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Structure and Expression of *STK*, a *src*-Related Gene in the Simple Metazoan *Hydra attenuata*

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Both cDNA clones and a genomic DNA clone encoding a 509-amino-acid protein that is 64% similar to chicken pp60$^{c\text{-}src}$ were isolated from the simple metazoan *Hydra attenuata*. We have designated this gene *STK*, for *src*-type kinase. Features of the amino acid sequence of the protein encoded by the *STK* gene suggest that it is likely to be myristoylated and regulated by phosphorylation in a manner similar to that found for pp60$^{c\text{-}src}$. The genomic sequence encoding the protein was found to be interrupted by at least two introns, one of which was located in a position identical to that of one of the introns in the chicken *src* gene. The *STK* gene was expressed during early development of *H. attenuata* and at high levels in the epithelial cells of adult polyps. Probing of *Hydra* proteins with an antibody to phosphotyrosine indicated that the major phosphotyrosine-containing protein in *H. attenuata* may be the *STK* protein itself. *H. attenuata* is the simplest organism from which a protein-tyrosine kinase gene has been isolated. The presence of such a gene in the evolutionarily ancient phylum Cnidaria suggests that protein-tyrosine kinase genes arose concomitantly with or shortly after the appearance of multicellular organisms.

The *src* proto-oncogene encodes a protein-tyrosine kinase (pp60$^{c\text{-}src}$) whose role in the cell is not yet known. *src* is the prototype of a group of closely related genes that make up the so-called *src* family (J. A. Cooper, *in* B. Kemp and P. F. Alewood, ed., *Peptides and Protein Phosphorylation*, in press). One approach to understanding the function of pp60$^{c\text{-}src}$ and other members of the *src* family is to define the level of organismal complexity at which *src*-like protein-tyrosine kinases arose during evolution. Such information would be useful for narrowing the scope of possible functions for these proteins and may point to additional useful biological systems for biochemical and molecular genetic analysis of these proteins. To date, *src*-type genes have been isolated from various vertebrates and from *Drosophila melanogaster* (reviewed in reference 28).

Schartl and Barnekow (57) and Barnekow and Müller (3) have shown that a protein-tyrosine kinase activity immunoprecipitable with pp60$^{v\text{-}src}$ antiserum from rabbits with Rous sarcoma virus-induced tumors is present in a wide range of metazoans, including the marine hydroid *Hydractinia*. Such activity was not found in protozoans or plants (57). The subsequent identification of such a protein-tyrosine kinase activity in the simplest metazoans, the sponges, lead to the suggestion that the presence of a *src*-like kinase is directly related to requirements imposed by multicellularity (4). Levin et al. (44) reported the isolation of two genes from yeast cells, *KIN1* and *KIN2*, that encode 117- and 126-kilodalton (kDa) proteins with some amino acid sequence motifs considered characteristic of protein-tyrosine kinases. Additional analysis of the sequences of these two proteins suggested, however, that they are more closely related to serine/threonine kinases than to protein-tyrosine kinases (28). Recent work has confirmed that these proteins are indeed serine/threonine kinases (C. Hammond, M. Tibbetts, and R. M. Donovan, J. Cell. Biol., **107**:278a, 1988). Evidence of phosphotyrosine-containing proteins in yeast cells has been reported (10, 58), although these data are not unequivocal (73).

We have chosen the simple freshwater animal *Hydra attenuata*, a member of the evolutionarily ancient phylum Cnidaria, to study the nature and possible functional roles of the protein or proteins responsible for the protein-tyrosine kinase activity detected in simple metazoa (3, 4, 57). *H. attenuata* has a simple body plan consisting of two cell layers, ectoderm and endoderm, separated by a basement membrane, the mesoglea (9). The endoderm is made up of gastrodermal digestive cells, with a small number of mucous and gland cells interspersed among them. The ectoderm is made up of epitheliomuscular cells, between which are distributed interstitial cells, nematocytes, and nerve cells. The nervous system is a net of ganglion and sensory cells that extends throughout the animal. Because of its structural simplicity and amenability to studies at the cytological, biochemical, and developmental levels, *H. attenuata* may be a particularly useful organism for analysis of protein-tyrosine kinase function.

Using the oligonucleotide screening procedure of Levin et al. (44), we have isolated from *H. attenuata* a *src*-related gene that we have named *STK*, for *src*-type kinase. The *Hydra STK* gene contains a number of the features characteristic of members of the *src* gene family, indicating that these features were fixed very early in the evolution of genes in this family. The *STK* gene is expressed in early embryos and at a high level in epithelial cells of adult polyps. Our finding of the *STK* gene supports the hypothesis that protein-tyrosine kinases appeared very early in the evolution of metazoans and suggests that such proteins are required for one or more cellular processes which are fundamental to all multicellular animals.

MATERIALS AND METHODS

Culture of animals and tissue preparation. Polyps of *H. attenuata*, *H. magnipapillata*, and *H. circumcincta* were cultured by standard methods (42). *H. magnipapillata* were depleted of interstitial cells and their progeny by culturing sf-1, a temperature-sensitive mutant of this species (47), at

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28°C for 4 weeks. Cell composition of the temperature-treated polyps was determined by the maceration method (15). Temperature-treated animals contained no interstitial cells, no nematocytes, 4% of the normal complement of nerve cells, and 10% of the normal complement of gland cells. Eggs and two to four cell-stage embryos were obtained by dissection from *H. circumcincta*.

Nucleic acid isolation. DNA was isolated from polyps by the method of Davis et al. (17). Total RNA was extracted from whole polyps or tissues by a guanidinium isothiocyanate method (12).

Hybridization analysis of DNA and RNA. DNA from whole polyps was digested with restriction enzymes, fractionated by gel electrophoresis, and transferred to nitrocellulose filters as described previously (46). Hybridization to filter-bound DNA was carried out in a solution containing 35% formamide, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinlypyrrolidone, 5× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate) 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, 100 μg of torula yeast RNA per ml, and 500 μg of herring sperm DNA per ml at 42°C overnight. After hybridization, filters were washed twice at room temperature in 2× SSC–0.1% SDS. An additional wash was carried out for 1 h at 50°C in 2× SSC–0.1% SDS (low stringency) or at 65°C in 0.1× SSC–0.1% SDS (high stringency). The filters were then given two washes at room temperature in 2× SSC, blotted dry, and exposed to X-ray film.

For hybridization analysis of RNA, samples of total RNA were electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane (BioTrace RP; Gelman Sciences, Inc.) essentially as described previously (46). The filter-bound RNA was hybridized in a solution containing the same components as that used for DNA hybridization except that the formamide concentration was 50%. After hybridization, the filters were washed under the high-stringency conditions described above for DNA hybridization. The DNA fragments used as hybridization probes were labeled with ^{32}P by the random primer method (20).

