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The Clk/Sty protein kinase phosphorylates SR splicing factors and regulates their intranuclear distribution

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Mammalian Clk/Sty is the prototype for a family of dual specificity kinases (termed LAMMER kinases) that have been conserved in evolution, but whose physiological substrates are unknown. In a yeast two-hybrid screen, the Clk/Sty kinase specifically interacted with RNA binding proteins, particularly members of the serine/arginine-rich (SR) family of splicing factors. Clk/Sty itself has an serine/arginine-rich non-catalytic N-terminal region which is important for its association with SR splicing factors. *In vitro*, Clk/Sty efficiently phosphorylated the SR family member ASF/SF2 on serine residues located within its serine/arginine-rich region (the RS domain). Tryptic phosphopeptide mapping demonstrated that the sites on ASF/SF2 phosphorylated *in vitro* overlap with those phosphorylated *in vivo*. Immunofluorescence studies showed that a catalytically inactive form of Clk/Sty co-localized with SR proteins in nuclear speckles. Overexpression of the active Clk/Sty kinase caused a redistribution of SR proteins within the nucleus. These results suggest that the Clk/Sty kinase directly regulates the activity and compartmentalization of SR splicing factors.

Keywords: ASF/protein phosphorylation/RS domains/SF2

Introduction

Clk/Sty is a mammalian protein kinase with the unusual property of phosphorylating both serine/threonine and tyrosine residues (for review, see Lindberg *et al.*, 1992; Douville *et al.*, 1994). Clk/Sty was identified in an anti-phosphotyrosine antibody screen designed to isolate cDNAs for enzymatically active tyrosine kinases (Ben-David *et al.*, 1991; Howell *et al.*, 1991). The protein is composed of a C-terminal kinase domain, most closely related to serine/threonine-specific kinases, preceded by an N-terminal non-catalytic region of 156 residues. When isolated from bacteria or mammalian cells, Clk/Sty has been shown to autophosphorylate on all three hydroxy-amino acids (Ben-David *et al.*, 1991; Howell *et al.*, 1991; Duncan *et al.*, 1995).

Clk/Sty is the prototypic member of the LAMMER family of protein kinases, which has been conserved through evolution (Yun *et al.*, 1994). This family includes KNS1 from *Saccharomyces cerevisiae* (Padmanabha *et al.*, 1991), AFC1, AFC2 and AFC3 from *Arabidopsis thaliana* (Bender and Fink, 1994), Doa from *Drosophila melanogaster* (Yun *et al.*, 1994), human Clk (Johnson and Smith, 1991), Clk2 (Hanks *et al.*, 1991; Hanes *et al.*, 1994) and Clk3 (Hanes *et al.*, 1994) as well as mouse Clk/Sty. In addition to a strong degree of homology across the kinase domain, these proteins share a highly conserved motif, 'EHLAMMERILGPLP', in a subdomain of the kinase domain. This region is not conserved among kinases in general (Hanks *et al.*, 1988) and may be involved in dictating substrate specificity (Yun *et al.*, 1994).

The best characterized member of this family from a genetic perspective is *Drosophila* Doa. The *doa* gene was isolated as a dosage-sensitive modifier of the *apricot* allele at the *white eye* locus (Rabinow and Birchler, 1989). Homozygous loss-of-function *doa* mutants die at the early larval stage, indicating that this gene is critical for development (Rabinow and Birchler, 1989). Stage-specific and tissue-specific *doa* transcripts have been identified in *Drosophila*, indicating that expression of the LAMMER family may be regulated at the post-transcriptional level (Yun *et al.*, 1994). Consistent with this notion, the expression of mouse Clk/Sty is developmentally regulated at the level of RNA processing, as illustrated by the generation of two alternatively spliced transcripts encoding a catalytically active kinase (Clk/Sty) and a truncated protein lacking the kinase domain (Clk/Sty^{t}) (Duncan *et al.*, 1995). These two transcripts are also observed in the three human Clk kinases (Hanes *et al.*, 1994; Duncan *et al.*, 1995). In mice, these two transcripts are present in rapidly proliferating cells and undifferentiated stem cells (Ben-David *et al.*, 1991; Howell *et al.*, 1991; Duncan *et al.*, 1995). In contrast, in adult mouse tissues or upon stem cell differentiation, larger transcripts are detected (Ben-David *et al.*, 1991; Howell *et al.*, 1991).

The biological functions of mammalian Clk/Sty are unknown, although a possible role in PC12 differentiation has been suggested (Myers *et al.*, 1994). No *in vivo* substrates of Clk/Sty, or other LAMMER kinases, have been identified. To address this question, we have employed a yeast two-hybrid screen to isolate Clk/Sty binding proteins (Fields and Song, 1989). We show here that Clk/Sty interacts with RNA binding proteins in yeast, notably members of the serine/arginine-rich (SR) family of splicing factors. Furthermore, Clk/Sty phosphorylates one such protein, ASF/SF2, *in vitro* at sites that are

also phosphorylated *in vivo*, and regulates the nuclear distribution of SR splicing factors *in vivo*. We propose that SR splicing factors are physiologically relevant substrates for the Clk/Sty protein kinase.

Results

Clk/Sty kinase interacts with splicing factors

To investigate Clk/Sty function, a yeast two-hybrid screen was performed to isolate proteins that interact with the mouse Clk/Sty kinase. To this end, the full-length Clk/Sty cDNA was fused to a cDNA encoding the GAL4 DNA binding domain to generate the pAS-Clk/Sty construct. The yeast strain Y153 carrying the pAS-Clk/Sty vector was transformed with an unstimulated murine T cell cDNA plasmid library in which cDNAs were fused to the coding sequence for the GAL4 DNA activation domain (Durfee *et al.*, 1993). Two and a half million colonies containing both Clk/Sty and cDNA plasmids were screened for stimulation of two reporter genes, β-galactosidase and *HIS3*. Eighty-six colonies were positive in the initial screen, indicating in each case a potential interaction between Clk/Sty and a protein from the cDNA library. The positive cDNA plasmids were then purified from yeast and selected in *Escherichia coli*.

To eliminate false positives, the yeast strain Y153 was transformed with the purified clones to check whether the encoded proteins stimulated the reporters on their own. In addition, Y153 carrying the clones were mated to Y187 carrying one of the following plasmids: pAS1, pAS-Clk/Sty, pAS-SNF1 and pAS-CDK2 (Harper *et al.*, 1993). Thirty-three clones that interacted specifically with Clk/Sty were analysed further.

At least 100 bp of every clone were sequenced, and the resulting sequences were used to search Genbank. Due to redundancy in the library, the actual number of different clones was 25. Of these cDNAs, five encode RNA binding proteins: hnRNP G (Soulard *et al.*, 1993), RNP S1 (Schmidt and Werner, 1993), ASF/SF2 (also known as SRp30a) (Ge *et al.*, 1991; Krainer *et al.*, 1991), X16 (also known as SRp20) (Ayane *et al.*, 1991) and clone 9.11 (Figure 1). Clone 9.11 is most similar to human SRp75 (Zahler *et al.*, 1993b) which, like X16 and ASF/SF2, is a member of the SR family of splicing factors (Zahler *et al.*, 1992). The protein encoded by clone 9.11 is 82% identical (92% similar) to human SRp75 over the region sequenced (Figure 1). In comparison, the human and mouse forms of both X16 and ASF/SF2 are 100% identical to one another at the amino acid level (Tacke *et al.*, 1992; Zahler *et al.*, 1992). The clone 9.11 also encodes 28 N-terminal amino acids not found in SRp75. Clone 9.11 may therefore represent a novel SR family member. Three independent clones of X16 and one clone of the other four RNA binding proteins were isolated. With a single exception, the fusion proteins encoded by these cloned cDNAs begin with the first 45 residues of the corresponding full-length proteins (Figure 1) and extend to their C-terminal ends. Clone 9.11, in contrast, is predicted to terminate within the serine/arginine-rich region (RS domain).

All five of these cloned proteins contain RNA binding domains known as RNA recognition motifs (RRM) at their N-termini (Figure 1) (for review, see Kenan *et al.*, 1991; Burd and Dreyfuss, 1994). The RNA binding region of ASF/SF2 and SRp75 contains a second domain, RRMH, homologous to the RRM. The two domains act synergistically to bind RNA (Caceres and Krainer, 1993; Zuo and Manley, 1993).

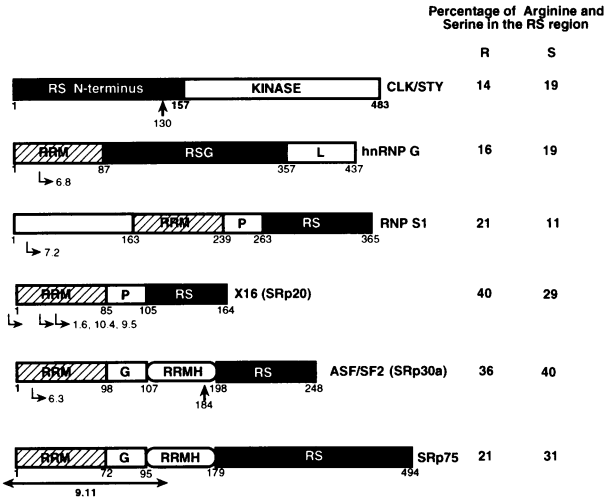

Fig. 1. A schematic of Clk/Sty and the five interacting RNA binding proteins. Each protein has an RS domain, and the percentage of arginine and serine residues is indicated. The five RNA binding proteins have an RNA binding domain known as the RNA recognition motif (RRM), whereas Clk/Sty has a kinase domain. The RNA binding regions of ASF/SF2 and SRp75 contain an additional motif, RRMH, that is similar to the RRM. The bent arrows indicate where the cloned fusion proteins start in relation to the intact proteins. The clones are numbered after the arrows. The vertical arrows in Clk/Sty and ASF/SF2 denote the corresponding amino acid position of alternative splice sites. The horizontal arrow on SRp75 indicates the portion of 9.11 that has been sequenced and found similar to human SRp75 (82% identity). Abbreviations: RS, arginine/serine rich; RRM, RNA recognition motif; RSG, arginine/serine/glycine rich; L, leucine rich; P, proline rich; G, glycine rich; RRMH, RNA recognition motif homologous domain.

