*2972–2980* *Nucleic Acids Research, 1999, Vol. 27, No. 14* 

# SR protein-specific kinase 1 is highly expressed in testis and phosphorylates protamine 1

**Stamatia Papoutsopoulou, Eleni Nikolakaki, George Chalepakis[1], Volker Kruft[2], Philippe Chevaillier[3] and Thomas Giannakouros***

Laboratory of Biochemistry, School of Chemistry, The Aristotelian University of Thessaloniki, Thessaloniki 54 006, Greece, [1]Department of Biology, The University of Crete, Heraclion 71 409, Crete, Greece, [2]PE Biosystems, Applied Biosystems GmbH, Brunnenweg 13, D-64331 Weiterstadt, Germany and [3]Laboratoire de Biologie Cellulaire, Université Paris-Val de Marne, 94010 Créteil, France

Received February 18, 1999; Revised April 12, 1999; Accepted May 25, 1999 DDBJ/EMBL/GenBank accession no. AJ224115

## ABSTRACT

**Arginine/serine protein kinases constitute a novel class of enzymes that can modify arginine/serine (RS) dipeptide motifs. SR splicing factors that are essential for pre-mRNA splicing are among the best characterized proteins that contain RS domains. Two SR protein-specific kinases, SRPK1 and SRPK2, have been considered as highly specific for the phosphorylation of these proteins, thereby contributing to splicing regulation. However, despite the fact that SR proteins are more or less conserved among metazoa and have a rather ubiquitous tissue distribution we now demonstrate that SRPK1 is predominantly expressed in testis. *In situ* expression analysis on transverse sections of adult mouse testis shows that *SRPK1* mRNA is abundant in all germinal cells but not in mature spermatozoa. RS kinase activity was found primarily in the cytosol and only minimal activity was detected in the nucleus. In a search for testis-specific substrates of SRPK1 we found that the enzyme phosphorylates human protamine 1 as well as a cytoplasmic pool of SR proteins present in the testis. Protamine 1 belongs to a family of small basic arginine-rich proteins that replace histones during the development of mature spermatozoa. The result of this progressive replacement is the formation of a highly compact chromatin structure devoid of any transcriptional activity. These findings indicate that SRPK1 may have a role not only in pre-mRNA splicing, but also in the condensation of sperm chromatin.**

## INTRODUCTION

Protein phosphorylation/dephosphorylation represents a major mechanism of mediation of cellular functions. The vast family of known protein kinases catalyzes phosphorylation of a multitude of proteins in the cell in response to various stimuli. Although in the last decade the control mechanisms for the regulation of numerous protein kinases have been delineated, the regulation as well as the specific mode of action of several other kinases, and especially those that are not activated by any known second messenger, remain more or less obscure. Arginine/serine protein kinases constitute a novel class of enzymes that specifically modify arginine/serine (RS) dipeptide motifs. Among the best characterized proteins that contain RS repeats are the superfamily of arginine/serine-rich (RS) domain-containing splicing factors (for a review see 1). The so-called SR proteins are characterized by a shared phospho epitope that cross-reacts with the monoclonal antibody mAb 104, at least one N-terminal RNA recognition motif and a basic C-terminal domain rich in arginine and serine residues, often arranged in tandem repeats. Due to their RS domains SR proteins play a critical role in selecting and pairing functional splice sites and therefore they are not only essential for constitutive splicing but can also affect alternative splicing (2–5). Furthermore, the RS domains function, in a phosphorylation-dependent manner, in the recruitment of the SR splicing factors from nuclear speckles, in which they are concentrated, to active sites of transcription (6,7).

Two SR protein-specific kinases, SRPK1 and SRPK2, have been reported to specifically phosphorylate the RS motifs of the SR family of splicing factors and play roles in spliceosome assembly and in mediating the trafficking of SR splicing factors in mammalian cells (8–10). SRPK1 may also be responsible for the redistribution of splicing factors as cells enter mitosis (8). Furthermore, mammalian Clk/Sty, considered as the prototype for a family of dual specificity kinases (termed LAMMER kinases), was also found to interact with members of the SR family of splicing factors in the yeast two-hybrid system and to efficiently phosphorylate ASF/SF2 (11). However, the Clk/Sty kinase modifies, at least *in vitro*, not only Ser/Arg but also Ser/Lys and Ser/Pro sites, suggesting that the enzyme has a broader substrate specificity than SRPKs (12,13).

Several other proteins were also found to contain RS domains that differ in length, number of arginine/serine dipeptides and content of other amino acids (for relevant information see

*To whom correspondence should be addressed. Tel: +30 31 997726; Fax: +30 31 997689; Email: giannako@ccf.auth.gr

14 and an automatic update of RS domain-containing proteins at World Wide Web site http://www.mann.embl-heidelberg.de/Services/PeptideSearch/PeptideSearchIntro.html ). In an attempt to determine whether SRPK1 has additional substrates, outside the SR family of splicing factors, we have recently shown that this kinase may also efficiently phosphorylate, the lamin B receptor (LBR), an integral protein of the inner nuclear membrane, that contains a stretch of RS repeats in its nucleoplasmic, N-terminal domain (15). In this report we extend our studies on the cellular role(s) of SRPK1 demonstrating that SRPK1 is highly expressed in human and mouse testis and that the enzyme phosphorylates human protamine 1 in an efficient and highly specific manner.

Protamines are small highly basic proteins that replace histones during spermatogenesis in almost all organisms, resulting in extreme chromatin condensation (for a review see 16). The protamine 1 (P1) genes are conserved in all vertebrates, even though they have undergone significant changes during the evolution from birds to mammals, gaining an intron in the DNA sequence (17,18), while protamine 2 (P2) has been described only in some species, including man, stallion, hamster and mouse (19). P1 protamines are divided into two classes: those that do not contain cysteine residues and are found in birds, reptiles and marsupial mammals; those of the placental mammals that contain six to nine cysteine residues, allowing the sperm nuclei to form covalent intermolecular disulfide bridges and thus become more stable (20–23). However, as shown below, all P1 protamines, and even more molluscan protamine-like proteins (24,25), contain RS domains that may serve as targets of phosphorylation by members of the SRPK family of protein kinases.

## MATERIALS AND METHODS

### Cloning of human and mouse SRPK1

RNA from HeLa cells was isolated using the guanidinium/isothiocyanate protocol (26). Poly(A)$^+$ RNA was selected by oligo(dT)$_{25}$–cellulose attached to Dynabeads according to the manufacturer's instructions [Dynabeads Oligo®(dT)$_{25}$; Dynal AS, Oslo, Norway] and used for solid phase cDNA synthesis with Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Eggenstein, Germany) for 45 min at 41°C, followed by the thermostable *Thermus thermophilus* reverse transcriptase (Boehringer Mannheim GmbH, Mannheim, Germany) for 5 min at 50°C and 10 min at 72°C. A 617 bp fragment was amplified by 30 cycles of PCR, using HeLa cDNA as template and two primers comprising part of the sequence of human *SRPK1* (8) (sense primer 5'-$^{370}$TGGGGACACTTTTCAACAGTATGG$^{393}$-3'; antisense primer 5'-$^{987}$CTCCATTTCCTCAATTTCCTGCAT$^{964}$-3'). Parameters for the first cycle of PCR were as follows: denaturation at 92°C for 1 min, annealing at 56°C for 3 min and extension at 72°C for 5 min, whereas for the remaining 29 cycles annealing was performed at 56°C for 40 s and extension at 72°C for 1 min. The PCR product was purified using the QIAEX gel extraction kit (Qiagen GmbH, Hilden, Germany), sequenced and then labeled with [α-$^{32}$P]dCTP using the Multiprime DNA labeling system (Amersham, Bacacos SA, Greece). This labeled fragment was subsequently used to screen a Lambda ZAP F9 cDNA library (kindly provided by Dr Scholer, EMBL, Heidelberg, Germany) and a Lambda ZAP Express™ human testis cDNA library (Stratagene, La Jolla, CA). Samples of $5 \times 10^5$ plaques were screened in each case by hybridization of Hybond filters (Amersham), using standard procedures (27), to yield nine positive clones from the F9 cDNA library, none of which was full-length, and two positive clones from the human testis cDNA library, one of which was full-length. The sequence of both strands was determined by a series of nested deletions using unidirectional exonuclease III digestion according to the manufacturer's instructions (Double-Stranded Nested Deletion kit; Pharmacia, Uppsala, Sweden). The EMBL databank accession number for the mouse sequence is AJ224115.

