially used exons and their immediate flanking sequences, consistent with the solo-exon class of models. Furthermore, by linker scanning analysis, we found that at least three distinct sequences within exon 4 are necessary for the tissue-specific splicing. Effects of exon sequences on splicing have been described previously (Somasekhar and Mertz, 1985; Reed and Maniatis, 1986; Mardon *et al.*, 1987; Cooper *et al.*, 1988; Kakizuka *et al.*, 1988), although in none of these cases were the effects of exon sequences on tissue-specificity of splicing studied. From the sequence data alone, it is not obvious how the exon sequences might affect splicing. Extensive secondary structures around the splice consensus sites have been demonstrated to affect the efficiency of splicing either *in vivo* or *in vitro* (Solnick, 1985; Solnick and Lee, 1987; Eperon *et al.*, 1988). However, we can not find any stable secondary structure within the region found to be sufficient to confer

the tissue-specific splicing (454 bp in and around exon 6 and 293 bp in and around exon 4). Nevertheless, one cannot exclude the possibility that apparently very weak secondary structures are stabilized *in vivo* by interactions with other factors such as spliceosome protein and/or ribonucleoprotein. Because both exon 4 and exon 6 can be spliced in a tissue-specific manner, it might be possible to identify essential sequences by comparing their sequences. Comparison of the sequences in and around exon 4 and exon 6 disclosed pairs of similar sequences (see Table II). On a statistical basis, the significance of these sequence similarities may be marginal at best. However, it should be noted that the sequences from exon 4 are located within one of the critical segments identified by linker scanning analysis. The functional significance of these sequences, whether they are binding sites for one or more *trans*-acting factors, or important for secondary structure formation, or a combination of the two, will require further study.

Trans-acting factor

Our results confirm previous conjecture (Hall *et al.*, 1988) that the tissue-specific splicing of the human LCA pre-mRNA is independent of the structure of the primary transcripts. The mini-LCA constructs use the only available promoter and one poly(A) attachment site, both derived from the SV40 genome (Figure 3B). Furthermore, our results with mini-LCA genes show that most of the human LCA gene, including the promoter region and poly(A) attachment site, is irrelevant to the tissue-specific splicing. Insofar as the same precursor mRNA is spliced differently in B cells and thymocytes, one must assume the existence of a *trans*-acting factor that modulates the splicing pattern. The involvement of *trans*-acting factors in differential splicing has been proposed previously (Leff *et al.*, 1986; Breitbart *et al.*, 1987). As for the chemical nature of the *trans*-acting factor, a diffusible factor, such as a protein or nucleoprotein, is the most obvious candidate, although the relevance of differences in pH and/or salt concentration (Schmitt *et al.*, 1987) cannot be excluded. In the nucleus, precursor mRNA forms heterogeneous nuclear ribonucleoprotein (hnRNP) by complexing with >20 different proteins (Beyer *et al.*, 1977; Pinol-Roma *et al.*, 1988). Some hnRNP proteins have been implicated in pre-mRNA splicing (Choi *et al.*, 1986; Mayrand *et al.*, 1986). Thus it is possible that some of the hnRNP proteins are involved in tissue-specific alternative splicing. Whatever the nature of the *trans*-acting factor, it must be evolutionarily conserved, because the mouse factor can operate properly on a human LCA pre-mRNA.

Three non-hematopoietic cell lines (HeLa, NIH-3T3 and L cells) that do not express endogenous LCA gene process

Table I. Summary of the effects of exon 4 linker scanning mutations on alternative splicing

Gene constructs	mini-LCA18	LS1	LS2	LS3	LS4	LS5	LS6
PAS in B cell	—	—	—	—	—	—	—
PAS in thymocyte	70	—	90	15	45	—	—
Gene constructs	LS7	LS8	LS9	LS10	LS11	LS12	LS13
PAS in B cell	—	—	—	—	—	—	—
PAS in thymocyte	—	—	—	35	15	35	30
Gene constructs	LS14	LS15	LS16	LS17	LS18		
PAS in B cell	—	—	—	—	—		
PAS in thymocyte	—	65	90	80	15		

The percentage of mini-LCA mRNA that does not contain exon 4 sequences is given for either B (300-19) or thymocyte (EL4) cells transfected with the linker scanning constructs LS1–LS18, and for the parental mini-LCA18 construct. Primer extension bands corresponding to mini-LCA RNAs excluding (EX; a doublet at 228 nt) or including (IN; at 426 nt) exon 4 were quantitated by densitometric scanning of the autoradiographs shown in Figure 7 as described in Materials and methods. The percentage alternative splicing (PAS) was calculated by the following formula, and rounded to the nearest 5%:

$$\text{PAS} = 100 \times \text{EX}/(\text{EX} + \text{IN}).$$

— undetectable.