A common feature of the five isolated RNA binding proteins is the presence of an RS domain characterized by stretches of alternating serine and arginine residues (Zahler *et al.*, 1992). ASF/SF2 is alternatively spliced to produce three isoforms, ASF1, ASF2 and ASF3, of which ASF2 and ASF3 lack the RS domain (Ge *et al.*, 1991). Sequence analysis confirmed that the ASF/SF2 isoform cloned through its interaction with Clk/Sty was ASF1, the splice variant which retains the RS domain.

Analysis of protein domains required for interactions of Clk/Sty with SR proteins

To investigate whether the interaction of Clk/Sty with RNA binding proteins is dependent on kinase activity, a mutant form of Clk/Sty lacking catalytic activity was tested for its binding properties in the two-hybrid system. In this mutant (pAS-Clk/Sty^{K190R}) the invariant lysine (Lys190) of the Clk/Sty kinase domain, which is required for kinase activity, was replaced with arginine (Ben-David *et al.*, 1991). In the two-hybrid assay, kinase-inactive Clk/Sty bound to the RNA binding proteins with similar efficiency to wild-type (data not shown).

In addition to the C-terminal kinase domain, Clk/Sty possesses an N-terminal non-catalytic region that is itself serine/arginine rich (Figure 1). To delineate further the

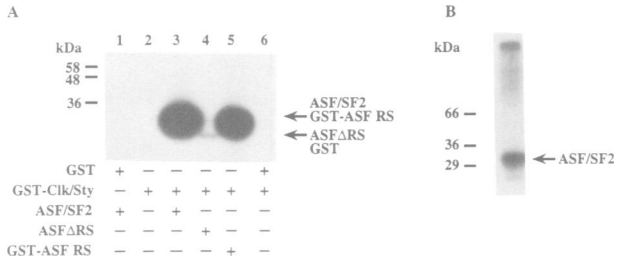

Fig. 2. (**A**) Clk/Sty phosphorylation of ASF/SF2 in an *in vitro* kinase assay. GST–Clk/Sty (500 ng) was incubated alone (lane 2), or with 1 μg of ASF/SF2 (lane 3), ASFΔRS (lane 4), GST–ASF RS (lane 5) or GST (lane 6). As a negative control, GST alone was incubated with ASF/SF2 (lane 1). The samples were resolved on a 12% SDS–polyacrylamide gel and exposed to autoradiography. (**B**) Phosphorylation of ASF/SF2 *in vivo*. ASF/SF2 was transfected into COS-1 cells, labelled with [^{32}P]orthophosphate, immunoprecipitated with an α-T7 Tag antibody, purified by SDS–PAGE and exposed to autoradiography.

sites of interaction between Clk/Sty and the RNA binding proteins, Clk/Sty was divided into its kinase domain and its N-terminal RS domain. The kinase domain alone (pAS-Clk/Sty^{KIN}) did not interact detectably with the RNA binding proteins (data not shown). In contrast, the N-terminal RS region of Clk/Sty (pAS-Clk/Sty^{RS}), when expressed in the two-hybrid system, associated with RNP S1, ASF/SF2 and X16, but not 9.11 or hnRNP G (data not shown).

RS domains have been implicated in protein–protein interactions (Wu and Maniatis, 1993; Amrein *et al.*, 1994; Kohtz *et al.*, 1994). To see if the RS domains of the RNA binding proteins were responsible for their interactions with Clk/Sty, the RS domains of X16 and ASF were tested for binding to the four pAS-Clk/Sty polypeptides in the two-hybrid screen. The X16 RS domain bound weakly to pAS-Clk/Sty and pAS-Clk/Sty^{K190R} but not to pAS-Clk/Sty^{KIN} or pAS-Clk/Sty^{RS} (data not shown). The RS domain of ASF/SF2 did not by itself bind to any of the four Clk/Sty proteins (data not shown). Since the RS domains of X16 and ASF/SF2 were not sufficient to bind Clk/Sty, the RNA binding domain of X16 was tested in the two-hybrid system. Like the RS domains, the RNA binding domain of X16 was unable to bind any of the four Clk/Sty constructs. These results indicate that although the RS domain of Clk/Sty is sufficient to bind a subset of the RNA binding proteins, more extensive contacts along both binding partners are in general required for efficient association of Clk/Sty with SR splicing factors.

Phosphorylation of ASF/SF2

The interaction between Clk/Sty and SR proteins suggests that these RNA binding proteins might be substrates for Clk/Sty phosphorylation. We tested, therefore, the ability of Clk/Sty to phosphorylate the SR protein ASF/SF2 *in vitro*. A bacterially expressed GST–Clk/Sty fusion protein (Duncan *et al.*, 1995) efficiently phosphorylated recombinant ASF/SF2 *in vitro* (Figure 2A, lane 3). To localize the regions of ASF/SF2 which were phosphorylated by Clk/Sty, ASF/SF2 was separated into its RNA binding and RS domains. For this purpose, two polypeptides were expressed in bacteria: ASFΔRS which retains the RNA binding domain but lacks the RS domain (Zuo and Manley, 1993) and GST–ASF RS, which contains only the RS domain. Phosphorylation of ASFΔRS by Clk/Sty was reduced dramatically compared with intact ASF/SF2 (Figure 2A, lane 4). In contrast, Clk/Sty phosphorylated GST–ASF RS, which possesses the isolated RS domain, to a similar level as intact ASF/SF2 protein (Figure 2A, lane 5). Phosphorylation of the GST–ASF RS polypeptide was probably specific to the RS domain, as GST alone was not phosphorylated by Clk/Sty (Figure 2A, lane 6). These results indicated that the ASF/SF2 RS domain contains the major sites of *in vitro* phosphorylation by Clk/Sty.

To confirm that ASF/SF2 is a phosphoprotein *in vivo*, COS-1 cells were transfected with an expression vector encoding ASF/SF2 with an N-terminal T7 epitope tag, and labelled with [^{32}P]orthophosphate. ASF/SF2 was immunoprecipitated with an α-T7 Tag antibody and the isolated protein was purified further by SDS gel electrophoresis. A single band of phosphorylated ASF/SF2 was detected (Figure 2B).

Specificity of Clk/Sty protein kinase activity

To determine if ASF/SF2 is a preferred substrate of Clk/Sty, other exogenous substrates were tested for phosphorylation by Clk/Sty in an *in vitro* kinase assay. Clk/Sty was unable to phosphorylate β-casein, enolase or a GST–c-Jun N-terminal fusion protein (containing Jun residues 5–89) (Figure 3A). Clk/Sty was able to phosphorylate the basic proteins H1 histone and myelin basic protein (MBP), although not to the same extent as ASF/SF2 (Figure 3A).

Previous work has shown that ASF/SF2 is a substrate for SRPK1 but not p34^{cdc2} (Gui *et al.*, 1994a). Given the key role of ASF/SF2 in alternative splicing, it is likely to be regulated by multiple kinases. We also tested the ability of other protein kinases, including p42/p44 MAP kinase (MAPK), protein kinase C (PKC), casein kinase II (CKII) and protein kinase A (PKA) to phosphorylate ASF/SF2 *in vitro*. ASF/SF2 has potential phosphorylation motifs for each of these kinases (Pearson and Kemp, 1991), with the exception of CKII, which has an acidic recognition site. Neither MAPK nor CKII were able to phosphorylate ASF/SF2. However, both PKA and PKC induced ASF/SF2 phosphorylation *in vitro* (Figure 3B). Taken together, these results suggest that ASF/SF2 is a preferred substrate for Clk/Sty (Figure 3B). It is apparent from these and previously published data that ASF/SF2 is potentially phosphorylated by multiple kinases (Woppmann *et al.*, 1993; Gui *et al.*, 1994a).

Phosphoamino acid analysis of ASF/SF2

Phosphoamino acid analysis showed that both full-length ASF/SF2 phosphorylated *in vitro* by Clk/Sty and ASF/SF2 isolated from [^{32}P]orthophosphate-labelled cells contained predominantly phosphoserine (Figure 4A and B; Gui *et al.*, 1994a). A trace amount of phosphothreonine was also seen in ASF/SF2 phosphorylated *in vivo* and *in vitro* by Clk/Sty. No phosphorylation on tyrosine was detected. ASFΔRS, which was phosphorylated *in vitro* to a very low level by Clk/Sty, contained phosphoserine and a lesser amount of phosphothreonine (Figure 4C). The GST–ASF RS polypeptide was phosphorylated *in vitro* exclusively on serine residues (Figure 4D).