Bacteriophage library construction and screening. An *H. attenuata* cDNA library in λgt11 was constructed essentially as described previously (32). Recombinant phage (1.5×10^6) were obtained and amplified on *Escherichia coli* Y1088 by the plate lysate method (46). For screening, the cDNA library was plated on *E. coli* LE392. A genomic library was constructed by partially digesting *H. attenuata* DNA with *Sau*3AI, size selecting 10- to 20-kilobase-pair (kbp) fragments on a low-melting-point agarose gel, and ligating these fragments to phosphatase-treated, *Bam*HI-digested EMBL3 bacteriophage arms (Promega Biotec). In vitro-packaged phage particles were plated on *E. coli* MB406, and the resulting 1.6×10^5 recombinant phage were screened without amplification. Phage were blotted onto nitrocellulose by the method of Benton and Davis (5). The cDNA library was initially screened with a mixture of synthetic oligodeoxynucleotides identical to the mixture of pools 1 and 2 described by Levin et al. (44). The oligonucleotide pools were synthesized by the Protein/Nucleic Acids Analysis Laboratory in the Biological Chemistry Department at the University of California, Irvine (UCI). The oligonucleotides were end labeled with ^{32}P by using T4 polynucleotide kinase (46). Hybridization with the oligonucleotide mixture was carried out overnight at 53°C in a solution containing 0.9 M NaCl, 0.18 M Tris (pH 8.0), 12 mM EDTA, 1% SDS, 1% powdered milk, and 250 μg of yeast RNA per ml. After hybridization, the filters were washed four times for 5 min each in 6× SSC–0.1% SDS at 15°C and then in a solution of the same composition for 1 min at 52°C. The filters were blotted dry and exposed to X-ray film. Positive plaques were picked and rescreened as described above until they were pure. Additional screening of the cDNA library with the initially isolated clone and screening of the genomic library were carried out essentially as described above except that hybridization and washing conditions were as described for hybridization analysis of genomic DNA blots.

DNA sequence analysis. For DNA sequence determination, restriction fragments were cloned into the phagemid vector pBS- (60). Single-stranded template DNA was obtained by superinfection with phage M13K07 (74) of *E. coli* BSJ72 (from Tom St. John, Fred Hutchinson Cancer Research Center) carrying the desired recombinant plasmid. DNA sequencing was done by using the chain termination method (56) and a modified form of T7 DNA polymerase (Sequenase; U.S. Biochemical Corp.). DNA and amino acid sequence data were analyzed by using the University of Wisconsin Genetics Computer Group programs (18).

In vitro transcription and translation. DNA fragments from cHySTK2 and gHySTK1 were combined in plasmid pSP65 (50) to generate a plasmid with a complete coding sequence for the *STK* protein.

Plasmid pSPSTK1 was constructed as follows. The 2.5-kbp *Kpn*I fragment of gHySTK1, which contains the 3′ end of the *STK* gene, was subcloned into the *Kpn*I site of pBS- (60). The 3′ portion of the *Kpn*I fragment was then excised from this plasmid by cleavage with *Bgl*II and *Sma*I. *Bgl*II cleaves 800 bp in from the 5′ end of the *Kpn*I fragment, and *Sma*I cleaves in the vector polylinker sequence adjacent to the 3′ end of the *Kpn*I fragment. The 5′ end of the gene was obtained by isolation of the 1.1-kbp *Ssp*I-*Bgl*II fragment from cHySTK2. The *Ssp*I site is located 53 nucleotides upstream of the ATG initiation codon. A plasmid containing a complete open reading frame was constructed by combining the 3′ *Bgl*II-*Sma*I fragment, the 5′ *Ssp*I-*Bgl*II fragment, and *Sma*I-cleaved pBS-. The combined 5′ and 3′ fragments were excised with *Sac*I and *Sma*I and then inserted into *Sac*I-*Sma*I-cleaved pSP65 (50).

The plasmid was linearized by restriction enzyme cleavage 3′ to the insert and transcribed with SP6 RNA polymerase essentially as described previously (40), with $m^7G(5')ppp(5')G$ included. In vitro-synthesized RNA was purified from the transcription reaction mixture by DNase digestion, phenol-chloroform extraction, and ethanol precipitation. In vitro translation of the purified RNA was carried out in a nuclease-treated rabbit reticulocyte lysate (Stratagene) in the presence of [^{35}S]methionine (Amersham Corp.). The reaction was carried out as specified by the supplier. After translation, an equal volume of 2× SDS sample buffer was added, and the sample was boiled for 5 min. The in vitro translation products were analyzed by electrophoresis in a 10% polyacrylamide–SDS gel (41). Labeled polypeptides were detected by fluorography with En^{3}Hance (Dupont, NEN Research Products).

Immunoblotting of proteins. A sample of proteins from epidermal growth factor (EGF)-treated A431 cells was generously provided by David Schlaepfer and Harry Haigler (Department of Physiology and Biophysics, UCI). Total *Hydra* protein samples (each containing about 150 μg of protein) were prepared by boiling polyps in SDS sample buffer. Proteins were fractionated by electrophoresis in a 10% polyacrylamide–SDS gel (41) and transferred onto a nitrocellulose filter by the method of Burnette (6). The filter

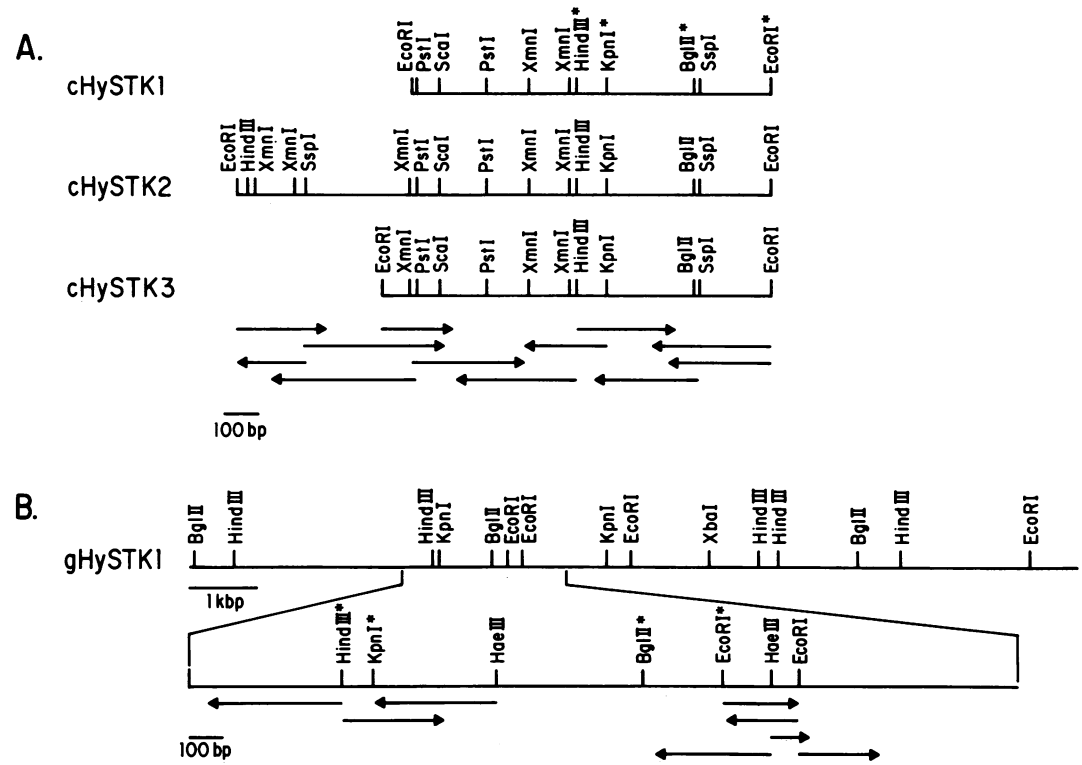

FIG. 1. Structures of *STK* cDNA and genomic clones. (A) Restriction enzyme cleavage maps of three *STK* cDNA clones. The sites marked by asterisks correspond to the sites marked by asterisks in the genomic clone map shown in panel B. Arrows at the bottom show the strategy used for DNA sequence analysis of the cDNA clones. (B) Restriction enzyme cleavage map of the genomic *STK* clone gHySTK1. A portion of this map is expanded to show the strategy used for DNA sequence analysis.

was probed with a monoclonal antibody against phosphotyrosine (provided by Mark Kamps and Bart Sefton, The Salk Institute). The antibody was raised against the same hapten used to raise the previously described polyclonal antiphosphotyrosine antibodies (34). Blocking and incubation of the filters with antibody were as described previously (34). Bound antibody was detected by sequential incubations with rabbit anti-mouse antibodies (Zymed) and ^{125}I-labeled protein A. Between each of these incubations, the filter was washed as after the incubation with the antiphosphotyrosine antibody (34). Bound protein A was detected by exposure of the filter to X-ray film. For blocking with phosphotyrosine, antibody was incubated for 30 min in the presence of 100 mM *o*-phospho-DL-tyrosine (Sigma Chemical Co.) in phosphate-buffered saline. Filter blocking and incubation buffers also contained *o*-phospho-DL-tyrosine at a concentration of 10 mM.