### Northern blot hybridization

RNA from different tissues of 6-month-old C3Hb mice was isolated using the guanidinium/isothiocyanate protocol (26). Poly(A)$^+$ RNA was selected as described above, denatured in formamide/formaldehyde at 65°C for 5 min prior to electrophoresis on denaturating 1.2% agarose/formaldehyde gels and blotted overnight onto GeneScreen Plus™ membrane (Du Pont-New England Nuclear) in 10× SSC (1× SSC is composed of 0.15 M NaCl plus 0.015 M sodium citrate). Prehybridization of membranes was carried out at 42°C for 3 h in 5× SSPE (20× SSPE is composed of 3 M NaCl, 176 mM $NaH_2PO_4$ and 20 mM EDTA), 5× Denhardts solution (50× Denhardts contains 5 g Ficoll, 5 g polyvinylpyrolidone and 5 g BSA in 500 ml $ddH_2O$), 40% (v/v) deionized formamide, 0.8% (w/v) SDS and 150 µg/ml boiled sonicated salmon sperm DNA. Fresh hybridization solution (as above) was added to the membrane as well as denatured (in boiling water for 5 min) random-primed DNA probe. Random-prime labeled probes ($5 \times 10^8$ c.p.m./µg, $10^6$ c.p.m./ml) were generated from 50 ng DNA using the Multiprime DNA labeling system (Amersham). Following overnight hybridization at 42°C the membranes were washed in 2× SSPE at room temperature for 20 min, twice in 0.5× SSPE, 0.5% SDS at 55°C for 20 min and then exposed to Kodak X-ray films with intensifying screens, at –70°C, for various lengths of time.

Human multiple tissue northern blot filters I and II (Clontech, Palo Alto, CA) were kindly provided by G. Mavrothalassitis (University of Crete, Heraklion, Crete) and processed as described above.

### *In situ* hybridization

For *in situ* hybridization experiments testes were obtained from adult NMRI mice, fixed in 4% paraformaldehyde (PFA) in PBS at 4°C, dehydrated (the following day) through ethanol/saline solutions and after xylene treatment embedded in Paraplast. Sections (0.8 mm) were cut and dried on chromalum–gelatin slices. The slices were processed through the following steps: dewaxing in xylene, dehydration, washing in PBS, refixing in 4% PFA, washing, protease K treatment (0.02 mg/ml), washing, 4% PFA treatment, washing, 0.1 M triethanolamine treatment, washing and dehydration. An 860 bp *Eco*RI–*Xho*I fragment in pBluescript SK$^+$, representing the insert of one of the mouse clones (comprising nucleotides 1–860 of the sequence of mouse *SRPK1*), was used as a template to prepare sense and antisense RNA probes. The sense probe was synthesized using T3 RNA polymerase and the plasmid was linearized with *Kpn*I, whereas the antisense probe was synthesized using T7 RNA polymerase and the plasmid was linearized with *Xba*I.

$^{35}$S-labeled probes ($1 \times 10^8$ c.p.m./ml) were dissolved in hybridization buffer [300 mM NaCl, 10 mM Tris–HCl, pH 7.4, 10 mM sodium phosphate, 5 mM EDTA, 100 mM dithiothreitol (DTT), 10% dextran sulfate, 50% formamide, 2 mg/ml bovine serum albumin, 2 mg/ml Ficoll and 2 mg/ml polyvinylpyrrolidone], heated at 80°C for 2 min, applied onto the sections and covered with siliconized coverslips. Following overnight hybridization at 55°C the slices were washed as follows: 5× SSC and 10 mM DTT for 30 min at 37°C; 50% formamide and 2× SSC for 30 min at 65°C; 0.5 NaCl, 10 mM Tris–HCl, pH 7.4 and 4 mM EDTA for 10 min at 37°C; 0.5 NaCl, 10 mM Tris–HCl, pH 7.4, 5 mM EDTA and 0.02 mg/ml RNase A for 10 min at 37°C; 2× SSC, 10 mM DTT and 50% formamide for 15 min at 37°C; 2× SSC for 15 min at 37°C; 0.1× SSC for 15 min at 37°C. For morphological identification the sections were stained with Giemsa and coverslips were mounted in Eukitt, whereas for autoradiography the slices were dipped in Kodak NTB-2 emulsion diluted 1:1 with water and exposed for 10 days.

### Biochemical fractionation of rat tissues

Various tissues from 5-month-old male Wistar rats were homogenized, by means of a Potter-Elvehjem tissue homogenizer, in 3 vol of an ice-cold solution containing 0.25 M sucrose, 50 mM Tris–HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 5 mM DTT and 3 mM PMSF. The homogenates were passed through two layers of surgical gauze and then centrifuged at 1000 *g* for 10 min at 4°C to yield a crude nuclear fraction. The supernatant was centrifuged at 100 000 *g* for 2 h to obtain the cytosolic fraction (S100). The nuclei were further purified from the crude nuclear fraction by centrifugation, through a cushion of 2.3 M sucrose, at 100 000 *g* for 90 min according to the method of Blobel and Potter (28). Nuclear pellets were resuspended in 10 mM $Na_2HPO_4/NaH_2PO_4$, pH 7.5, 2 mM $MgCl_2$ and 1 mM PMSF and digested with 100 µg/ml DNase I for 45 min at room temperature. NaCl to a final concentration of 1 M was then added and the mixture was further incubated at 4°C for 2 h, on a rotator. Nuclear extracts were collected by centrifugation at 10 000 *g* for 30 min at 4°C and contained >90% of the total RS kinase activity present in the nuclei of the different tissues.

### Purification of sperm nuclear proteins

Basic nuclear proteins were prepared from purified sperm nuclei of human spermatozoa as previously described (29). Briefly, sperm nuclei were washed in 20 mM Tris–HCl, pH 7.5, 0.25 M sucrose, 150 mM NaCl, 2 mM EDTA and 0.5 mM PMSF and then incubated for 1 h at 4°C, under nitrogen, in 50 mM Tris–HCl, pH 8.8, 2 mM EDTA and 10 mM DTT. Iodoacetamide was added up to 20 mM and incubation was continued for 1 h under the same conditions. Sperm nuclei were pelleted and proteins were extracted overnight with 0.25 M HCl. Acid-soluble proteins, henceforth referred to as sperm nuclear basic proteins, were precipitated with 20% trichloroacetic acid, recovered by centrifugation and washed with acetone.