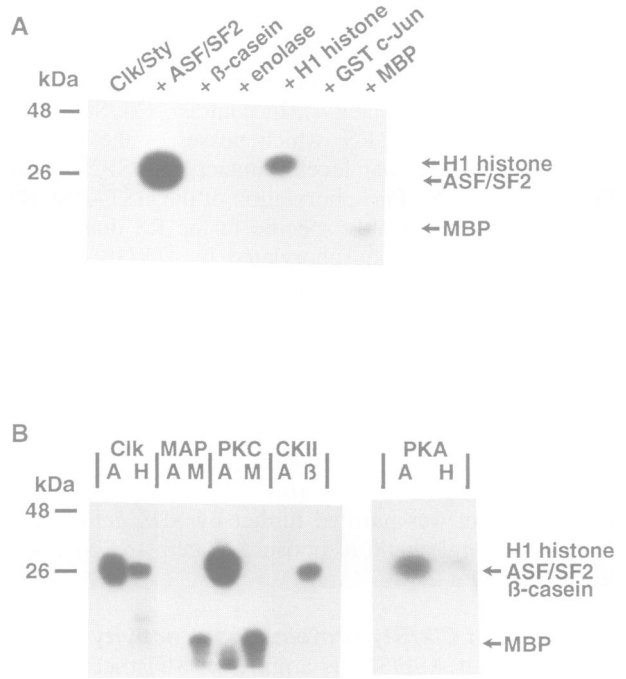

Fig. 3. Specificity of the phosphorylation of ASF/SF2 by Clk/Sty. (**A**) Clk/Sty (150 ng) was incubated alone (lane 1) or with ASF/SF2 (lane 2), β-casein (lane 3), acid-treated enolase (lane 4), H1 histone (lane 5), GST–c-Jun 5–89 (lane 6) and myelin basic protein (MBP, lane 7). (**B**) Several kinases (150 ng) were tested for their ability to phosphorylate ASF/SF2 or a known substrate of the respective kinase. Lanes 1 and 2, Clk/Sty incubation with ASF/SF2 and H1 histone; lanes 3 and 4, MAPK incubation with ASF/SF2 and MBP; lanes 5 and 6, PKC incubation with ASF/SF2 and MBP; lanes 7 and 8, CKII incubation with ASF/SF2 and β-casein; lanes 9 and 10, PKA incubation with ASF/SF2 and H1 histone. Kinase assays were terminated after 30 min by addition of 2× SDS sample buffer and the samples were loaded onto a SDS–polyacrylamide gel. Abbreviations: Clk, Clk/Sty; MAP, MAPK; A, ASF/SF2; H, H1 histone; M, MBP; β, β-casein.

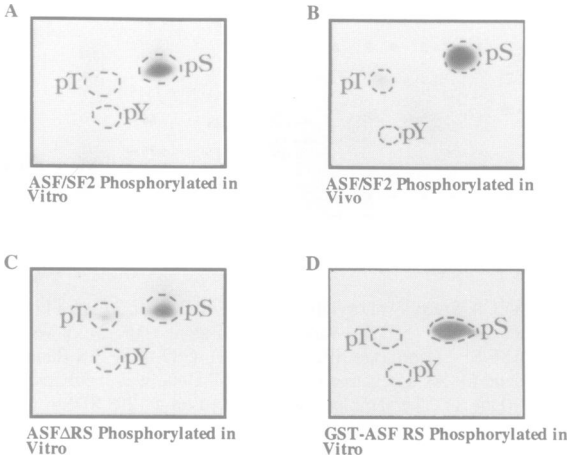

Fig. 4. Phosphoamino acid analysis of ASF/SF2. Samples were hydrolysed in 5.7 M HCl for 1 h at 100°C and the amino acids were separated by two-dimensional electrophoresis (Boyle *et al.*, 1991). (**A**) ASF/SF2 phosphorylated *in vitro* by Clk/Sty. (**B**) *In vivo* phosphorylated ASF/SF2. (**C**) ASFΔRS phosphorylated *in vitro* by Clk/Sty. (**D**) GST–ASF RS phosphorylated *in vitro* by Clk/Sty. The circles indicate the migration of non-radioactive standards. Abbreviations: pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine.

Comparative tryptic phosphopeptide mapping of ASF/SF2

To investigate ASF/SF2 phosphorylation sites in more detail, full-length ASF/SF2 was phosphorylated *in vitro* by Clk/Sty and then digested with trypsin. *In vitro* phosphorylated ASF/SF2 generated 12 phosphorylated tryptic peptides (Figure 5A). A subset of these peptides probably represents partial tryptic digestion products of ASF/SF2 due to the presence of adjacent arginine residues (in tandem), as well as the inability of trypsin to cut after an arginine in phosphoserine–X–arginine motifs (Hardie *et al.*, 1993). Indeed, the presence of five of the 12 phosphopeptides (peptides A–D, and peptide 2, Figure 5F) was variable from one experiment to another (compare Figure 5A with B). The location of the phosphopeptides identified in full-length ASF/SF2 phosphorylated by Clk/Sty was investigated by preparing a tryptic phosphopeptide map of GST–ASF RS which had been phosphorylated *in vitro* by Clk/Sty (Figure 5C). All of the phosphopeptides detected in full-length ASF/SF2 co-migrated with phosphopeptides from GST–ASF RS which only contains the RS domain. There are two extra peptides in the map of GST–ASF RS that may represent phosphorylation of an RS dipeptide generated by the fusion of GST to the RS domain. These results confirm that ASF/SF2 is phosphorylated primarily within its RS domain by Clk/Sty.

A tryptic phosphopeptide map of ASF/SF2 labelled *in vivo* (Figure 5D) showed several peptides (1, 2, 5, 6, 7, 8 and 9) in common with ASF/SF2 phosphorylated *in vitro* by Clk/Sty. *In vivo* labelled ASF/SF2 lacked peptides E and F found in the *in vitro* phosphorylated ASF/SF2 and contained three additional peptides (3, 4 and 10). The lack of peptides E and F in the *in vivo* tryptic map may be due to inaccessibility of these sites owing to the formation of protein complexes *in vivo*, phosphatase activity, or to reduced phosphorylation due to ASF/SF2 overexpression in COS-1 cells (Romac and Keene, 1995). A mix of tryptic digests of ^{32}P-labelled ASF/SF2 phosphorylated either *in vivo* or *in vitro* (Figure 5E) illustrates the presence of the co-migrating phosphopeptides. In aggregate, these results suggest that Clk/Sty phosphorylates ASF/SF2 at physiological sites within the RS domain.

Subnuclear localization of Clk/Sty and SR proteins in vivo

If Clk/Sty interacts with SR proteins in mammalian cells then these polypeptides might be anticipated to have a shared subcellular localization. During interphase, pre-mRNA splicing factors, including the SR proteins, are found localized in nuclear structures termed speckles (Spector *et al.*, 1983; Verheijen *et al.*, 1986; Fu and Maniatis, 1990). To investigate this point, COS-1 cells were transiently transfected with plasmids encoding either full-length Clk/Sty (M-Clk/Sty) or a kinase-inactive Clk/Sty (M-Clk/Sty^{K190R}) (Duncan *et al.*, 1995). These proteins were fused to the Myc epitope to allow detection by mAb 9E10 (Evan *et al.*, 1985). Cells were probed subsequently with mAb 104 which recognizes SR proteins (Roth *et al.*,

