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Multiple *src*-related kinase genes, *srk*1–4, in the fresh water sponge *Spongilla lacustris*

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In one of the simplest metazoan organisms, the sponge *Spongilla lacustris*, at least four different *src*-related kinase genes (*srk*1–4) are expressed, all of which show a high degree of similarity to the c-*src* genes of vertebrates. Whereas *srk*2 and *srk*3 are clearly unrelated at the nucleic acid level, *srk*1 and *srk*4 share identical sequences in the 5′ parts of their cDNAs. The cloning of several primer extension clones and genomic polymerase chain reaction experiments confirmed the hypothesis of an alternative splicing of tandemly arranged carboxy-terminal parts of *srk*1 and *srk*4. The genomic sequence encoding both proteins was found to be interrupted at the splice point by an intron which is located in the same position as one of the introns in the chicken *src* gene, which is the only gene conserved in invertebrates and vertebrates. All four *srk* genes are expressed in adult sponges as mRNA transcripts of about 2.2 kb. Tyrosine kinase activity of a *src*-related kinase could be detected in adult sponges but not in their resting form (gemmulae), and may reflect the activity of the *srk* protein products. *Spongilla lacustris* is the simplest organism from which a protein tyrosine kinase gene has been isolated. The presence of at least four such genes in the evolutionary ancient and primitive phylum Porifera suggests that tyrosine kinase genes arose concomitantly with or shortly after the appearance of multicellular organisms and that their activity may be involved in aggregation and cell–cell recognition.

Introduction

Protein phosphorylation on tyrosine residues plays a central role in cellular signal transduction. Numerous proteins with phosphotyrosine kinase (PTK) activity have been identified in higher vertebrates (reviewed by Hanks *et al.*, 1988), most of them constituting closely related families of transmembrane receptors or cytosolic non-receptors. PTK-related sequences and/or PTK enzymatic activity have been found in all multicellular animals. In prokaryotes, the existence of PTKs has not been shown convincingly. In unicellular eukaryotes, such as yeast, PTKs are found, but they are capable of phosphorylating tyrosine equally as well as serine and/or threonine (Featherstone & Russell, 1991; Stern *et al.*, 1991). This has led to the assumption that tyrosine-specific PTKs have emerged with the metazoa and grown in number in step with the increasing complexity of multicellular organisms. The group of *src*-related PTKs has been studied most extensively with respect to evolutionary questions (for review see Barnekow, 1989). The prototypic c-*src* gene in chicken encodes a phosphoprotein with a molecular weight of 60 000 Da, pp60$^{c\text{-}src}$. The enzymatic activity resides within a carboxy-terminal domain ('kinase' domain or '*src* homology region 1', SH1) which is highly conserved between the members of the *src* multigene family. The amino-terminal half of the protein is thought to be more involved in regulation. It contains, besides a myristylation consensus at its amino terminus that is involved in membrane anchorage, two conserved regions (SH2, SH3) which have been shown to be important for the transforming function of oncogenic *src* and are involved in protein–protein interaction leading to kinase activation (for review, see Cantley *et al.*, 1991).

The c-*src* gene is preferentially expressed in neural tissues of all animals examined so far (Cotton & Brugge, 1983; Schartl & Barnekow, 1984; Maness, 1986; Raulf *et al.*, 1989a). Consequently, a c-*src*-related gene has been isolated from the most primitive metazoa with nerve cells, the coelenterate *Hydra* (Bosch *et al.*, 1989), and has shown to be expressed at that early evolutionary level in neural cells (Schartl *et al.*, 1989), consistent with a proposed physiological function of pp60$^{c\text{-}src}$ in differentiated neurones (Barnekow *et al.*, 1990). Sponges, the simplest metazoan organisms, which are thought to precede the coelenterates on the phylogenetic level or are at least derived from an earlier common ancestor, also clearly display a pp60$^{c\text{-}src}$-related PTK activity (Barnekow & Schartl, 1984). In addition, a carboxy-terminal chicken c-*src* antipeptide antibody precipitates a specific phosphoprotein (Hirsch-Behnam & Barnekow, 1988). Interestingly, plants, algae, and protozoa do not contain detectable *src*-like kinases (Schartl & Barnekow, 1982).