RESULTS

***H. attenuata* contains a gene that encodes a protein closely related to pp60$^{c\text{-}src}$.** To screen a *Hydra* cDNA library for *src*-related genes, we applied the oligonucleotide hybridization strategy used by Levin et al. (44). In this approach, the hybridization probe consists of a mixture of 96 different 17-nucleotide-long synthetic oligonucleotides. The mixture contains all possible DNA sequences encoding the amino acid sequence Asp-Val-Trp-Ser-Phe-Gly (the third nucleotide of the glycine codon is not included in the oligonucleotides). Screening of an *H. attenuata* cDNA library with this probe mixture resulted in the isolation of the single cDNA clone cHySTK1. Rescreening of the cDNA library with the insert from cHySTK1 resulted in the isolation of a number of additional clones, two of which were cHySTK2 and cHySTK3. The identities of the restriction maps of the three clones in their regions of overlap (Fig. 1A) indicated that the three cDNAs were derived from the same gene.

Initial DNA sequence analysis of cHySTK1 indicated the presence of an open reading frame encoding a polypeptide with a high degree of similarity to chicken pp60$^{c\text{-}src}$. To determine the exact nature of the protein product of the *Hydra* gene, the entire DNA sequence included in the cDNA clones was determined. We found that all three cDNA clones shown in Fig. 1 lacked the sequence encoding the carboxyl-terminal portion of the protein. This was apparently the result of a failure of *Eco*RI site methylation during library construction and subsequent cleavage at an internal *Eco*RI site during cleavage of the synthetic *Eco*RI linkers added to the cDNA termini. Therefore, to complete the sequence, a clone was isolated from a genomic library of *H. attenuata* by screening with the 208-bp 3′ *Ssp*I-*Eco*RI fragment of cHySTK1. A restriction map of this clone, gHySTK1, and its relationship to the cDNA clones are shown in Fig. 1B.

The nucleotide and predicted protein sequences obtained by combined analysis of the cDNA and genomic clones are shown in Fig. 2. (The nucleotide sequence is being deposited in the GenBank/EMBL Data Bank under accession no. M25245.) Analysis of the sequence at the 5′ end of cHySTK2 revealed that the sequence of nucleotides 4 to 198 in the 5′ untranslated region of the clone was an inverted copy of the sequence of nucleotides 828 to 1022 in the coding region. Artifacts of this type have been seen in other cDNA clones,

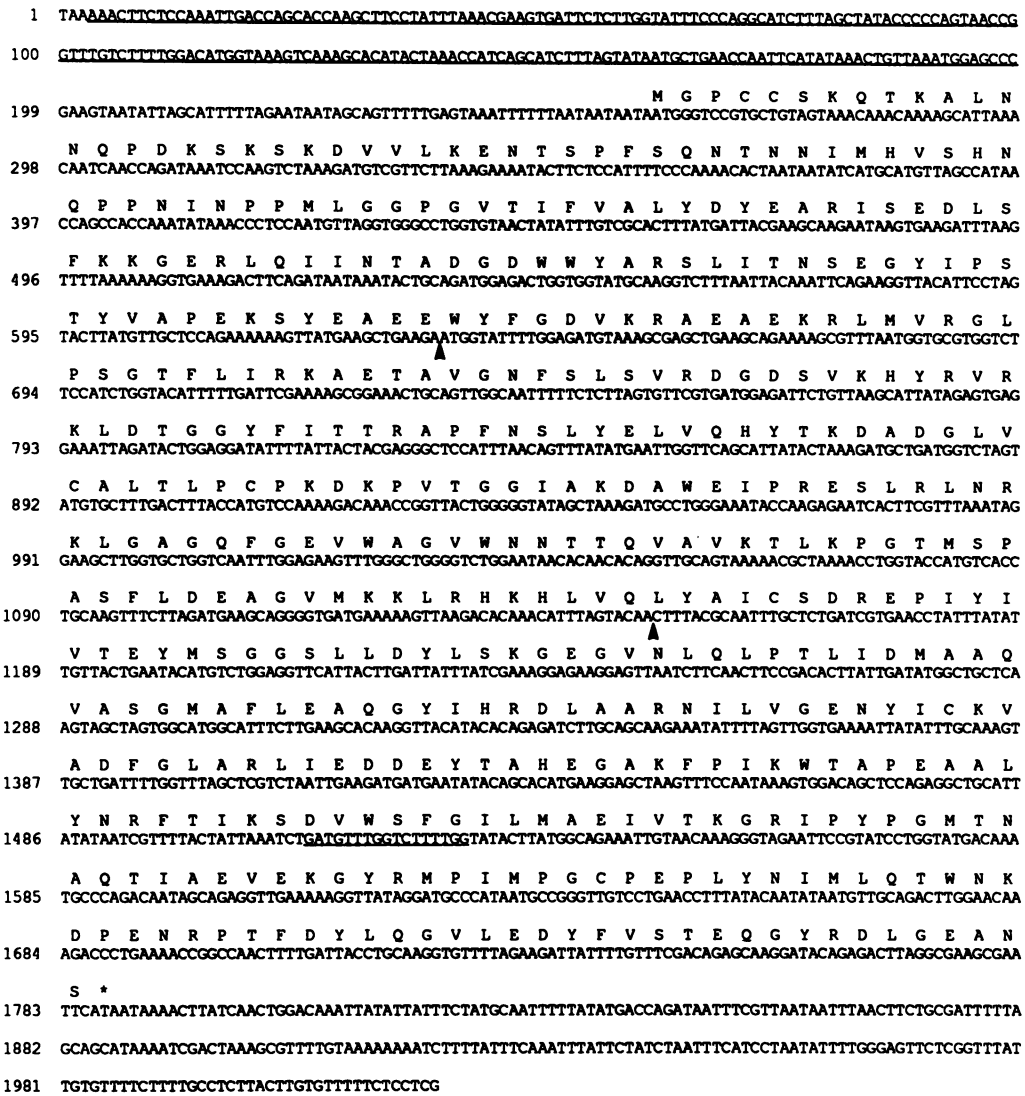

FIG. 2. *STK* cDNA sequence. Nucleotides 1 to 1565 of the sequence were derived from analysis of the three cDNA clones shown in Fig. 1A. The remaining portion of the sequence was obtained by analysis of the genomic clone gHySTK1 (Fig. 1B). The sequence of the polypeptide encoded by the DNA sequence is indicated in one-letter amino acid code. The underlined nucleotides from positions 4 to 198 correspond to the inverted repeat sequence described in the text. Arrowheads mark the locations of the two introns that have been identified from analysis of gHySTK1. The underlined nucleotides at positions 1511 to 1527 match one of the sequences in the oligonucleotide probe mixture used to isolate cHySTK1.

and possible mechanisms for their formation during cDNA synthesis have been proposed (22, 75). We therefore consider only the sequence starting at nucleotide 199 to reflect the sequence as it occurs in *STK* mRNA.