The non-phosphorylated form of protamine P1 was purified from sperm nuclear basic proteins by chromatography on carboxymethylcellulose and characterized by its amino acid composition and partial amino acid sequence, as previously described (19,29).

### Purification of SR proteins from rat testis cytosol

SR proteins were prepared from rat testis cytosol according to the method of Zahler *et al.* (30). The identity of the proteins was confirmed by western blotting using the mAb 104 monoclonal antibody (culture supernatant, kindly provided by Juan Valcarcel, EMBL). Portions of SR proteins (~5 µg) were dephosphorylated in 50 mM Tris–HCl, pH 7.5, 0.2 mM $MnCl_2$, 0.1 mM $Na_2EDTA$, 5 mM DTT and 0.01 mM Brij 35 using 40 U of Lambda protein phosphatase (New England Biolabs Ltd, Bishops Stortford, UK). Samples were incubated for 60 min at 30°C in a total volume of 15 µl. Phosphatase was inactivated by adding 0.2 mM $NaVO_3$ to the reaction mixture and heating at 70°C for 10 min. To determine the sequence of SC35, SR proteins were analyzed by SDS–PAGE, transferred to PVDF membranes and the 30 kDa band was excised from the blot and digested for 6 h, at room temperature, in 25 µl of 25 mM Tris–HCl, pH 8.0, to which 1% Zwittergent 3-16 (Calbiochem, La Jolla, CA) and 0.2 U endoproteinase Lys-C (sequencing grade; Boehringer, Mannheim GmbH) were added. The peptides were separated on an ABI 173A microblotter (PE Biosystems, Foster City, CA), with automatic transfer of the eluent onto ProBlott membrane. Membrane sections corresponding to peptide peaks were excised, coated with 50 µg of biobrene (PE Biosystems) and subjected to N-terminal sequencing employing the Applied Biosystems model 494CLC sequencing system.

### Construction of plasmids and expression of fusion proteins

The pGEX-2T bacterial expression vector (Pharmacia LKB Biotechnology Inc.) was used to express human *SRPK1* fused with glutathione *S*-transferase (GST) in *Escherichia coli*. To this purpose oligonucleotides corresponding to 21 nt of the 5'- and 3'-complementary coding regions of human *SRPK1* with additional *Bam*HI and *Hin*dIII sites at the 5'- and 3'-ends, respectively, were prepared and PCR was performed as described previously (31). The product was digested with *Bam*HI and *Hin*dIII, repurified and cloned into the *Bam*HI and *Hin*dIII sites of pGEX-2T. GST–SRPK1 was produced in bacteria and purified using glutathione–Sepharose (Pharmacia), as described (31).

The same vector was also used to construct plasmids that encode the wild-type (wtNt) and four mutated forms ($wtNtA^{78}$, $wtNtA^{80}$, $wtNtA^{82}$ and $wtNtA^{84}$) of the N-terminal domain of chicken LBR (32) fused with GST (for pertinent information see 31). A fusion protein missing the RS motifs (deletion of residues 75–84, construct GST–ΔRSNt) was generated as described previously (31).

### *In vitro* kinase assays

RS kinase activity was determined by measuring the incorporation of $PO_4^{3-}$ from [γ-$^{32}$P]ATP (6000 Ci/mmol; ICN Pharmaceuticals Ltd, UK) to sperm nuclear basic proteins or SR proteins or bacterially expressed LBR. Routine assays were carried out at 30°C in a total volume of 25 µl containing 25 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 200 mM NaCl, 50 µM [γ-$^{32}$P]ATP, 2–6 µg of the appropriate substrate and an aliquot of the enzyme as indicated. Samples were incubated for 30 min at 30°C and the reaction was stopped by adding 5 µl of 5× Laemmli buffer (33) and heating at 95°C for 3 min.

For the determination of $K_m$ the amount of substrate in the reaction mixture was varied between 0.1 and 5 µg and the

concentration of ATP was raised to 100 μM. Incorporation of radioactivity was measured by excising the respective radioactive bands from a SDS–PAGE gel and scintillation counting. The $K_m$ values were calculated using the MicroCAl Origin (v.2.94) program.

### Other methods

SDS–PAGE was performed according to Laemmli (33). Dried gels were exposed to Kodak X-ray film with intensifying screens. Protein concentration was determined by the method of Bradford (34).

Histones were purchased from Boehringer Mannheim GmbH, while myelin basic protein (MBP) was obtained from Sigma (Sigma Chemical Co., Deisenhofen, Germany). All other chemicals were purchased from Sigma.

## RESULTS

SRPK1 was originally purified and cloned from HeLa cells and has been characterized as highly specific for the RS-rich superfamily of splicing factors (8,9,12). Yet, we have recently shown that this kinase may also efficiently phosphorylate LBR, a ubiquitous component of the inner nuclear membrane (15). To further elucidate the cellular role(s) exhibited by the RS kinases we decided to study the tissue distribution of SRPK1. To this purpose we have isolated the complementary DNA clone of both human and mouse SRPK1 (for details see Materials and Methods) and performed northern blot analyses on poly(A)$^+$-selected RNA from various human and mouse tissues. As shown in Figure 1A in a survey of 16 human tissues one transcript of 4.5 kb, which was predominantly expressed in testis but was also present at low levels in most of the tissues examined, and a testis-specific transcript of 2.7 kb were detected. In the various mouse tissues tested only poly(A)$^+$ RNA extracted from testis contained significant levels of the 4.5 kb transcript, whereas the 2.7 kb transcript was barely detectable (Fig. 1B). Taking into account that the size of the isolated complementary DNA of human SRPK1 is 4.3 kb (see also 8), the 4.5 kb poly(A)$^+$ mRNA probably corresponds to SRPK1, whereas the 2.7 kb transcript could result from use of an alternative polyadenylation signal. Consistent with this idea the size of the isolated mouse clone was 2.5 kb and contained a significantly shorter 3'-untranslated region (UTR) (for pertinent information see EMBL databank accession no. AJ224115). In agreement with our data, Wang *et al.* (10) provided an almost identical expression pattern of human SRPK1 in heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas, while Kuroyanagi *et al.* (35) detected multiple mouse *SRPK1* transcripts, three of which, with sizes of 3.4, 3.0 and 1.4 kb, respectively, were highly expressed in testis. To investigate whether *SRPK1* mRNA accumulation in testis is restricted to certain cells we applied the RNA *in situ* hybridization technique on transverse sections of adult mouse testis. Figure 2 shows that SRPK1 is present in all germinal cells present in the seminiferous tubules but not in mature spermatozoa. In agreement with these data we have been unable to detect any RS kinase activity in extracts from human sperm cells (data not shown).

