Do lampreys have lymphocytes? The Spi evidence

Seikou Shintani*, Janos Terzic[†], Akie Sato*, Mirna Saraga-Babic[†], Colm O'hUigin*, Herbert Tichy*, and Jan Klein*[‡]

*Max-Planck-Institut für Biologie, Abteilung Immungenetik, Correnstrasse 42, D-7076 Tübingen, Germany; and [†]Departments of Physiology and Histology and Embryology, University of Split School of Medicine, Split, Croatia

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It is generally accepted that living jawless vertebrates (lampreys and hagfishes) lack the capability of mounting an adaptive immune response. At the same time, however, there are reports describing histological evidence for the presence in agnathan tissues of lymphocytes, the key players in adaptive immunity. The question therefore arises whether the cells identified morphologically as lymphocytes are true lymphocytes in terms of their genetic developmental program. In this study, evidence is provided that the lampreys express a member of the purine box 1 (*PU.1*)/spleen focus-forming virus integration B (*Spi-B*) gene family known to be critically and specifically involved in the differentiation of lymphocytes in jawed vertebrates. The lamprey gene is expressed in the lymphocyte-like cells of the digestive tract and inexplicably also in the ovary.

he immune response of vertebrates has two arms, the adaptive and the nonadaptive (1). The adaptive response is effected by lymphocytes that express antigen-specific receptors on their surfaces, either the B cell (Ig) or the T cell receptors. The latter recognize peptides presented to them by molecules encoded in the MHC genes. Ig, T cell receptors, and MHC molecules are known only from jawed vertebrates (Gnathostomata); all efforts to isolate them or clone the encoding genes in jawless vertebrates (Agnatha), the lampreys and the hagfish, have failed (2). On the strength of this negative evidence, it is generally accepted that jawless vertebrates lack an adaptive immune system. However, this conclusion seems to be contradicted by the presence of cells in both the lamprey and the hagfish that morphologically resemble lymphocytes of jawed vertebrates (3-5). The agnathan lymphocyte-like cells are mainly accumulated in the subepithelial layers of the digestive tract and in the branchial arches (3-5). In the lamprey, the branchial accumulations of lymphocyte-like cells have even been referred to as the "thymus," a designation otherwise reserved for the organ of gnathostome T lymphocyte development (6, 7). The question therefore arises: Are the lymphocyte-like cells of the agnathans genetically related to lymphocytes or is the morphological similarity the result of evolutionary convergence?

Gnathostome lymphocytes are known to arise from pluripotential hemopoietic stem cells in a genetically programmed sequence of transformations, each step involving a specific combination of transcription factors (8). Some of the transcription factors are also involved in the differentiation of other cell types, whereas others appear to be restricted largely to the lymphocyte differentiation pathway (9). Prominent in the latter category are three related transcription factors, Spi-1 (=PU.1; refs. 10 and 11), Spi-B (12), and Spi-C (13). All three factors are members of the E26 transformation-specific (Ets) family of ~ 45 proteins characterized by the possession of the Ets DNA-binding domain in the C-terminal part of the molecule (14). The ≈ 85 residues of the domain interact with DNA sequences containing the purine-rich core motif 5'-GAGGAA-3', the PU box. The Spi-1 and Spi-B proteins show low sequence similarity to each other throughout their entire length (12), whereas the Spi-C protein has significant sequence similarity to the Spi-1 and Spi-B proteins (as well as to other members of the Ets family) in the DNA-binding domain, but not outside of this region (13). The Spi-1 and Spi-B proteins share two other domains, the glutamine-rich domain in the N-terminal half and the PEST (proline, glutamic acid, serine, and threonine-rich) domain in the center. The glutamine-rich domain and several acidic residues in the N-terminal half of the protein are necessary for *trans*-activation (15), whereas the PEST domain is involved in protein–protein interaction (16).

The expression of Spi-1 is relatively widespread in hemopoietic cells and includes progenitor cells as well as myeloid (granulocyte, monocyte, and osteoclast) and lymphoid (B and immature T lymphocyte) lineages (11, 12, 17–20). The expression of Spi-B appears to be restricted to lymphoid cells with high levels found in B cells and low levels in T cell progenitors (12, 18, 19). A high level of the Spi-C protein has been found in mature B cells and lower levels in macrophages (13). The *Spi* genes appeared therefore to be good markers for testing whether the agnathan lymphocyte-like cells are genetically related to gnathostome lymphocytes. The aim of the present study was to determine whether homologs of these genes are present in the lamprey and, if so, in what cells they are expressed. To obtain a better understanding of their evolution, *Spi* genes of a bony fish, an amphibian, and a reptile were also characterized.

Materials and Methods

Animals and RNA/DNA Sources. Two species of lamprey were used, ammocoetes larvae and adults of the sea lamprey, *Petromyzon marinus*, from Lakes Huron and Champlain in the United States, and juveniles of freshwater lampreys, *Lampetra fluviatilis*, from the Rhine River near Karlsruhe, Germany. For comparison, one bony fish and two tetrapod species were also included in the study, the cichlid *Aulonocara hansbaenschi*, the smooth-fronted caiman, *Paleosuchus palpebrosus*, and the African clawed toad, *Xenopus laevis*. Four cDNA libraries were used. The total RNA for their construction was isolated from the gut of *P. marinus* larva (21), the spleen and hepatopancreas of a cichlid fish (22), the whole jaws of a 3-day-old caiman (23), and the jaws of the clawed toad (23). Genomic DNAs were isolated from the liver of *P. marinus* and *L. fluviatilis* by the phenol-chloroform extraction method.

PCR Amplification. Conditions of the PCR were described in an earlier publication (24). Partial coding sequences were obtained from the cDNA libraries using primers based on conserved regions of *Spi* genes in combination with vector anchor primers. The complete coding sequences could then be obtained using primers based on the partial fragments. The lamprey Spi primer was PU-G6 (5'-AACTGGTAGGTBAGCTTCTTCTT-3'); the fish Spi-1 and Spi-C primers were PS-G3 (5'-GNGCCAKCT-TCTGATAGGTCAT-3') and SB-G1 (5'-CTTGCGGTTSC-CCTTCTGCTG-3'), respectively; the toad Spi-B primer was

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Abbreviations: Ets, E26 transformation specific; AAR, amino acid residue; indel, insertion/ deletion; PU, purine box; Spi, spleen focus-forming virus integration (site); UTR, untranslated region.

See commentary on page 6924.

[‡]To whom reprint requests should be addressed. E-mail: klein@tuebingen.mpg.de.