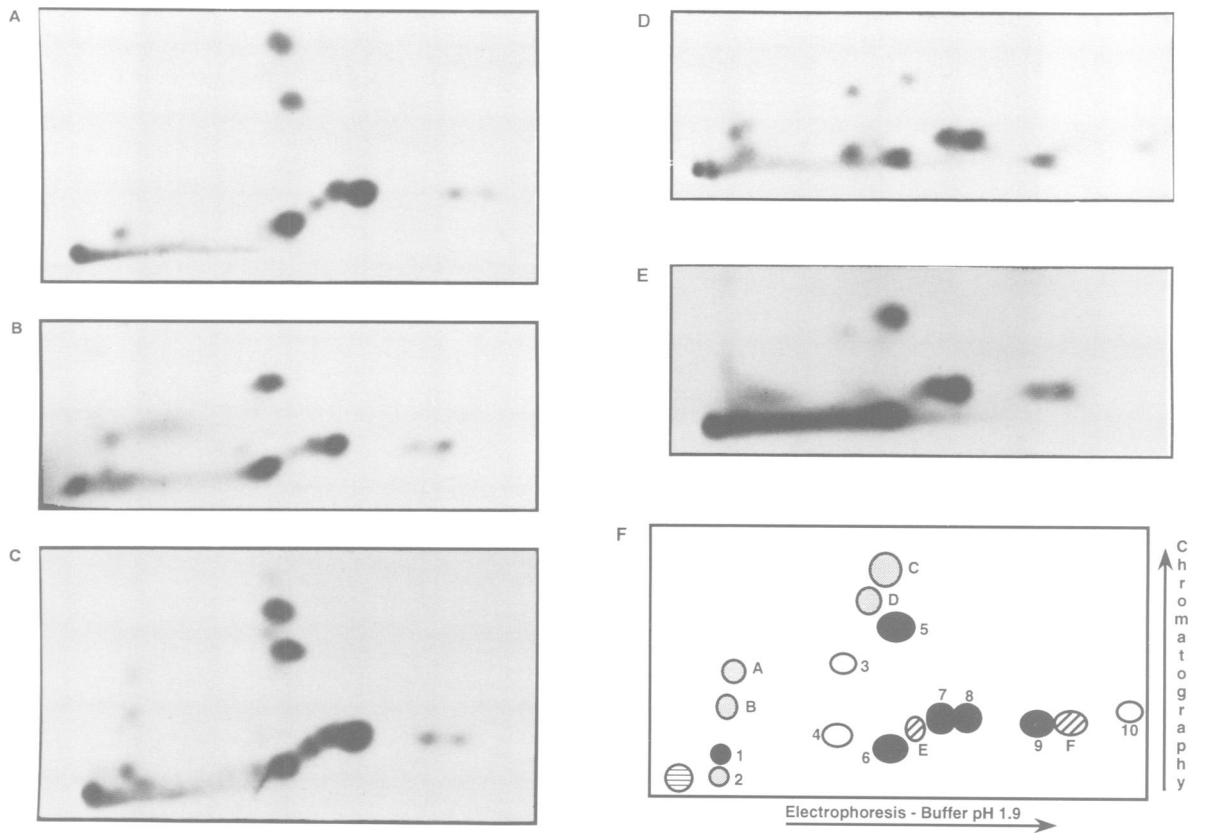

Fig. 5. Tryptic phosphopeptide maps of ASF/SF2. Samples were eluted from SDS–polyacrylamide, precipitated and digested with trypsin. Individual peptides were resolved on thin layer cellulose plates as described (Boyle *et al.*, 1991). (**A**) and (**B**) ASF/SF2 phosphorylated *in vitro* by Clk/Sty. (**C**) GST–ASF RS phosphorylated *in vitro* by Clk/Sty. (**D**) *In vivo* phospholabelled ASF/SF2, and (**E**) a mix of ASF/SF2 phospholabelled *in vitro* and *in vivo*. (**F**) A schematic representation of the phosphopeptide map. Open circles correspond to peptides found only in ASF/SF2 phosphorylated *in vivo*. Closed circles represent peptides phosphorylated both *in vitro* and *in vivo*. Striped circles indicate peptides found only in ASF/SF2 phosphorylated *in vitro* by Clk/Sty. Grey circles symbolize peptides that are variable from one experiment to the next. The origin is denoted by a horizontally striped circle.

1990) and mAb 9E10 to detect transfected Clk/Sty proteins, and analysed by indirect immunofluorescence followed by confocal microscopy imaging. Representative fields are shown in Figure 6. M-Clk/Sty^{K190R} was found predominantly in nuclear speckles, where it co-localized with endogenous SR proteins (Figure 6d–i). In contrast, cells expressing catalytically active M-Clk/Sty displayed a diffuse nucleoplasmic staining pattern for both the kinase and SR proteins (Figure 6j–o). Untransfected cells adjacent to those expressing M-Clk/Sty displayed a normal distribution of SR proteins in nuclear speckles (Figure 6a–c). As a control, the experiment was repeated using a haemagglutinin (HA)-tagged Clk/Sty to demonstrate that Clk/Sty, and not the Myc epitope tag, is responsible for the localization (data not shown). Identical results were also obtained using an antibody specific for a single SR protein family member, SC35 (data not shown) (Fu and Maniatis, 1990).

Since anti-SC35 and anti-mAB104 recognize phosphoepitopes, it is conceivable that the dispersion of the immunofluorescence signal representing the SR family seen when Clk/Sty is overexpressed may be due to phosphorylation of SR members outside of the speckle once Clk/Sty is released from the speckles. To ensure that overexpression of Clk/Sty caused disassembly of speckles and redistribution of SR family members rather than phosphorylation of SR members outside the speckles, the anti-Sm antibody which recognizes components of the snRNPs was used (Spector *et al.*, 1983). When M-Clk/Sty^{K190R} is overexpressed, the Sm proteins are co-localized with M-Clk/Sty^{K190R} in speckles (data not shown). Overexpression of M-Clk/Sty causes a redistribution of the Sm proteins similar to the SR family, indicating that Clk does indeed cause disassembly of nuclear speckles and subsequent redistribution of speckle proteins (data not shown).

The apparently contrasting subnuclear localization of M-Clk/Sty and M-Clk/Sty^{K190R} proteins as detected by immunofluorescent staining was supported by the observation that these two proteins display differential solubility in a non-ionic detergent. COS-1 cells transfected with either M-Clk/Sty or M-Clk/Sty^{K190R} were separated into Triton X-100-soluble and -insoluble fractions, as described in Materials and methods, and analysed by immunoblotting (Figure 7A). While essentially all of the M-Clk/Sty protein was soluble in Triton X-100, a significant portion of the M-Clk/Sty^{K190R} protein remained insoluble. It is of interest to note that the active kinase, M-Clk/Sty, migrates as a broad band while the inactive point mutant, M-Clk/Sty^{K190R}, migrates as a discrete band following SDS–

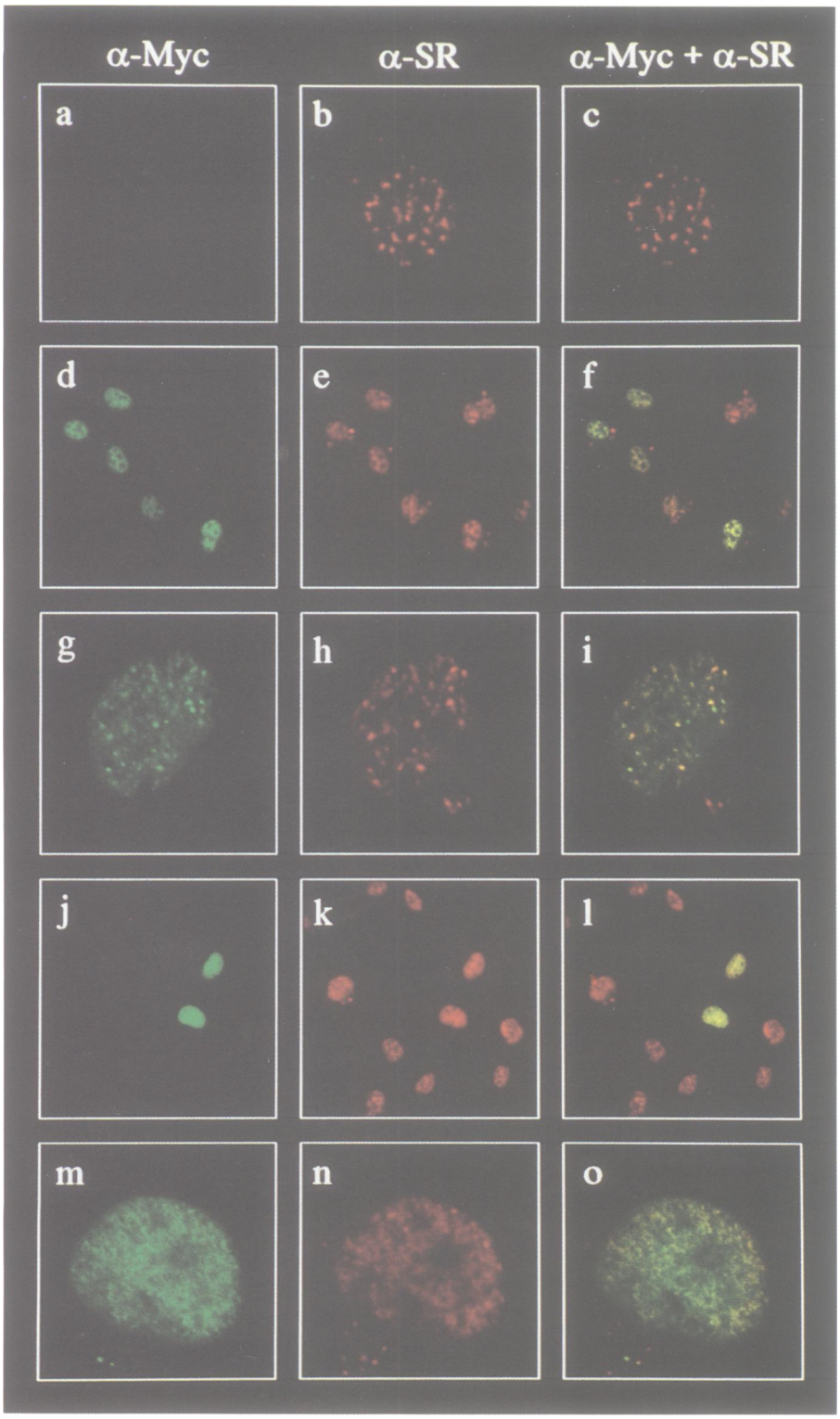

Fig. 6. Nuclear localization of Clk/Sty and its effects on the distribution of SR proteins. Indirect immunofluorescent staining of transfected COS-1 cells using the anti-Myc 9E10 mAB (α-Myc, **a**, **d**, **g**, **j** and **m**), anti-SR mAB 104 (α-SR, **b**, **e**, **h**, **k** and **n**), or an overlay of the two signals (α-Myc + α-SR, **c**, **f**, **i**, **l** and **o**). Alignment of green and red signals appears yellow. Both low power magnification (25×) of M-Clk/Sty^{K190R}-transfected COS-1 cells (d–f) and M-Clk/Sty-transfected cells (j–l) and high power magnification (63×) of untransfected COS-1 cells (a–c), M-Clk/Sty^{K190R}- (g–i) and M-Clk/Sty (m–o)-transfected cells is shown.

PAGE. This banding pattern is suggestive of multiple phosphorylation states of the active kinase, as has been observed for other kinases such as MAPK and Wee1 (Gotoh *et al.*, 1991; Tang *et al.*, 1993).