To contribute to an understanding of the origin of PTKs, we have cloned and analysed c-*src*-related genes from the freshwater sponge *Spongilla lacustris*. Additionally we have undertaken this evolutionary approach to contribute to an understanding of the function of *src*-related genes. Determination of amino acid residues or motifs – besides those generally diagnostic for tyrosine

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kinases – which are conserved over large evolutionary distances might help to delineate functionally important structures.

Results

To isolate *src*-related sequences from sponges, two cDNA libraries of the freshwater sponge *Spongilla lacustris* were constructed and screened with a probe derived from the highly conserved protein kinase domain of the viral *src* gene. Restriction enzyme and sequence analysis of 27 clones indicated that they belong to four different classes of *src*-related sequences. All contain stretches with high similarity to the tyrosine kinase domain of v-*src* and all members of the *src* family, both vertebrate and invertebrate, and are therefore referred to as *src*-related kinases, *srk*1–4 (Figure 1).

Apparently all clones were incomplete for the 5′ coding sequences. Therefore, to obtain the complete cDNA sequence of one of the *srk* genes by primer extension cloning, an oligonucleotide which was derived from *srk*1 was synthesized as the largest and first-completed sequence. Later it turned out that this primer sequence was also *srk*4 specific owing to an identical 5′ part of *srk*1 and *srk*4 (see below). Restriction analysis and partial sequence analysis of 10 clones demonstrated that all clones overlap *srk*1 and *srk*4 (Figure 1), and contain the complete N-terminal region of the predicted protein. The 355-bp sequence of one clone was sequenced from both strands and named clone *srk*1/4PE.

The combined sequence of *srk*1 (cDNA clone *srk*1 and primer extension clone *srk*1/4PE) translates into a predicted protein of 505 amino acids, corresponding to a relative molecular mass of 57 693 Da. All of the amino acids that have been shown to play functional roles in pp60$^{c\text{-}src}$ of higher vertebrates are conserved in *srk*1 (Figure 2).

The *srk* proteins show a high degree of similarity to

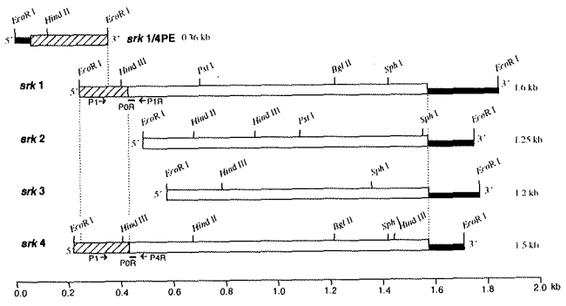

Figure 1 Restriction maps of *Spongilla lacustris* cDNA clones *srk1*, *srk*2, *srk*3 and *srk*4 aligned at the end of their predicted open reading frames represented by the open bars. Hatched areas indicate the 5′ identical portions of the *srk*1 and *srk*4 cDNA clones, and the black lines indicate the 3′ and 5′ untranslated regions. The primer extension clone *srk*1/4PE is aligned above. kb, kilobase pairs. (All EcoRI sites are derived from the cloning adaptors.) The positions of oligonucleotides used in PCR and hybridization experiments are shown below *srk*1 and *srk*4. (The nucleotide sequences are deposited in the GenBank/EMBL Data Bank under accession nos. X61601–X61604)