The sequence in Fig. 2 contains a long open reading frame of 1527 nucleotides which begins at nucleotide 260, the position of the 5′-most ATG triplet in the sequence. This ATG triplet is preceded by an A residue 3 nucleotides upstream and is immediately followed by a G, which conforms to the consensus translation initiation sequence described by Kozak (39). The amino acid sequence predicted from this open reading frame is 509 residues long, terminating with a TAA codon at position 1787. The predicted protein product of the gene has a molecular size of 56,885 kDa. Confirmation of this predicted coding potential was obtained by in vitro translation of synthetic *STK* RNA. By using fragments from cHySTK2 and gHySTK1, a plasmid that could be transcribed in vitro to produce *STK* RNA was constructed (Fig. 3A). In vitro translation of the synthetic RNA yielded the predicted 57-kDa polypeptide product (Fig. 3B, lane 2).

Comparison of the amino acid sequence of the *Hydra STK* protein with sequences of the members of the *src* family by using the algorithm of Needleman and Wunsch (51) resulted in the similarity values shown in Table 1. The *STK* protein was found to be more similar to vertebrate *src* and *yes* proteins than to any other members of the family. Interestingly, the *Hydra* protein was more like any of the vertebrate proteins than either of the *Drosophila* proteins. Because we do not know with which of the *src* family members the *Hydra* protein is functionally homologous, we have decided to name the gene encoding this protein *STK*, for *src*-type kinase.

Figure 4 shows a comparison of the sequences of the *STK* protein and chicken $pp60^{c\text{-}src}$. The level of sequence identity between the two proteins was found to be relatively uniform

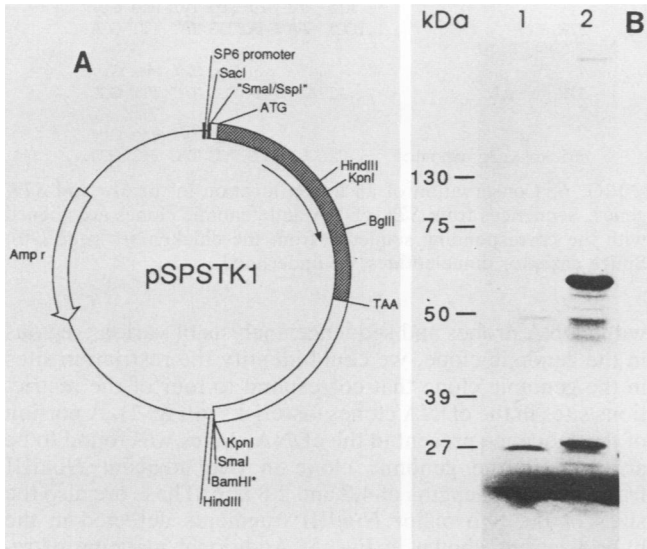

FIG. 3. In vitro synthesis of the *STK* protein. (A) Map of plasmid pSPSTK1, designed to allow synthesis of *STK* RNA in vitro. Symbols: ▩, *STK* coding sequence; ▭, noncoding sequences contained in either the cHySTK2 or gHySTK1 fragments; ▮, region derived from the polylinker of pBS-. The *Bam*HI site (*) was cleaved to linearize the plasmid for RNA synthesis. (B) In vitro translation products of RNA transcribed from pSPSTK1. Lanes: 1, results obtained from a nuclease-treated reticulocyte lysate to which no RNA was added; 2, products obtained when in vitro-synthesized *STK* RNA was added to the lysate.

except in the region near the amino terminus. This result is not unexpected, given the finding that among vertebrates the amino-terminal region of pp60$^{c\text{-}src}$ has diverged more rapidly than the rest of the protein (49, 70, 71; R. E. Steele, T.F. Unger, M. J. Mardis, and J. B. Fero, J. Biol. Chem., in press). All of the amino acids that have been shown to have functional roles in chicken pp60$^{c\text{-}src}$ are conserved in the *STK* sequence. The glycine at position 2 in chicken pp60$^{c\text{-}src}$ is the site of attachment of the myristic acid moiety that is involved in anchoring pp60$^{c\text{-}src}$ to the plasma membrane (33). The attachment of myristate has been shown to be required for membrane localization (8). In addition, both pp60$^{c\text{-}src}$ and

TABLE 1. Similarities between *STK* protein and *src*-type kinases[a]

Protein	% Similarity to *STK*
Chicken *src*	64.3
Human *yes*	64.0
Human *fyn*	62.0
Human *fgr*	61.5
Human *lyn*	56.5
Human *hck*	56.5
Mouse *lck*	53.8
Drosophila src64	51.0
Drosophila src28C	43.9

[a] Comparisons of amino acid sequences were made with the algorithm of Needleman and Wunsch (51), using the University of Wisconsin Genetics Computer Group programs (18). The gap weight for all of the comparisons was 1.000, and the length weight was 0.10. The amino acid sequences for the various proteins were obtained by translation of the appropriate DNA sequence entries in the GenBank/EMBL Data Bank. References for the sequences: chicken *src* (70), human *yes* (69), human *fyn* (38, 59), human *fgr* (37), human *lyn* (80), human *hck* (54, 82), mouse *lck* (48, 76), *Drosophila src64* (61), *Drosophila src28C* (26).

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STK       1 MGPCCSKQTKALNNQPDKSKSKDVVLKENT..SPF..SQNTNNIMHVSHN 46
            ||  ||  |           |         |    |  ||  |
src       1 MGSSKSKP.KDPSQRRR...SLEP..PDSTHHGGFPASQTPNKTAAPDTH 44

STK      47 QPPN.......INPPMLGG.........P.........GVTIFVALYDYEA 72
              |           |   ||          |         ||| ||||||||
src      45 RTPSRSFGTVATEPKLFGGFNTSDTVTSPQRAGALAGGVTTFVALYDYES 94

STK      73 RISEDLSFKKGERLQIINTADGDWWYARSLITNSEGYIPSTYVAPEKSYE 122
            |  ||||||||||||  |    ||||  | ||  |    |||||  ||||  |
src      95 RTETDLSFKKGERLQIVNNTEGDWWLAHSLTTGQTGYIPSNYVAPSDSIQ 144

STK     123 AEEWYFGDVKRAEAEKRLMV.RGLPSGTFLIRKAETAVGNFSLSVRD... 168
            |||||||   | | |  ||      | ||||  |   ||  |   |||  |
src     145 AEEWYFGKITRRESE.RLLLNPENPRGTFLVRESETTKGAYCLSVSDFDN 193

STK     169 ..GDSVKHYRVRKLDTGGYFITTRAPFNSLYELVQHYTKDADGLVCALTL 216
              |  ||||  |||| ||  || |  | ||  ||  | | ||||   ||
src     194 AKGLNVKHYKIRKLDSGGFYITSRTQFSSLQQLVAYYSKHADGLCHRLTN 243

STK     217 PCPKDKPVTGGIAKDAWEIPRESLRLNRKLGAGQFGEVWAGVWNNTTQVA 266
            ||  || | | ||||||||||||||  ||| | ||||| | || || ||
src     244 VCPTSKPQTQGLAKDAWEIPRESLRLEVKLGQGCFGEVWMGTWNGTTRVA 293