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ATG	CTG	GCA	ATG	CAC	ACC	ATG	CAG	CCC	ATG	TAT	GCC	GAG	CTG	CGA	GAC	GCA	TTA	GCG	TCA	AGC	TAT	GAC	GAT	GAA
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CTG	AGC	TCG	GCG	GAC	GAC	GCC	GAT	TAC	GAC	AGC	GAC	GTC	CCG	ACG	CTG	CAG	ATC	TCC	GAC	ACG	GAG	AGC	GAG	GAC
L	S	S	A	D	D	A	D	Y	D	S	D	v	Р	Т	\mathbf{L}	0	I	S	D	т	E	S	Е	D
	-	-		130			-							140		~								150
GAG	GAG	GAG	GAG	GAG	GAG	GAG	GGG	GAC	GAG	GAG	GGC	TGC	CGG	AGG	AGG	TGC	CGC	CCG	GTT	GAG	CTG	CCG	CCC	TTC
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GGC	AGC	GTC	GCT	GGC	GGC	GAC	GGC	CAA	CAG	CAG	CAG	CAG	CAG	CAG	GGC	CGC	CAC	CGC	CAC	CAC	CAG	CAG	CAC	CAC
GGC G	AGC S	GTC V	GCT A	GGC G	GGC G	GAC D	GGC G	CAA Q	CAG Q	CAG Q	CAG Q	CAG Q	CAG Q	CAG Q	GGC G	CGC R	CAC H	CGC R	CAC H	CAC H	CAG Q	CAG Q	CAC H	CAC H
GGC G	AGC S	GTC V	GCT A	GGC G 180	GGC G	GAC D	GGC G	CAA Q	CAG Q	CAG Q	CAG Q	CAG Q	CAG Q	CAG Q 190	GGC G	CGC R	CAC H	CGC R	CAC H	CAC H	CAG Q	CAG Q	CAC H	CAC H 200
GGC G CAC	AGC S CAG	GTC V CAG	GCT A CAG	GGC G 180 CAG	GGC G CAG	GAC D CAG	GGC G CTA	CAA Q CCT	CAG Q CGT	CAG Q GGT	CAG Q GTG	CAG Q ACC	CAG Q GTG	CAG Q 190 AAG	GGC G AGG	CGC R GAG	CAC H AAA	CGC R GGA	CAC H GTG	CAC H GGG	CAG Q AAG	CAG Q CGA	CAC H GGC	CAC H 200 AAG
GGC G CAC H	AGC S CAG	GTC V CAG	GCT A CAG Q	GGC G 180 CAG Q	GGC G CAG Q	GAC D CAG Q	GGC G CTA L	CAA Q CCT P	CAG Q CGT R	Q Q GGT G	CAG Q GTG V	CAG Q ACC T	CAG Q GTG V	CAG Q 190 AAG K	GGC G AGG R	CGC R GAG E	H AAA K	CGC R GGA G	CAC H GTG	CAC H GGG G	CAG Q AAG K	CAG Q CGA R	CAC H GGC G	CAC H 200 AAG K
GGC G <u>CAC</u> H	AGC S CAG Q	GTC V CAG Q	GCT A CAG Q	GGC G 180 CAG Q	GGC G CAG Q	GAC D CAG Q 210	GGC G CTA L	CAA Q CCT P	CAG Q CGT R	Q Q GGT G	Q Q GTG V	CAG Q ACC T	CAG Q GTG V	CAG Q 190 AAG K	GGC G AGG R	CGC R GAG E 220	CAC H AAA K	CGC R GGA G	CAC H GTG V	CAC H GGG G	CAG Q AAG K	CAG Q CGA R	CAC H GGC G	H 200 AAG K
GGC G CAC H ACG	AGC S CAG Q AGG	GTC V CAG Q TTG	GCT A CAG Q TAC	GGC G 180 CAG Q CAG	GGC G CAG Q TTC	GAC D CAG Q 210 CTC	GGC G CTA L ATG	CAA Q CCT P GAG	CAG Q CGT R ATC	CAG Q GGT G CTC	CAG Q GTG V CAG	CAG Q ACC T GAC	CAG Q GTG V CAG	CAG Q 190 AAG K AGG	GGC G AGG R ATG	CGC R GAG E 220 TGC	H AAA K CAC	CGC R GGA G TGC	CAC H GTG V ATC	CAC H GGG G TGG	CAG Q AAG K TGG	CAG Q CGA R GTG	CAC H GGC G GAC	H 200 AAG K GAG
GGC G <u>CAC</u> H ACG T	AGC S CAG Q AGG R	GTC V CAG Q TTG L	GCT A CAG Q TAC Y	GGC G 180 CAG Q CAG Q	GGC G CAG Q TTC F	GAC D CAG Q 210 CTC L	GGC G CTA L ATG M	CAA Q CCT P GAG E	CAG Q CGT R ATC I	Q Q GGT G CTC L	CAG Q GTG V CAG Q	CAG Q ACC T GAC D	CAG Q GTG V CAG Q	CAG Q 190 AAG K AGG R	GGC G AGG R ATG M	CGC R GAG E 220 TGC C	CAC H AAA K CAC H	CGC R GGA G TGC C	ATC	CAC H GGG G TGG W	CAG Q AAG K TGG W	CAG Q CGA R GTG V	CAC H GGC G GAC D	H 200 AAG K GAG E
GGC G CAC H ACG T	AGC S CAG Q AGG R	GTC V CAG Q TTG L	GCT A CAG Q TAC Y	GGC G 180 CAG Q CAG Q 230	GGC G CAG Q TTC F	GAC D CAG Q 210 CTC L	GGC G CTA L ATG M	CAA Q CCT P GAG E	CGT R ATC I	CAG Q GGT G CTC L	CAG Q GTG V CAG Q	CAG Q ACC T GAC D	CAG Q GTG V CAG Q	CAG Q 190 AAG K AGG R 240	GGC G AGG R ATG M	CGC R GAG E 220 TGC C	CAC H AAA K CAC H	CGC R GGA G TGC C	ITO CAC H GTG V ATC I	CAC H GGG G TGG W	CAG Q AAG K TGG W	CAG Q CGA R GTG V	CAC H GGC G GAC D	H 200 AAG K GAG E 250
GGC G CAC H ACG T CGC	AGC S CAG Q AGG R CAG	GTC V CAG Q TTG L GGC	GCT A CAG Q TAC Y ATC	GGC G 180 CAG Q CAG Q 230 TTC	GGC G CAG Q TTC F CAG	GAC D CAG Q 210 CTC L TTC	GGC G CTA L ATG M TCG	CAA Q CCT P GAG E TCG	CGT R ATC CAG	CAG Q GGT G CTC L CAC	CAG Q GTG V CAG Q AAG	CAG Q ACC T GAC D GAG	CAG Q GTG V CAG Q GAG	CAG Q 190 AAG K AGG R 240 CTG	GGC G AGG R ATG M GCG	CGC R GAG E 220 TGC C AAG	CAC H AAA K CAC H AAG	CGC R GGA G TGC C TGG	H GTG V ATC I GGC	CAC H GGG G TGG W CAG	CAG Q AAG K TGG W CGG	CAG Q CGA R GTG V AAG	CAC H GGC G GAC D GGC	H 200 AAG K GAG E 250 AAC
GGC G H ACG T CGC R	AGC S Q AGG R CAG Q	GTC V CAG Q TTG L GGC G	GCT A Q TAC Y ATC I	GGC G 180 CAG Q CAG Q 230 TTC F	GGC G Q TTC F CAG Q	GAC D CAG Q 210 CTC L TTC F	GGC G CTA L ATG M TCG S	CAA Q CCT P GAG E TCG S	CGT R ATC I CAG Q	CAG Q GGT G CTC L CAC H	CAG Q GTG V CAG Q AAG K	CAG Q ACC T GAC D GAG E	CAG Q GTG V CAG Q GAG E	CAG Q 190 AAG K AGG R 240 CTG L	GGC G AGG R ATG M GCG A	CGC R GAG E 220 TGC C AAG K	CAC H AAA K CAC H AAG K	CGC R GGA G TGC C TGG W	GTG V ATC GGC G	CAC H GGG G TGG W CAG Q	CAG Q AAG K TGG W CGG R	CAG Q CGA R GTG V AAG K	GGC G GAC D GGC G	CAC H 200 AAG K GAG E 250 AAC N