Pursuing this point further we examined the levels of RS kinase activity in several rat tissues, using as a substrate a fusion protein consisting of GST and the N-terminal domain of

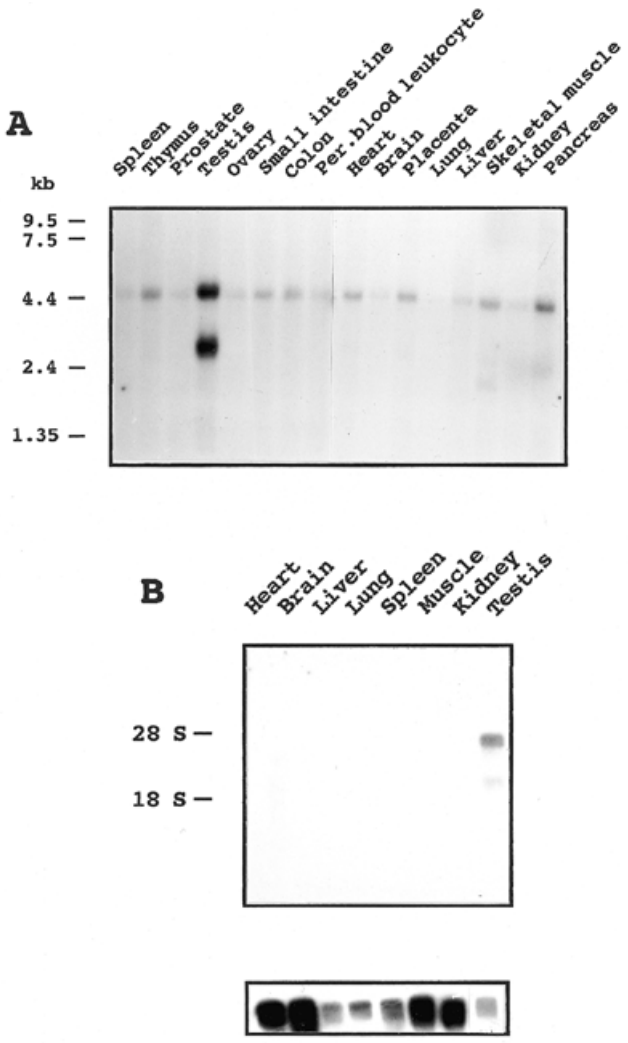


**Figure 1.** Northern blot analysis of SRPK1 expression in human and mouse tissues. (**A**) Human multiple tissue northern blot filters I and II were purchased from Clontech. The filters were hybridized with a cDNA probe comprising nt 1–860 of the sequence of mouse *SRPK1* and processed as described in Materials and Methods. Positions of molecular size markers in kb are indicated. (**B**) Equal amounts of poly(A)$^+$ RNA (2 μg) from the indicated mouse tissues were run on 1.2% agarose/formaldehyde gels, transferred to GeneScreen Plus™ membrane and hybridized with the same probe as in (A) (for details see Materials and Methods). Migration of 28S (4712 bp) and 18S rRNA (1869 bp) in parallel lanes of poly(A)$^+$ RNA is indicated. (Lower panel) Hybridization with glyceraldeyde 3-phosphate dehydrogenase (GAPDH) cDNA. The 860 bp mouse fragment is 92% identical at the DNA level (98% at the protein level) to the respective human fragment and 60% identical to the respective sequence of human SRPK2. Note in both blots the very low signal obtained in lung and brain, indicating that the probe does not cross-react with SRPK2, which, as previously reported (10,35), is highly expressed in testis, lung and brain.

LBR (residues 1–205, construct GST–wtNt). For control purposes we also measured the phosphorylation of a similar fusion protein missing the RS motifs (deletion of residues 75–84, construct GST–ΔRSNt; data not shown) and the respective values were subtracted. In agreement with the results obtained from the northern blots the highest activity was found in testis

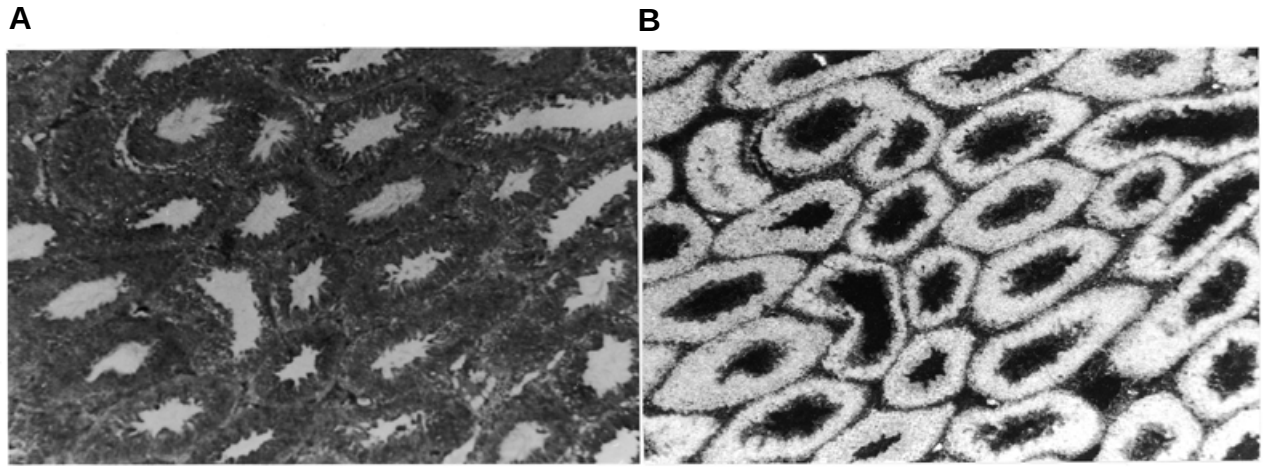


**Figure 2.** *In situ* expression analysis of SRPK1 on transverse sections of adult mouse testis. (**A**) Dark field image of the tissue section; (**B**) bright field image. Antisense and sense probes were prepared as described in Materials and Methods, using as template a cDNA of 860 bp comprising nt 1–860 of the sequence of mouse *SRPK1*. No signal was obtained with the sense probe.

(Fig. 3). However, lung, brain, spleen and thymus also contain significant activity, indicating that other kinases may also target the RS dipeptide motifs of LBR. In this respect it should be noted that the tissue distribution of a previously identified LBR kinase (31,36) is not known and that SRPK2, which is highly related to SRPK1 in sequence, kinase activity and substrate specificity, is highly expressed in testis, but also in brain and lung (10,35). Furthermore, several LAMMER kinases may also phosphorylate LBR (E.Nikolakaki, T.Giannakouros and L.Rabinow, unpublished observations). In all tissues examined the kinase activity was found primarily in the cytosol and only minimal activity is present in the nuclei (Fig. 3). Yet, the specific activities of the nuclear extracts were 2- (testis) to 10-fold (thymus) higher compared to those of the respective cytoplasmic extracts.

Given that the main function of the testis is to produce male germ cells we sought to investigate whether any proteins involved in the process of spermatogenesis are phosphorylated by SRPK1. As a first step we searched for RS domain-containing proteins that are present at high levels in testis, at World Wide Web site http://www. mann.embl-heidelberg.de/Services/PeptideSearch/PeptideSearchIntro.html , using peptide queries with three, four or five RS repeats. The finding that P1 protamines satisfy the substrate specificity requirements of SRPK1 (Table 1), coupled with the fact that most, if not all, of these proteins are known to be phosphoproteins (19,37,38) made it appear possible that they could serve as SRPK1 substrates. Consistent with this hypothesis Ser10 and Ser8 ($^{7}$R**S**Q**S**RSR$^{13}$) were previously identified as the phosphorylation sites of mono- and di-phosphorylated human P1 protamine (19). Accordingly, we examined total nuclear basic proteins extracted from purified sperm nuclei of human spermatozoa as well as purified P1 protamine (for details see Materials and Methods) to see whether or not recombinant GST–SRPK1 would phosphorylate them *in vitro*. To assess the activity of the recombinant enzyme we used as substrates purified LBR and

