Coupling of signal transduction to alternative pre-mRNA splicing by a composite splice regulator

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Alternative splicing of pre-mRNA is a fundamental mechanism of differential gene expression in that it can give rise to functionally distinct proteins from a single gene, according to the developmental or physiological state of cells in multicellular organisms. In the pre-mRNA of the cell surface molecule CD44, the inclusion of up to 10 variant exons (v1–v10) is regulated during development, upon activation of lymphocytes and dendritic cells, and during tumour progression. Using minigene constructs containing CD44 exon v5, we have discovered exonic RNA elements that couple signal transduction to alternative splicing. They form a composite splice regulator encompassing an exon recognition element and splice silencer elements. Both type of elements are necessary to govern cell type-specific inclusion of the exon as well as inducible inclusion in T cells after stimulation by concanavalin A, by Ras signalling or after activation of protein kinase C by phorbol ester. Inducible splicing does not depend on *de novo* protein synthesis. The coupling of signal transduction to alternative splicing by such elements probably represents the mechanism whereby splice patterns of genes are established during development and can be changed under physiological and pathological conditions.

Keywords: CD44/inducible splice regulator/minigene/
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Introduction

In multicellular organisms, alternative pre-mRNA splicing can give rise to functionally distinct proteins from a single gene. It can be regulated dependent on sex (Baker, 1989; Rio, 1993), developmental stage or tissue (Rio, 1993; Sharp, 1994; Wang and Manley, 1997) and in response to extracellular stimuli such as growth factors (Wang *et al.*, 1991; Shifrin and Neel, 1993; Metheny and Skuse, 1996; Fichter *et al.*, 1997), hormones (Kosaki and Webster, 1993; Chalfant *et al.*, 1995; Metheny and Skuse, 1996), cytokines (Mackay *et al.*, 1994; McKay *et al.*, 1994; Eissa *et al.*, 1996), extracellular pH (Borsi *et al.*, 1995) or membrane depolarization (Zacharias and Strehler, 1996). Alternative splicing is thus a fundamental mechanism of differential gene expression. Coupling of pre-mRNA splicing to extracellular signals is not only crucial for

altering splice patterns according to the physiological state of cells but it may also be important in establishing and changing splice patterns during development and cell differentiation. In contrast to *cis*-acting DNA elements in promoters and enhancers of genes that are able to regulate transcription in an inducible manner, signal-responsive RNA elements and the mechanism whereby alternative pre-mRNA splicing is linked to cell signalling are as yet unknown. We have addressed this important issue by investigating alternative pre-mRNA splicing of the cell surface molecule CD44, which is regulated both in a cell type-specific manner and in response to extracellular stimuli. Alternative splicing generates variant CD44 isoforms by the inclusion of up to 10 variant exons (v1–v10) during embryonic development and during tumour progression (reviewed in Sherman *et al.*, 1996), as well as upon activation of lymphocytes (Arch *et al.*, 1992; Koopman *et al.*, 1993; Mackay *et al.*, 1994; Screaton *et al.*, 1995) and dendritic cells (Weiss *et al.*, 1997). In an attempt to understand the mechanisms underlying the cell type-specific and inducible inclusion of CD44 variant exons, we have sought to identify splice regulatory elements in the CD44 pre-mRNA. We report here on the identification of a composite exonic splice control unit which combines an exon recognition element with splice silencer elements and which governs alternative splicing in a cell type-specific manner and in response to activation of protein kinase C (PKC) or Ras signalling pathways. The discovery of signal-responsive splice elements provides a link between extracellular signals and differential gene expression through alternative pre-mRNA splicing.

Results and discussion

The principle of the analysis

In a previous study, we showed by cell fusion experiments that alternative splicing of CD44 is controlled by *trans*-acting factors (König *et al.*, 1996). The CD44 gene spans ~50 kb, and multiple variant exons can be included by alternative splicing in various combinations (Screaton *et al.*, 1992; Tölg *et al.*, 1993). To explore how individual exons are selected and which sequence elements are targeted by *trans*-acting factors and influenced by extracellular stimuli, we reduced the complexity of the CD44 gene. To this end, a minigene construct was generated which carries only one variant exon plus surrounding intron sequences in an exon-trap vector. Our approach was first to transfect the minigene into cell lines which differ in CD44 variant exon inclusion in order to test whether the variant exon of the minigene is alternatively spliced in the same way as the variant exon of the endogenous CD44 gene. If so, mutation analyses of the minigene should then be used to define sequence elements which regulate alternative splicing. Two conditions were

to be met: the selection of suitable recipient cell lines and the choice of a variant exon for the minigene construct whose endogenous counterpart was under pronounced regulation and which, in the minigene context, was still alternatively spliced.

Cell type-specific and inducible alternative splicing of CD44 pre-mRNA

We chose a cell line that constitutively expresses CD44 variant isoforms, the murine carcinoma cell line KLN205, and a cell line which shows inducible expression of variant isoforms, the mouse T-lymphoma cell line LB17 2.3. As shown by exon-specific PCR analysis (see Figure 1A), the KLN205 carcinoma cell line expresses predominantly CD44 variant mRNAs (Figure 1B, lanes 11 and 12), including one RNA species that carries all variant exon sequences, v1–v10 (Figure 1B, lanes 1–10). In contrast, in the T-lymphoma line LB17 2.3, variant exons are skipped, giving rise almost exclusively to mRNA lacking variant exon sequences (Figure 1C, upper panel, lane 11). Expression of transcripts carrying single exon sequences of v1, v4, v7 or v10 were only weakly detectable (lanes 1, 4, 7 and 10). Inclusion of variant exons was, however, induced upon treatment of the LB17 cells with the phorbol ester 12-*o*-tetradecanoylphorbol-13-acetate (TPA) and the calcium ionophore ionomycin. Although representing only a fraction of total CD44 mRNA, transcripts with single exon sequences of either v1, v3, v4, v5, v7 or v10 were now easily detectable (Figure 1C, lower panel, lanes 1, 3–5, 7 and 10). In addition, one transcript occurred in which exon v4 is linked to an additional downstream exon (lane 4).

A minigene model for cell type-specific and signal-induced alternative splicing

For the minigene construct to be used for transfection into the two cell lines and for the subsequent identification of regulatory splice elements, we chose CD44 exon v5. We found this exon frequently to be included during tumour progression and upon T-cell activation, and its inclusion was tightly regulated by phorbol ester in the T-cell line to be used as one of the recipients (see Figure 1C). A part of the murine genomic CD44 region including exon v5 and intron sequences on either side was cloned into the exon-trap vector pL53In, between the pre-proinsulin exons 2 and 3 and their adjacent intron sequences (see Figure 2A). The insulin exons provided a means of distinguishing the minigene transcripts from the endogenous CD44 mRNA.