GGC G H ACG T CGC R	AGC S CAG Q AGG R CAG Q	GTC V CAG Q TTG L GGC G	GCT A Q TAC Y ATC I	GGC G 180 CAG Q CAG Q 230 TTC F	GGC G CAG Q TTC F CAG Q	GAC D CAG Q 210 CTC L TTC F	GGC G CTA L ATG M TCG S	CAA Q CCT P GAG E TCG S	Q CGT R ATC I CAG Q 260	Q GGT G CTC L CAC H	CAG Q GTG V CAG Q AAG K	Q Q ACC T GAC D GAG E	CAG Q GTG V CAG Q GAG E	CAG Q 190 AAG K AGG R 240 CTG L	GGC G AGG R ATG M GCG A	R GAG E 220 TGC C AAG K	H AAA K CAC H AAG K	R GGA G TGC C TGG W	ATC GGC GGC G 270	H GGG G TGG W CAG Q	Q AAG K TGG W CGG R	Q Q CGA R GTG V AAG K	H GGC G GAC D GGC G	CAC H 200 AAG K GAG E 250 AAC N
GGC G H ACG T CGC R CGC	AGC S Q AGG R CAG Q AAG	GTC V CAG Q TTG L GGC G GCG	GCT A CAG Q TAC Y ATC I ATG	GGC G 180 CAG Q 230 TTC F ACC	GGC G Q TTC F CAG Q TAC	GAC D CAG Q 210 CTC L TTC F CAG	GGC G CTA L ATG M TCG S AAG	CAA Q CCT P GAG E TCG S CTG	Q CGT R ATC I CAG Q 260 GCG	Q GGT G CTC L CAC H CGG	CAG Q GTG V CAG Q AAG K GCG	CAG Q ACC T GAC D GAG E CTG	CAG Q GTG V CAG Q GAG E CGG	CAG Q 190 AAG K AGG R 240 CTG L AAC	GGC G AGG R ATG M GCG A TAC	R GAG E 220 TGC C AAG K GAG	H AAA K CAC H AAG K GCG	R GGA G TGC C TGG W ACG	ATC GGC GGC GGC GGC GGC	H GGG G TGG W CAG Q GAG	CAG Q AAG K TGG W CGG R ATC	CAG Q CGA R GTG V AAG K CGC	H GGC G GAC D GGC G AAG	H 200 AAG K GAG E 250 AAC N ATC
GGC G H ACG T CGC R CGC R	AGC S Q AGG R CAG Q AAG K	GTC V Q TTG GGC G GCG A	GCT A Q TAC Y ATC I ATG M	GGC G 180 CAG Q 230 TTC F ACC T	GGC G Q TTC F CAG Q TAC Y	GAC D CAG Q 210 CTC L TTC F CAG Q	GGC G CTA L ATG M TCG S AAG K	CAA Q CCT P GAG E TCG S CTG L	Q CGT R ATC I CAG Q 260 GCG A	CAG Q GGT G CTC L CAC H CAC H CAG R	CAG Q GTG V CAG Q AAG K GCG A	CAG Q ACC T GAC D GAG E CTG L	CAG Q GTG V CAG Q GAG E CGG R	CAG Q 190 AAG K AGG R 240 CTG L AAC N	GGC G AGG R ATG M GCG A TAC Y	R GAG E 220 TGC C AAG K GAG E	H AAA K CAC H AAG K GCG A	CGC R GGA G TGC C TGG W ACG T	ATC GGC GGC GGC GGC GGC GGC GGC	CAC H GGG G TGG W CAG Q GAG E	CAG Q AAG K TGG W CGG R ATC I	CAG Q CGA R GTG V AAG K CGC R	CAC H GGC G GAC D GGC G AAG K	H 200 AAG K GAG E 250 AAC N ATC I
GGC G H ACG T CGC R CGC R	AGC S Q AGG R CAG Q AAG K	GTC V Q TTG L GGC G GCG A	GCT A Q TAC Y ATC I ATG M	GGC G 180 CAG Q 230 TTC F ACC T 280	GGC G Q TTC F CAG Q TAC Y	GAC D CAG Q 210 CTC L TTC F CAG Q	GGC G CTA L ATG M TCG S AAG K	CAA Q CCT P GAG E TCG S CTG L	Q CGT R ATC I CAG Q 260 GCG A	Q GGT G CTC L CAC H CGG R	CAG Q GTG V CAG Q AAG K GCG A	CAG Q ACC T GAC D GAG E CTG L	CAG Q GTG V CAG Q GAG E CGG R	CAG Q 190 AAG K AGG R 240 CTG L AAC N	GGC G AGG R ATG M GCG A TAC Y 290	R GAG E 220 TGC C AAG K GAG E	H AAA K CAC H AAG K GCG A	R GGA G TGC C TGG W ACG T	ATC GGC GGC GGC GGC GGC G	H GGG G TGG W CAG Q GAG E	Q AAG K TGG W CGG R ATC I	Q CGA R GTG V AAG K CGC R	H GGC G GAC D GGC G AAG K	H 200 AAG K GAG E 250 AAC N ATC I
GGC G H ACG T CGC R CGC R AAG	AGC S Q AGG R CAG Q AAG K AAG	GTC V Q TTG L GGC G GCG A AAG	GCT A Q TAC Y ATC I ATG M CTG	GGC G 180 CAG Q 230 TTC F ACC T 280 ACG	GGC G Q TTC F CAG Q TAC Y TAC	GAC D CAG Q 210 CTC L TTC F CAG Q CAG	GGC CTA L ATG M TCG S AAG K TTC	CAA Q CCT P GAG E TCG S CTG L GGC	Q CGT R ATC I CAG Q 260 GCG A GCC	Q GGT G CTC L CAC H CGG R AAG	CAG Q GTG V CAG Q AAG K GCG A TG	CAG Q ACC T GAC D GAG E CTG L ATG	CAG Q GTG V CAG Q GAG E CGG R GGC	CAG Q 190 AAG K AGG R 240 CTG L AAC N GCC	GGC G AGG R ATG M GCG A TAC Y 290 TTC	R GAG E 220 TGC C AAG K GAG E AGG	CAC H AAAA K CAC H AAG K GCG A CGA	CGC R GGA TGC C TGG W ACG TGA	H GTG V ATC I GGC G GGC G GGC G	H GGG G TGG W CAG Q GAG E	Q AAG K TGG W CGG R ATC I	Q CGA R GTG V AAG K CGC R	H GGC G GAC D GGC G AAG K	H 200 AAG K GAG E 250 AAC N ATC I
GGC G H ACG T CGC R CGC R AAG K	AGC S Q AGG R CAG Q AAG K AAG K	GTC V Q TTG L GGC G GCG A AAG K	GCT A Q TAC Y ATC I ATG M CTG L	GGC G 180 CAG Q CAG Q 230 TTC F ACC T 280 ACG T	GGC G Q TTC F CAG Q TAC Y TAC Y	GAC D CAG Q 210 CTC L TTC F CAG Q CAG Q	GGC CTA L ATG M TCG S AAG K TTC F	CAA Q CCT P GAG E TCG S CTG CTG GGC G	Q CGT R ATC I CAG Q 260 GCG A GCC A	CAG Q GGT CTC L CAC H CGG R AAG K	CAG Q GTG V CAG Q AAG K GCG A TG M	CAG Q ACC T GAC D GAG E CTG L ATG M	CAG Q GTG V CAG Q GAG E CGG R GGC G	CAG Q 190 AAG K AGG R 240 CTG L AAC N GCC A	GGC G AGG R ATG M GCG A TAC Y 290 TTC F	CGC R GAG E 220 TGC C AAG K GAG E AGG R	CAC H AAAA K CAC H AAG K GCG A CGA R	CGC R GGA TGC C TGG W ACG T TGA *	H GTG V ATC I GGC G GGC G GGC G	H GGG G TGG W CAG Q GAG E	CAG Q AAG K TGG W CGG R ATC I	CAG Q CGA R GTG V AAG K CGC R	H GGC G GAC D GGC G AAG K	CAC H 200 AAG K GAG E 250 AAC N ATC I