Table II.

Exon 4 (45–72)	C	C	C	T	T	A		C	C	T	A	C	T	C	A	C	A	C	C	A	C	T	G	C	A		T	T	C	T
Exon 6 (67–95)	C	C	C	T	C	A	G	C	C	T	T		G	C	A	C	A	C	C	A	C	A	G	C	T	C	T	G	C	T
Exon 4 (46–64)	C	C	T	T	A	C	C	T	A	C	T	C	A	C	A	C	C	A	C											
Exon 6 (97–115)	C	C	T	T	A	C	C	T	G	C	A	C	G	C	A	C	C	T	C											
Exon 4 (68–79)	G	C	A	A	G	C	A	C	C	T	T	T																		
Exon 6 (24–35)	G	C	C	A	G	C	A	C	C	T	T	T																		

Asterisks indicate identical nucleotides.

pre-mRNA of mini-LCA genes the same way as thymocytes (EL4). If the *trans*-acting factor is a diffusible factor, these results might suggest that B cells rather than thymocytes have the *trans*-acting factor. However, the alternative possibility that thymocytes rather than B cells have the *trans*-acting factor cannot be excluded, because the same *trans*-acting factor might be used to regulate the splicing of other genes that happen to be expressed in the non-hematopoietic cells used in this study.

During the differentiation of T cells, the splicing pattern of the LCA gene changes in a complex manner. Thymocytes express an LCA encoded by mRNA that lacks exons 4, 5 or 6 (Morimoto *et al.*, 1985a; Woollett *et al.*, 1985; Lefrancois *et al.*, 1986; Streuli *et al.*, 1988b). As thymocytes mature, a subset of T cells develop that express LCA encoded by mRNA that includes exons 4, 5 and 6. Upon stimulation of the latter cells with either antigens or ConA, the expression of LCA mRNA containing exons 4, 5 or 6 is again down-regulated, while the expression of LCA mRNA without these exons is up-regulated (Takeuchi *et al.*, 1987; Clement *et al.*, 1988). These developmental changes can now be interpreted in terms of the induction or repression of the expression of specific *trans*-acting splice factors.

Models

There are several possible variations of the solo-exon class of models that will explain the tissue-specific splicing of the human LCA pre-mRNA. We propose two alternative models that we believe are more plausible than others. The first model, the positive regulation model, assumes a positive *trans*-acting factor expressed in B cells but not in thymocytes, NIH-3T3, HeLa or L cells. With this model, the alternatively used exons are inactive as such: the basic splicing machinery alone cannot recognize the exons and skips them. The B-cell-specific *trans*-acting factor interacts with the pre-mRNA and/or spliceosome to allow the inclusion of the exons. Nevertheless the alternatively used exons are apparently not defective, because their sequences conform to the standard consensus splice site sequence (Shapiro and Senapathy, 1987; Streuli *et al.*, 1987a; Hall *et al.*, 1988). Furthermore, small changes (i.e. linker scanning mutations) within exon 4 convert it to a constitutively used exon. Therefore, it seems that sequences within exon 4 (and probably exon 6) somehow interfere with the usage of these exons by the splicing machinery, perhaps by interacting with other nuclear components and/or by forming secondary structures. The B-cell-specific factor overrides the effect of the exon sequences. The deletion and substitution mutations eliminate the inhibitory sequences from the exon and make the usage of exon 4 independent of the B-cell-specific factor.

The second model, the negative regulation model, assumes that a negative *trans*-acting factor is expressed in thymocytes and in several non-hematopoietic cells, but not in B cells. With this model, we assume that the differentially used exons are normal in all aspects of splicing, except that they can be bound by a regulatory factor that interferes with the usage of these exons by the spliceosome. According to this model, the *cis*-elements within exon 4 (and 6) are binding sites for the inhibitory *trans*-acting factor. Deletion or substitution mutations in exon 4 eliminate the binding site for the negative factor and convert exon 4 into a constitutive exon.