STK     267 VKTLKPGTMSPASFLDEAGVMKKLRHKHLVQLYAICSDREPIYIVTEYMS 316
            ||||||||||  || || |||||||  ||||||  |  ||||||||||||
src     294 IKTLKPGTMSPEAFLQEAQVMKKLRHEKLVQLYAVVSE.EPIYIVTEYMS 342

STK     317 GGSLLDYLSKGE.GVNLQLPTLIDMAAQVASGMAFLEAQGYIHRDLAARN 365
            |||||  |||  |  || | ||||| |||||  |    | |||| | |
src     343 KGSLLDFL.KGEMGKYLRLPQLVDMAAQIASGMAYVERMNYVHRDLRAAN 391

STK     366 ILVGENYICKVADFGLARLIEDDEYTAHEGAKFPIKWTAPEAALYNRFTI 415
            ||||||  ||||||||||||||| ||||  ||||||||||||||||| ||||
src     392 ILVGENLVCKVADFGLARLIEDNEYTARQGAKFPIKWTAPEAALYGRFTI 441

STK     416 KSDVWSFGILMAEIVTKGRIPYPGMTNAQTIAEVEKGYRMPIMPGCPEPL 465
            ||||||||||  |  |||| |||||  |       || |||||  | ||| |
src     442 KSDVWSFGILLTELTTKGRVPYPGMVNREVLDQVERGYRMPCPPECPESL 491

STK     466 YNIMLQTWNKDPENRPTFDYLQGVLEDYFVSTEQGYRDLGEANS 509
              | |  ||| |||| |||  ||||| |||   |   || |
src     492 HDLMCQCWRRDPEERPTFEYLQAFLEDYFTSTEPQYQP.GE.NL 533
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FIG. 4. Comparison of the predicted *STK* protein amino acid sequence with the amino acid sequence of chicken pp60$^{c\text{-}src}$. The sequences were aligned by using the algorithm of Needleman and Wunsch (51) as executed by the GAP program in the University of Wisconsin Genetics Computer Group program package (18). The gap weight was set at 1.000, and the length weight was set at 0.10. Gaps introduced into the alignment are indicated by dots, and amino acid matches are indicated by vertical lines. The chicken pp60$^{c\text{-}src}$ sequence is from reference 70.

the *STK* protein have a serine at position 6 and a lysine at position 7. These two residues play a critical role in the recognition of pp60$^{c\text{-}src}$ by myristoyltransferase (7, 36, 72). Therefore, it appears likely that the *STK* protein will be found to be myristoylated and localized to the plasma membrane in a manner similar to that of pp60$^{c\text{-}src}$. Various residues in pp60$^{c\text{-}src}$ have been demonstrated or predicted to be involved in binding of ATP. The sequence Gly-X-Gly-X-X-Gly at positions 274 to 279 in chicken pp60$^{c\text{-}src}$ is thought, on the basis of modeling studies, to interact with the ribose of ATP (66). This sequence is conserved in the *STK* protein at positions 247 to 252. Chemical modification studies (35) have shown that a lysine at position 295 in pp60$^{c\text{-}src}$ participates in the phosphotransfer reaction. A lysine is present at the equivalent position (position 268) in the *STK* protein. Furthermore, the tyrosine at position 501 in the *STK* protein is likely to be homologous to tyrosine 527 in pp60$^{c\text{-}src}$. This tyrosine residue has been shown to be the site of a phosphorylation that inhibits the kinase activity of pp60$^{c\text{-}src}$ (13). This tyrosine in pp60$^{c\text{-}src}$ and all other vertebrate members of the *src* family is flanked on each side by a glutamine (Cooper, in press). In the *STK* protein, this tyrosine is in the sequence Gly-Tyr-Arg. In this regard, the *STK* protein is more like the *Drosophila src* protein, which has the sequence Pro-Tyr-Arg (61). Also conserved is the tyrosine residue at position 390 in the *STK* protein, which is

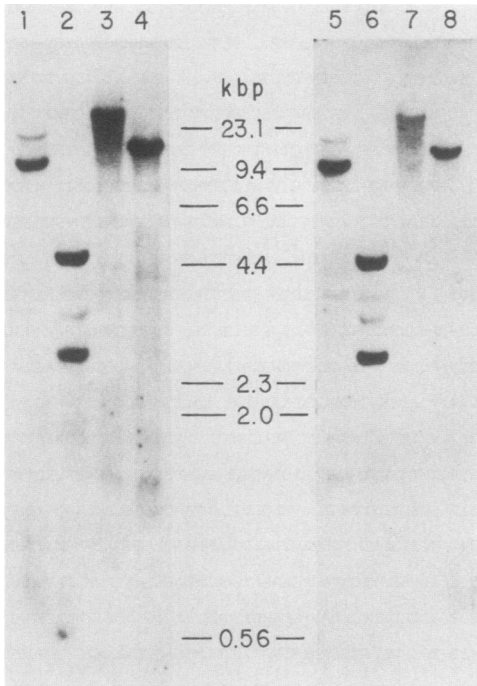

FIG. 5. Hybridization of an *STK* cDNA probe to *H. attenuata* DNA. Total DNA from *H. attenuata* (20 μg per lane) was probed at low stringency with the insert from cHySTK1. After hybridization, the filters were washed at low (lanes 1 to 4) or high (lanes 5 to 8) stringency as described in Materials and Methods. The restriction enzymes used to cleave the DNA were *Eco*RI (lanes 1 and 5), *Hin*dIII (lanes 2 and 6), *Bam*HI (lanes 3 and 7), and *Pst*I (lanes 4 and 8).

equivalent to tyrosine residue 416 in pp60$^{c\text{-}src}$, the major site of autophosphorylation (53, 62).

Because we have not yet analyzed the *STK* protein directly, we have no direct proof that it has protein-tyrosine kinase activity. We infer that such activity is present because of the great degree of similarity between the *STK* protein and members of the *src* family. In addition, the *STK* protein contains the sequence Asp-Leu-Ala-Ala-Arg-Asn at positions 360 to 365. This sequence has been shown to be an indicator of the specificity of a kinase for tyrosine as a substrate (28).

Structural organization of the *STK* gene. To gain information about the structural organization of the *STK* gene and to determine whether related genes may be present in the *Hydra* genome, restriction digests of total *H. attenuata* DNA were probed with the insert from the cDNA clone cHySTK1. Hybridizations to duplicate filters were performed at low stringency, and each of the filters was then washed under either high- or low-stringency conditions. The patterns of fragments to which the probe hybridized were not obviously different at the two stringencies (Fig. 5). In both cases, one or two fragments were prominently labeled. The minor fragments visible were present at both stringencies. These results suggest that the *STK* gene is not part of a large family of closely related genes, in contrast to the case for the *src* family in vertebrates.