To test whether the CD44 exon v5 of the minigene was alternatively spliced according to exon v5 of the endogenous CD44 gene, the minigene construct was transiently transfected into the two cell lines which differ in CD44 v5 exon inclusion. Because of the low transfection efficiency of the cell lines, transcripts from the minigene constructs were analysed by RT-PCR. PCR primers were derived from the insulin exons (see Figure 2A). In the KLN205 carcinoma cells, the vast majority of the minigene mRNA included the CD44 exon v5 sequence, whereas in the LB17 T-lymphoma cell line exon v5 was predominantly absent (Figure 2B). Co-regulation of exon inclusion and exon skipping in the minigene mRNA and in the endogenous CD44 mRNA was also observed in human cell

Fig. 1. Cell type-specific and inducible inclusion of CD44 variant exons. (A) Simplified scheme of murine CD44 gene organization indicating the positions of the PCR primers (arrowheads) used. Open boxes, constant CD44 gene sequences; filled boxes, variant exons v1–v10 (Tölg *et al.*, 1993); lines, intron sequences. Endogenous CD44 transcripts were analysed by exon-specific RT-PCR in (B) mouse KLN205 carcinoma cells and (C) mouse LB17 2.3 T-lymphoma cells treated with TPA (40 ng/ml) plus ionomycin (0.4 µg/ml) for 20 h (TPA+Ion) or, as a control (C), with DMSO. Exon-specific PCRs were performed using the 5' primers specific for the variant exons indicated (lanes 1–10) and the constant region 5' primer c13 (lanes 11 and 12), respectively, in combination with the 3' C2A primer (König *et al.*, 1996). Lane 12 in (B) shows a longer exposure of lane 11. CD44 mRNA lacking all variant exons gives rise to a 594 bp PCR product. M = 123 bp ladder (Life Technologies). Asterisks denote an unspecific PCR product (König *et al.*, 1996). All PCR products were resolved on ethidium bromide-containing 2% agarose gels and visualized by UV irradiation.

lines: exon skipping in 293 embryonic kidney cells and in KG-1 acute myelogenous leukaemia cells versus exon inclusion in HaCaT keratinocytes or in HT-3 cervix carcinoma cells (data not shown). Thus, in the minigene pre-mRNA, the exon v5 sequence is included or skipped in a cell type-specific manner, similarly to the v5 sequence of the endogenous CD44 pre-mRNA. Furthermore, activation of the LB17 T-lymphoma cell line by TPA resulted

Fig. 2. Cell type-specific and inducible inclusion of CD44 exon v5 from a minigene construct upon transient transfection. (A) Scheme showing the minigene construct pETv5 (bottom) and the location of the included genomic CD44 sequences in the murine CD44 gene (Tölg *et al.*, 1993) (top). To reduce complexity, exon v4 was deleted. RSV, Rous sarcoma virus; MCS, multiple cloning site; SV40 PolyA, simian virus 40 polyadenylation sequence. Symbols for genomic CD44 region are as in the legend to Figure 1. Arrowheads indicate the positions of the primers used for RT-PCR in (B) and (C). Skipping of exon v5 results in a 244 bp PCR product, and v5 inclusion in a 361 bp product. After transient transfection, inclusion of exon v5 in minigene transcripts was analysed by RT-PCR of cytoplasmic RNA prepared from (B) KLN205 carcinoma cells and LB17 2.3 T-lymphoma cells and (C) LB17 2.3 T-lymphoma cells treated as indicated for 7 h. In lanes 12 and 13, the minigene construct was co-transfected with either an empty expression plasmid (RSV) or a plasmid expressing the activating Ha-ras mutant Leu61 (RSV Ha-ras) (Medema *et al.*, 1991). M, 123 bp ladder (Life Technologies); C, control (DMSO); TPA (40 ng/ml); Ion, ionomycin (0.4 µg/ml); An, anisomycin (100 µM); CHX, cycloheximide (100 µg/ml); CsA, cyclosporin A (1 µg/ml); ConA, concanavalin A (14 µg/ml).

in a marked enhancement of exon v5 inclusion (Figure 2C, lanes 1 and 2), again reflecting the splicing behaviour of the endogenous CD44 v5 exon. Inclusion of exon v5 was also induced by treatment of LB17 cells with concanavalin A (Figure 2C, lane 11), a lectin that triggers T-cell activation (Tsien *et al.*, 1982). Ionomycin, known to synergize with phorbol esters in T-cell activation (Truneh *et al.*, 1985), did not induce exon inclusion and did not enhance the degree of inclusion triggered by TPA treatment alone, neither in the minigene nor in the endogenous gene transcripts (Figure 2C, lanes 3 and 4; and data not shown). Consistent with this finding, cyclosporin A, which inhibits a calcium-dependent step in T-cell activation (Liu *et al.*, 1991), did not affect exon

inclusion upon treatment by TPA plus ionomycin (Figure 2C, lanes 9 and 10). We can thus conclude that the minigene is suitable for analysing both cell type-specific and signal-induced alternative splicing of CD44 exon v5.

Protein synthesis-independent activation of CD44 v5 exon inclusion

To investigate whether the activation of alternative splicing depends on *de novo* protein synthesis, we tested phorbol ester inducibility of v5 exon inclusion in the presence of the protein synthesis inhibitors anisomycin or cycloheximide. When applied alone, both inhibitors stimulated exon inclusion to some extent (Figure 2C, lanes 5 and 7). This could be due to activation of the mitogen-activated kinase (MAPK) and stress-activated kinase (SAPK) cascades by these inhibitors (Zinck *et al.*, 1995; Iordanov *et al.*, 1997). Additional treatment with TPA and ionomycin, however, resulted in markedly higher levels of v5 exon inclusion (Figure 2C, lanes 6 and 8), comparable with the level obtained after TPA/ionomycin treatment alone (Figure 2C, lane 4). Thus, neither inhibitor impaired phorbol ester-induced exon inclusion, suggesting that post-translational modifications of pre-existing splice regulatory factors rather than the expression of new factors are involved. Similarly, changes of splice patterns during neural induction (neural-specific c-src mRNA; Collett and Steele, 1993) and in response to elevated intracellular Ca^{2+} (plasma membrane Ca-ATPase; Zacharias and Strehler, 1996) do not require protein synthesis. In contrast, growth factor-induced generation of phosphotyrosine phosphatase PTP-1B-mRNA isoforms has been reported to depend on *de novo* protein synthesis (Shifrin and Neel, 1993).