When Triton X-100-treated COS-1 cells were subjected to analysis by immunofluorescence, the speckled pattern of M-Clk/Sty^{K190R} remained intact (Figure 7B) while the wild-type protein staining pattern was lost (data not

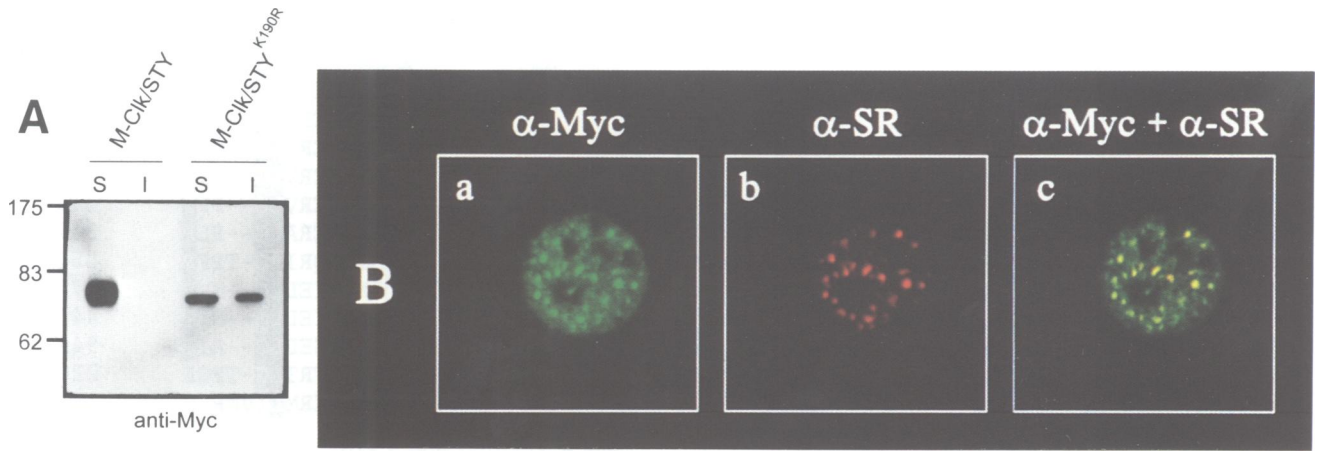

Fig. 7. Active and inactive Clk/Sty kinases display differential solubility in non-ionic detergent. (**A**) COS-1 cells transfected with M-Clk/Sty or a catalytic mutant M-Clk/Sty^{K190R} were lysed in Triton X-100-containing buffer (see Materials and methods) to yield a Triton-soluble fraction (S) and a Triton-insoluble fraction (I). Extracts were analysed by SDS–PAGE and immunoblotting with the anti-Myc antibody. Positions of molecular mass markers (kDa) are indicated to the left. (**B**) The Triton X-100-insoluble M-Clk/Sty^{K190R} co-localizes with SR splicing proteins. COS-1 cells transfected with M-Clk/Sty^{K190R} and grown on coverslips were extracted with Triton X-100-containing buffer. Following extractions, cells were fixed and processed for indirect immunofluorescence by confocal microscopy using the anti-Myc antibody (α-Myc) and the anti-SR antibody (α-SR). (a) α-Myc, (b) α-SR, (c) overlay of the α-Myc and α-SR signals. Alignment of the green and red signals appears yellow.

shown). These results are consistent with the idea that the catalytic activity of M-Clk/Sty is involved in the regulation of its subnuclear localization.

Discussion

Clk/Sty interacts with RNA binding proteins

We have isolated potential binding partners for the Clk/Sty protein kinase by screening a T cell cDNA library using the yeast two-hybrid system. Five of the clones isolated are RNA binding proteins, and three are members of the SR family of essential splicing factors. Additional clones may represent novel serine/arginine-rich proteins (K.Colwill and T.Pawson, unpublished results). The function of hnRNP G or RNP S1 has not been ascertained. hnRNP G is a member of the heterogenous nuclear ribonucleoprotein family that is involved in pre-mRNA processing (for review, see Dreyfuss *et al.*, 1993). SR proteins, such as ASF/SF2, X16 and SRp75, are required for splicing and play a role in selection at the 5′ splice site (Zahler *et al.*, 1992, 1993b).

While the Clk/Sty kinase domain was not sufficient for a positive interaction with these RNA binding proteins, the isolated N-terminal RS region of Clk/Sty associated with ASF/SF2, X16 and RNP S1. This result is consistent with previous data implicating RS domains in protein–protein interactions (Wu and Maniatis, 1993; Amrein *et al.*, 1994; Kohtz *et al.*, 1994). A characteristic of previously described RS domains is the presence of repeating RS/SR dipeptides. Although Clk/Sty does not contain long stretches of RSRS repeats, it contains 10 RS/SR dipeptides and one RSRS motif. However, the N-terminal domain of Clk/Sty possesses one of the main features of known RS domains, which is an ability to interact with SR proteins. The RS domain of Clk corresponds to an alternatively spliced truncated Clk/Sty polypeptide (Clk/Sty^t) whose function is unknown (Duncan *et al.*, 1995). The tight association of the Clk/Sty RS domain with SR proteins suggests that this splice variant could be involved in the regulation of SR protein compartmentalization. This could occur by the formation of inactive Clk/Sty–Clk/Sty^t heterodimers or by the sequestering of SR proteins by Clk/Sty^t (Reith *et al.*, 1991; Thomis and Samuel, 1993).

Although the Clk/Sty kinase domain on its own could not interact detectably with any of the RNA binding proteins, it probably participates in binding as not all the RNA binding proteins could interact with the isolated Clk/Sty RS domain. The finding that an inactive Clk/Sty protein was still able to bind the RNA binding proteins is not surprising, as contacts between a kinase and its substrate are expected to extend throughout the kinase domain (Knighton *et al.*, 1991).

In reciprocal experiments, the RS domains of X16 and ASF as well as the RNA binding domain of X16 were not sufficient for the efficient interaction of either protein with Clk/Sty, indicating that both domains are required for recognition by Clk/Sty. Taken together, these results suggest that the RS domain of Clk/Sty is important for its recognition of SR splicing factors, but that extensive contacts are probably made between the kinase and its binding partners.

ASF/SF2 is a candidate physiological substrate of Clk/Sty

Phosphorylation/dephosphorylation of splicing factors may be an important aspect of splicing regulation (Mermoud *et al.*, 1992; Tazi *et al.*, 1992), since SR protein function *in vitro* can be affected by phosphorylation (Gui *et al.*, 1994a; Mermoud *et al.*, 1994). *In vitro*, Clk/Sty efficiently phosphorylated ASF/SF2, predominantly in the ASF/SF2 RS domain. This conclusion is based on the observation that the isolated ASF/SF2 RS domain was phosphorylated to a level comparable with the full-length protein, whereas ASFΔRS, which lacks the RS domain, was only very weakly phosphorylated. In addition, all of the tryptic phosphopeptides observed in full-length ASF/SF2 phosphorylated by Clk/Sty can be accounted for by the RS domain alone. However, ASF/SF2 phosphorylated *in vivo* or *in vitro* by Clk/Sty displayed a trace amount of

phosphothreonine. Since, phosphothreonine was only seen in the RNA binding domain and not in the RS domain, it is likely that the RNA binding domain was phosphorylated to a minor extent by Clk/Sty.

Other potential protein substrates were used to test the specificity of Clk/Sty. Clk/Sty phosphorylated the basic proteins H1 histone and MBP, although more weakly than ASF/SF2, but not more acidic substrates such as β-casein, enolase or a GST fusion protein containing the c-Jun N-terminal region. It seems likely that Clk/Sty recognizes the site to be phosphorylated in the context of basic amino acids, as found in the RSRS stretch of ASF/SF2. These results indicate that ASF/SF2 is a preferential substrate for Clk/Sty.

The finding that the tryptic phosphopeptide maps of ASF/SF2 labelled *in vivo* or phosphorylated *in vitro* by Clk/Sty are similar to one another is consistent with the suggestion that Clk/Sty phosphorylates ASF/SF2 in intact cells, and may, therefore, regulate its activity. There are additional sites phosphorylated *in vivo* that cannot be accounted for by Clk/Sty phosphorylation, suggesting that Clk/Sty is not the only kinase that phosphorylates SR proteins *in vivo*. Indeed, two other kinases have been identified that can phosphorylate ASF/SF2 *in vitro*, SRPK1 (Gui *et al.*, 1994a) and an uncloned U1 70K kinase (Woppmann *et al.*, 1993). Also, we have shown here that PKC and PKA are able to phosphorylate ASF/SF2 *in vitro*. It remains to be seen if either of these latter two kinases, like Clk/Sty, is able to phosphorylate ASF/SF2 on sites that are also phosphorylated *in vivo*. SRPK1, the first SR family kinase to be identified, phosphorylates ASF/SF2 on sites *in vitro* that comprise the phosphoepitope recognized by anti-mAB104, indicating that SRPK1 is likely to regulate ASF/SF2 function *in vivo* (Gui *et al.*, 1994b).

Comparison of Clk/Sty and SRPK1

SRPK1 is most closely related to a *Schizosaccharomyces pombe* kinase DSK1 and a *Caenorhabditis elegans* Clk/Sty-like hypothetical kinase (CEHK) (Takeuchi and Yanagida, 1993; Wilson *et al.*, 1994). Interestingly, DSK1 autophosphorylates on all three amino acids, identifying it as a dual specificity kinase similar to Clk/Sty (Takeuchi and Yanagida, 1993).

Across the kinase domain, there are interesting relationships between Clk/Sty and the SRPK1 family. The entire EHLAMMERILGPLP sequence is not conserved, but there are two invariant amino acids that correspond to His386 and Pro398 of Clk/Sty (Figure 8). Clk3 has a valine at position 388, which is a conservative change from the alanine found in the other family members. These three amino acids are not conserved among other kinases. Two other protein kinases, CDK2 and SNF1, did not interact with RNA binding proteins in the two-hybrid assay (data not shown) and their equivalent sequences are shown for comparison.