At present, we cannot choose between these two variations

of the solo-exon model. The analysis of non-hematopoietic cells favors the positive regulatory model. However, the negative regulatory model benefits from its simplicity. Furthermore, there is no simple explanation for the fact that various degrees of inclusion of the exon 4 or 6 sequence in mRNA occurs for some mini-LCA constructs even in thymocytes. Perhaps, a more subtle and complex modification to the models will emerge from further work. To resolve these problems, and to establish the nature of the *trans*-factor, use of lymphocyte-derived cell-free splicing system is essential. Whichever model turns out to be correct, the data presented here demonstrate that multiple *cis*-elements located within the alternatively spliced exons and a tissue-specific *trans*-acting factor are required for regulation of LCA gene expression.

Materials and methods

Buffers and media

Tris-saline: 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl. Buffer A: 0.32 M sucrose, 10 mM Tris-HCl (pH 7.4), 3 mM MgCl₂, 250 µg/ml heparin, 1 mM spermidine. Kinase buffer: 50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 5 mM DTT, 0.1 mM spermidine-HCl and 0.1 mM EDTA. Pre-PE buffer: 10 mM Tris-HCl (pH 8.0), 100 mM NaCl and 1 mM EDTA. PE buffer: 40 mM Tris-HCl (pH 8.0), 8 mM MgCl₂, 5 mM DTT and 0.5 mM each of dATP, dCTP, dGTP and dTTP. TBE buffer: 50 mM Tris base, 50 mM boric acid and 1 mM EDTA (pH 8.0). TE buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. TNE buffer: 50 mM Tris-HCl (pH 7.5), 100 mM NaCl and 5 mM EDTA. Sequencing dye buffer: 80% (v/v) formamide, 1 × TBE, 0.1% (w/v) bromophenol blue and 0.1% (w/v) xylene cyanide FF. Medium A: RPMI medium (Gibco), 10% fetal calf serum (HyClone Laboratories), 50 µM 2-mercaptoethanol, 2 mM L-glutamine, and 50 µg/ml gentamicin sulfate (Whittaker MA Bioproducts).

Plasmid vectors

The plasmid vectors used in this work, pSV-SP2 and pSV2neo-SP were constructed from segments of pSV2neo (Southern and Berg, 1982) and pSP65 (Meltan *et al.*, 1984) via several intermediates. pSV-SP2 is composed of, in this order: 342-bp *Pvu*II-*Hind*III fragment from pSV2neo containing the SV40 enhancer/promoter, the 32-bp *Hind*III-*Sma*I fragment from pSP65 containing multiple restriction sites including *Sal*I, the 884-bp *Hap*I-*Eco*RI fragment from pSV2neo, which contains the poly(A) attachment site for the early SV40 RNA and the 2777-bp *Eco*RI-*Pvu*II fragment of pSP65 containing *amp*^r and *ori*. The salient feature of pSV-SP2 is that the unique *Hind*III, *Xba*I and *Sal*I sites are located in between the SV40 promoter and the SV40 poly(A) attachment site. pSV2neo-SP was constructed, in effect, by replacing the 2295-bp *Eco*RI-*Pvu*II fragment in pSV2neo, which derives from pBR322, with the 2777-bp *Eco*RI-*Pvu*II fragment from pSP65. The use of the pSP65-derived replication origin, instead of that of the pBR322 origin, resulted in much better plasmid yields.