Fig. 1. Nucleotide sequence of the lamprey *Spi* gene. Numbering shows codon positions. The coding region is displayed by codons and its translation is given in the single-letter amino acid code. The underlined nucleotide regions show the two repeats, one consisting of glutamic acid and the other of glutamine and histidine residues. Vertical lines indicate exon borders.

PS-G1 (5'-TTCCAGTTCTCSTCSAAGCACAA-3'); and the caiman Spi-1 and Spi-B primers were PU-G2 (5'-ATGTC-CAGVAGGAACTGRTACAG-3') and SPB-T2 (5'-TTTTCT-GGTAGGTCATTTTCTTGC-3'), respectively. Cloning and sequencing were carried out as described earlier (24).

In Situ Hybridization. The animals were killed and cut at \approx 1-cm intervals. The tissue blocks thus obtained were fixed in 4%paraformaldehyde in PBS and embedded in paraffin. Tissue blocks were cut transversally and mounted on glass slides coated with chrome alum (Serva) or on SuperFrost Plus slides (Schutt Labortechnik). A ³⁵S-labeled antisense RNA probe was generated by using T7 RNA polymerase according to the manufacturer's (Roche Molecular Biochemicals) specifications. For the probe preparation, the 393-bp lamprey Spi cDNA insert, covering exons 1, 2, 3, and part of exon 4, was used. For control experiments, sense RNA was generated from the same cDNA insert by using SP6 RNA polymerase. Prehybridization, hybridization, and washing procedures were as described previously (25). For autoradiography, slides were dipped into the NTB-2 emulsion (Kodak). Sections were exposed for up to 4 wk, developed in D-19 solution (Kodak), and stained with Giemsa. Photomicrographs were taken with a Zeiss microscope using bright-field and phase-contrast optics with a polarizing filter. In parallel experiments, paraffin sections were stained with hema-toxylin and eosin by standard methods.

Data Analysis. Nucleotide and protein sequences were aligned with the aid of the SeqPup (ref. 26: University of Indiana, Bloomington, http://iubio.bio.indiana.edu/soft/molbio), the GenDoc (ref. 27: Pittsburg Supercomputing Center, Pittsburg, www.cris.com/~ketchup/genedoc.shtml), or the Clustal W (28) computer programs. BLAST searches (29) of protein databases were used to identify the most closely related outgroup sequence, the human ELF5 (E74-like factor 5) transcription factor. Phylogenetic reconstructions were made using the neighborjoining method (30) of the MEGA program (31) based on distances derived from the proportion of identical amino acids following omission of gapped sites.

Results and Discussion

The lamprey cDNA sequence is 1,272 bp long and contains an ORF for 293 amino acid residues (AAR) beginning with the translation start site at 224 bp (Fig. 1). The stop codon at 1,105 bp is followed by a 3' untranslated region (UTR) of 167 bp, which however seems to lack the polyadenylation signal. The cichlid



Fig. 2. Comparison of the exon-intron organization of lamprey, human (32), mouse (33, 44), and fowl (chicken; ref. 45) *Spi* genes. Boxed regions show exons (E) and numbers inside the boxes exon sizes in bp. The inserts in the lamprey Spi sequence indicate two repeat regions, one consisting of glutamic acid and the other of glutamine and histidine residues.

Spi-1 cDNA sequence is 2,663 bp long and contains an ORF for 264 AAR beginning with the translation start site at 303 bp. The stop codon at 1,095 bp is followed by a 3' UTR of 1,566 bp with the putative polyadenylation signal AATAAA at 2,655 bp. The cichlid Spi-C cDNA sequence is 1,723 bp long and contains an ORF for 264 AAR. The stop codon at 794 bp is followed by a 3' UTR of 927 bp; however, this clone seems to lack the translation start site and the putative polyadenylation signal. The toad Spi-B cDNA sequence is 1,446 bp long and contains an ORF for 281 AAR beginning with the translation start site at 49 bp. The stop codon at 892 bp is followed by a 3' UTR of 552 bp, which seems to lack a polyadenylation signal, however. The caiman Spi-1 cDNA sequence is 1,497 bp long and contains an ORF for 266 AAR beginning with the translation start site at 207 bp. The stop codon at 1,005 bp is followed by a 3' UTR of 490 bp with the putative polyadenylation signal AATAAA at 1,485 bp. The caiman Spi-B cDNA sequence is 1,563 bp long and contains an ORF for 266 AAR beginning with the translation start site at 40 bp. The stop codon at 835 bp is followed by a 3' UTR of 726 bp with the putative polyadenylation signal AATAAA at 1,540 bp.

To determine the exon-intron organization of the lamprey *Spi* gene, a series of primer pairs corresponding to stretches spaced at short intervals along the cDNA sequence was used. The products obtained by PCR amplification of lamprey genomic DNA with these primers were partially sequenced and the exon-intron borders assigned by comparison with the cDNA sequence (Fig. 1). Like the mouse and human *Spi-B* genes (32, 33), the lamprey homolog has six exons separated by five introns (Fig. 2). Of these, however, only exon 6 has the same length in the three species. Lamprey exon 4 is much shorter and lamprey exon 5 is longer than the corresponding mouse and human elements; the remaining three lamprey exons are longer than their mouse and human counterparts. The phases of introns 1 through 5 of the lamprey gene are the same as those of the mammalian *Spi-B* genes (0, 0, 1, 0, 1, respectively).

A unique feature of the lamprey gene is the presence of two repetitive sequences in exon 5. One sequence is 45 bp long and consists of 11 glutamic acid GAG codons interspersed with glycine GGC, aspartic acid GAC, and serine AGC codons. The second sequence lies 66 bp downstream, is 72 bp long, and consists of 15 glutamine CAG codons interspersed with 6 histidine CAC and some arginine CGC and glycine GGC codons. Both repeats may be microsatellite derived. Structural predictions suggest that the peptide specified by both repeats forms an α -helix. The function of the repeats is unclear, but since similar sequences are also present in a related species (*L. fluviatilis*, data not shown) and in unrelated genes of other metazoans (34–36), they do not seem to be without function. Possibly the glutaminerich repeat is analogous to a glutamine-rich region found in exon 3 of *Spi-1* genes and used in transactivation of *Spi-1* (37).

Sequence similarity of the lamprey Spi to mammalian Spi-B and Spi-1 proteins is limited to the Ets domain encoded in the last exon of the gene (Fig. 3). The dissimilarity of the non-Ets part could mean either that the cloned gene is not Spi or that the gene's upstream part evolves under low selection pressure and at a rapid evolutionary rate. It was the desire to test this latter possibility that led us to extend the study to Spi sequences of species from other vertebrate classes. If the non-Ets part is evolving rapidly, clear evidence for an increased evolutionary rate should be found by comparing Spi sequences from classes separated by progressively longer divergence times. This expectation has indeed been borne out by the comparison of the mammalian, reptilian, amphibian, and bony fish sequences. The human-mouse, mammalian-reptile, and amphibian-tetrapod Spi-B protein sequences are 78%, 44%, and 38% identical, respectively. Dramatic decrease in Spi-B sequence similarity is also reflected in the number of insertion/deletions (indels) required for optimal alignments. The human-mouse, caimanmammal, and toad-mammal comparisons require the introduction of 2, 10, and 15 indels into the alignments, respectively. All of the 15 indels are in the non-Ets part. Given the considerably longer divergence times of agnathans from tetrapods in comparison to divergence times within tetrapods, the low similarity levels found (10%) are to be expected. The rapid divergence of the non-Ets part appears to be a characteristic of the Spi-B proteins; the Spi-1 sequences, by contrast, retain much of their similarity over long evolutionary periods. The human-mouse, mammal-caiman, and fish-tetrapod sequences are 85%, 74%, and 41% identical, respectively. Similarly, only four indels are required to align all of the tetrapod sequences used. The Spi-C sequences, however, are only 28% identical between fish and mouse in the non-Ets region. The rapid evolution of the Spi-B protein is also reflected in the phylogenetic tree in Fig. 4, in which the branch lengths within the Spi-1 cluster are shorter than those within the Spi-B cluster. The difference in evolutionary rates probably influences the placement of the lamprey and toad sequences and is responsible for the difficulty in rooting the divergence between the Spi-1 and Spi-B lineages.