Besides the residues conserved in the LAMMER motif, there are other key amino acids that are identical between Clk/Sty and SRPK1. In the catalytic loop (subdomain 6), an arginine which is otherwise highly conserved among protein kinases is replaced by a threonine in both Clk/Sty and SRPK1 (Thr286 of Clk/Sty). In the cAMP-dependent kinase structure, this arginine residue interacts with phosphorylated Thr197 to form a pocket for the amino acid

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mClk-Sty   EHLAMMERILG--PLP   398
huCLK      EHLAMMERILG--PLP   369
huCLK2     EHLAMMERILG--PIP   401
huCLK3     EHLVMMEKILG--PIP   394
DOA        EHLAMMERILG--QIP   394
AFC1       EHLAMMERVLG--PLP   359
AFC2       EHLAMMERVLG--PFP   340
AFC3       EHLAMMERALG--PLP   307
KNS1       EHMAMMQRING-TPFP   564
SRPK1      DHIALIIELLG--KVP   577
DSK1       DHIAQIIELLV--NYP   440
CEHK       DHLAHISELLG--AIP   943
CDK2       DQLFRIFRILG-TPDE   224
SNF1       GAAGLIKRMLIVNPLN   293
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Fig. 8. Alignment of the LAMMER motif of the LAMMER family kinases with SRPK1, DSK1 the *C.elegans* kinase, CDK2 and SNF1. The last amino acid in the motif is indicated on the right. Amino acids that are conserved among 80% of the aligned kinases are highlighted in black. Amino acids that are identical in 60–80% of these kinases are in bold. Abbreviations: mClk-Sty, mouse Clk/Sty; huCLK, human CLK; CEHK, *C.elegans* hypothetical kinase.

(P+1) following the hydroxyamino acid being phosphorylated (Knighton *et al.*, 1991). The substitution of this arginine for threonine may affect the pocket for the P+1 amino acid and possibly the orientation of a peptide substrate in the active site.

The Clk/Sty and SRPK1 kinase families also have a conserved arginine corresponding to amino acid 345 of Clk/Sty. In the ERK2 structure, this arginine has been shown to form part of the P+1 binding site (Zhang *et al.*, 1994a). The conserved regions in these two sets of kinase families may help explain why the two kinases phosphorylate the same protein.

Regulation of SR splicing factors by Clk/Sty in vivo

It has been shown previously that Clk/Sty is localized to the nucleus (Duncan *et al.*, 1995). In this study, we show that a kinase-inactive mutant of Clk/Sty was localized to discrete speckles within the nucleus, where SR proteins are located. In contrast to the Clk/Sty kinase-inactive mutant, wild-type Clk/Sty displayed a more diffuse nuclear distribution. *In vivo*, kinase-inactive Clk/Sty appeared to be hypophosphorylated, whereas the wild-type protein was hyperphosphorylated, presumably as a consequence of autophosphorylation (Duncan *et al.*, unpublished results, and Figure 7). This result indicates that phosphorylation may oppose retention in the speckles. This hypothesis is further borne out by the loss of SR proteins from the speckles when wild-type Clk/Sty was overexpressed. We propose that the SR proteins and Clk/Sty are targeted to the speckles through their RS domain (Li and Bingham, 1991) but are released from these sites upon phosphorylation. This redistribution after phosphorylation was first reported in permeabilized cells that overexpress the SRPK1 protein kinase, indicating that speckle disassembly may be a common phenomenon after SR family phosphorylation (Gui *et al.*, 1994a).

Nuclear speckles have been proposed to act as storage sites for splicing components (Fakan *et al.*, 1984; Spector, 1993; Huang *et al.*, 1994; Zhang *et al.*, 1994b). There is no evidence that speckles are disassembled prior to the

onset of splicing (Zhang *et al.*, 1994) and this may only occur when the kinase responsible for the release of factors is overexpressed. One possibility is that at physiological levels of Clk/Sty only a subset of SR factors are phosphorylated and released from the speckles. The availability of a subset of SR factors may be a significant regulatory mechanism, given the influence of the relative concentration of individual splicing factors on pre-mRNA processing (Ge and Manley, 1990; Krainer *et al.*, 1990; Fu, 1993; Zahler *et al.*, 1993a). Protein phosphatase 1 has been shown to affect 5′ splice site selection *in vitro*, indicating that phosphorylation plays a role in alternative splicing (Cardinali *et al.*, 1994). Indeed, preliminary results indicate that overexpression of Clk/Sty in 293 cells can alter 5′ splice site selection *in vivo* (J.Prasad, K.Colwill, T.Pawson and J.L.Manley, unpublished data).

Due to the complexity of factors involved in pre-mRNA processing, it seems likely that multiple kinases, perhaps including other members of the LAMMER family, regulate the many facets of splicing. We propose that Clk/Sty and SRPK1 are two members of a family of kinases that regulate SR protein trafficking and activity.

Material and methods

DNA subcloning

The following Clk/Sty constructs were amplified by PCR from plasmid pE3.1 (Ben-David *et al.*, 1991) using *Bam*HI-tagged primers and inserted into vector pAS1 (Durfee *et al.*, 1993) in-frame with the DNA binding domain: pAS-Clk/Sty (corresponding to aa 1–483), pAS-Clk/Sty^{RS} (aa 1–156) and pAS-Clk/Sty^{KIN} (aa 157–483). To generate pAS-Clk/Sty^{K190R}, the plasmid pE3-2KR-6 (Ben-David *et al.*, 1991), with Lys190 mutated to Arg, was cut with *Hin*dIII and *Eco*RI to release fragment 1 (nt 265–1701). Fragment 2 (nt 70–504) was generated by PCR from pE3.1 with *Eco*RI-tagged primers and cut with *Hin*dIII to generate a smaller fragment (nt 70–264). The two fragments were ligated into the *Eco*RI site of the yeast plasmid pRD54 (gift of R.Deschenes). A *Sal*I fragment (nt 277–1521) was released from pAS-Clk/Sty and a similar *Sal*I fragment from pRD54-Clk/Sty^{K190R} was inserted in its place. To construct pACT-X16 RS, X16 (aa 86–164) was amplified from pX16.12S5 (Ayane *et al.*, 1991) by PCR with *Nco*I–*Eco*RI-tagged primers and cloned into *Nco*I–*Eco*RI-digested pACTII vector (gift of S.Elledge). pACT-X16 RNA binding domain (aa 1–85) was constructed similarly. To construct pACT-ASF RS, ASF/SF2 (aa 198–248) was amplified by PCR from pDS-H6F1 (Ge *et al.*, 1991) with *Eco*RI–*Sal*I-tagged primers and cloned into the *Eco*RI–*Xho*I sites of pACTII. To generate GST–ASF RS, the RS domain of ASF/SF2 was amplified by PCR using *Bam*HI–*Sal*I-tagged primers and cloned into the *Bam*HI–*Sal*I-digested pGEX-4T-2 vector (from Pharmacia). To construct RC-ASF, pDS-H6F1 was digested with *Bam*HI releasing full-length ASF/SF2 (aa 1–248) that was ligated into *Bam*HI-digested pET-28a (Novagen), resulting in plasmid pET-ASF that coded for a T7 Tag epitope at the N-terminus of ASF/SF2. The ASF/SF2 insert was digested with *Eco*RI, blunt ended with Klenow, digested with *Xba*I and inserted into *Xba*I-blunt end sites of pRC/CMV (Invitrogen). All PCR products were sequenced to confirm their sequence integrity.

Two-hybrid screen

Yeast strain Y153 (Durfee *et al.*, 1993) was transformed with pAS-Clk/Sty. Expression of the construct was confirmed by Western analysis using the monoclonal antibody α-12CA5F (available from BABCO) which recognizes an HA epitope tag preceding the N-terminus of Clk/Sty. The resulting strain was co-transformed with an unstimulated murine T cell cDNA library fused to the GAL4 activation domain in the vector pACT (Durfee *et al.*, 1993). Putative interacting clones were identified on selective plates containing 50 mM 3-amino triazole and screened for β-galactosidase activity (Durfee *et al.*, 1993). Positive clones were purified in bacteria selecting for their LEU marker and re-transformed into the yeast strain Y153. Any clone that activated the reporter on its own was eliminated. To eliminate further false positives, Y153 containing the cDNA clones were mated with yeast strain Y187 transformed with one of the following plasmids: pAS1, pAS-Clk/Sty, pAS-CDK2, pAS-SNF1 (Harper *et al.*, 1993). To define the domains of interaction, Y153 was transformed with pACT-ASF RS and pACT-X16 RS. These transformants, along with the library clone transformants, were mated to yeast strain Y187 containing one of the following: pAS-Clk/Sty^{K190R}, pAS-Clk/Sty^{KIN} and pAS-Clk/Sty^{RS} and assayed for activation of reporter genes.

Sequencing of clones

Clones that interacted specifically with Clk/Sty were partially sequenced with [^{35}S]ATP or fluorescent primers using the Automated Laser Fluorescence DNA sequencer.

Protein purification

GST–Clk/Sty (Duncan *et al.*, 1995) was induced into bacteria using 1 mM IPTG, harvested after 3 h of induction in buffer A [50 mM NaPi pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 1% Triton X-100, 2 mM dithiothreitol (DTT), 2 mM benzamadine], and bound to glutathione–agarose. GST–Clk/Sty was eluted from the glutathione–agarose using 30 mM glutathione, and concentrated into buffer B (buffer A without 1% Triton X-100). GST–Clk/Sty^{K190R} was isolated as previously described (Duncan *et al.*, 1995). GST and GST–ASF RS were induced in bacteria, harvested in phosphate-buffered saline (PBS) with 1% Triton X-100 and 1% Tween, eluted from glutathione–agarose with 10 mM glutathione, and concentrated into 40 mM HEPES pH 7.5. ASF/SF2 and ASFΔRS (Zuo and Manley, 1993) were purified by nickel chromatography as described previously (Ge *et al.*, 1991).