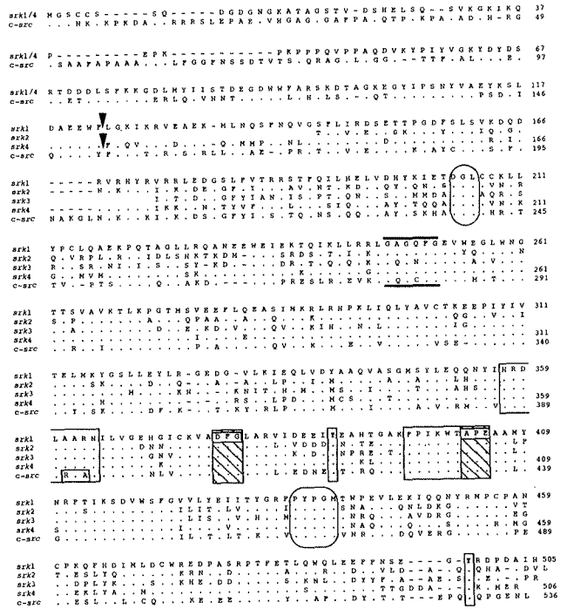

Figure 2 Alignment of the predicted carboxy-terminal amino acid sequences of *Spongilla lacustris srk*1, *srk*2, *srk*3 and *srk*4 cDNA clones. The four sequences were aligned using the program GAP (Devereux *et al.*, 1984); dots represent identical amino acid residues and dashes represent gaps in the alignment. For comparison the human pp60$^{c\text{-}src}$ sequence (Anderson *et al.*, 1985; Parker *et al.*, 1985) is shown below. Residues diagnostic for tyrosine-specific protein kinase catalytic domains according to Hanks *et al.* (1988) are marked by open boxes, the common kinase-specific motifs by shadowed boxes. The ATP binding site is marked by bars and the regulatory tyrosine residues are bold printed and boxed. Two other *src*-specific motifs are circled (see discussion). The arrows between the predicted amino acids 124 and 125 of *srk*1 and *srk*4 mark the border between the identical amino-terminal and the specific carboxy-terminal parts

the *src* and *src*-related genes of vertebrates and slightly less similarity to the *Drosophila src*-related genes *Dsrc*64B (Hoffmann *et al.*, 1983; Simon *et al.*, 1985) and *Dsrc*28C (Gregory *et al.*, 1987), with the highest values in the catalytic domain (Table 1). Interestingly, the highest degree of overall similarity is found to the *Hydra STK* gene (Bosch *et al.*, 1989). Sequence comparisons of the predicted tyrosine kinase domains of the clones *srk*1–4 are shown in Table 2. All four *srk* clones show a considerable similarity to each other throughout the kinase domain with a remarkably close relationship between *srk*1 and *srk*4. However, in the 3′-untranslated region *srk*1–4 do not display any significant similarity at all. This indicates that at least four different genomic loci encoding *src*-related genes are present in the sponge *Spongilla lacustris*.

The nucleotide sequence of *srk*1 revealed an identical 5′ overlap to the 5′ part of *srk*4. The identity extends to the predicted amino acid 122, which is located in the amino-terminal part of the SH2 domain exactly where all *src* family genes of vertebrates contain an intron. To test the hypothesis that this site corresponds to a homologous exon–intron border in sponges, polymerase chain reaction (PCR) analysis was performed. Using a primer from the common amino-terminal region (P1) and primers 88 bp downstream specific for

Table 1 Similarities between the *Spongilla lacustris src*-related proteins and tyrosine kinases from other organisms*

	Amino acid identity to sponge protein of (%)			
	srk*1*	srk*4*	srk*2*	srk*3*
Protein of kinase gene (reference)	*(entire protein†/tyrosine kinase domain alone‡)*		*(tyrosine kinase domain only‡)*	
Human *src* (Anderson *et al.*, 1985; Parker *et al.*, 1985)	56.9/63.2	56.8/62.6	62.2	63.0
Chicken *src* (Takeya & Hanafusa, 1983)	57.7/63.5	57.5/63.0	64.5	62.7
Xenopus src 1 (Steele *et al.*, 1989)	57.1/63.5	56.9/63.9	65.6	63.0
*Xiphophorus src***	55.2/62.2	54.6/61.8	62.2	61.0
Hydra STK (Bosch *et al.*, 1989)	57.4/62.7	59.1/64.0	64.4	63.7
*Drosophila Dsrc*64B (Simon *et al.*, 1985)	50.1/53.5	50.2/52.5	55.3	51.5
*Drosophila Dsrc*28C (Gregory *et al.*, 1987)	43.9/50.2	44.2/49.8	48.6	52.1
Human *yes* (Sukegawa *et al.*, 1987)	57.6/62.2	58.8/63.4	65.1	63.1
Human *fyn* (Kawakami *et al.*, 1986; Semba *et al.*, 1986)	55.0/59.8	56.9/61.7	64.4	60.3
Human *fgr* (Katamine *et al.*, 1988)	55.4/62.0	56.5/63.6	63.9	60.8
Human *lyn* (Yamanashi *et al.*, 1987)	53.0/57.8	54.5/58.7	59.7	55.5
Human *hck* (Quintrell *et al.*, 1987; Ziegler *et al.*, 1987)	54.3/56.6	55.0/57.6	59.9	56.4
Mouse *lck* (Marth *et al.*, 1985; Voronova & Sefton, 1986)	51.4/57.1	51.1/55.7	60.1	54.8
Mouse *blk* (Dymecki *et al.*, 1990)	53.6/58.7	52.4/56.1	59.3	55.9
Human *erb*B (Ullrich *et al.*, 1984)	40.4/43.9	38.8/38.4	39.5	38.9