Activation of alternative splicing by Ras

Phorbol esters are not physiological activators of T cells. Their action on exon inclusion indicates, however, that signalling pathways involving the phorbol ester target PKC (Castagna *et al.*, 1982) can regulate alternative splicing. PKC is known to be a downstream signalling molecule of the T-cell receptor and to activate $p21^{ras}$ in T cells (Valge *et al.*, 1988; Downward *et al.*, 1990). Activation of $p21^{ras}$ can also be achieved by PKC-independent mechanisms and seems to be a necessary step in T-cell activation (Cantrell, 1996). To test whether activation of $p21^{ras}$ can induce inclusion of CD44 exon v5, we transfected the minigene into LB17 cells together with either a plasmid expressing activated Ha-Ras or the empty expression vector. Figure 2C (lanes 12 and 13) shows that activated $p21^{ras}$ indeed induces v5 exon inclusion, indicating coupling of Ras signalling pathways to alternative splicing. Since mutational activation of *ras* genes is a frequent event in human tumours (Rodenhuis, 1992), Ras signalling may also account for the expression of variant CD44 isoforms observed during tumour progression of certain human tumours (Sherman *et al.*, 1996).

Cell type-specific and inducible splice regulatory elements in CD44 exon v5

The results described above suggest that regulatory sequences necessary for both cell type-specific and inducible splicing of CD44 exon v5 are present in the genomic CD44 sequences of the minigene construct. To identify these regulatory sequences, we generated deletion

Fig. 3. Sequences within CD44 exon v5 determine its inclusion. (A) Schematic representation of the sequence exchanges performed within exon v5 of the minigene constructs indicated. Black boxes, CD44 exon v5 sequences; grey box, polylinker sequence from pBluescript SK (Stratagene); hatched box, sequence from the bacterial ampicillin resistance gene (see Materials and methods). RT-PCR analysis upon transient transfection of the minigene constructs shown in (A) into (B) KLN205 carcinoma cells and (C) LB17 2.3 T-lymphoma cells which were either left untreated (–) or treated with TPA (+) for 7 h. M, 123 bp ladder (Life Technologies).

and linker-scan mutants. Deletion of intron sequences upstream or downstream of exon v5 (leaving 5' and 3' splice sites intact) did not affect splice regulation in KLN205 carcinoma cells (data not shown), suggesting that the CD44 intron sequences contain no elements necessary for v5 exon inclusion. We therefore tested the possibility that sequences within the alternatively spliced exon itself governed exon inclusion. With this reasoning, the CD44 v5 exon sequence was replaced by unrelated sequences of approximately the same length from either a polylinker region (v5blue) or the bacterial ampicillin resistance gene (v5amp; see Figure 3A). The four 5' most and the three 3' most base pairs of exon v5 were maintained in these substituted exons to leave the splice sites unchanged. (That the splice sites remain functional is demonstrated below by the linker-scan mutants ls1 and ls11 which are spliced efficiently; see Figure 4A and B.) Both substituted exons were no longer included in the

minigene mRNA, neither in KLN205 cells (Figure 3B) nor in TPA-treated LB17 cells (Figure 3C). Thus, exon v5 contains sequences necessary for both cell type-specific and inducible alternative splicing.

Linker-scan mutations define distinct regulatory elements in exon v5

To identify the sequences within exon v5 that govern alternative splicing, we generated linker-scan (ls) mutants by consecutively replacing 10 bp segments within the 117 bp v5 exon by a 10 bp linker sequence (Figure 4A). Again, the exon ends were left unchanged as in the substitution mutants. After transient transfection into KLN205 cells, mutations ls1–ls8 and ls10 did not impair exon v5 inclusion. Mutation ls9 and, to a somewhat lesser extent, ls11 inhibited exon v5 inclusion as compared with the unmutated v5 exon (Figure 4B). Moreover, in LB17 lymphoma cells which spontaneously omit v5 (see Figure 2B), the ls9 mutant decreased the low basal level of exon inclusion even further and almost completely abolished TPA inducibility (Figure 4C). Mutant ls11 also impaired induction of exon inclusion upon phorbol ester treatment. Thus, the two mutants, ls9 and ls11, apparently interrupted positively acting splice regulatory sequences in the 3' region of the exon.

Interestingly, all ls mutations upstream of ls9, except for ls5, resulted in strong enhancement of exon inclusion in LB17 cells in the absence of TPA (Figure 4C) which could not be markedly increased by TPA. The strong enhancement of v5 inclusion is not due to a splice enhancer activity of the linker sequence since ls5 does not show this up-regulation, and a mutant containing a deletion instead of the linker in the position of ls7 had the same effect as the potent ls7 linker-scan mutant (data not shown). These findings indicate the existence of splice silencer elements upstream of ls9 which repress exon inclusion in non-stimulated LB17 cells. These silencers appear to be inactive in KLN205 cells and to be inactivated upon TPA treatment of LB17 cells. The minor effect of these mutants in the KLN205 cells (Figure 4B) is consistent with the already very high level of CD44 exon v5 inclusion in these cells (see Figure 2B).

The exon recognition element

The finding that the ls9 and ls11 mutations individually did not completely inhibit exon inclusion in KLN205 cells (see Figure 4B) suggested that changing 10 bp in this positively acting region might not suffice to prevent splice activation completely. This may be due to a high abundance of binding factors in these cells so that an insufficiently mutated binding site could still be recognized to some extent. According to this hypothesis, the functional loss should be more pronounced if the positively acting region were removed completely. We therefore deleted 40 bp, including the sequences affected by the ls9 and ls11 mutations, from the 3' region of exon v5, maintaining, as in previous constructs, the three 3' most nucleotides (see Figure 5A, mutant ΔR). This deletion completely abolished exon inclusion in the KLN205 cells (Figure 5A, lane 4) and in TPA-treated LB17 cells (Figure 5A, lanes 11 and 12). The loss of exon inclusion is not due merely to the change in exon length since replacing the 3' region by a polylinker sequence of the same length has the same effect

Fig. 4. Identification of positively and negatively acting regulatory sequences within CD44 exon v5 by linker-scan mutagenesis. (A) Sequence of CD44 exon v5 indicating the positions of the introduced linker-scan mutants (Is1–Is11). Segments flanked by vertical lines were replaced consecutively by a 10 bp *Mlu*I linker sequence (boxed). Upper case lettering, exon sequence; lower case lettering, intron sequences. The Is mutants were transiently transfected into (B) KLN205 carcinoma cells and (C) LB17 2.3 T-lymphoma cells. Where indicated, LB17 cells were treated with TPA (40 ng/ml) for 7 h. Exon inclusion was quantified by phosphorimaging following blotting and hybridization of the RT-PCR products with a radioactively labeled probe containing the pre-proinsulin exon sequences. Exon inclusion of the mutants is expressed as a percentage relative to v5 wild-type exon inclusion. In (C), TPA-induced exon inclusion was used as reference value. Data represent the means of two independent experiments.