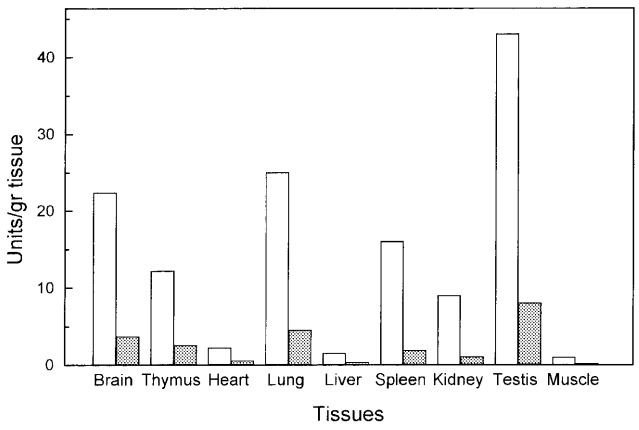


**Figure 3.** Distribution of SR protein kinase activity in rat tissues. Aliquots of 15 µg of cytosolic and nuclear extracts (prepared as described in Materials and Methods) were assayed for RS kinase activity toward 0.12 mg/ml GST–wtNt in the presence of 50 µM [$\gamma$-$^{32}$P]ATP, 10 mM $MgCl_2$ and 200 mM NaCl. Samples were analyzed by SDS–PAGE on 12% gels and autoradiographed. The radioactive bands corresponding to GST–wtNt were excised and the radioactivity was measured by scintillation counting. As controls we measured the phosphorylation of GST–ΔRSNt and the respective values were subtracted. SR kinase activity of different fractions is expressed as U/g tissue. One unit is the amount of enzyme required to catalyze the transfer of 0.1 nmol phosphate to 2 µg GST–wtNt in 30 min at 30°C. Open columns, cytoplasmic activity; shaded columns, nuclear activity. Under the assay conditions minimal (or no detectable) phosphorylation was observed in the absence of GST–wtNt or GST–ΔRSNt.

GST–wtNt, which were both efficiently phosphorylated, while, as expected, GST–ΔRSNt, which did not contain any RS motifs, was not modified (Fig. 4C). Figure 4C shows that P1 protamine was phosphorylated by GST–SRPK1 in an efficient and highly specific manner, since GST–SRPK1 did not

**Table 1.** Alignment of the RS domain of P1 protamines from various vertebrates and of protamine-like proteins from marine bivalve molluscs

| Taxa | Species | Sequence |
|---|---|---|
| Bivalve molluscs | Razor shell (EM1) (24) | SKS**RSRSRSRSRS**SKS |
| | Blue mussel (PHI-2B) (46) | RKS**RSRSRSRS**KSP |
| | California mussel (PL-II) (59) | PSR**RSRSRSRSRS**KS |
| Reptiles | American alligator (60) | ERN**RSRSRS**RRR |
| | Echidna (23) | RPS**RSRSRS**LYR |
| | Southeastern quoll (61) | YRR**RSRSRSRS**RYR |
| Marsupials | Eastern gray kangaroo (61) | YRH**SRSRSRSR**YRR |
| | Flat-skulled marsupial mouse (62) | RRH**SRSRSRSR**NQC |
| | Short-nosed bandicoot (61) | YRN**SRSRSRSR**FRR |
| Fish | Dogfish (protamine Z3) (63) | A**RSRS**R**RS**YGR |
| Birds | Rooster (23) | YR**RSRTRSRS**PRS |
| Mammals | Human (22) | RCC**RS**Q**SRSR**YYR |
| | Mouse (23) | RCC**RS**K**SRSR**CRR |

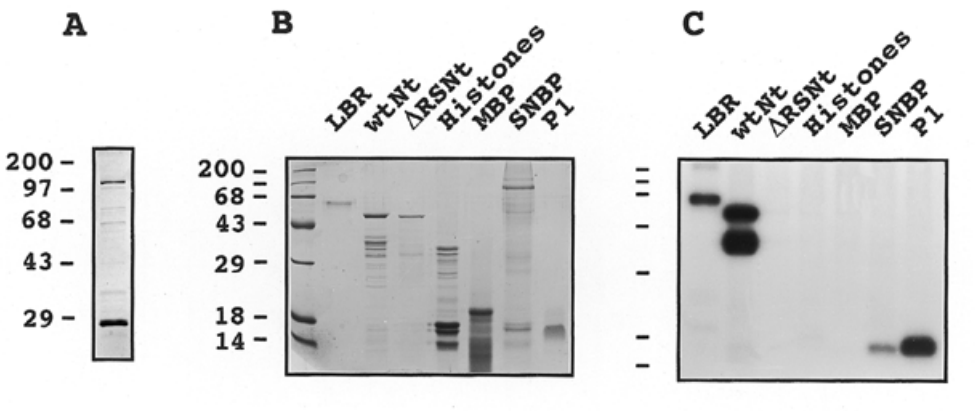


**Figure 4.** Phosphorylation of human sperm nuclear basic proteins and purified P1 protamine by GST–SRPK1. (**A**) SDS–PAGE analysis on a 10% gel and Coomassie blue staining of bacterially expressed SRPK1 fused with glutathione *S*-transferase. (**B**) SDS–PAGE analysis on a 12.5% gel and Coomassie blue staining of electro-eluted LBR (1.5 μg), bacterially produced GST–wtNt (4 μg), GST–ΔRSNt (3 μg), histones (6 μg), MBP (5 μg), sperm nuclear basic proteins (SNBP, 4 μg) and purified P1 protamine (2 μg). Electroeluted LBR was obtained from urea-insoluble turkey erythrocyte nuclear envelopes as described previously (31). Nuclear basic proteins were extracted from purified sperm nuclei of human spermatozoa as described in Materials and Methods. Full-length GST–wtNt migrates with an apparent molecular mass of 51 kDa. The lower bands represent degradation products (see also 31,58). Bars on the left indicate molecular masses (in kDa); the second bar corresponds to 97 kDa. (**C**) *In vitro* phosphorylation of the above-mentioned substrates by GST–SRPK1 (0.5 μg). The protein level of GST–SRPK1 was based on SDS–PAGE analysis and Coomassie blue staining of the material eluted from the glutathione–agarose affinity column (A). Assays were performed as described in Materials and Methods. The samples were analyzed by SDS–PAGE on 12.5% gels and autoradiographed. Bars on the left indicate the same molecular masses as in (B).

phosphorylate any other basic proteins of the human sperm nuclei. Furthermore, the recombinant enzyme did not act on myelin basic protein (MBP), which was previously shown to be moderately modified by purified SRPK1 from HeLa cells (9,39) as well as by Dsk1 kinase, which is the fission yeast homolog of SRPK1 (39). The apparent $K_m$ value displayed by the fusion kinase for the phosphorylation of human P1 protamine (5 μM) was higher compared to the $K_m$ values observed for the phosphorylation of ASF/SF2 by *in vitro* translated SRPK1 fused with a FLAG tag sequence (0.28 μM; 12), and for the phosphorylation of LBR by bacterially expressed GST–SRPK1 (0.4 μM; 15).