* Comparisons of amino acid sequences were made with the algorithm of Needlemen & Wunsch (1970) using the University of Wisconsin Genetics computer group program GAP (Devereux *et al.*, 1984). The gap weight was set to 1.0, and the length weight was 0.1

† Entire protein means the predicted amino acid sequence of the combined nucleic acid sequence of cDNA *srk*1 (or *srk*4) and the primer extension clone *srk*1/4PE

‡ As tyrosine kinase domain sequence was used the predicted amino acid sequence of chicken exons 7–12 or its analogue

** c-*src* sequence from the teleost fish *Xiphophorus* (F. Raulf, PhD thesis, Ludwig-Maximilians-University, Munich, 1991; GenBank/EMBL accession no. X64658).

Table 2 Sequence comparison of the putative tyrosine kinase domain of the four *Spongilla lacustris src*-related cDNA clones

	srk*1*	srk*2*	srk*3*	srk*4*	
*srk*1	—	78.0 (63.2)	80.1 (64.2)	88.4 (82.1)	Amino acids*
*srk*2	66.3	—	77.3 (63.3)	78.5 (64.6)	
*srk*3	65.5	63.1	—	79.1 (61.3)	
*srk*4	80.7	66.6	65.1	—	
	NA†				

* Similarity values of amino acid sequence comparisons include conservative changes according to the GCG program GAP (Devereux *et al.*, 1984); the values in brackets are percentage identity only; algorithm as in Table 1.

† Comparisons of nucleic acid sequences were also made with the GAP program but using the gap weight 5.0 and the length weight 0.3

*srk*1 (P1R) or *srk*4 (P4R), both located 3′ to amino acid 123, different reaction products of 0.45 kb (*srk*1) and 2.5 kb (*srk*4) were obtained with *Spongilla lacustris* genomic DNA. This shows that an intron indeed separates the common 5′ parts from the *srk*1- and *srk*4-specific parts of the genes. Hybridization of the PCR products with the internal oligonucleotide P0R verified another specific 1.8-kb PCR product of the primer pairing P1/P4R that is slightly visible in ethidium bromide-stained gels. Hybridization of the same reaction products with the oligonucleotide P1R showed that the 2.5- and 1.8-kb *srk*4-specific PCR products also contain the sequence of this *srk*1-specific oligonucleotide. This may be explained either by an alternative splicing of one gene with two different carboxy termini or by the existence of two independent genes with identical 5′ exon(s) owing to a recent gene duplication/exon-shuffling event, one residing in an intron of the second gene.

Northern blot analysis of *Spongilla lacustris* poly(A)$^+$ RNA with all four *srk*s always revealed a single transcript of 2.2 kb (data not shown). This corresponds in size to the transcript that was previously detected with the heterologous v-*src* kinase domain probe (Barnekow & Schartl, 1984).