Mini-gene constructs

The prototype LCA mini-gene, pSP-mini-LCA1, was constructed by ligating the following DNA fragments together: the 1.4-kb *Bgl*II-*Sma*I fragment of pSP65, the 1.2-kb *Sph*I-*Hind*III fragment from the human LCA exon 2-intron 2 region (Hall *et al.*, 1988; the *Sph*I site is within exon 2 and was made blunt by T4 DNA polymerase in order to make it compatible with the *Sma*I site), the 3.9-kb *Hind*III-*Eco*RI fragment containing the human LCA exon 3 and its flanking regions (Hall *et al.*, 1988), the 18-bp *Eco*RI-*Bam*HI fragment from pSP65, the 2.85-kb *Bam*HI-*Eco*RI fragment containing the human LCA exons 6, 7 and 8 and their flanking regions (Streuli *et al.*, 1987a) and the 1.63-kb *Eco*RI-*Bgl*II fragment from pSP64. In the pSP-mini-LCA1 construct, the human LCA exons 2, 3, 6, 7 and 8 are included in an 8-kb fragment flanked at both ends by *Sal*I sites. This 8-kb *Sal*I fragment was inserted into the unique *Sal*I site in pSV-SP2 so that the orientation of the transcription from the SV40 early promoter coincides with the orientation of the LCA mini-gene. The resultant plasmid was named pSV-mini-LCA1. In pSV-mini-LCA1, the LCA exon 2 and the first exon of the SV40 large T gene are fused to make a hybrid exon of 201 nt. (The early SV40 promoter has another slightly weaker initiation site 4 bp downstream of the primary initiation site. The usage of the second initiation site results in an exon of 197 nt in length.) The other pSV-mini-LCA constructs

are derived from pSV-mini-LCA1, in many cases via intermediates, by deletion and/or sequence replacements (see Figure 1).

Oligonucleotides

Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer at the core molecular biology facility at the Dana-Farber Cancer Institute, and purified by acrylamide gel electrophoresis. The following six oligonucleotides complementary to indicated segments of the human LCA exons (Streuli *et al.*, 1987; Hall *et al.*, 1988) were used as primers for primer extension experiments:

- E3 AGTGGGGGAAGGTGTTGGGCTTTG (nucleotides 3–26 of exon 3)
 E4 CACTGGGCATCTTTGCTGTAGTCAATC (nucleotides 1–27 of exon 4)
 E6 GCTGTACTCCTCTCTCCTGGGACAT (nucleotides 1–25 of exon 6)
 E7 GGTGTTTCAGAGGCATTAAGGTAGGC (nucleotides 3–29 of exon 7)
 E8 CACATGTTGGCTTAGATGGAGTAGTAG (nucleotides 1–27 of exon 8)
 E9 GTAATCCACAGTGATGTTTGCATATTTTTC (nucleotides 3–32 of exon 9)

The following four oligonucleotides were used as hybridization probes and sequencing primers for the construction of linker scanning mutants:

- I3a CTATAGTGGACTGGGGAGTT (nucleotides –100 to –81 of intron 3)
 I3b GTCCTTCTCCATTTTCCAT (nucleotides –30 to –11 of intron 3)
 I4a GACATTCCCTGATATTTAAG (complementary to nucleotides 23–42 of intron 4)
 I4b CACATACTTAGAACTGTGC (complementary to nucleotides 90–109 of intron 4)

Clal linker, CCATCGATGG, used for linker scanning was purchased from New England Biolabs.

Construction of linker scanning mutants

Linker scanning mutants were constructed essentially according to McKnight and Kingsbury (1982). Briefly, pSV-mini-LCA18 (see Figure 1) was initially linearized with either *NdeI* or *BglII*. The unique *NdeI* site is located 5' of exon 4, while the unique *BglII* site is located 3' of exon 4. After limited digestion with *Bal31*, ends were made blunt with T4 DNA polymerase, and *Clal* linkers (CCATCGATGG) were added to the ends using T4 DNA ligase. DNA was digested with *Clal*, purified by agarose gel electrophoresis, covalently circularized by T4 DNA ligase and used to transform competent *Escherichia coli* DH1 (Hanahan, 1983). Plasmid DNAs from individual transformed cells were prepared by a modification of the alkali lysis method (Birnbom and Doly, 1979), denatured by boiling, bound to nitrocellulose membranes using the Hybri-Dot Manifold (BRL), and hybridized to either end-labeled oligonucleotide probe I3b or I4a. Plasmid DNAs that hybridized to one of these probes but not to the other had one of the deletion endpoints within or very near exon 4. These candidates were initially sequenced by the chain termination method of Sanger *et al.* (1977) using chemically modified T7 DNA polymerase (Sequenase™, US Biochemical) (Tabor and Richardson, 1987). Selected pairs of plasmid DNAs, one derived from *NdeI*-digested DNA and another from *BglII*-digested DNA, were digested with *Clal* and *NheI*. The *NheI*–*Clal* fragments from *BglII* plasmids that contain the 5' half of exon 4 were ligated to *Clal*–*NheI* fragments from *NdeI* plasmids that contain the 3' half of exon 4. Resultant plasmids, pSV-mini-LCA18-LS1 through -LS18 (in the text abbreviated as LS1–LS18), were sequenced to confirm the structure around the *Clal* linker.