Fig. 5. Regulated inclusion of CD44 exon v5 requires an exon recognition sequence and splice silencer elements. (A) RT-PCR analysis of KLN205 carcinoma cells and LB17 2.3 T-lymphoma cells transiently transfected with the deletion mutants of the CD44v5 minigene shown on the left. L, M and R, 40 bp segments of CD44 exon v5 which were deleted as indicated; the four 5' most and the three 3' most nucleotides were retained. (B) and (C) RT-PCR analysis of LB17 2.3 T-lymphoma cells transiently transfected with the CD44v5 minigenes shown on the left. Black boxes, CD44 exon v5 sequences; grey boxes, pBluescript polylinker sequences. Where indicated, cells were treated with TPA (40 ng/ml) for 7 h.

(see Figure 5C, mutant ΔR blue, lanes 5 and 6; data not shown for KLN205 cells). Thus, the 3' 40 bp of the exon seem to be absolutely necessary for exon recognition. Interestingly, the sequences within this region which are replaced in the ls9 and ls11 mutants (see Figure 4A) are very similar to purine-rich sequence elements (so-called exon recognition sequences) (Watakabe *et al.*, 1993; Tanaka *et al.*, 1994) and A/C-rich splicing enhancers (Coulter *et al.*, 1997), respectively. Purine-rich elements are known to bind members of the family of serine/arginine-rich (SR) proteins (Fu, 1995; Manley and Tacke, 1996; Valcarel and Green, 1996), which is possibly also true for the exon recognition element in exon v5.

The linker-scan mutations in the 5' region of the v5 exon suggest that the positive element(s) in the 3' part of the exon suffice to retain the exon in the transcript. We have confirmed the autonomy of the enhancer elements by placing the 3' 40 bp into the artificial polylinker-substituted v5blue exon (see Figure 3 and Figure 5B, lanes 3 and 4) to create a chimaeric exon (v5bls7; see Figure 5B). This chimaeric exon was now included very efficiently in both KLN205 (not shown) and in LB17 lymphoma cells (Figure 5B), indicating that the 3' 40 bp of CD44 exon v5 indeed act as an exon recognition element. Inclusion of the chimaeric exon in the LB17 lymphoma cells occurred already without phorbol ester stimulation (Figure 5B, lanes 5 and 6). This result suggests that both cell type-specific and inducible exon usage require v5 sequences upstream of the exon recognition element which, in the v5bls7 exon, were replaced by polylinker sequences. Furthermore, the finding confirms the result of the linker-scan analysis (see Figure 4C) which also suggested negative regulatory sequences in this region.

Silencer elements are necessary for cell type-specific and inducible exon usage

Interestingly, all linker-scan mutants within the stretch of 80 bp upstream of the exon recognition sequence resulted in constitutive exon inclusion, with the exception of ls5. The linker-scan mutations in this region apparently fall into two areas separated by ls5. Both areas in the wild-type exon configuration participate in suppressing exon usage. To test whether the two areas indeed represent two regulatory subdomains in the 80 bp silencer region, we generated 40 bp deletions in either the 5' ('left') part (subdomain L, whereby the four 5' most base pairs were maintained) or the 'middle' part (subdomain M) of the exon (see mutants ΔL and ΔM , Figure 5A). Whereas, as expected from the linker-scan analysis, deletion of subdomain L resulted in a high level of exon inclusion in the LB17 T-lymphoma cells, which can only be enhanced slightly by TPA treatment (Figure 5A, lanes 7 and 8), deletion of subdomain M did not impair regulation of exon inclusion (Figure 5A, lanes 9 and 10). Thus, in contrast to subdomain M, subdomain L suffices to keep the basal level of exon inclusion low and allows regulated exon inclusion in this setting. This result is at variance with the results of the linker-scan analysis: linker-scan mutants in subdomain M led to constitutive v5 exon usage (see ls6–8, Figure 4C).

The discrepancy between linker-scan mutants and deletion of subdomain M led us to postulate that subdomain M exerted a specific function in conjunction with sub-

domain L. This function would be needed for regulation only if subdomain L were located at its original position, 40 bp apart from the exon recognition element (subdomain R, see previous paragraph). To test this assumption, we moved subdomain L back to its original position by inserting 40 bp of polylinker sequence between the L and R subdomains (mutant ΔM blue, see Figure 5C). Subdomain L was now no longer sufficient to regulate exon inclusion (Figure 5C, lanes 3 and 4), suggesting that, within the complete exon, subdomain M is required together with subdomain L to regulate exon usage. Possibly, the cooperating components of the composite splice unit must be direct neighbours in order to form a functional complex.

Silencer versus enhancer: which element responds to signal transduction?

The substitution mutants in the silencer region indicated that the silencer sequences are necessary for regulated exon inclusion since these mutants resulted in constitutive exon usage (see Figure 5B and C). However, the mutants do not allow distinction between whether the silencer elements respond to phorbol ester and the 3' enhancer element acts only in a constitutive manner, or whether the activity of the 3' enhancer is modulated by phorbol ester activation. To test whether the 3' enhancer can respond to phorbol ester activation, we generated a mutant exon that, like the v5bls7 exon (see Figure 5B), contained no silencer sequences but, due to a reduction in exon size, should be recognized less efficiently. Figure 6A shows that a mini-exon containing only the 3' enhancer element (v5ls1/8) is not recognized at all (lanes 3 and 4), probably because it is too small. In contrast, a mini-exon containing the 3' enhancer plus 30 bp of polylinker sequence (v5ls1/8blue20) instead of 80 bp as in the v5bls7 exon (see Figure 5B) is included to a low degree in the LB17 T-lymphoma cells (Figure 6A, lane 5). After TPA treatment of the cells (mutant ΔM in lanes 1 and 2 served as a positive control for TPA action), inclusion of this mini-exon is enhanced (Figure 6A, lanes 5 and 6), suggesting that the 3' enhancer element can respond to TPA activation, at least in this setting.