In a following step we looked for testis cytoplasmic proteins that may also serve as substrates of SRPK1. To this purpose we incubated the cytosolic fraction (S100) of rat testis with bacterially expressed GST–SRPK1. Endogenous protein kinase activity of the S100 fraction was inactivated by heating to 70°C for 10 min, prior to incubation with GST–SRPK1. As shown in Figure 5B, several phosphorylated bands were observed. As a first step towards the characterization of the cytoplasmic substrates of

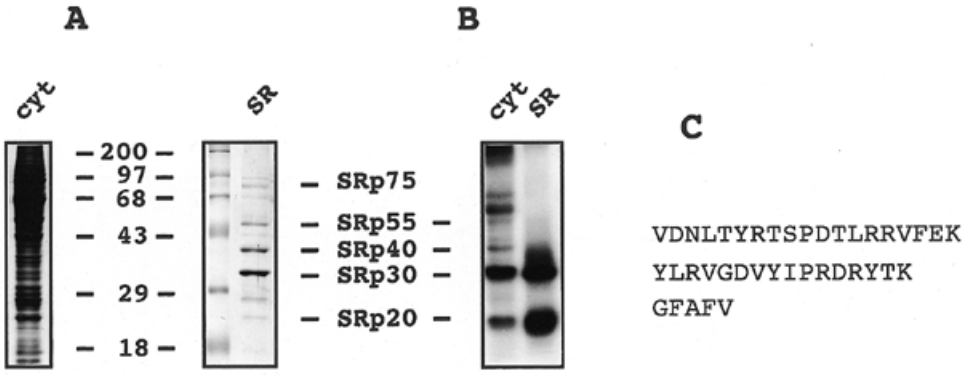


**Figure 5.** Phosphorylation of rat testis cytoplasmic proteins by GST–SRPK1. *In vitro* phosphorylation of total cytoplasmic extracts of rat testis (50 µg) and dephosphorylated SR proteins from rat testis cytosol (~5 µg) by bacterially produced GST–SRPK1. The samples were analyzed by SDS–PAGE on 12% gels and stained with Coomassie blue (**A**) or autoradiographed (**B**). No phosphorylation was observed when pre-heated cytoplasmic extracts were incubated with [$\gamma$-$^{32}$P]ATP in the absence of exogenously added GST–SRPK1. SR proteins were purified from rat testis cytosol and dephosphorylated as described in Materials and Methods. (**C**) Internal peptide sequences obtained from the 30 kDa protein. For more details see Materials and Methods.

SRPK1 we proceeded to isolate RS domain-containing proteins from rat testis cytosol using the method of Zahler *et al.* (30). Analysis of the purified proteins by SDS–PAGE revealed that significant amounts of SR proteins are present in the cytosol of rat testis (Fig. 5A). The identity of SR proteins was established by size (30) and by western blotting using mAb 104 (data not shown). Following dephosphorylation by lambda protein phosphatase we used the SR proteins as substrates for GST–SRPK1. As shown in Figure 5B, the two major phosphorylated bands of rat testis cytosol with apparent molecular masses of 30 and 20 kDa correspond to SRp30 and SRp20, respectively, whereas SRp40 and SRp55 are phosphorylated to a lesser extent. Interestingly, two of the phosphorylated proteins, one with moderate intensity at 58 kDa and a minor one at 67 kDa, probably represent RS domain-containing proteins that do not belong to the family of SR splicing factors. The 30 kDa band has been reported to contain two distinct polypeptides, SRp30a and SRp30b, which have also been described as ASF/SF2 and SC35, respectively. To determine the identity of the 30 kDa phosphoprotein in our preparation the blotted protein was digested with endoproteinase Lys-C and the resulting peptides were separated and subjected to N-terminal sequencing (for details see Materials and Methods). All peptides matched the reported amino acid sequences of human and chicken SC35, confirming that the 30 kDa band corresponds to SC35 (Fig. 5C, human SC35 residue numbers from the N- to the C-terminus 18–36, 37–52 and 56–60).

## DISCUSSION

SRPK1 was originally described as a kinase highly specific for the RS-rich superfamily of splicing factors, which commit precursor mRNA to splicing and promote spliceosome assembly (8). In a recent study we demonstrated that SRPK1 may also phosphorylate additional substrates, such as LBR, with similar kinetics as observed for the SR proteins (15), indicating that members of the SRPK family may play a broader regulatory role than was initially proposed. Pursuing this thought further, it is reasonable to suggest that if SRPK1 interacts with and phosphorylates SR proteins and LBR in mammalian cells then these polypeptides are anticipated to have a shared tissue distribution. In this respect there have been no reports up to now on tissue- or cell-specific expression of LBR, while SR proteins are more or less conserved among metazoa and have a rather ubiquitous tissue distribution (1,4,40). Yet, using northern blot analysis, together with *in situ* hybridization assays, we now show that SRPK1 is highly expressed in testis and, more specifically, in all germinal cells, but not in mature spermatozoa. Furthermore, when a survey was made of the RS kinase activity, in extracts made from various tissues of adult male rats, the highest activity was found in testis.

In a search for testis-specific substrates of SRPK1 we found that the enzyme phosphorylates human protamine 1 (HP1) *in vitro* in a highly specific and efficient manner. HP1 contains a short RS domain composed of one RS and two SR dipeptides, with a glutamine residue between them (**RS**Q**SRSR**; see also Table 1). Consistent with the observation that HP1 may serve as a substrate for SRPK1, we have previously shown that GST–SRPK1 could efficiently phosphorylate different mutants of the N-terminal domain of LBR, where in each case one of the Ser residues of the RS motif was mutated to Ala or Gly (**RSR**G**RSRSRS**, **RSRSR**A**RSRS** and **RSRSRSR**A**RS**; for pertinent information see 15). It should be noted that not all RS domain-containing proteins are substrates for SRPK1. For example, the LAMMER kinases, which contain two RS repeats (**RSRS** or **RS**X**RS**, X = Gly in the case of Clk-1 and Lys in the case of DOA) could not be phosphorylated *in vitro* by GST–SRPK1 (our unpublished data in collaboration with L.Rabinow). Taken together, these data indicate that phosphorylation mediated by SRPK1 requires at least three RS or SR repeats.

P1 protamine is a very attractive candidate as an *in vivo* substrate for SRPK1. Besides being phosphorylated *in vitro* by SRPK1 with high specificity, several research groups have shown the existence of phosphorylated P1 protamines in mammals

(16,19,29,37), while Ser10 and Ser8 of the RS domain ($^{7}$R**S**Q**S**RSR$^{13}$) were previously identified as the phosphorylation sites of mono- and di-phosphorylated HP1 (19). Furthermore, the alternating RS or SR dipeptides are highly conserved in all vertebrate P1 protamines examined to date and even more in several molluscan protamine-like proteins (Table 1), indicating that they play a crucial role in the condensation of sperm chromatin and that members of the SRPK family of protein kinases and/or other RS-targeting kinases might be important regulators of this process.

Shortly after their synthesis in haploid, round spermatids (41), protamines are highly phosphorylated (37,38), while later in mature spermatozoa they are partially dephosphorylated (16,19,29). It has been proposed that their phosphorylation could facilitate correct binding to DNA, whereas their partial dephosphorylation increases chromatin condensation in sperm nuclei (16,29). Alternatively, phosphorylation of P1 protamines, mediated by members of the SRPK family might be a prerequisite for correct chromatin condensation. Ordinarily, one would expect phosphorylation to destabilize highly condensed sperm chromatin. However, taking into acount that a large number of protamines and other highly basic proteins accumulate around DNA, phosphorylation of certain well-defined residues might reduce the repelling forces between neighboring positive charges.