Transfections

The Abelson virus-transformed, murine pre-B lymphocyte line, 300-19 (Alt *et al.*, 1984), murine thymocyte line EL4, L cells, HeLa cells and NIH-3T3 cells were grown in medium A. Cells were transfected by electroporation (Chu *et al.*, 1987) with the mini-gene construct plasmids using the Cell-Porator Electroporation System (Bethesda Research Laboratories) essentially as described by the manufacturer. Mini-LCA constructs and pSV2neo-SP were linearized by digestion with *PvuI* before transfection. The parameters for electroporation were ~50 µg mini-LCA plasmid DNA and ~5 µg pSV2neo-SP DNA/5 × 10⁶ cells/0.5 ml DME medium (Gibco) at 0°C, 1.6 millifarads, 250 V/0.4 cm and using the high ohm setting. Transfected cells were selected in medium A supplemented with Geneticin (Gibco) (2.0 mg/ml) for 300-19, 0.25 mg/ml for EL4, 0.75 mg/ml for L cells, 1.0 mg/ml for HeLa cell and 0.5 mg/ml for NIH-3T3 cell). Geneticin-

resistant clones were maintained in medium A supplemented with Geneticin (0.5 mg/ml for 300-19, L cell and HeLa cell, and 0.25 mg/ml for EL4 and NIH-3T3).

Isolation of cytoplasmic RNA

Cells were washed twice with Tris-saline and resuspended in Buffer A at ~10⁷ cells/ml. Cells were lysed by adding NP40 to a final concentration of 0.5%. After a 5-min incubation on ice, lysed cells were centrifuged at 1000 g for 5 min in a Beckman JS4.2 rotor. Supernatants were adjusted to 0.1% SDS and 10 mM EDTA and extracted with phenol/chloroform once at 65°C and twice at 20°C. Extracted RNA was precipitated with ethanol and redissolved in H₂O.

Primer extensions

Oligonucleotide primers were 5' end-labeled in a 50-µl reaction containing 5 pmol oligonucleotide, 5 µl of 10 × kinase buffer, 0.15 mCi [³²P]ATP (7000 Ci/mmol, New England Nuclear) and 20 U of T4 polynucleotide kinase (US Biochemical). After incubation at 37°C for 30 min, labeled oligonucleotide was purified by Sephadex G-25 (Pharmacia Fine Chemical) column chromatography. The specific activities of the oligonucleotide primers were ~5 × 10⁶ c.p.m./pmol. The end-labeled primer was hybridized to mRNA in a 50-µl reaction containing 25 µg of cytoplasmic RNA, 5 µl of 10 × pre-PE buffer and 0.15 pmol of end-labeled primer. The mixture was heated to 70°C for 10 min, cooled gradually (~4 h) to 42°C and incubated at 42°C for 12 h. Extension of the primer was initiated by the addition of 6.25 µl of 10 × PE buffer, 0.78 µl RNasin (40 U/µl, Promega), 1.56 µl avian myeloblastosis virus reverse transcriptase (20 U/µl, Seikagaku America), 0.625 µl Actinomycin D (1 mg/ml) and 3.3 µl H₂O. The 62.5-µl reaction mixture was incubated at 42°C for 60 min. The reaction was stopped by adding 10 µl of 10 × TNE and 27.5 µl of 25 µg/ml sonicated denatured salmon sperm DNA. Nucleic acids were extracted with phenol/chloroform, precipitated with ethanol and redissolved in 50 µl TE containing 40 µg/ml RNase A. After incubating at 37°C for 15 min, 50 µl 2 × TNE was added. Nucleic acids were again extracted with phenol/chloroform, precipitated with ethanol and dissolved in 2 µl sequencing dye buffer. Samples were heated to 90°C for 2 min, quickly chilled on ice and 1 µl of the samples were electrophoresed through a 6% polyacrylamide gel (acrylamide:bisacrylamide = 20:1, 8 M urea, 2 × TBE buffer). Autoradiography was at –80°C for 2–7 days with an intensifying screen. Appropriate primer extension bands were quantitated by densitometric scanning of autoradiographs using an LKB laser densitometer (model Ultrascan XL).

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