To investigate coupling of signal transduction to the silencer and/or enhancer sequences in the original context (full exon length), we made use of silencer substitution mutants in which subdomains L or M were replaced by a heterologous sequence of the same length (see Figure 6B). In contrast to the ΔL blue and ΔM blue mutants (see Figure 5C), these substitutions (ΔL a30 and ΔM a30) did not interfere with basal repression of exon inclusion in the LB17 T-lymphoma cells (Figure 6B, lanes 1, 3 and 5, and Figure 6C). The reason for this difference in behaviour of the ΔL blue and the $\Delta a30$ mutants is not clear, but it might be related to short sequence elements which we found shared between the substitution sequence of the $\Delta a30$ mutants and subdomain L of the silencer region. (When substituting the 3' enhancer region with this sequence, exon recognition was ablated, as was observed with the ΔR blue mutant; compare Figure 6B, lanes 7 and 8, and Figure 5C, lanes 5 and 6.) Although the enhancer element is present in the silencer substitution mutants, these mutants show an impaired TPA response as compared with the wild-type exon (Figure 6B, lanes 1–6, and Figure

Fig. 6. The 3' exon recognition sequence can respond to phorbol ester-induced signal transduction in a CD44 v5 mini-exon but is not sufficient for full TPA response in the complete v5 exon. (A) RT-PCR analysis was performed from cytoplasmic RNA of LB17 T-lymphoma cells transiently transfected with the mutant constructs indicated on the left. Black boxes, v5 sequences; grey boxes, linker sequences. (B) RT-PCR analysis of the silencer substitution mutants shown on the left after transient transfection into LB17 T-lymphoma cells. Where indicated, cells were treated with TPA (40 ng/ml) for 7 h. Black boxes, CD44 exon v5 sequences; hatched boxes, a30 substitution sequence (see Materials and methods); L, M and R, 40 bp subdomains of CD44 exon v5 which were substituted as indicated. (C) Quantification of the RT-PCR analysis shown in (B). RT-PCR bands were scanned and logarithmic density values of pixel data were determined using the Adobe Photoshop programme. Results are expressed as the percentage of v5-containing RT-PCR products relative to total RT-PCR products.

6C). The result indicates that, in the context of the complete v5 exon, phorbol ester activation via the enhancer element does not suffice for the full phorbol ester response, and suggests that at least part of the TPA response is mediated by the silencer region. However, one cannot totally exclude the possibility that TPA-induced signal transduction targets the enhancer region and that the impaired TPA response is caused by a defect in enhancer-silencer communication (evoked by the silencer mutation and not affecting basal communication). A role for the 3' enhancer element in the phorbol ester response in the complete v5 exon would be consistent with the behaviour of the linker-scan mutants ls9 and ls11, which showed impaired TPA responsiveness (see Figure 4C).

Conclusions and implications

Our results have supplied evidence for a composite exonic splice regulator which is responsive to cell signalling. Retention or excision of CD44 exon v5 from pre-mRNA is regulated by distinct types of *cis*-acting elements: an exon recognition element and a silencer element composed of two subdomains, L and M (see Figure 7). Whereas subdomain L can work independently, subdomain M is able to act only in conjunction with subdomain L. Given the necessity for both silencer and enhancer elements for regulated exon inclusion in the complete exon, there currently is no definitive answer to the question of whether one of the elements or both sense phorbol ester activation. An answer can probably only be obtained after cloning of the factors binding to these elements.

We have shown previously by cell fusion experiments that *trans*-acting factors govern alternative splicing of CD44 pre-mRNA (König *et al.*, 1996). These factors could bind to the elements identified here, either directly or indirectly, or they might represent factors able to modify proteins recognizing these elements. Regulation could be established by the cooperative binding of silencer proteins

Fig. 7. Schematic representation of the regulatory domains within CD44 exon v5 and of the postulated factors. Open boxes, L, M and R subdomains; hatched and grey box within R, purine-rich and A/C-rich elements, respectively. +, positive regulatory factors; -, strong negative regulatory factors; -, weak negative regulatory factors (for details see text).

to subdomains L and M of the silencer region which might impair the binding or function of positively acting factors at the exon recognition element (subdomain R) (see Figure 7). In cells showing high constitutive inclusion of CD44 exon v5, the silencer factors might be present in very low amounts or, alternatively, they could be modified in such a way that either their RNA binding or their interaction with enhancer factors is abolished. In cells in which the exon is skipped, the silencer factors would bind to their cognate elements and thereby displace the exon recognition factors or interfere with their activating function. Activation of signal transduction pathways such as PKC or Ras signalling could modify either the silencer factors, the enhancer factors or both, thus affecting their RNA-binding capacity or their ability to interact with each other and with components of the splicing machinery.

Changes in phosphorylation recently have been shown to affect protein-protein and protein-RNA interactions of SR proteins (Tacke *et al.*, 1997; Xiao and Manley, 1997) and RNA binding of heterogeneous nuclear ribonucleo-

proteins (hnRNPs) (Mayrand *et al.*, 1993; Municio *et al.*, 1995). Interestingly, some hnRNPs are known to be phosphorylated in response to extracellular signals (Municio *et al.*, 1995; van Seuning *et al.*, 1995). Furthermore, SR protein kinases can affect redistribution of SR proteins in the nucleus (Gui *et al.*, 1994; Colwill *et al.*, 1996), and one of them, Clk1, has been shown to influence alternative splice patterns *in vivo* (Duncan *et al.*, 1997). Whether the activity of SR protein kinases can be regulated by signal transduction pathways is as yet unknown. Both SR proteins and hnRNPs can bind RNA in a cooperative fashion (Kumar *et al.*, 1990; Lynch and Maniatis, 1996; McAfee *et al.*, 1996) and can affect alternative splicing positively and negatively (for recent reviews, see Chabot, 1996; Wang and Manley, 1997), making them candidates for the factors postulated here to regulate alternative splicing.

The composite exonic splice regulator, demonstrated here for CD44 exon v5, may represent a prototype of RNA elements used in controlling alternative splicing. A juxtaposition of exon recognition elements and exonic silencer sequences has been described previously for the fibronectin EDA exon (Caputi *et al.*, 1994) and for viral transcripts of human immunodeficiency virus type 1 and bovine papilloma virus type 1 (Amendt *et al.*, 1995; Staffa and Cochrane, 1995; Zheng *et al.*, 1996). However, the composite splice unit in CD44 exon v5 described here represents, to our knowledge, the first example of splice regulatory elements responsive to cell signalling. The coupling of signal transduction to alternative splicing by such RNA elements could be the basis for establishing tissue-specific splice patterns during development and for switching splice patterns under physiological or pathological conditions.