Analysis of the genomic clone gHySTK1 has made it possible to obtain information on the structure of the *STK* gene that bears on its evolutionary relationship to the vertebrate *src* family genes and on the question of how many *src*-type genes are present in *H. attenuata*. By hybridization

```
                                Ala Glu Glu Trp Tyr Phe Gly
STK cDNA                  ...GCT GAA GAA TGG TAT TTT GGA...

                                    u Trp Tyr Phe Gly
STK genomic               ...ATTATTTAGA TGG TAT TTT GGA...

                                    u Trp Tyr Phe Gly
chicken src genomic       ...CTCCCACAGG TGG TAC TTT GGG...
```

FIG. 6. Conservation of an intron location in the *src* and *STK* genes. Sequences from *STK* cDNA and genomic clones are aligned with the corresponding sequence from the chicken *src* gene (70). Splice acceptor dinucleotides are underlined.

with cDNA probes and sequence analysis of various regions in the genomic clone, we could identify the restriction sites in the genomic clone that correspond to four of the restrictions sites in the cDNA clones (asterisks in Fig. 1). A portion of the sequence present in the cDNA clones was found to be arranged in the genomic clone in two adjacent *Hin*dIII fragments with lengths of 4.7 and 2.8 kbp. These are also the sizes of the two major *Hin*dIII fragments detected in the hybridizations shown in Fig. 5. Additional mapping of restriction sites in gHySTK1 has indicated that this clone does not contain all of the *STK* gene. Therefore, at least some of the additional minor fragments seen in the *Hin*dIII pattern in Fig. 5 must have resulted from hybridization to genomic DNA containing the portion of the *STK* gene present in the cDNA probe but absent from gHySTK1. It is therefore possible that all of the fragments seen in the hybridizations in Fig. 5 resulted from the presence of the *STK* gene alone. It appeared that there could be at most only one or two other genes that are closely related to *STK*.

The distance between the *Bgl*II and *Kpn*I sites in gHySTK1 is greater than the distance between the corresponding sites in the cDNA clone, indicating the presence of an intron in this portion of the gene. Sequence analysis (data not shown) showed that this intron was located between nucleotides 1150 and 1151 in the cDNA sequence (arrowhead in Fig. 2). This is not an intron site in any members of the *src* family for which the exon-intron arrangement has been determined. The distances between the *Bgl*II and *Eco*RI sites and the *Hin*dIII and *Kpn*I sites are identical in the cDNA and genomic clones, suggesting that introns are absent from the regions located between these sites. With sequence data from the region of gHySTK1 extending upstream of the *Hin*dIII site marked with an asterisk in Fig. 1, it was possible to locate the site of a second intron in the *STK* gene between nucleotides 633 and 634 in Fig. 2. Comparison of the sequence containing the splice acceptor site of this intron with the sequence at the splice acceptor site of exon 5 in the chicken *src* gene showed that the *STK* gene contains an intron at a location identical to that of the intron which separates exons 4 and 5 in the chicken (70), human (71), and *Xenopus* (R. E. Steele and J. B. Fero, unpublished observation) *src* genes (Fig. 6).

Expression of the *STK* gene in *Hydra* tissues. To obtain information relating to the possible function of the *STK* gene product in *Hydra* cells, we attempted to determine in which tissues the gene is expressed. RNAs were isolated from *H. magnipapillata* polyps (strain sf-1) that were depleted of all cells of the interstitial cell lineage and from *H. magnipapillata* polyps that had been decapitated and allowed to regenerate for 2 h. Strain sf-1 carries a temperature-sensitive mutation (47) that causes loss of interstitial cells and their differentiation derivatives (nematocytes, nerve cells, mucous cells, gland cells, and germ cells) when polyps are incubated at the nonpermissive temperature (28°C). The

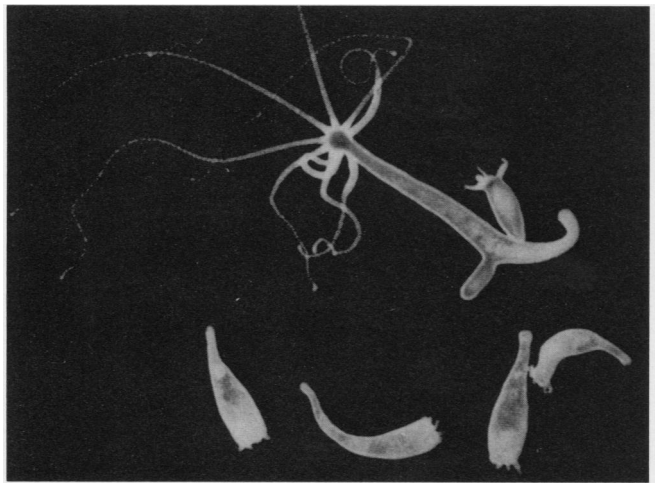

FIG. 7. Morphology of *H. magnipapillata* sf-1 mutants cultured at the nonpermissive temperature. Shown are a single individual of strain sf-1 (47) as it appears when cultured at the permissive temperature (18°C) and four individuals of strain sf-1 after culturing for 4 weeks at the nonpermissive temperature of 28°C. The normal animal contains two buds.

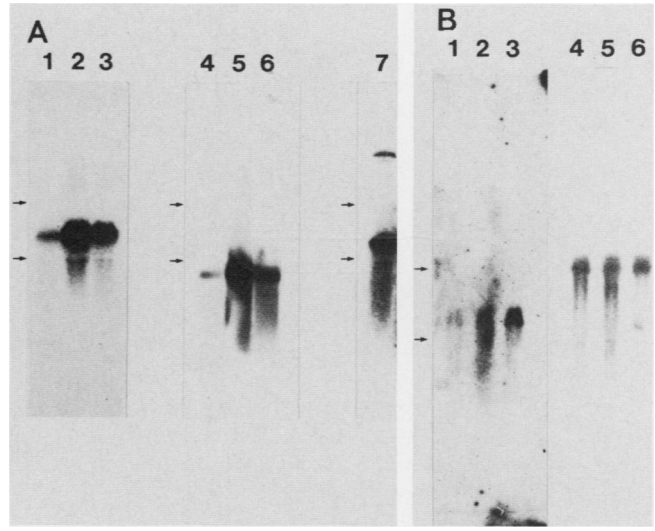

FIG. 8. Expression of *STK* RNA. Total RNAs from various sources were fractionated in formaldehyde-agarose gels, transferred onto nylon filters, and probed with an *STK* cDNA fragment, a *Hydra* actin cDNA fragment, or a 4.65-kbp *Eco*RI fragment of *X. laevis* ribosomal DNA that consisted mostly of 28S rRNA sequence (65). (A) Lanes: 1 and 4, RNAs from *H. magnipapillata* sf-1 cultured at 18°C; 2 and 5, RNAs from epithelial *H. magnipapillata* generated by incubation of strain sf-1 at 28°C for 4 weeks; 3 and 6, RNAs from *H. magnipapillata* sf-1 polyps that had been decapitated and allowed to regenerate for 2 h; 7, RNA from a mixture of eggs and two to four cell embryos of *H. circumcincta*. Probes were the *STK* cDNA fragment (lanes 1 to 3 and 7) and the *Hydra* actin cDNA fragment (lanes 4 to 6). (B) Results of an experiment similar to that shown in panel A. Lanes: 1 to 3, RNAs from normal, regenerating, and epithelial polyps, respectively, probed with the *STK* cDNA fragment (relative hybridization levels were as for the experiment shown in panel A); 4 to 6, results obtained when the *STK* probe was removed and the filter was reprobed with the *X. laevis* ribosomal DNA fragment. Arrows indicate positions of *X. laevis* 18S and 28S rRNA markers. *X. laevis* 18S rRNA is 1,825 nucleotides long (55), and the 28S rRNA is 4,110 nucleotides long (77).

resulting polyps contain only epithelial cells (Fig. 7). In addition, RNA was isolated from a mixture of eggs and two to four cell embryos of *H. circumcincta*. This species was used because it produces eggs more readily than do most *Hydra* species.