H1 histone and enolase were purchased from Boehringer Mannheim and MBP and β-casein were purchased from Sigma. GST–c-Jun 5–89 was a gift of Jim Woodgett. Enolase was acid treated as described (Sadowski and Pawson, 1987).

In vitro kinase reaction

GST–Clk/Sty (500 ng) was incubated in 50 μl of 40 mM HEPES pH 7.5, 10 mM $MgCl_2$, 20 μM ATP, 2 mM DTT and 2 μCi of [γ-^{32}P]ATP for 30 min at room temperature. The reaction was terminated by boiling in 50 μl of 2× SDS sample buffer. For exogenous substrates, 1 μg of substrate was added. For the kinase specificity assay, 150 ng of Clk/Sty, PKA, PKC, CKII or p42/p44 MAPK were used to phosphorylate 1 μg of ASF/SF2 or an exogenous substrate as indicated. PKC, MAPK and CKII were a gift of Jim Woodgett, and PKA was purchased from Sigma.

In vivo labelling

COS-1 cells were transfected with RC-ASF using lipofectin. Approximately 68 h post-transfection, the medium was replaced with 5 mCi of [^{32}P]orthophosphate in DMEM–phosphate media plus 10% fetal bovine serum. The cells were incubated for 4 h and then harvested in NP-40–RIPA buffer [150 mM NaCl, 50 mM Tris–HCl pH 7.5, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS, 10 mM NaF, 1 mM DTT, 2 mM EDTA, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 200 μg/ml phenylmethylsulfonyl fluoride (PMSF)]. The supernatant was incubated with an α-T7 Tag antibody (from Novagen) bound to mouse protein A–Sepharose for 1.5 h. The immunoprecipitates were washed four times with NP-40–RIPA buffer and boiled in 2× SDS sample buffer.

Phosphoamino acid analysis

The samples were resolved by SDS–PAGE and subsequently transferred to PVDF membrane. After exposure to autoradiography, the ^{32}P-labelled bands were excised, hydrolysed and subjected to two-dimensional electrophoresis (Boyle *et al.*, 1991) in the presence of non-radioactive markers.

Tryptic mapping

Pertinent bands were excised from the polyacrylamide gel. The proteins were then eluted, precipitated and trypsin digested (Boyle *et al.*, 1991). After digestion, the samples were spotted onto a thin layer cellulose plate and subjected to electrophoresis for 60 min at 1000 V in pH 1.9 buffer, followed by chromatography using phosphochromatography buffer (Boyle *et al.*, 1991). For the mixes, equal amounts of radioactivity of each were mixed and spotted on the same plate.

Immunofluorescence assay

COS-1 cells were transfected with plasmids encoding M-Clk/Sty or M-Clk/Sty^{K190R}, both of which contain the Myc epitope (Duncan *et al.*, 1995). Cells were fixed, and probed as described (Duncan *et al.*, 1995). For double staining by indirect immunofluorescence, fixed cells were incubated with mAB 9E10 (α-Myc, 25 μl of culture supernatant) (Evan

et al., 1985), followed by secondary fluorescein-conjugated goat α-mouse IgG (1:100, Jackson Immunoresearch Laboratories) specific for mouse IgG, and incubated subsequently with mAb 104 (α-SR, 25 μl culture supernatant) (Roth *et al.*, 1990) followed by secondary biotin-labelled α-mouse IgM (1:100, Amersham) and streptavidin coupled to Texas Red (1:100, Amersham). Cells were viewed by confocal microscopy using an upright Leica Confocal Laser Scanning Microscope equipped with a 55 mW krypton/argon air-cooled laser and a 25× or 63× Plan Apo oil immersion lens.

Triton X-100 extraction

Transfected cells were extracted on coverslips (for immunofluorescence) or in microcentrifuge tubes (for immunoblotting) with extraction buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 2 mM NaF, 2 mM sodium pyrophosphate, 500 μM sodium vanadate, 200 μg/ml PMSF, 2 μg/ml aprotinin, 5 μg/ml leupeptin) for 30 min at 4°C. For immunofluorescence, coverslips were washed with PBS, and fixed and processed as above. For immunolobotting, lysates were cleared by centrifugation. Supernatants (Triton-extracted fractions) and pellets (Triton-insoluble fraction) were boiled in SDS buffer and samples resolved by 10% SDS–PAGE and transferred to nitrocellulose membrane. Membranes were probed with anti-Myc mAb as described above.

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References

Amrein,H., Hedley,M.L. and Maniatis,T. (1994) The role of specific protein–RNA and protein–protein interactions in positive and negative control of pre-mRNA splicing by *Transformer 2*. *Cell*, **76**, 735–746.

Ayane,M., Preuss,U., Kohler,G. and Nielsen,P.J. (1991) A differentially expressed murine RNA encoding a protein with similarities to two types of nucleic acid binding motifs. *Nucleic Acids Res.*, **19**, 1273–1278.

Ben-David,Y., Letwin,K., Tannock,L., Bernstein,A. and Pawson,T. (1991) A mammalian protein kinase with potential for serine/threonine and tyrosine phosphorylation is related to cell cycle regulators. *EMBO J.*, **10**, 317–325.

Bender,J. and Fink,G.R. (1994) AFC1, a LAMMER kinase from *Arabidopsis thaliana*, activates STE12-dependent processes in yeast. *Proc. Natl Acad. Sci. USA*, **91**, 12105–12109.

Boyle,W.J., van der Geer,P. and Hunter,T. (1991) Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol.*, **201**, 110–149.

Burd,C.G. and Dreyfuss,G. (1994) Conserved structures and diversity of functions of RNA-binding proteins. *Science*, **265**, 615–621.

Caceres,J.F. and Krainer,A.R. (1993) Functional analysis of pre-mRNA splicing factor SF2/ASF structural domains. *EMBO J.*, **12**, 4715–4726.

Cardinali,B., Cohen,P.T.W. and Lamond,A.I. (1994) Protein phosphatase 1 can modulate alternative 5′ splice site selection in a HeLa splicing extract. *FEBS Lett.*, **352**, 276–280.

Douville,E.M.J., Duncan,P.I., Abraham,N. and Bell,J.C. (1994) Dual specificity kinases—a new family of signal transducers. *Cancer Metastasis Rev.*, **13**, 1–7.

Dreyfuss,G., Matunis,M.J., Pinol-Roma,S. and Burd,C.G. (1993) hnRNP proteins and the biogenesis of mRNA. *Annu. Rev. Biochem.*, **62**, 289–321.

Duncan,P.I., Howell,B.W., Marius,R.M., Drmanic,S., Douville,E.M.J. and Bell,J.C. (1995) Alternative splicing of Sty, a nuclear dual specificity kinase. *J. Biol. Chem.*, **270**, 21524–21531.

Durfee,T., Becherer,K., Chen,P.-L., Yeh,S.-H., Yang,Y., Kilburn,A.E., Lee,W.-H. and Elledge,S.J. (1993) The retinoblastoma protein associates with the protein phosphatase type I catalytic subunit. *Genes Dev.*, **7**, 555–569.

Evan,G.I., Lewis,G.K., Ramsay,G. and Bishop,J.M. (1985) Isolation of monoclonal antibodies specific for human c-*myc* proto-oncogene product. *Mol. Cell. Biol.*, **5**, 3610–3616.

Fakan,S., Leser,G. and Martin,T.E. (1984) Ultrastructural distribution of nuclear ribonucleoproteins as visualized by immunocytochemistry on thin sections. *J. Cell Biol.*, **98**, 358–363.

Fields,S. and Song,O.-K. (1989) A novel genetic system to detect protein–protein interactions. *Nature*, **340**, 245–246.

Fu,X.-D. (1993) Specific commitment of different pre-mRNAs to splicing by single SR proteins. *Nature*, **365**, 82–85.

Fu,X.-D. and Maniatis,T. (1990) Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. *Nature*, **343**, 437–441.

Ge,H. and Manley,J.L. (1990) A protein factor, ASF, controls cell-specific alternative splicing of SV40 early pre-mRNA *in vitro*. *Cell*, **62**, 25–34.

Ge,H., Zuo,P. and Manley,J.L. (1991) Primary structure of the human splicing factor ASF reveals similarities with *Drosophila* regulators. *Cell*, **66**, 373–382.

Gotoh,Y., Moriyama,K., Matsuda,S., Okumura,E., Kishimoto,T., Kawasaki,H., Suzuki,K. and Nishida,E. (1991) *Xenopus* M phase MAP kinase: isolation of its cDNA and activation by MPF. *EMBO J.*, **10**, 2661–2668.

Gui,J.-F., Lane,W.S. and Fu,X.-D. (1994a) A serine kinase regulates intracellular localization of splicing factors in the cell cycle. *Nature*, **369**, 678–682.

Gui,J.-F., Tronchere,H., Chandler,S.D. and Fu,X.-D. (1994b) Purification and characterization of a kinase specific for the serine- and arginine-rich pre-mRNA splicing factors. *Proc. Natl Acad. Sci. USA*, **91**, 10824–10828.

Hanes,J., von der Kammer,H., Klaudiny,J. and Scheit,K.H. (1994) Characterization by cDNA cloning of two new human protein kinases. *J. Mol. Biol.*, **244**, 665–672.

Hanks,S.K., Quinn,A.M. and Hunter,T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science*, **241**, 42–52.

Hanks,S.K., Quinn,A.M. and Hunter,T. (1991) Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. *Methods Enzymol.*, **200**, 38–62.