Materials and methods

Cell culture and transfections

LB17 2.3 is a subline derived from the murine LB17 T-lymphoma cell line (Zahalka *et al.*, 1995); culture conditions were as described elsewhere (Zahalka *et al.*, 1995). KLN205 mouse carcinoma cells (ATCC CRL-1453), 293 human embryonal kidney cells (ATCC CRL-1573), HT-3 human cervix carcinoma cells (ATCC HTB-32), KG-1 human acute myelogenous carcinoma cells (ATCC CRL-8031) and HaCaT immortalized human keratinocytes (Boukamp *et al.*, 1988) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine, at 37°C and 6% CO₂. KLN205, HaCaT and HT-3 cells were transfected with 10 µg of plasmid DNA by electroporation using a Bio-Rad Gene Pulser (0.2 cm gap cuvettes, 250 µF, 380 V for KLN205, HT-3 and HaCaT cells; 0.4 cm gap cuvettes, 960 µF, 320 V for KG-1 cells). Transfections of LB17 2.3 cells were performed using 5 µg of DNA and a polycationic reagent (SuperFect, Qiagen) according to the manufacturer's instructions. In co-transfection experiments, 4 µg of the minigene plasmid were co-transfected with either 4 µg of RSV-ras (leucine 61) (Medema *et al.*, 1991) or the empty expression vector.

RT-PCR analysis

RT-PCR analysis of the minigene transcripts was performed as described (König *et al.*, 1996) using 0.6 µg of DNase-treated cytoplasmic RNA and the insulin exon primers N5Ins GAGGGATCCGCTTCTGCCCC and N3Ins CTCCCGGGCCACCTCCAGTGCC. Thirty PCR cycles (60 s at 94°C, 60 s at 59°C, 90 s at 72°C) were performed using cDNA of transfected KLN205 cells and 35 cycles for cDNA of transfected LB17 2.3 cells. PCR reactions were in the linear phase (not in the plateau phase) under these conditions, as verified by using different amounts of cDNA. Cytoplasmic RNA was prepared according to Wirth and Baltimore (1988). Exon-specific RT-PCRs (24 cycles) were performed

with rat CD44 primers as described previously (König *et al.*, 1996). Only for exon v6, the 5' primer pv6mouse (CTAATAGTACAGCAGAA-GCA) was used.

Plasmid constructions and linker-scan mutagenesis

CD44 v5 minigene construct. A 3 kb fragment of the mouse genomic CD44 sequence, containing exons v4, v5 and corresponding intron sequences was cloned into the exon-trap vector pL53In (Mobictec). The 3 kb genomic sequence was cut out as a *NotI*-*EcoRV* fragment from a genomic CD44 subclone in pBluescript (kindly provided by Jürgen Moll) and cloned into the multiple cloning site (*NotI*-*SacI* blunt) of pL53In. Subsequently, exon v4 was deleted by PCR, resulting in the construct pETv5.

pETv5 blue and pETv5amp. A 105 bp *SacI*-*KpnI* (blunt) fragment of the pBluescript SK (Stratagene) polylinker sequence (for v5blue) or a 111 bp *SacI*-*PvuI* (blunt) fragment from the bacterial ampicillin resistance gene (for v5amp) were cloned into the *MluI* site (blunt) of pETIs1/11. pETIs1/11 was obtained by PCR-mediated deletion of the v5 sequence from pETv5 except for the four 5' most and the three 3' most nucleotides. By the PCR primers, a 10 bp *MluI* linker had been introduced.

Linker scan mutants pETv5ls1-ls11. Ten base pairs of the 117 bp v5 exon (see Figure 4A) were replaced consecutively with a 10 bp *MluI* linker (CGACGCGTCG) by PCR. Eleven PCR primer pairs were used that deleted 10 bp each throughout exon v5 (except for the four 5' most and the three 3' most nucleotides). At their 5' ends, the primers contained the 10 bp *MluI* linker sequence. Primer sequences and further details are available on request.

Deletion mutants ΔL, ΔM and ΔR. Mutants were generated by combining corresponding PCR fragments used for linker-scan mutagenesis. In each mutant, 40 bp were removed from the 5' (ΔL, segments ls1-ls4; see Figure 4A), the middle (ΔM, ls5-ls8) or the 3' (ΔR, ls8-ls11) portion of the original v5 sequence (whereby 10 bp of the deleted 40 bp were replaced by the *MluI* linker).

Mutants ΔLblue, ΔMblue, ΔRblue, ΔLa30, ΔMa30 and ΔRa30. To obtain the Δblue mutants, the oligonucleotides 5'-CGCGAGCTTGATATCG-AATTCCTGCAGCCC-3' (upper strand) and 5'-CGCGGGGCTGCA-GGAATTCGATATCAAGCT-3' (lower strand), derived from the polylinker region of pBluescript SK (Stratagene), were cloned into the *MluI* site of the ΔL, ΔM and ΔR constructs. The Δa30 mutants were generated similarly, using the oligonucleotides 5'-CGCGTAT-CATGGTTATGGCAGCACTGCATA-3' (upper strand, derived from the non-coding strand of the bacterial ampicillin resistance gene) and 5'-CGCGTATGCAGTGCTGCCATAACCATGATA-3' (lower strand).

Mutant pETv5ls7. An *MluI*-*EcoNI* fragment from linker-scan mutant ls7 (see Figure 4A), spanning the 3' 43 bp of exon v5 and 109 bp of the downstream intron, was used to replace a corresponding *SmaI*-*EcoNI* fragment of pETv5blue, spanning the 3' 46 bp of the v5blue exon plus the 109 bp intron sequence.

Mutants pETv5ls1/8 and pETIs1/8bl20. pETIs1/8 was obtained by PCR-mediated deletion of the v5 sequences spanning linker-scan segments ls1-ls8 (see Figure 4A). By the PCR primers, a 10 bp *MluI* linker had been introduced. To generate pETIs1/8bl20, the oligonucleotides 5'-CGCGATATCGAATTCCTGCAGCCC-3' (upper strand) and 5'-CGCGGGGCTGCAGGAATTCGATAT-3' (lower strand), derived from the pBluescript polylinker, were cloned into the *MluI* site of pETIs1/8.

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References

- Amendt, B.A., Si, Z.-H. and Stoltzfus, C.M. (1995) Presence of exon splicing silencers within human immunodeficiency virus type I tat exon 2 and tat-rev exon 3: evidence for inhibition mediated by cellular factors. *Mol. Cell. Biol.*, **15**, 4606-4615.