Although the non-Ets part is not highly conserved, there are some conserved residues that are shared between the lamprey protein and other Spi-family members. At position 22 of the alignment (Fig. 3), Spi-B and -C family members have a Y or F, the lamprey sequence a Y, whereas the Spi-1 group has S or T residues. At position 24, all Spi sequences have a D or E (D in lamprey) and again at position 33, D or E is found (except for Spi-C sequences which contain an indel). At position 68, a conserved H is found in Spi-1, an A or G in Spi-B (G in the lamprey), whereas Spi-C has none of these residues. At position 77, F is found in Spi-1 sequences and Y in Spi-B and Spi-C (lamprey has Y). A P residue is found in all Spi-B sequences and in lamprey at position 104. At position 121, the lamprey protein shares an L residue with all Spi-1 and Spi-B sequences.

The lamprey Spi sequence also contains regions in the non-Ets part that may be equivalent functionally to the domains of the mammalian proteins. Thus, there are serine residues at positions 20, 21, 27, 31, 32, 107, 108, 116, and 124 in the lamprey sequence, some of which may represent the phosphorylation sites for kinases, corresponding to those in the mammalian Spi proteins (38). Similarly, although no PEST domain (16) as such can be identified in the lamprey sequence, a region rich in glutamic acid residues is present at positions 126–141. Finally, the inserted repeat at positions 164–188 contains numerous glutamine residues which may carry out a function equivalent to that of the glutamine-rich domain in the mammalian Spi proteins (15).

The lamprey Ets domain shows the highest (and approximately equal) sequence similarity with the corresponding gna-