A *src*-type tyrosine kinase activity could be immunoprecipitated from total sponge extracts by using at least two different TBR sera (sera from Rous sarcoma virus tumor-bearing rabbits), all with the same result, as has been reported previously (Barnekow & Schartl, 1984). Here we confirm and expand these analyses to gemmulae of *Spongilla lacustris*, and to another species of freshwater sponge, *Ephydatia fluviatilis*. Gemmulae of *Spongilla lacustris*, the resting permanent forms of sponges that contain exclusively totipotent binucleated embryonic stem cells, thesocytes, displayed no tyrosine kinase activity (Figure 3), whereas significant amounts of tyrosine kinase activity could be detected in total extracts of adult sponges from both freshwater species. To analyse if there is cell type-specific expression of the *srk*s, cell separation techniques (Müller *et al.*, 1976) were used in preliminary experiments. Although no pure fractions containing only a single cell type could be obtained, fractions enriched for archaeocytes, the omnipotent stem cells of adult sponges that are instrumental in regeneration, remodelling and aggregation, exhibited considerable specific pp60$^{c\text{-}src}$-like tyrosine kinase activity (data not shown). This indicates that the *src*-related kinase activity is not uniformly distributed in the sponge but might indeed be cell type specifically regulated. Further analyses including *in situ* hybridization will be necessary to detect a cell type-specific expression of the different *srk* genes in sponges.

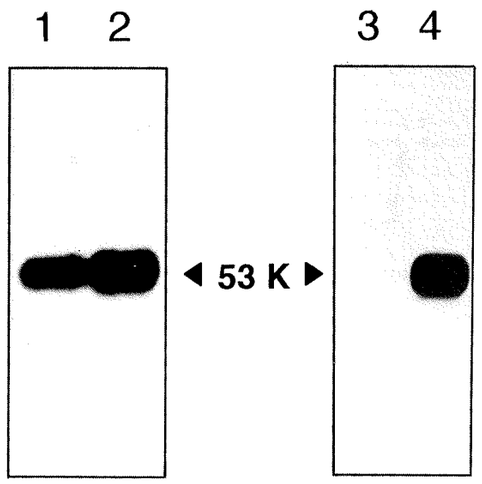

Figure 3 *src*-like tyrosine kinase activity in adult *Ephydatia fluviatilis* (1), *Spongilla lacustris* (2 and 4) and in gemmulae of *Spongilla lacustris* (3). 53 K indicates the heavy chain of immunoglobulin G phosphorylated by the pp60$^{c\text{-}src}$-related kinase

Discussion

Sponges, the simplest multicellular animals, are the lowest organisms of the phylogenetic tree that have been found to contain a *src*-type tyrosine kinase activity (Schartl & Barnekow, 1982). We have now isolated several different cDNAs that exhibit all the hallmarks of functional *src*-like tyrosine kinases. It is surprising to find a multigene family consisting of at least 3–4 members of divergent *src*-related genes in such a primitive organism, which is composed of about eight different cell types at most or perhaps only three (Rasmont, 1975). In contrast, the coelenterate *Hydra*, which follows the sponges on the next higher level of organismic organization in phylogenesis, contains only one *src*-related gene, and even in *Drosophila* the *src* gene family consists only of *Dsrc*64B and *Dsrc*28C. From this it is clear that the *srk* gene family was generated and diverged during the evolution of the sponges as a phylum. Sponges are unique with their marked ability for cell–cell recognition and aggregation. It will be interesting to determine if extension of the *srk* gene family somehow relates to this phenomenon.

The sponge-specific evolution of the *srk* gene family precludes deductions about the homology of *srk*1–4 to individual members of the *src* family of vertebrates. Nevertheless, the sequence comparisons suggest a closer relationship to c-*src*, c-*yes*, c-*fgr* and *fyn* than to the rest of the *src* gene family. These four protein kinases constitute a separate branch in the deduced phylogenetic tree of the *src* gene family (Hanks *et al.*, 1988) and therefore should be more representative of the ancestral enzyme.

Sponges existed as long ago as 1.5 billion years, therefore the *srk* genes and the vertebrate *src* gene family have been subjected to 3×10^9 years of independent evolution. Comparison of the consensus sequence for all four *srk* proteins with the vertebrate pp60$^{c\text{-}src}$ allows a more precise definition of the conserved and thus functionally relevant amino acid motifs, e.g. the protein kinase-specific motif DFG is surrounded by distinct *src*-specific sequences. In addition, motifs that are common to sponge *srk*s and vertebrate c-*src* become apparent besides those that have been defined as protein kinase specific in general (Hanks *et al.*, 1988). For instance, between kinase subdomain IX and X a motif with the core PYPGM is located, and between the SH2 domain and the tyrosine kinase domain the motif DGL is conserved. It is tempting to assume that such motifs are involved in specifying the *src*-type protein kinase activity. Site-directed mutagenesis offers a way to clarify this.