Hardie,D.G., Campbell,D.G., Caudwell,F.B. and Haystead,T.A.J. (1993) Analysis of sites phosphorylated *in vivo* and *in vitro*. In Hardie,D.G. (ed.), *Protein Phosphorylation—A Practical Approach*. IRL Press, Oxford, pp. 61–85.

Harper,J.W., Adami,G.R., Wei,N., Keyomarsi,K. and Elledge,S.J. (1993) The p21 CDK-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*, **75**, 805–816.

Howell,B.W., Afar,D.E.H., Lew,J., Douville,E.M.J., Icely,P.L.E., Gray,D.A. and Bell,J.C. (1991) STY, a tyrosine-phosphorylating enzyme with sequence homology to serine/threonine kinases. *Mol. Cell. Biol.*, **11**, 568–572.

Huang,S., Deerinck,T.J., Ellisman,M.H. and Spector,D.L. (1994) *In vivo* analysis of the stability and transport of nuclear poly(A)$^+$ RNA. *J. Cell Biol.*, **126**, 877–899.

Johnson,K.W. and Smith,K.A. (1991) Molecular cloning of a novel human cdc2/CDC28-like protein kinase. *J. Biol. Chem.*, **266**, 3402–3407.

Kenan,D.J., Query,C.C. and Keene,J.D. (1991) RNA recognition: towards identifying determinants of specificity. *Trends Biochem. Sci.*, **16**, 214–220.

Knighton,D.R., Zheng,J., Ten Eyck,L.F., Xuong,N.-H., Taylor,S.S. and Sadowski,J.M. (1991) Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science*, **253**, 414–424.

Kohtz,J.D., Jamison,S.F., Will,C.L., Zuo,P., Lührmann,R., Garcia-Blanco,M.A. and Manley,J.L. (1994) Protein–protein interactions and 5′-splice-site recognition in mammalian mRNA precursors. *Nature*, **368**, 119–124.

Krainer,A.R., Conway,G.C. and Kozak,D. (1990) The essential pre-mRNA splicing factor SF2 influences 5′ splice site selection by activating proximal sites. *Cell*, **62**, 35–42.

Krainer,A.R., Mayeda,A., Kozak,D. and Binns,G. (1991) Functional expression of cloned human splicing factor SF2: homology to RNA-binding proteins, U1 70K, and *Drosophila* regulators. *Cell*, **66**, 383–394.

Li,H. and Bingham,P.M. (1991) Arginine/serine-rich domains of the *su(w^a)* and *tra* RNA processing regulators target proteins to a subnuclear compartment implicated in splicing. *Cell*, **67**, 335–342.

Lindberg,R.A., Quinn,A.M. and Hunter,T. (1992) Dual-specificity protein kinases: will any hydroxyl do? *Trends Biochem. Sci.*, **17**, 114–119.
Mermoud,J.E., Cohen,P. and Lamond,A.I. (1992) Ser/Thr-specific protein phosphatases are required for both catalytic steps of pre-mRNA splicing. *Nucleic Acids Res.*, **20**, 5263–5269.
Mermoud,J.E., Cohen,P.T.W. and Lamond,A.I. (1994) Regulation of mammalian spliceosome assembly by a protein phosphorylation mechanism. *EMBO J.*, **13**, 5679–5688.
Myers,M.P., Murphy,M.B. and Landreth,G. (1994) The dual-specificity CLK kinase induces neuronal differentiation of PC12 cells. *Mol. Cell. Biol.*, **14**, 6954–6961.
Padmanabha,R., Gehrung,S. and Snyder,M. (1991) The KNS1 gene of *Saccharomyces cerevisiae* encodes a nonessential protein kinase homologue that is distantly related to members of the CDC28/cdc2 gene family. *Mol. Gen. Genet.*, **229**, 1–9.
Pearson,R.B. and Kemp,B.E. (1991) Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. *Methods Enzymol.*, **200**, 62–81.
Rabinow,L. and Birchler,J.A. (1989) A dosage-sensitive modifier of retrotransposon induced alleles of the *Drosophila white* locus. *EMBO J.*, **8**, 879–889.
Reith,A.D., Ellis,C., Lyman,S.D., Anderson,D.M., Williams,D.E., Bernstein,A. and Pawson,T. (1991) Signal transduction by normal isoforms and *W* mutant variants of the Kit receptor tyrosine kinase. *EMBO J.*, **10**, 2451–2459.
Romac,J.M.J. and Keene,J.D. (1995) Overexpression of the arginine-rich carboxy-terminal region of U1 snRNP 70K inhibits both splicing and nucleocytoplasmic transport of mRNA. *Genes Dev.*, **9**, 1400–1410.
Roth,M.B., Murphy,C. and Gall,J.G. (1990) A monoclonal antibody that recognizes a phosphorylated epitope stains lampbrush chromosome loops and small granules in the amphibian germinal vesicle. *J. Cell Biol.*, **111**, 2217–2223.
Sadowski,I. and Pawson,T. (1987) Catalytic and non-catalytic domains of the Fujinami sarcoma virus $P130^{gag-fps}$ protein-tyrosine kinase distinguished by the expression of v-*fps* polypeptides in *Escherichia coli*. *Oncogene*, **1**, 181–191.
Schmidt,G. and Werner,D. (1993) Sequence of a complete murine cDNA reflecting an S phase-prevalent transcript encoding a protein with two types of nucleic acid binding motifs. *Biochim. Biophys. Acta*, **1216**, 317–320.
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.
Soulard,M. *et al.* (1993) hnRNP G: sequence and characterization of a glycosylated RNA-binding protein. *Nucleic Acids Res.*, **21**, 4210–4217.
Spector,D.L. (1993) Macromolecular domains within the cell nucleus. *Annu. Rev. Cell Biol.*, **9**, 265–315.
Spector,D.L., Schrier,W.H. and Busch,H. (1983) Immunoelectron microscopic localization of snRNPs. *Biol. Cell.*, **49**, 1–10.
Tacke,R., Boned,A. and Goridis,C. (1992) ASF alternative transcripts are highly conserved between mouse and man. *Nucleic Acids Res.*, **20**, 5482.
Takeuchi,M. and Yanagida,M. (1993) A mitotic role for a novel fission yeast protein kinase dsk1 with cell cycle stage dependent phosphorylation and localization. *Mol. Biol. Cell*, **4**, 247–260.
Tang,Z., Coleman,T.R. and Dunphy,W.G. (1993) Two distinct mechanisms for negative regulation of the Wee1 protein kinase. *EMBO J.*, **12**, 3427–3436.
Tazi,J., Daugeron,M.C., Cathala,G., Brunel,C. and Jeanteur,P. (1992) Adenosine phosphorothioates (ATP alpha S and ATP tau S) differentially affect the two steps of mammalian pre-mRNA splicing. *J. Biol. Chem.*, **267**, 4322–4326.
Thomis,D.C. and Samuel,C.E. (1993) Mechanism of interferon action: evidence for intermolecular autophosphorylation and autoactivation of the interferon-induced, RNA-dependent protein kinase PKR. *J. Virol.*, **67**, 7695–7700.
Verheijen,R., Kuijpers,H., Vooijs,P., van Venrooij,W. and Ramaekers,F. (1986) Distribution of the 70K U1 RNA-associated protein during interphase and mitosis. Correlation with other U RNP particles and proteins of the nuclear matrix. *J. Cell Sci.*, **86**, 173–190.
Wilson,R. *et al.* (1994) 2.2 Mb of contiguous nucleotide sequence from chromosome II of *C.elegans*. *Nature*, **368**, 32–38.
Woppmann,A., Will,C.L., Kornstadt,U., Zuo,P., Manley,J.L. and Lührmann,R. (1993) Identification of an snRNP-associated kinase activity that phosphorylates arginine/serine rich domains typical of splicing factors. *Nucleic Acids Res.*, **21**, 2815–2822.
Wu,J.Y. and Maniatis,T. (1993) Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. *Cell*, **75**, 1061–1070.
Yun,B., Farkas,R., Lee,K. and Rabinow,L. (1994) The *Doa* locus encodes a member of a new protein kinase family and is essential for eye and embryonic development in *Drosophila melanogaster*. *Genes Dev.*, **8**, 1160–1173.
Zahler,A.M., Lane,W.S., Stolk,J.A. and Roth,M.B. (1992) SR proteins: a conserved family of pre-mRNA splicing factors. *Genes Dev.*, **6**, 837–847.
Zahler,A.M., Neugebauer,K.M., Lane,W.S. and Roth,M.B. (1993a) Distinct functions of SR proteins in alternative pre-mRNA splicing. *Science*, **260**, 219–222.
Zahler,A.M., Neugebauer,K.M., Stolk,J.A. and Roth,M.B. (1993b) Human SR proteins and isolation of a cDNA encoding SRp75. *Mol. Cell. Biol.*, **13**, 4023–4028.
Zhang,F., Strand,A., Robbins,D., Cobbs,M.H. and Goldsmith,E.J. (1994a) Atomic structure of the MAP kinase ERK2 at 2.3 Å resolution. *Nature*, **367**, 704–710.
Zhang,G., Taneja,K.L., Singer,R.H. and Green,M.R. (1994b) Localization of pre-mRNA splicing in mammalian nuclei. *Nature*, **372**, 809–812.
Zuo,P. and Manley,J.L. (1993) Functional domains of the human splicing factor ASF/SF2. *EMBO J.*, **12**, 4727–4737.

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Note added in proof

Clone 9-11 is the murine homolog of the recently cloned human SRp55 (Screaton *et al.*, 1995). Over the region sequenced, clone 9-11 is 95% identical (98% similar) to human SRp55 (K.Colwill, E.Aippersbach and T.Pawson, unpublished results).