		20	40	60	80
Human Spi-1	MLQACKMEGFP****	VPPPSEDLV**PY	DTDLYQRQTHEYYPYLS***	**SDGESHSDHYWDFHP	H*HVHS**EFESF
Mouse Spi-1	MLQACKMEGFS****	TAPPSDDLV**TY	DSELYORPMHDYYSFVG***	**SDGESHSDHYWDFSA	H*HVHNN*EFENF
Fowl Spi-1	MLQACKMEGFP****	IPPPSEDMV**SY	ESDLYROP*HDYYQYLN***	**SDGDSHGDHYWEYHP	H*HVHS**EFETF
Caiman Spi-1	MLOACKMEGFP****	IPPPSEDMV**TY	ESDLYROP*HDYYOYLN***	**SDGDSHGEHYWDYHP	H*HMHS**EFENF
Fish Spi-1	MFHPYRMESY*****	IOPHTDEI****Y	DPEIYRHOIPEYSYPYV***	**LDPESOAEH*WDYHA	HPHVHPA*EFENL
Human Spi-B	MTALEAAOLOG****PH	FSCINPDGV**FY	DIDSCKHS**SYPD*****	****SEGAPDSLWDWTV	APPVPAT*PYFAF
Mouse Spi-B	MTALEAAOLOG****PH	USCINPEGV**FY	DI DSCKPF**SVPD*****	****SDGGLDSTWGWTE	
Caiman Sni-B	MTTLEASOLOG****PH	IPSYMESDSS**FY	DI DSCKPI.P*TFPH*****	****CIMEAEPPTDPCA	GWLELAEPGVEPE
Toad Spi-B	MT CLOSI DEDN*****	FCOVDDAT**I	DI FAFNKUCNEVSO**T***	**SESDTUTDI I WNI TE	A**EDD**CVETV
Mouse Spi-C	**MTCCIDODS*****		TI *TOOGA **************	*******CESOVSSEN	
Fich Spi-C	**VI TCI DCD*******	INCUEODAT***D	11 10024	+++++++MICYDODY	C++++++VVENT
Fish Spi-C	MIAMUTMODMYAEIDDA	TACEVODEACT		THETOKCONSTIDED	CODUTT C*CVECC
ramprey spr	MLAMHIMQPMIAELRDA	THASSIDDEASLII	PSDIDNSLKIDDPLDIIDQI	TTHELORCONSPRIETS.	GSDVILS~GIEGS
		100	120	140	160
Human Cri-1	A + + + ENNETETO CUODE			CHONEYI DEMCI ONE	CI + + + + CDAOD + C
Mouco Spi-1	D***ENHETELOSVOF		*MEL * COMULT DTDMUDDUT	CL CHOVEYMPPMCEDYO	TI ****SPAUCOS
Foul Coi 1	C+++DNUETELOSVOPE		+MEL+EQMHVEDIPMVPPHI	GLSHQVSIMPRMCFFIQ	1L****SPARQUS
rowi Spi-i	G***DNHFTELQSVQPF		*MEI*EQMHVEDSAIPTTHI	GLIHA AND OUS	******SPROP*S
Caiman Spi-i	G***DNHFTELQSVQPE	Q · · · · LQQLIRH ·	*MEI*EQMHVLDLGLPAPHI	GLNHQVSYLPRMCLQYP	States SPHQP S
Fish Spi-1	Q***ESNFTELQNVQAL	H****PSGLLRHD	TLRY*EAENLLDPNLAAHSH	VIOOPVILEEPR**TLXP	P*****HISQR*S
Human Spi-B	DP*AAAAFSHPQAAQLC	Y****EPPTYSP*.	AGNL*ELAPS EAPGPGLPA	YP**TENFASOTLVPPA	Y****APYPSPVL
Mouse Spi-B	DP*ATAAFSHSQTVQLC	YSHGPNPSTYSP*	MGTL*DPAPSLEAPGPGLQV	YP**PEDFTSQTLGSLA	Y****APYPSPVL
Caiman Spi-B	DS*GQLAPLHTVTVPYG	H****GP**YPP*.	APS**DAIYSLEGPLPAPSH	CPVLPEEYGAQPYTLYS	P****CPLPSTPL
Toad Spi-B	E***NIQLTSLQNVQLF	PY****LPGTYPQ*	*YCP*DTVQNLDGTVPCPAP	CEMPEDLYIAAPYPSYA	SYSMPGNV <mark>PS</mark> PP <mark>L</mark>
Mouse Spi-C	I****NPYPHVRGNAN*	********YYG*	*******MSPTENPLYDWRG	VTN***********	*******GSADL
Fish Spi-C	G****SHHQSLQCQISC	C******LVTH*	*******QSEVPTPVYDWND	MAQ***********	********SWPQV
Lamprey Spi	ESSYQSLLDEPHTAPSV	P****SPTLSSAD	DADYDSDVPTLQISDTESED	EEEEEEGDEEGCRRRC	RPVELPPFGSVAG
		100	200	220	c 240
	B	180	200	220	c 240
Human Spi-1	B SDEEEG*ERQS**PPLE	180 VSDG*EADGLEPG	200 PG*LLP <mark>GET</mark> GSKK** <mark>KIRL</mark> Y	220 QFLLDLLRSGDMKDSIW	C 240
Human Spi-1 Mouse Spi-1	B SDEEEG*EROS**PPLE SDEEEG*EROS**PPLE	180 VSDG*EADGLEPG VSDG*EADGLEPG	200 PG*LLP <mark>GET</mark> SSKK**KIRLY PG*LLHGETSSKK**KIRLY	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW	C 240 WVDKDKGTFQFSS WVDKDKGTFQFSS
Human Spi-1 Mouse Spi-1 Fowl Spi-1	B SDEEEG*ERQS**PPLE SDEEEG*ERQS**PPLE SDEEDI*ERQS**PPLE	180 VSDG*EADGLEPG VSDG*EADGLEPG VSDG*ETDGVDPG	200 PG*LLP <mark>GETGSKK**KTRLY</mark> PG*LLHG <mark>ETGSKK**KTRLY</mark> PG*IMHGET <mark>GSKK**KTRLY</mark>	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW	C 240 WVDKDKGTFQFSS WVDKDKGTFQFSS WVDKEKGTFQFSS
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1	B SDEEEG*ERQS**PPLE SDEEEG*ERQS**PPLE SDEEDI*ERQS**PPLE SDEEDM*ERQS**PPLE	180 VSDG*EADGLEPG VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT	200 PG*LLPGETGSKK** <mark>KIRL</mark> Y PG*LLHGETGSKK**KIRLY PG*IMHGETGSKK**KIRLY PG*IMHGEPGSKK**KIRLY	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRNGDMKDSIW	C 240 WVDKDKGTFOFSS WVDKDKGTFOFSS WVDKEKGTFOFSS WVDKEKGTFOFSS
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1	B SDEEEG*ERQS**PPLE SDEEEG*ERQS**PPLE SDEEDI*ERQS**PPLE SDEEDM*ERQS**PPLE SDEEDH*GARS**PPLE	180 VSDG*EADGLEPG VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSD**ECLRDRV	200 PG*LLPGETGSKK** <mark>KIRL</mark> Y PG*LLHGETGSKK**KIRLY PG*IMHGETGSKK**KIRLY PG*IMHGEPGSKK**KIRLY SY*IPTGELGNKK**KIRLY	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRNGDMKDSIW QFLLDLLRNGDMKDSIR	C 240 WVDKDKGTFOFSS WVDKDKGTFOFSS WVDKEKGTFOFSS WVDRDKGTFOFSS
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B	B SDEEEG*ERQS**PPLE SDEEDI*ERQS**PPLE SDEEDI*ERQS**PPLE SDEEDM*ERQS**PPLE SDEEDH*GARS**PPLE SDEEDL*PLDS**PALE	180 VSDG*EADGLEPG VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSD**EECLRDRV VSDS*E <mark>SDE</mark> ALVA	200 PG*LLPGETGSKK** <mark>KIRLY</mark> PG*LLHGETGSKK**KIRLY PG*IMHGETGSKK**KIRLY PG*IMHGEPSSKK**KIRLY SY*IPTGELGNKK**KIRLY GPEGKGSEAGTRK**KIRLY	220 QFLLDLIRSGDMKDSIW QFLLDLIRSGDMKDSIW QFLLDLIRSGDMKDSIW QFLLDLIRNGDMKDSIW QFLLDLIRNGDMKDSIR QFLLDLIRNGDMKDSIR	C 240 WVDKDKGTFQFSS WVDKCKGTFQFSS WVDKEKGTFQFSS WVDRDKGTFQFSS WVDRDKGTFQFSS
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B	B SDEEEG*ERQS**PPLE SDEEDI*ERQS**PPLE SDEEDI*ERQS**PPLE SDEEDM*ERQS**PPLE SDEEDM*GARS**PPLE SEEEDL*PLDS**PALE SEEEDI*RLDS**PALE	180 VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSDG*EECLRDRV VSDS*ESDEALVA VSDS*ESDEALVA	200 PG*LLPGETGSKK** <mark>KIRLY</mark> PG*LLHGETGSKK**KIRLY PG*IMHGEPGSKK**KIRLY SY*IPTGELGNKK**KIRLY GPEGKGSEAGTRK**KLRLY GSEGRGSEAGARK**KLRLY	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRNGDMKDSIW QFLLDLLRNGDMKDSIR QFLLGLLRGDMRECVW	C 240 WVDKDKGTFQFSS WVDKCKGTFQFSS WVDKEKGTFQFSS WVDRDKGTFQFSS WVEPGAGVFQFSS WVEPGAGVFQFSS