An interesting observation is that the core motif of subdomain VI which reads DLRAAN in *src* family kinases of vertebrates has a central inversion DLAARN in the *srk*s. The latter is also found in the vertebrate *abl* and *fes* kinases as well as in all receptor tyrosine kinases (Hanks *et al.*, 1988). The sponge motif is also present in the *src*-type kinases of other invertebrates, *Hydra STK* (Bosch *et al.*, 1989) and *Drosophila Dsrc*64B (Hoffmann *et al.*, 1983; Simon *et al.*, 1985) and *Dsrc*28c (Gregory *et al.*, 1987). This lends support to the notion that the protein kinases associated with the inner surface of the cell membrane and the receptor tyrosine kinases indeed have a common ancestor and are not the result of convergent evolution.

The c-*src* gene of vertebrates belongs to those genes whose tissue-specific functions can be regulated via alternative splicing (Levy *et al.*, 1987; Martinez *et al.*, 1987; Wang *et al.*, 1987; Pyper & Bolen, 1989). But the neural-specific insertion has been shown to be confined to vertebrates (Raulf *et al.*, 1989b), and so far no alternative splicing of *src*-related genes has been detected in non-vertebrate organisms. Unexpectedly, we obtained two cDNAs that share an identical amino terminus. The identity ends soon (seven nucleotides or two amino acids) after an exon–intron splice point conserved in all vertebrate *src* genes (nucleotides 415/416 of *srk*1). We could show that in the same region an intron is also located in sponge *srk*1 and 4. Therefore, alternative splicing of tandemly arranged carboxy-terminal parts seems a reasonable explanation. The fact that the identity of *srk*1 and *srk*4 extends minimally into the next exon may be easily explained by the high degree of similarity between *srk*1 and 4 throughout the rest of the sequence (the highest similarity of all possible *srk* pairs throughout the kinase domain, see Table 2). The location of the alternative splice point requires some consideration because it localizes to the amino-terminal border of the SH2 domain which is involved in protein–protein interaction. The postulated alternative splicing would generate two *srk*s that have different SH2 domains (70.9% identity) as well as different kinase domains (82.1% identity) but a common SH3 domain. Although the function of the SH3 domain in protein–protein interaction is less defined, it seems reasonable to propose that such two proteins could couple different signal transduction pathways as long as they are present in the same cell, i.e. by interaction of the common SH3 domain with the same protein and interaction of the diverged SH2 domain with different effector/substrate/regulatory molecules. If they are present in different cell types the two isoforms could be responsible for a cell type-specific response to a common signal.

It is intriguing that the intron that separates the common amino termini from the rest of the gene is just the single intron whose location with respect to the protein product is conserved from sponge, *Hydra* (Bosch *et al.*, 1989), *Dsrc*28C (Gregory *et al.*, 1987; but not in *Dsrc*64B, Simon *et al.*, 1985), to all vertebrate members of *src*-related kinase genes (between exons 4 and 5 in chicken c-*src*; Takeya & Hanafusa, 1983). There are no data contradicting the possibility that in higher vertebrates also there exist alternatively spliced forms of a *src* family member with different carboxy termini, although no such cDNAs have been isolated so far. The conservation of the placement of this intron suggests that it either has an important function as a structural element in the generation of the mature mRNA or contains so far unknown information.

Materials and methods

Animals

Freshwater sponges and gemmulae of the species *Spongilla lacustris* and *Ephydatia fuviatilis* were collected from the River Sieg (Germany). Cell suspensions in calcium- and magnesium-free water and separation of different cell types by Ficoll-density gradient centrifugation were performed according to Müller *et al.* (1976).