- Arch,R., Wirth,K., Hofmann,M., Ponta,H., Matzku,S., Herrlich,P. and Zöller,M. (1992) Participation in normal immune response of a splice variant of CD44 that encodes a metastasis-inducing domain. *Science*, **257**, 682–685.
- Baker,B.S. (1989) Sex in flies: the splice of life. *Nature*, **340**, 521–524.
- Borsi,L., Balza,E., Gaggero,B., Allemanni,G. and Zardi,L. (1995) The alternative splicing pattern of tenascin C pre-mRNA is controlled by extracellular pH. *J. Biol. Chem.*, **270**, 6243–6245.
- Boukamp,P., Petrussevska,R.T., Breitkreutz,D., Hornung,J., Markham,A. and Fusenig,N.E. (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.*, **106**, 761–771.
- Cantrell,D. (1996) T cell antigen receptor signal transduction pathways. *Annu. Rev. Immunol.*, **14**, 259–274.
- Caputi,M., Casari,G., Guenzi,S., Tagliabue,R., Sidoli,A., Melo,C.A. and Baralle,F.E. (1994) A novel bipartite splicing enhancer modulates the differential processing of the human fibronectin EDA exon. *Nucleic Acids Res.*, **22**, 1018–1102.
- Castagna,M., Takai,Y., Kaibuchi,K., Sano,K., Kikkawa,U. and Nishizuka,Y. (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.*, **257**, 7847–7851.
- Chabot,B. (1996) Directing alternative splicing: cast and scenarios. *Trends Genet.*, **12**, 472–478.
- Chalfant,C.E., Mischak,H., Watson,J.E., Winkler,B.C., Goodnight,J., Farese,R.V. and Cooper,D.R. (1995) Regulation of alternative splicing of protein kinase C beta by insulin. *J. Biol. Chem.*, **270**, 13326–13332.
- Collett,J.W. and Steele,R.E. (1993) Alternative splicing of a neural-specific Src mRNA (Src⁺) is a rapid and protein synthesis independent response to neural induction in *Xenopus laevis*. *Dev. Biol.*, **158**, 487–495.
- Colwill,K., Pawson,T., Andrews,B., Prasad,J., Manley,J.L., Bell,J.C. and Duncan,P.I. (1996) The Clk/Sty protein kinase phosphorylates SR splicing factors and regulates their intracellular distribution. *EMBO J.*, **15**, 265–275.
- Coulter,L.R., Landree,M.A. and Cooper,T.A. (1997) Identification of a new class of exonic splicing enhancers. *Mol. Cell Biol.*, **17**, 2143–2150.
- Downward,J., Graves,J.D., Warne,P.H., Rayter,S. and Cantrell,D.A. (1990) Stimulation of p21^{ras} upon T-cell activation. *Nature*, **346**, 719–723.
- Duncan,P.I., Stojdl,D.F., Marius,R.M. and Bell,J.C. (1997) *In vivo* regulation of alternative pre-mRNA splicing by the Clk1 protein kinase. *Mol. Cell Biol.*, **17**, 5996–6001.
- Eissa,N.T., Strauss,A.J., Haggerty,C.M., Choo,E.K., Chu,S.C. and Moss,J. (1996) Alternative splicing of human inducible nitric-oxide synthase mRNA: tissue-specific regulation and induction by cytokines. *J. Biol. Chem.*, **271**, 27184–27187.
- Fichter,M., Hinrichs,R., Eissner,G., Scheffer,B., Classen,S. and Ueffing,M. (1997) Expression of CD44 isoforms in neuroblastoma cells is regulated by PI 3-kinase and protein kinase C. *Oncogene*, **14**, 2817–2824.
- Fu,X.-D. (1995) The superfamily of arginine/serine-rich splicing factors. *RNA*, **1**, 663–680.
- Gui,J., Lane,W.S. and Fu,X.-D. (1994) A serine kinase regulates intracellular localization of splicing factors in the cell cycle. *Nature*, **369**, 678–682.
- Iordanov,M.S., Pribnow,D., Magun,J.L., Dinh,T.H., Pearson,J.A., Chen,S.L. and Magun,B.E. (1997) Ribotoxic stress response: activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the alpha-sarcin/ricin loop in the 28S rRNA. *Mol. Cell Biol.*, **17**, 3373–3381.
- König,H., Moll,J., Ponta,H. and Herrlich,P. (1996) *Trans*-acting factors regulate the expression of CD44 splice variants. *EMBO J.*, **15**, 4030–4039.
- Koopman,G., Heider,K.-H., Horst,E., Adolf,G.R., van den Berg,F., Ponta,H., Herrlich,P. and Pals,S.T. (1993) Activated human lymphocytes and aggressive non-Hodgkin's lymphomas express a homologue of the rat metastasis-associated variant of CD44. *J. Exp. Med.*, **177**, 897–904.
- Kosaki,A. and Webster,N.J.G. (1993) Effect of dexamethasone on the alternative splicing of the insulin receptor mRNA and insulin action in HepG2 hepatoma cells. *J. Biol. Chem.*, **268**, 21990–21996.
- Kumar,A., Casas-Finet,J.R., Luneau,C.J., Karpel,R.L., Merrill,B.M., Williams,K.R. and Wilson,S.H. (1990) Mammalian heterogeneous nuclear ribonucleoprotein A1. Nucleic acid binding properties of the COOH-terminal domain. *J. Biol. Chem.*, **265**, 17094–17100.
- Liu,J., Farmer,J.D., Jr, Lane,W.S., Friedman,J., Weissman,I. and Schreiber,S.L. (1991) Calcineurin is a common target of cyclophilin–cyclosporin A and FK506 complexes. *Cell*, **66**, 807–815.
- Lynch,K.M. and Maniatis,T. (1996) Assembly of specific SR protein complexes on distinct regulatory elements of the *Drosophila* doublesex splicing enhancer. *Genes Dev.*, **10**, 2089–2101.
- Mackay,C.R., Terpe,H.-J., Stauder,R., Martson,W.L., Stark,H. and Günther,U. (1994) Expression and modulation of CD44 variant isoforms in humans. *J. Cell Biol.*, **124**, 71–82.
- Manley,J.L. and Tacke,R. (1996) SR proteins and splicing control. *Genes Dev.*, **10**, 1569–1579.
- Mayrand,S.H., Dwen,P. and Pederson,T. (1993) Serine/threonine phosphorylation regulates binding of C hnRNP proteins to pre-mRNA. *Proc. Natl Acad. Sci. USA*, **90**, 7764–7768.
- McAfee,J.G., Soltaninassab,S.R., Lindsay,M.E. and LeSturgeon,W.M. (1996) Proteins C1 and C2 of heterogeneous nuclear ribonucleoprotein complexes bind RNA in a highly cooperative fashion: support for their contiguous deposition on pre-mRNA during transcription. *Biochemistry*, **35**, 1212–1222.
- McKay,N.G., Hunter,D.J., Haites,N.E. and Power,D.A. (1994) Regulation of alternative splicing of the fibronectin III CS domain by cytokines. *Biochem. Biophys. Res. Commun.*, **199**, 1005–1011.
- Medema,R.H., Wubbolts,R. and Bos,J.L. (1991) Two dominant inhibitory mutants of p21^{ras} interfere with insulin-induced gene expression. *Mol. Cell Biol.*, **11**, 5963–5967.
- Metheny,L.J. and Skuse,G.R. (1996) NF1 mRNA isoform expression in PC12 cells: modulation by extrinsic factors. *Exp. Cell Res.*, **228**, 44–49.
- Municio,M.M., Lozano,J., Sanchez,P., Moscat,J. and Diaz-Meco,M.T. (1995) Identification of heterogeneous ribonucleoprotein A1 as a novel substrate for protein kinase ζ . *J. Biol. Chem.*, **270**, 15884–15891.
- Rio,D.C. (1993) Splicing of pre-mRNA: mechanism, regulation and role in development. *Curr. Opin. Genet. Dev.*, **3**, 574–584.
- Rodenhuis,S. (1992) Ras and human tumors. *Semin. Cancer Biol.*, **3**, 241–247.
- Screaton,G.R., Bell,M.V., Jackson,D.G., Cornelis,F.B., Gerth,U. and Bell,J.I. (1992) Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. *Proc. Natl Acad. Sci. USA*, **89**, 1260–12164.
- Screaton,G.R., Caceres,J.F., Mayeda,A., Bell,M.V., Plebanski,M., Jackson,D.G., Bell,J.I. and Krainer,A.R. (1995) Identification and characterization of three members of the human SR family of pre-mRNA splicing factors. *EMBO J.*, **14**, 4336–4349.
- Sharp,P.A. (1994) Split genes and RNA splicing. *Cell*, **77**, 805–815.
- Sherman,L., Sleeman,J., Dall,P., Hekele,A., Moll,J., Ponta,H. and Herrlich,P. (1996) The CD44 proteins in embryonic development and in cancer. *Curr. Top. Microbiol. Immunol.*, **213**, 249–269.
- Shifrin,V.I. and Neel,B.G. (1993) Growth factor-inducible alternative splicing of nontransmembrane phosphotyrosine phosphatase PTP-1B pre-mRNA. *J. Biol. Chem.*, **268**, 25376–25384.
- Staffa,A. and Cochrane,A. (1995) Identification of positive and negative splicing regulatory elements within the terminal tat–rev exon of human immunodeficiency virus type I. *Mol. Cell Biol.*, **15**, 4597–4605.
- Tacke,R., Chen,Y. and Manley,J.L. (1997) Sequence-specific RNA binding by an SR protein requires RS domain phosphorylation: creation of an SRp40-specific splicing enhancer. *Proc. Natl Acad. Sci. USA*, **94**, 1148–1153.
- Tanaka,K., Watakabe,A. and Shimura,Y. (1994) Polypurine sequences within a downstream exon function as a splicing enhancer. *Mol. Cell Biol.*, **14**, 1347–1354.
- Tölg,C., Hofmann,M., Herrlich,P. and Ponta,H. (1993) Splicing choice from ten variant exons establishes CD44 variability. *Nucleic Acids Res.*, **21**, 1225–1229.
- Truneh,A., Albert,F., Golstein,P. and Schmitt-Verhulst,A.-M. (1985) Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. *Nature*, **313**, 318–320.
- Tsien,R.Y., Pozzan,T. and Rink,T.J. (1982) T-cell mitogens cause early changes in cytoplasmic free Ca²⁺ and membrane potential in lymphocytes. *Nature*, **295**, 68–71.
- Valcarel,J. and Green,M.R. (1996) The SR protein family: pleiotropic functions in pre-mRNA splicing. *Trends Biochem. Sci.*, **21**, 296–301.
- Valge,V.A., Wong,J.G.P., Datlof,B.M., Sinskey,A.J. and Rao,A. (1988) Protein kinase C is required for responses to T cell receptor ligands but not to interleukin-2 in T cells. *Cell*, **55**, 101–112.