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B Caiman Spi-B	B SDEEEG*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDM*ERQS** PPLE SDEEDM*GARS** PPLE SDEEDH*GARS** PALE SEEEDL*PLDS** PALE SEEEDI*MLDS** PALE SEEEDI*MLDS** PALE	180 VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSD*ECLRDRV VSDS*ESDEALVA VSDS*ESDEALLA VSDS*DSDENLSP	200 PG*LLPGETGSKK** <mark>KIRLY</mark> PG*LHHGETGSKK**KIRLY PG*IMHGEPGSKK**KIRLY SY*IPTGELSNKK**KIRLY GPEGKGSEAGTKK**KIRLY GSEGRGSEAGARK**KLRLY GSSLDLDSGSRR**KLRLY	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRNGDMKDSIW QFLLDLLRNGDMKDSIR QFLLGLLRGDMRECVW QFLLGLLLRGDMRECVW QFLLGLLLRGDMRECVW	C 240 WVDKDKGTFOFSS WVDKKGTFOFSS WVDKEKGTFOFSS WVDRCKGTFOFSS WVDRDKGTFOFSS WVEPGAGVFOFSS WVEPGAGVFOFSS WVEPGAGVFOFSS
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B Caiman Spi-B Toad Spi-B	B SDEEG*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDH*GARS** PPLE SEEEDH*GARS** PPLE SEEEDI*PLDS** PALE SEEEDI*MLDS** PALE SEDDF*PTDA** PALE SEEEDFRHAES** PPLE	180 VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSDS*ECLRDRV VSDS*ESDEALVA VSDS*ESDEALLA VSDS*DSDENLSP VSDS*ESDENLSP	200 PG*LLPGETSSKK**KIRLY PG*LHHGETSSKK**KIRLY PG*IMHGEPSSKK**KIRLY SY*IPTGELSNKK**KIRLY GPEGKGSEASTKK**KIRLY GSEGRGSEAGARK**KIRLY GSEGRGSEAGARK**KIRLY GS*SLDLDSGSRR**KIRLY GE*CLGYDPGVRK**KVRLY	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRNGDMKDSIW QFLLDLLRNGDMKDSIR QFLLGLLRGDMKDSIR QFLLGLLRGDMKDCIW QFLLGLLQRGDMQECVW KFLLELLLNGDMRDCIW	C 240 WVDKDKGTFOFSS WVDKCKGTFOFSS WVDKEKGTFOFSS WVDRCKGTFOFSS WVDRDKGTFOFSS WVEPGAGVFOFSS WVEPGAGVFOFSS WVEPGAGVFOFSS WVEHDSGVFOFSS WLDRERGTFOFSS
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B Caiman Spi-B Toad Spi-B Mouse Spi-C	B SDEEG*ERQS** PPLE SDEED1*ERQS** PPLE SDEEDM*ERQS** PPLE SDEEDM*GARS** PPLE SEED1*PLDS** PALE SEED1*MLDS** PALE SEED1*MLDS** PALE SEEDFRHAES** PPLE YLGGGF*HQS*****	180 VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSDS*EECLRDRV VSDS*ESDEALVA VSDS*ESDEALVA VSDS*ESDEALLA VSDS*ESDEALLA VSDS*ESDEALSP VSDS*ESDENESP VQNIAESQLVQPP	200 PG*LLPGETSSKK**KIRLY PG*LHHGETSSKK**KIRLY PG*IMHGEPGSKK**KIRLY SY*IPTGELSNKK**KIRLY GPEGKGSEAGTRK**KIRLY GSEGRGSEAGARK**KIRLY GG*SLDLDSGSRR**KLRLY GE*CIGYDPGVRK**KVRLY FF*QQKGGRGRRK***LRLF	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRNGDMKDSIW QFLLGLLRSDMKDSIR QFLLGLLRSDMKDSIR QFLLGLLRSDMKDCIW QFLLGLLQRSDMQECVW KFLLELLLNGDMRDCIW EYLFESLCNSEMVSCIQ	C 240 WVDKDKGTFOFSS WVDKKGTFOFSS WVDKEKGTFOFSS WVDRKGTFOFSS WVEPGACVFOFSS WVEPGACVFOFSS WVEPGACVFOFSS WVEPGACVFOFSS WVEHDSCVFOFSS WLDRERGTFOFSS WVDKARAIFOFIS
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B Caiman Spi-B Toad Spi-B Mouse Spi-C Fish Spi-C	B SDEEG*ERQS** PPLE SDEEDT*ERQS** PPLE SDEEDM*ERQS** PPLE SDEEDH*GARS** PPLE SEEEDT*PLDS** PALE SEEEDT*NLDS** PALE SEEEDFRHAES** PPLE SEEEDFRHAES** PPLE YLGGGF**HQS******	180 VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSDS*ESDEALVA VSDS*ESDEALVA VSDS*ESDEALLA VSDS*ESDEALLA VSDS*ESDEALLA VSDS*ESDEALLA VSDS*ESDEALSP VQNIAESQLVQPP VQN**ESPHFYSI	200 PG*LLPGET SSKK**KIRLY PG*LHGET SSKK**KIRLY PG*IMHGET SSKK**KIRLY SY*IPTGELSNKK**KIRLY GPEGKGSEAGTRK**KIRLY GSEGRGSEAGARK**KIRLY GSESLDLDSGSRR**KIRLY GE*CIGYDPGVRK**KVRLY FF*QQKGGRGRRK***LRLF LP*PQRNGKGRKK***LRLY	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRNGDMKDSIW QFLLGLLTRGDMKDSIR QFLLGLLRGDMREOVW QFLLGLLRGDMREOVW QFLLGLLQRGDMQEOVW KFLLELLLNGDMRDOIW EYLFESLCNSEMVSCIQ EYLHEALNDPNMGDSIQ	C 240 WVDKDKGTFOFSS WVDKEKGTFOFSS WVDKEKGTFOFSS WVDRKGTFOFSS WVEPGAGVFOFSS WVEPGAGVFOFSS WVEPGAGVFOFSS WVEPGAGVFOFSS WVEPGAGVFOFSS WVERGTFOFSS WVERGTFOFSS WVDKARAIFOFIS WTDSGSGTFHFIS
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B Caiman Spi-B Toad Spi-B Mouse Spi-C Fish Spi-C Lamprey Spi	B SDEEG*ERQS** PPLE SDEED1*ERQS** PPLE SDEED1*ERQS** PPLE SDEED1*GARS** PPLE SDEED1*GARS** PPLE SEED1*PLDS** PALE SEED1*MLDS** PALE SEEDFRHAES** PPLE YLGGGF**HQS****** IPDVAL*SHS******	180 VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSDS*ESDEALVA VSDS*ESDEALVA VSDS*ESDEALLA VSDS*ESDEALLA VSDS*ESDEALLA VSDS*ESDEALLA VSDS*ESDEALLA VSDS*ESDEALLA VSDS*ESDEALLA VSDS*ESDEALLA VSDS*ESDEALLA VSDS*ESDEALLA VSDS	200 PG*LLPGETSSKK**KIRLY PG*LHGETSSKK**KIRLY PG*IMHGEPGSKK**KIRLY SY*IPTGELGNKK**KIRLY GPEGKGSEAGTRK**KIRLY GSEGRGSEAGARK**KIRLY GSECTGYDPGVRK**KIRLY GE*CIGYDPGVRK**KVRLY FF*QQKGGRGRRK***LRLF LP*PQRNGKGRKK***LRLY RGVTVKREKGVGKRGKTRLY	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRNGDMKDSIW QFLLGLLTRGDMKDSIW QFLLGLLRGDMREOVW QFLLGLLRGDMREOVW QFLLGLLQRGDMQEOVW KFLLEILLNGDMRDOIW EYLFESLCNSEMVSCIQ EYLHEALNDPNMGDSIQ QFLMEILQDQRMCHCIW	C 240 WVDKDKGTFOFSS WVDKEKGTFOFSS WVDKEKGTFOFSS WVDRKGTFOFSS WVEPGAGVFOFSS WVEPGAGVFOFSS WVEPGAGVFOFSS WVEPGAGVFOFSS WVEPGAGVFOFSS WVERGTFOFSS WVDKARAIFQFIS WVDKARAIFQFSS
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B Toad Spi-B Mouse Spi-C Fish Spi-C Lamprey Spi	B SDEE G*ERQS** PPLE SDEE DI*ERQS** PPLE SDEE DI*ERQS** PPLE SDEE DI*ERQS** PPLE SDEE DI*ERQS** PPLE SDEE DI*RLDS** PALE SEED I*MLDS** PALE SEED F*PTDA** PALE SEED F*PTDA** PALE SEED F*HAES** PPLE SEED F*HAES** PPLE	180 VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSDS*ESDEALVA VSDS*ESDEALVA VSDS*ESDEALLA VSDS*ESDENLSP VQNIAESQLVQPP VQNIAESQLVQPP VQN+*ESPHFYSI QHHH*QQQQQQLP	200 PG*LLPGET SSKK**KIRLY PG*LHHGET SSKK**KIRLY PG*IMHGEP SSKK**KIRLY SY*IPTGELGNKK**KIRLY GPEGKGSEAGTRK**KIRLY GSEGRGSEAGARK**KIRLY GG*SLDLDS SSR**KIRLY GE*CIGYDPGVRK**KVRLY FF*QQKGGRGRRK***LRLF LP*PQRNGKGRKK***LRLY RGVTVKREKGVGKRGKTRLY	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRNGDMKDSIW QFLLGLLRGDMKDSIW QFLLGLLLRGDMRECVW QFLLGLLQRGDMQECVW KFLLEILLNGDMRCOIW EYLFESLCNSEWVSCIQ QFLMEILQDQRMCHCIW	C 240