Equal amounts of RNAs (as determined by A_{260} and ethidium bromide staining) from normal, epithelial, and regenerating polyps were fractionated in a formaldehyde-agarose gel, blotted onto a nylon filter, and probed with the *STK* fragment from cHySTK1. A duplicate filter was probed with a fragment from a *Hydra* actin cDNA clone (D. Fisher and H. Bode, Gene, in press). RNAs from normal polyps, epithelial polyps, and regenerating polyps were probed with the *STK* fragment from cHySTK1 (Fig. 8A, lanes 1 to 3). When the *STK* fragment was used as a probe, we detected a single 2.5-kb species in all three samples. The fact that we detected *STK* RNA in the epithelial animals indicated that this gene is expressed in adult epithelial cells. In fact, the level of *STK* RNA found in epithelial animals was considerably higher than that in normal polyps, suggesting that *STK* RNA constitutes a greater proportion of the RNA population in epithelial cells than in the total cell population. It is not yet possible to say whether the *STK* gene is also expressed in nonepithelial cell types. We will be able to answer this question when in situ hybridization methods are perfected for *Hydra* species. We also found higher than normal levels of *STK* RNA in animals that had been decapitated and allowed to regenerate for 2 h (Fig. 8A, lane 3). Surprisingly, the level of actin RNA appeared to increase in parallel with the level of *STK* RNA. The actin RNA level was expected to be identical in all samples and thus serve as a control for equal loading and transfer of RNA. Because of the variation in actin RNA levels, we repeated the experiment by using a fragment of *Xenopus laevis* ribosomal DNA as a control probe. The fragment contained mostly 28S rRNA sequence. After initial hybridization with the *STK* probe, the filter was reprobed with the ribosomal DNA probe (Fig. 8B, lanes 4 to 6). As expected, the probe hybridized to the 28S rRNA, and the signal was of equal intensity in all three samples. This result confirmed that both *STK* and actin RNA levels were increased in epithelial and regenerating polyps. In addition to examining somatic tissues, we looked for *STK* expression during early development. In RNA from a mixture of eggs and two to four cell embryos, we detected a major *STK* RNA species of the same size as that in whole polyps (Fig. 8A, lane 7). However, we also detected an additional 8.7-kb RNA that was not seen in any of the other samples. We suspect that this large RNA was an incompletely processed primary transcript from the *STK* gene. An alternative explanation is that this RNA was derived from an *STK*-related gene that is expressed at high levels only in early embryos. Given the evidence suggesting that additional genes closely related to *STK* are absent from the *Hydra* genome (Fig. 5), the latter explanation seems unlikely.

Phosphotyrosine-containing proteins in *H. attenuata*. The finding of what appeared to be a single gene for a *src*-type kinase in *H. attenuata* suggested that the search for substrates for this kinase may be simpler in *Hydra* species than in vertebrates, which have a large number of diverse tyrosine kinases. To approach the question of *STK* substrates, we examined *Hydra* proteins with an antibody directed against phosphotyrosine (Fig. 9). As a positive control, proteins from A431 human epidermal carcinoma cells treated with EGF were used EGF treatment of A431 cells induces the protein-tyrosine kinase activity of the EGF receptor and causes the phosphorylation of a variety of proteins on

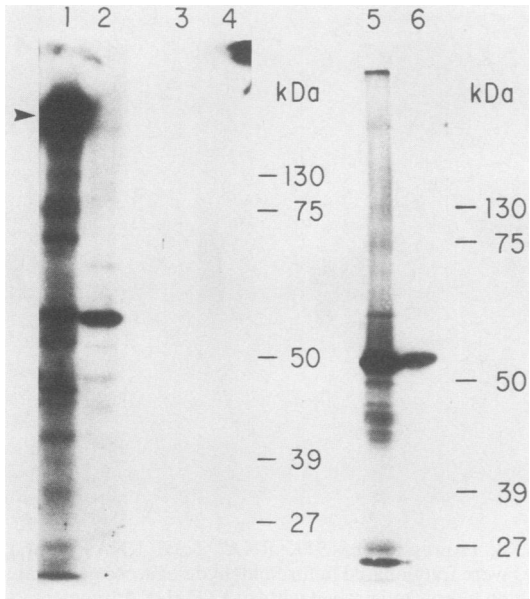

FIG. 9. Phosphotyrosine-containing proteins in *H. attenuata*. Total protein from *H. attenuata* polyps was fractionated in a 10% polyacrylamide–SDS gel, blotted onto nitrocellulose, and probed with a monoclonal antibody to phosphotyrosine (provided by Mark Kamps and Bart Sefton, The Salk Institute) as described in Materials and Methods. Lanes: 1, pattern of phosphotyrosine-containing proteins detected in A431 human epidermal carcinoma cells treated with EGF (▶, position of autophosphorylated EGF receptor [19, 31]); 2, *H. attenuata* protein; 3 and 4, portions of the same samples contained in lanes 1 and 2 except that antibody binding was blocked by the addition of *o*-phospho-DL-tyrosine; 5 and 6, results obtained when whole polyp protein (lane 6) and in vitro-synthesized, ^{35}S-labeled *STK* protein (lane 5) were fractionated in the same gel and transferred to a nitrocellulose filter. The phosphotyrosine-containing proteins in the whole polyp sample were detected by probing the filter with antiphosphotyrosine antibody.

tyrosine, including the EGF receptor itself (19, 31). The strong signal in lane 1 of Fig. 9 is the 170-kDa EGF receptor. A number of other proteins in A431 cells were also detected by the antibody. In the *Hydra* sample (lane 2), the pattern of phosphotyrosine-containing proteins was much simpler than that seen in A431 cells. A single protein species predominated, although a few minor species were also detected. To verify that the antibody was specifically recognizing phosphotyrosine-containing proteins, a second filter was probed with the antibody in the presence of *o*-phospho-DL-tyrosine. Lanes 3 and 4 show that such treatment abolished binding of antibody to all of the proteins detected in lanes 1 and 2.

The major species of phosphotyrosine-containing protein detected in *H. attenuata* had an apparent molecular size of 56 kDa, about the size predicted for the *STK* product (56.9 kDa). This result suggested that the major phosphotyrosine-containing protein in *H. attenuata* might be the *STK* product itself. To determine whether the major phosphotyrosine-containing protein in *H. attenuata* is in fact identical in size to the *STK* protein, we fractionated both whole polyp protein and the ^{35}S-labeled translation product of in vitro-synthesized *STK* RNA in the same gel. The proteins were transferred onto a nitrocellulose filter and probed with the antiphosphotyrosine antibody. The translation product of in vitro-synthesized *STK* RNA (Fig. 9, lane 5) and the major phosphotyrosine-containing protein in whole polyps (lane 6) had indistinguishable mobilities in the gel. This finding suggests but does not prove that the two proteins are identical.