- Van Seuninghen,I., Ostrowski,J. and Bomsztyk,K. (1995) Description of an IL-1-responsive kinase that phosphorylates the K protein. Enhancement of phosphorylation by selective DNA and RNA motifs. *Biochemistry*, **34**, 5644–5650.
- Wang,A., Cohen,D.S., Palmer,E. and Sheppard,D. (1991) Polarized regulation of fibronectin secretion and alternative splicing by transforming growth factor. *J. Biol. Chem.*, **266**, 15598–15601.
- Wang,J. and Manley,J.L. (1997) Regulation of pre-mRNA splicing in metazoa. *Curr. Opin. Genet. Dev.*, **7**, 205–211.
- Watakabe,A., Tanaka,K. and Shimura,Y. (1993) The role of exon sequences in splice site selection. *Genes Dev.*, **7**, 407–418.
- Weiss,J.M. *et al.* (1997) An essential role for CD44 variant isoforms in epidermal Langerhans cell and blood dendritic cell function. *J. Cell Biol.*, **137**, 1137–1147.
- Wirth,T. and Baltimore,D. (1988) Nuclear factor NF- κ B can interact functionally with its cognate binding site to provide lymphoid-specific promoter function. *EMBO J.*, **7**, 3109–3113.
- Xiao,S.H. and Manley,J.L. (1997) Phosphorylation of the ASF/SF2 RS domain affects both protein–protein and protein–RNA interactions and is necessary for splicing. *Genes Dev.*, **11**, 334–344.
- Zacharias,D.A. and Strehler,E.E. (1996) Change in plasma membrane Ca^{2+} -ATPase splice-variant expression in response to a rise in intracellular Ca^{2+} . *Curr. Biol.*, **6**, 1642–1652.
- Zahalka,M.A., Okon,E., Gossler,U., Holzmann,B. and Naor,D. (1995) Lymph node (but not spleen) invasion by murine lymphoma is both CD44- and hyaluronate-dependent. *J. Immunol.*, **154**, 5345–5355.
- Zheng,Z.M., He,P. and Baker,C.C. (1996) Selection of the bovine papilloma virus type 1 nucleotide 3225 3' splice site is regulated through an exonic splicing enhancer and its juxtaposed exonic splicing suppressor. *J. Virol.*, **70**, 4691–4699.
- Zinck,R., Cahill,M.A., Kracht,M., Sachsenmaier,C., Hippskind,R.A. and Nordheim,A. (1995) Protein synthesis inhibitors reveal differential regulation of mitogen-activated protein kinase and stress-activated protein kinase pathways that converge on Elk-1. *Mol. Cell. Biol.*, **15**, 4930–4938.

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