Consistent with this idea, hyperphosphorylation of histone H1 and phosphorylation of histone H3 correlate well with the G2 phase to metaphase condensation of chromosomes (42). Furthermore, okadaic acid, a specific inhibitor of serine/threonine protein phosphatases (43), induces rounding of mammalian cells and premature chromatin condensation (44). SRPK1 activity, assayed using SC35 or ASF/SF2 as substrate, has been reported to be ~5-fold higher in extracts from metaphase compared to interphase cells (8). The break-up of the speckled pattern and the redistribution of splicing factors throughout the cytoplasm has been considered as the main mitotic function of SRPK1 (8). Interestingly, previous genetic analyses have implicated Dsk1, which is the fission yeast homolog of SRPK1 and is also similarly cell cycle regulated, in the regulation of chromosome segregation at the metaphase/anaphase transition (39,45). Is the role of SRPK1 in cell cycle regulation accomplished through the splicing pathway or by acting directly on the cell cycle control system at metaphase, or by both? To answer this question it is necessary to clearly determine the *in vivo* targets of SRPK1.

Several fold higher RS kinase activity was detected in the cytosolic extracts from all tissues examined as compared to the respective nuclear extracts. In agreement with our observations, Gui *et al.* purified and characterized SRPK1 from cytosolic extracts of HeLa cells that were found to contain high levels of the kinase activity (9), whereas recently Wang *et al.*, using immunofluorescence studies, observed that both SRPK1 and 2 are localized in cytoplasmic structures that remain to be characterized (10). On the other hand, Dsk1 was found to contain a cytoplasmic retention signal and to remain cytoplasmic in interphase cells, migrating to the nucleus before mitosis (45). If this is also characteristic of SRPK1, then one might assume that these enzymes are maintained stored in a cytoplasmic pool from which they are recruited to the nucleus in order to modify their substrates. Alternatively, the above findings might suggest that the role of these enzymes in cellular regulation is accomplished through the phosphorylation of a number of cytoplasmic proteins. In this respect it will be interesting to determine whether phosphorylation of protamines occurs inside the nucleus or whether these proteins are fully or partially modified en route to their final destination.

Looking further for potential cytoplasmic substrates of SRPK1 we found that testis cytoplasm contains significant amounts of SR proteins and that the kinase is able to phosphorylate mainly SC35 and SRp20 and to a lesser extent SRp40 and SRp55. It is not clear yet whether the presence of SR proteins in testis cytosol is due to their direct involvement in cytoplasmic events as opposed to having leaked from the nuclei during the fractionation procedure. However, consistent with our findings, Zahler *et al.* also reported a few years ago that HeLa cytoplasmic S100 extracts contain the five most prominent SR proteins (SRp20, SRp30a/b, SRp40, SRp55 and SRp75) at lower concentrations than the respective nuclear extracts (46). Furthermore, Caceres *et al.* recently reported that some human SR proteins shuttle rapidly and continuously between the nucleus and the cytoplasm (47), indicating that these family members may facilitate mRNA transport or have cytoplasmic functions, such as mRNA localization, mRNA stability or translational regulation. Even more interestingly, such regulatory events seem to be important during spermatogenesis (for reviews see 48–50). The mouse testis contains a large proportion of non-polysomal poly(A)$^{+}$ RNA which may represent a large pool of mRNA stored in a translationally repressed form (51). In addition, studies of individual genes encoding mouse germ cell-specific proteins have shown that transcription can precede translation by several days. The mRNAs for protamines are transcribed and then stored in a translationally inactive form for up to 7 days before becoming associated with polysomes and undergoing poly(A) tail shortening (52,53). Braun *et al.* have shown that the 156 nt of the mouse P1 3'-UTR are sufficient to confer mouse P1 translational regulation (54), probably through the formation of stem–loop structures with testis proteins (55). A similar mechanism has recently been reported to regulate the sexual fates in the *Caenorhabditis elegans* hermaphrodite germline (56). More specifically, a cytoplasmic protein that contains a RNA recognition motif and several SR repeats binds specifically to the *fem-3* 3'-UTR and mediates the sperm/oocyte switch. Further evidence that molecules involved in RNA processing or translational control may be critical in spermatogenesis is also provided by the data reported by Ma *et al.* (57). A mutation creating human azoospermia was mapped to a gene encoding a protein (azoospermia factor) that was presumed to be a component of the RNA processing machinery. Even more interestingly, this protein contains several repeats of a serine-arginine-glycine-tyrosine (SRGY) tetrapeptide.

Taken together, our data strongly support the concept that SRPK kinases play a broader role in cellular regulation than was initially thought, as molecules involved in pre-mRNA splicing. The complete characterization of the members of this family, together with the identification of additional substrates specifically modified by them, and the delineation of the control mechanisms that regulate their activity will ultimately contribute to an understanding of the functions of these enzymes.

## ACKNOWLEDGEMENTS

We thank H. Scholer for providing us with the cDNA library from F9 cells, G. Mavrothalassitis for the human multiple tissue northern blot filters I and II, G. Blobel, H. J. Worman and S. D. Georgatos for the LBR cDNA clone and J. Valcarcel for providing us with the mAb 104 monoclonal antibody. We also thank J. G. Georgatsos, S. D. Georgatos and L. Rabinow for useful discussions and comments on the manuscript. This work was supported in part by grants from the Greek Secretariat of Research and Technology (ΠΕΝΕΔ no. 1644) and the Greek Ministry of Health (ΚΕΣΥ no. 7264) to T.G.