DISCUSSION

A gene for a *src*-type protein-tyrosine kinase was present in the ancestor of all modern metazoans. Using the oligonucleotide screening strategy of Levin et al. (44), we have isolated a gene from the simple metazoan *Hydra* that encodes a protein which is 64% identical to chicken pp60$^{c\text{-}src}$. We have termed the *Hydra* gene *STK*, for *src*-type kinase. The high degree of sequence identity between the *STK* and *src* genes and the fact that the *Hydra* gene contains an intron in exactly the same location as in *src* genes of vertebrates provides compelling evidence that both the *Hydra* gene and the vertebrate *src* genes are descendants of the same ancestral gene. Hybridization analysis suggests that *STK* is likely to be the only gene of its type in *Hydra* species, and it therefore almost certainly encodes the *Hydra* version of the protein-tyrosine kinase that has been detected in various simple metazoa by immunoprecipitation with antiserum against pp60$^{v\text{-}src}$ (3, 4, 57). Since this activity has been detected in sponges but not in plants or protozoans (4, 57), it suggests that proteins of this type carry out a function that is necessitated by multicellularity in the metazoans. In addition, these findings argue that the organism from which all modern metazoans evolved contained a gene encoding a *src*-related tyrosine kinase. Furthermore, this distribution of occurrence of *src*-related genes argues strongly against the conclusion of Field et al. (21) that the cnidarians evolved from a different progenitor than did other metazoans and that cnidarians are most closely related to plants, fungi, and ciliates.

Having information on the structure of a *src*-related gene from a group of animals so much simpler than and so highly diverged from vertebrates as the cnidarians makes it possible to discern what features of *src*-type proteins are likely to be critical for proper structure or function. Among the amino acids with known function in pp60$^{c\text{-}src}$, all are conserved in the *STK* protein. These include amino acids involved in myristoylation (7, 33, 36), ATP binding (35), autophosphorylation (53, 62), substrate recognition (28), and regulation of kinase activity (13). The finding of these conserved elements strongly suggests that the *STK* protein is myristoylated and regulated by phosphorylation in the same manner as is pp60$^{c\text{-}src}$.

A feature of the *STK* gene that has not been completely conserved in the *src* gene is the distribution of introns. Although we have not yet determined the structure of the entire *STK* gene, we have detected a total of only two introns in a portion of the gene for which the homologous region in vertebrate *src* genes is interrupted by eight introns (1, 70, 71). One of the two *STK* introns is in the same location as the intron between exons 4 and 5 in vertebrate *src* genes. The other *STK* intron is at a site lacking an intron in vertebrate *src* genes. The only other *src*-related gene from an invertebrate for which intron locations have been examined is the *Drosophila* gene (29). Sequence from a *Drosophila src* genomic clone corresponding to nucleotides 881 to 1789 of the *Hydra STK* cDNA (Fig. 2) has been determined. As with the *STK* gene, this region of the *Drosophila src* gene contains a single intron. However, this intron is located 21 nucleotides downstream of the site of the *STK* intron. Given that the *Drosophila src* and *Hydra STK* genes both contain fewer introns than do vertebrate *src* genes, we would argue that intron acquisition has occurred in the vertebrate *src*

genes. An alternative argument would be that the *Hydra* and *Drosophila* genes have lost introns. Given its small genome, this might seem reasonable for *Drosophila* species. However, the *H. attenuata* genome is large (1.6×10^9 bp [16]), and therefore this organism would seem to be under little pressure to compact its genes. Sequence analysis of additional invertebrate *src*-related genes will help in determining the phylogeny of the intron-exon structure of this gene family.

An additional interesting feature of the *STK* gene is the unusual nature of its codon usage. For those codons for which there is a choice of nucleotides at the third position, A or T is chosen 82% of the time. This extreme A+T bias was first noted in *H. attenuata* actin genes (Fisher and Bode, in press) and is in keeping with the fact that the base composition of *H. attenuata* nuclear DNA is 71.4% A+T (Joseph Gall, personal communication). This extreme base composition may present problems for attempts to isolate *Hydra* genes by using cloned genes from other organisms as probes; therefore, screening with mixtures of oligonucleotides tailored to *Hydra* codon usage may prove to be a better approach.

STK mRNA is abundant in adult epithelial cells and is present during early development. Given that *src*-type genes appear to be present in all metazoa, what does this suggest about possible functions for the protein-tyrosine kinases encoded by these genes? Much has been made of the high levels of expression of the *src* gene and its relatives in the nervous systems of vertebrates and *Drosophila* species (14, 24, 61, 63, 67, 68). Processes in the nervous system in which the products of *src*-type genes have been suggested to be involved include secretion of neurotransmitters (25, 52), neuronal process outgrowth (45), and regulation of ion channel activity (30). We find that *Hydra* cells devoid of nerve cells still contain substantial levels of *STK* RNA, and sponges contain a *src*-type protein while entirely lacking a nervous system (4). These results suggest that *src*-type genes evolved in early metazoa to provide an essential function in cells that was required before the evolution of nerve cells.

The finding that $pp60^{c\text{-}src}$ is associated with chromaffin granule membranes (25, 52) suggests that *src*-type protein-tyrosine kinases may play a role in secretion. The presence of high levels of *STK* RNA in epithelial *Hydra* cells would seem to rule out the possibility that the *STK* protein is involved primarily in secretory processes, since epithelial polyps lack gland, mucous, and neurosecretory cells. Studies with cultured fibroblasts have shown that the degree of coupling of cells through gap junctions can be regulated by $pp60^{c\text{-}src}$ (2). Gap junctions are particularly abundant in the membranes of *Hydra* epithelial cells (23, 27, 79), cells that also contain a significant amount of the *STK* mRNA in the polyp. These observations suggest a possible role for $p57^{STK}$ in regulation of gap junction-mediated cell-cell communication in *Hydra* species.

The presence of *STK* RNA in eggs and early embryos of *Hydra* species suggests that the *STK* protein plays an important role in one or more processes during oogenesis, early development, or both. RNAs for *src*-type proteins have been found in oocytes and eggs from organisms as diverse as insects and amphibians (26, 43, 61, 64; R. E. Steele, M. Y. Irwin, C. L. Knudsen, J. W. Collett, and J. B. Fero, Oncogene Res., in press). Taken together with our findings for *Hydra* species, these results suggest a highly conserved role for *src*-type proteins in the egg or the embryo. Interestingly, gap junctions have been shown to be involved in coupling of blastomeres during early development and in communication between oocytes and follicle cells in both invertebrates and vertebrates (11).

The increase in *STK* RNA level that is associated with decapitation and regeneration is at present of unknown significance. It is likely that a variety of physiological responses accompany regeneration, one or more of which may be dependent on the increased production of $p57^{STK}$.

The *STK* protein may be the major phosphotyrosine-containing protein in *Hydra* species. By probing *Hydra* proteins with an antibody against phosphotyrosine, we had hoped to gain a measure of the complexity of the potential $p57^{STK}$ substrate population. Surprisingly, we found that most of the steady-state phosphotyrosine is contained in a single polypeptide. Furthermore, this polypeptide is identical in size to $p57^{STK}$. This finding suggests the possibility that much of the steady-state phosphotyrosine in *Hydra* cells is contained in the $p57^{STK}$ molecule itself. If this is the case, we would expect that most of the phosphorylation would be on Tyr-501, where it would serve to keep $p57^{STK}$ in an inactive state. By using the antiphosphotyrosine antibody as an affinity chromatography reagent for isolation of ^{35}S-labeled proteins from *Hydra* polyps, it should be possible to isolate sufficient quantities of the major phosphotyrosine-containing protein to allow its protease cleavage pattern to be compared with that of the *STK* protein synthesized by in vitro translation of synthetic *STK* mRNA. This will allow us to determine whether the two proteins are indeed identical.

Since *Hydra* cells have *src*-type protein-tyrosine kinases, it would be of interest to determine whether this organism also has other classes of protein-tyrosine kinases, particularly those of the receptor class (81). It has recently been shown that the polymerase chain reaction technique can be used to isolate DNAs encoding all classes of protein-tyrosine kinases (78). This approach seems ideally suited as a means of determining how many different protein-tyrosine kinases *Hydra* species make use of.

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