DNA and RNA isolation

Genomic DNA and total RNA were isolated by the guanidinium isothiocyanate method (Davis *et al.*, 1986). Briefly, sponges were quick frozen, powdered in liquid nitrogen and resuspended in 4 M guandinium isothiocyanate buffer for 15 min with gentle shaking at room temperature. The suspension was centrifuged (5000 r.p.m., SS34 rotor) and the supernatant subjected to a caesium chloride gradient. Poly(A)$^+$ RNA was prepared using oligo(dT) columns according to Davis *et al.* (1986). A 10-μg aliquot of poly(A)$^+$ RNA was analysed by Northern blot hybridization as previously described (Hannig *et al.*, 1991).

Construction and screening of Spongilla lacustris *cDNA libraries*

Synthesis of cDNA was carried out using 5 μg of poly(A)$^+$ RNA and a cDNA synthesis kit (Pharmacia/LKB) according to the supplier's instructions. cDNAs were ligated to EcoRI adaptors and subcloned into phosphatased λgt10 phage arms (Promega). For construction of the library the phage DNA was packaged using the 'Gigapack Gold' kit (Stratagene). Approximately 2×10^5 recombinant bacteriophages were screened under low-stringency conditions (hybridization conditions: 35% deionized formamide, 1 M sodium chloride, 50 mM Tris–Cl pH 7.5, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 1% SDS, 100 μg ml^{-1} heat-denatured calf thymus DNA, 42°C; washing conditions: 2 × SSC, 1% SDS, 42°C; SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) using as probe the viral *src* 612-bp PstI fragment F of RSV-SRA2 (DeLorbe *et al.*, 1980) encompassing most of the tyrosine kinase domain. The inserts of positive clones were excised with EcoRI and subcloned into Bluescript KS$^+$ (Stratagene).

Construction of a specifically primed cDNA library

The synthetic 20-mer oligonucleotide used in the primer extension is complementary to nucleotides 111–130 of the original *srk*1 clone (5′-TCGGAATGTAGCCTTCCTTC-3′), and to nt 356–375 of *srk*1 and *srk*4. A 5-μg aliquot of total RNA and 200 pmol of the oligonucleotide were incubated at 65°C for 10 min, then cooled gradually to 37°C. The cDNA synthesis was carried out using the Pharmacia cDNA synthesis kit (Pharmacia/LKB). The cDNA was subsequently subcloned into λgt10. A total of 5×10^4 recombinant bacteriophages were screened under high-stringency conditions with the 0.2-kb *srk*1 EcoRI/HindIII fragment (hybridization conditions: the same as above except that the formamide concentration was 50%; washing conditions: 1 × SSC, 0.1% SDS, 60°C).

DNA sequence analyses

Nucleotide sequences were obtained by the dideoxy chain-termination technique (Sanger *et al.*, 1977) using a modified T7 DNA polymerase (Sequenase, United States Biochemicals) and specific oligonucleotide primers. Oligonucleotides were synthesized on a 380A DNA synthesizer (Applied Biosystems, Foster City, CA, USA). All sequence data were analysed using the GCG program of the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984).

PCR analysis

Conventional genomic PCR (Saiki *et al.*, 1988) was performed as previously described (Raulf *et al.*, 1989b) except for using 250 ng genomic DNA of *Spongilla lacustris* and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer/Cetus) in a total volume of 100 μl of TAPS buffer [12.5 mM *N*-Tris-(hydroxymethyl)methyl-3-aminopropane sulphonic acid pH 9.3, 2 mM magnesium chloride, 50 mM potassium chloride, 1 mM 2-mercaptoethanol], and 35 cycles [1 min at 92°C, 1 min at the annealing temperature (40–60°C for different primers) and 1.5 min at 72°C]. The following oligonucleotide primers were used (for location see Figure 1): P1, 5′-GTCA-AAAGACACTGCCGGGAAGGAAGGCTA-3′; P0R, 5′-ACACGCTTAACTTGTCCCAAGAACCA-3′; P1R, 5′-AA-GATTGGATTAGCATT-3′; and P4R 5′-ATGGCATCATT-AACTGC-3′. Sequencing of PCR products was performed as previously described (Kocher *et al.*, 1989).

Preparation of antisera, cell extracts and immunoprecipitation

Preparation of TBR sera and cell extracts and the performance of immunoprecipitations followed by protein kinase assays were exactly as described by Schartl *et al.* (1989).

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