## REFERENCES

1. Fu,X.-D. (1995) *RNA*, **1**, 663–680.
2. Mayeda,A. and Krainer,A.R. (1992) *Cell*, **68**, 365–375.
3. Fu,X.-D. (1993) *Nature*, **365**, 82–85.
4. Zahler,A.M., Neugebauer,K.M., Lane,W.S. and Roth,M.B. (1993) *Science*, **260**, 219–222.
5. Caceres,J.F., Stamm,S., Helfman,D.M. and Krainer,A.R. (1994) *Science*, **265**, 1706–1709.
6. Misteli,T., Caseres,J.F., and Spector,D.L. (1997) *Nature*, **387**, 523–527.
7. Misteli,T., Caseres,J.F., Clement,J.Q., Krainer,A.R., Wilkinson,M.F. and Spector,D.L. (1998) *J. Cell Biol.*, **143**, 297–307.
8. Gui,J.-F., Lane,W.S. and Fu,X.-D. (1994) *Nature*, **369**, 678–682.
9. Gui,J.-F., Tronchere,H., Chandler,S.D. and Fu,X.-D. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 10824–10828.
10. Wang,H.-Y, Lin,W., Dyck,J.A., Yeakley,J.M., Songyang,Z., Cantley,L.C. and Fu,X.-D. (1998) *J. Cell Biol.*, **140**, 737–750.
11. Colwill,K., Pawson,T., Andrews,B., Prasad,J., Manley,J.L., Bell,J.C. and Duncan,P.I. (1996) *EMBO J.*, **15**, 265–275.
12. Colwill,K., Feng,L.L., Yeakley,J.M., Gish,G.D., Caceres,J.F., Pawson,T. and Fu,X.-D. (1996) *J. Biol. Chem.*, **271**, 24569–24575.
13. Lee,K., Du,C., Horn,M. and Rabinow,L. (1996) *J. Biol. Chem.*, **271**, 27299–27303.
14. Birney,E., Kumar,S. and Krainer,A.R. (1993) *Nucleic Acids Res.*, **21**, 5803–5816.
15. Papoutsopoulou,S., Nikolakaki,E. and Giannakouros,T. (1999) *Biochem. Biophys. Res. Commun.*, **255**, 602–607.
16. Oliva,R. and Dixon,G.H. (1991) *Prog. Nucleic Acid Res. Mol. Biol.*, **40**, 25–94.
17. Oliva,R., Goren,A. and Dixon,G.H. (1989) *J. Biol. Chem.*, **264**, 17627–17630.
18. Oliva,R. and Dixon,G.H. (1989) *J. Biol. Chem.*, **264**, 12472–12481.
19. Chirat,F., Arkhis,A., Martinage,A., Jaquinot,M., Chevaillier,P. and Sautière,P. (1993) *Biochim. Biophys. Acta*, **1203**, 109–114.
20. Bloch,D.P. (1969) *Genetics*, **61** (suppl.), 93–111.
21. Johnson,P.A., Peschon,J.J., Yelick,P.C., Palmiter,R.D. and Hecht,N.B. (1988) *Biochim. Biophys. Acta*, **950**, 45–53.
22. Domenjoud,L., Nussbaum,G., Adham,I.M., Greeske,G. and Engel,W. (1990) *Genomics*, **8**, 127–133.
23. Retief,J.D., Winkfein,R.J. and Dixon,G.H. (1993) *Eur. J. Biochem.*, **218**, 457–461.
24. Giancotti,V., Buratti,E., Santucci,A., Neri,P. and Crane-Robinson,C. (1992) *Biochim. Biophys. Acta*, **1119**, 296–302.
25. Carlos,S., Jutglar,L., Borrell,I., Hunt,D.F. and Ausio,J. (1993) *J. Biol. Chem.*, **268**, 185–194.
26. Puissant,C. and Houdebine,L.M. (1990) *BioTechniques*, **8**, 148–149.
27. Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
28. Blobel,G. and Potter,V.R. (1966) *Science*, **154**, 1662–1665.
29. Gusse,M., Sautière,P., Bélaiche,D., Martinage,A., Roux,C., Dadoune,J.-P. and Chevaillier, P. (1986) *Biochim. Biophys. Acta*, **884**, 124–134.
30. Zahler,A.M., Lane,W.S., Stolk,J.A. and Roth,M.B. (1992) *Genes Dev.*, **6**, 837–847.
31. Nikolakaki,E., Simos,G., Georgatos,S.D. and Giannakouros,T. (1996) *J. Biol. Chem.*, **271**, 8365–8372.
32. Worman,H.J., Evans,C.D. and Blobel,G. (1990) *J. Cell Biol.*, **111**, 1535–1542.
33. Laemmli,U.K. (1970) *Nature*, **227**, 685–688.
34. Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248–254.
35. Kuroyanagi,N., Onogi,H., Wakabayashi,T. and Hagiwara,M. (1998) *Biochem. Biophys. Res. Commun.*, **242**, 357–364.
36. Nikolakaki,E., Meier,J., Simos,G., Georgatos,S.D. and Giannakouros,T. (1997) *J. Biol. Chem.*, **272**, 6208–6213.
37. Marushige,Y. and Marushige,K. (1975) *J. Biol. Chem.*, **250**, 39–45.
38. Marushige,Y. and Marushige,K. (1975) *Biochim. Biophys. Acta*, **518**, 440–449.
39. Tang,Z., Yanagida,M. and Lin,R.-J. (1998) *J. Biol. Chem.*, **273**, 5963–5969.
40. Valcarcel,J. and Green,M.R. (1996) *Trends Biochem. Sci.*, **21**, 296–301.
41. Kleene,K.C., Distel,R.J. and Hecht,N.B. (1988) *Dev. Biol.*, **105**, 71–79.
42. Ajiro,K., Yoda,K., Utsumi,K. and Nishikawa,Y. (1996) *J. Biol. Chem.*, **271**, 13197–13201.
43. Cohen,P. (1989) *Annu. Rev. Biochem.*, **58**, 453–508.
44. Glicksman,N.R., Parsons,S.F. and Salmon,E.D. (1992) *J. Cell Biol.*, **119**, 1271–1276.
45. Takeuchi,M. and Yanagida,M. (1993) *Mol. Biol. Cell*, **4**, 247–260.
46. Zahler,A.M., Neugebauer,K.M., Stolk,J.A. and Roth,M.B. (1993) *Mol. Cell. Biol.*, **13**, 4023–4028.
47. Caceres,J.F., Screaton,G.R. and Krainer,A.R. (1998) *Genes Dev.*, **12**, 55–66.
48. Braun,R.E., Lee,K., Schumacher,J.M. and Fajardo,M.A. (1995) *Recent Prog. Hormone Res.*, **50**, 275–286.
49. Hecht,N.B. (1995) *Dev. Genet.*, **16**, 95–103.
50. Schafer,M., Nayernia,K., Engel,W. and Shafer,U. (1995) *Dev. Biol.*, **172**, 344–352.
51. Gold,B. and Hecht,N.B. (1981) *Biochemistry*, **20**, 4871–4877.
52. Kleene,K.C. (1989) *Development*, **106**, 367–373.
53. Haynes,S.R., Cooper,M.T., Pype,S. and Stolow,D.T. (1997) *Mol. Cell. Biol.*, **17**, 2708–2715.
54. Braun,R.E., Peschon,J.J., Behringer,R.R., Brinster,R.L. and Palmiter,R.D. (1989) *Genes Dev.*, **3**, 793–802.
55. Kwon,Y. and Hecht,N.B. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 3584–3588.
56. Zhang,B., Gallegos,M., Puoti,A., Durkin,E., Fields,S., Kimble,J. and Wickens,M.P. (1997) *Nature*, **390**, 477–484.
57. Ma,K., Inglis,J.D., Sharkey,A., Bickmore,W.A., Hill,R.E. Prosser,E.J., Speed,R.M., Thomson,E.J., Jobling,M., Taylor,K., Wolf,J., Cooke,H.J., Hargreave,T.B. and Chandley,A.C. (1993) *Cell*, **75**, 1287–1295.
58. Ye,Q. and Worman,H.J. (1994) *J. Biol. Chem.*, **269**, 11306–11311.
59. Carlos,S., Jutglar,L., Borrell,I., Hunt,D.F. and Ausio,J. (1993) *J. Biol. Chem.*, **268**, 185–194.
60. Hunt,J.G., Kasinsky,H.E., Elsey,R.M., Wright,C.L., Rice,P., Bell,J.E., Sharp,D.J., Kiss,A.J., Hunt,D.F., Arnott,D.P., Russ,M.M., Shabanowitz,J. and Ausio,J. (1996) *J. Biol. Chem.*, **271**, 23547–23557.
61. Retief,J.D., Krajewski,C., Westerman,M., Winkfein,R.J. and Dixon,G.H. (1995) *Proc. R. Soc. Lond. B Biol. Sci.*, **259**, 7–14.
62. Krajewski,C., Blacket,M., Buckley,L. and Westerman,M. (1997) *Mol. Phylogenet. Evol.*, **8**, 236–248.
63. Kouach,M., Jaquinod,M., Belaéche,D., Sautière,P., van Dorsselaer,A., Chevaillier,P. and Briand,G. (1993) *Biochim. Biophys. Acta*, **1162**, 99–104.