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B Toad Spi-B Mouse Spi-C Fish Spi-C Lamprey Spi	B SDEEEG*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*RQS** PPLE SEEEDI*PLDS** ALL SEEDI*PLDS** ALL SEEDF*PTDA** ALL SEEDF*PTDA** PPLE SEEDF*PTDA** ALL SEEDF*PTDA** ALL SEEDF*HQS****** GDGQQQQQQGRHRHHQ	180 VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSDS*ESDEALVA VSDS*ESDEALVA VSDS*ESDEALLA VSDS*ESDENFSP VQNIAESQLVQPP VQNIAESPHFYSI QCHHH*QQQQQLP 260	200 PG*LLPGET SSKK**KIRLY PG*LHGET SSKK**KIRLY PG*IMHGET SSKK**KIRLY SY*IPTGELSNKK**KIRLY GPEGKGSEAGTRK**KIRLY GSEGRGSEAGARK**KIRLY GG*SLDLDSSSR**KIRLY GE*CIGYDPGVRK**KVRLY FF*QQKGGRGRRK***LRLF LP*PQRNGKGRKK***LRLY RGVTVKREKGVGKRGKTRLY 280	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRNGDMKDSIW QFLLGLLRGDMKDSIW QFLLGLLRGDMREGVW QFLLGLLQRGDMQEGVW KFLLGLLQRGDMQEGVW KFLLEILLNGDMRDGIW EYLFESLCNSEMVSCIQ QFLMEILQDQRMCHCIW 3000	C 240
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B Toad Spi-B Mouse Spi-C Fish Spi-C Lamprey Spi	B SDEEG*ERQS** PPLE SDEED1*ERQS** PPLE SDEED1*ERQS** PPLE SDEED1*ERQS** PPLE SDEED1*GARS** PPLE SEED1*PLDS** PALE SEED1*MLDS** PALE SEEDFT*MLDS** PPLE SEEDFT*MLDS** PPLE SEEDFT	180 VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSDS*ESDEALVA VSDS*ESDEALVA VSDS*ESDEALLA VSDS*ESDENLSP VQSDS*ESDENLSP VQNIAESQLVQPP VQNIAESQLVQPP 260 KKMTYQKMARALR	200 PG*LLPGETGSKK**KIRLY PG*IMHGETGSKK**KIRLY PG*IMHGEPSSKK**KIRLY SY*IPTGELGNKK**KIRLY GPEGKGSEAGTRK**KIRLY GS*SLDLDSGSSR**KIRLY GG*SLDLDSGSR**KIRLY GE*CIGYDPGVRK**KVRLY FF*QQKGGRGRRK***LRLY LP*PQRNGKGRKK***LRLY 280 NYGKTGEVKKVKKKLTYQFS	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRNGDMKDSIW QFLLGLLRGDMRECVW QFLLGLLLRGDMRECVW QFLLGLLLRGDMRECVW QFLLGLLLNGDMRCCVW KFLLELLNGDMRCCIW EYLFESLCNSEMVSCIQ QFLMEILQDQRMCHCIW 300 GEVLGRGGLAERRHPPH	C 240
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B Caiman Spi-B Mouse Spi-C Fish Spi-C Lamprey Spi Human Spi-1 Mouse Spi-1	B SDEEG*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*RARS** PPLE SEEEDI*NLDS** PALE SEEEDI*NLDS** PALE SEEEDFRHAES** PPLE SEEEDFRHAES** PPLE SEEEDFRHAES** PLE SEEEDFRHAES** PLE SEEEFFRHAES** PLE SEEEDFRHAES** PLE SEEEFFRHAES** PLE SEEEFFRHAES** PLE SEEEFFRHAES** PLE SEEFFRHAES** PLE SE	180 VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSDG*ETDGVDPT VSDS*ESDEALLA VSDS*ESDEALLA VSDS*ESDEALLA VSDS*ESDEALLA VSDS*ESDEALLA VSDS*ESDENLSP VQNIAESQLVQPP VQNIAESQLVQPP 260 KKMTYQKMARALR KKMTYQKMARALR	200 PG*LLPGETGSKK**KIRLY PG*IMHGETGSKK**KIRLY PG*IMHGEPSSKK**KIRLY SY*IPTGELGNKK**KIRLY GPEGKGSEAGTRK**KIRLY GSEGRGSEAGARK**KIRLY GG*SLDLDSGSR**KIRLY GG*CIGYDPGVRK**VIRLY FF*QQKGGRGRRK**LRLF FF*QQKGGRGRRK**LRLF LP*PQRNGKGRKK**LRLY RGVTVKREKGVGKRGKTRLY 280 NYGKTGEVKKVKKKLTYQFS	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLRNGDMKDSIW QFLLGLLRGDMRECVW QFLLGLLRGDMRECVW QFLLGLLQRGDMQECVW KFLLELLLNGDMRDCIW EYLFESLCNSEMVSCIQ QFLMEILQDQRMCHCIW 300 GEVLGRGGLAERRHPPH GEVLGRGGLAERRHPPH	C 240
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B Caiman Spi-B Mouse Spi-C Fish Spi-C Lamprey Spi Human Spi-1 Mouse Spi-1 Fowl Spi-1	B SDEEG*ERQS** PPLE SDEED1*ERQS** PPLE SDEED1*ERQS** PPLE SDEEDM*ERQS** PPLE SDEEDH*GARS** PPLE SEED1*PLDS** PALE SEED1*MLDS** PALE SEEDFRHAES** PPLE YLGGF**HQS****** GDGQQQQQQQGRHRHHQ KHKEALAHRWGIQKGNE KHKEALAHRWGIQKGNE	180 VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSDS*EDGALVA VSDS*ESDEALVA VSDS*ESDEALVA VSDS*ESDEALLA VSDS*ESDENLSP VQNIAESQLVQPP VQNIAESQLVQPP VQNIAESQLVQPP 260 KKMTYQKMARALR KKMTYQKMARALR	200 PG*LLPGETGSKK**KIRLY PG*IMHGETGSKK**KIRLY PG*IMHGEPGSKK**KIRLY SY*IPTGELGNKK**KIRLY GPEGKGSEAGTRK**KIRLY GSEGRGSEAGARK**KIRLY GG*SLDLDSGSRR**KIRLY GG*CLGYDPGVRK**VRLY FF*QQKGGRGRRK***LRLF LP*PQRNGKGRKK***LRLY RGVTVKREKGVGKRGKTRLY 280 NYGKTGEVKKVKKKLTYQFS NYGKTGEVKKVKKKLTYQFS	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLRNGDMKDSIW QFLLGLLRNGDMKDSIW QFLLGLLRGDMRECVW QFLLGLLLRGDMRECVW QFLLGLLLRGDMRECVW QFLLGLLQRGDMQECVW KFLEILLNGDMRDCIW EYLFESLCNSEMVSCIQ QFLMEILQDQRMCHCIW 300 GEVLGRGGLAERRHPPH GEVLGRGGLAERRHPPH GEVLGRGGLAERRHPPH	C 240
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B Caiman Spi-B Mouse Spi-C Fish Spi-C Lamprey Spi Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1	B SDEEG*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDM*ERQS** PPLE SDEEDM*GARS** PPLE SEEDI*PLDS** PALE SEEDI*NLDS** PALE SEEDFRHAES** PPLE YLGGGF**HQS******* GDGQQQQQQQGRRHHQ KHKEALAHRWGIQKGNF KHKEALAHRWGIQKGNF KHKEALAHRWGIQKGNF	180 VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSDS*EDDALVA VSDS*ESDEALLA VSDS*ESDEALLA VSDS*ESDENLSP VSDS*ESDENLSP VQD1AESQLVQPP VQD**ESPHFYSI QHHH*QQQQQQLP 260 CKKMTYQKMARALR KKMTYQKMARALR KKMTYQKMARALR	200 PG*LLPGETGSKK**KIRLY PG*INHGETGSKK**KIRLY PG*INHGEPGSKK**KIRLY SY*IPTGELGNKK**KIRLY GPEGKGSEAGTKK**KIRLY GG*SLDLDSGSRK**KIRLY GG*SLDLDSGSRK**KIRLY GF*QQKGGRGRK**KIRLY FF*QQKGGRGRK***LRLF LP*PQRNGKGRKK***LRLY RGVTVKREKGVGKRGKTRLY 280 NYGKTGEVKKVKKKLTYQFS NYGKTGEVKKVKKKLTYQFS	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRNGDMKDSIW QFLLGLLRGDMKDSIW QFLLGLLRGDMQECVW QFLLGLLQRGDMQECVW KFLLEILLNGDMRCOIW EYLFESLCNSEMVSCIQ QFLMEILQDQRMCHCIW 300 GEVLGRGGLAERRHPPH GEVMGRA*SSDRKHYPH	C 240
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B Caiman Spi-B Toad Spi-B Mouse Spi-C Fish Spi-C Lamprey Spi Human Spi-1 Fowl Spi-1 Fish Spi-1	B SDEEG*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDM*ERQS** PPLE SDEEDM*GARS** PPLE SEEDI*PLDS** PALE SEEDI*MLDS** PALE SEEDFRHAES** PPLE SEEDFRHAES** PPLE SEEDFR	180 VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSDS*EDDALVA VSDS*ESDEALVA VSDS*ESDEALLA VSDS*ESDENLSP VQNIAESQLVQP VQNIAESQLVQP VQN	200 PG*LLPGETGSKK**KIRLY PG*ILHGETGSKK**KIRLY PG*IMHGEPGSKK**KIRLY SY*IPTGELSNKK**KIRLY GPEGKGSEAGTKK**KIRLY GSEGRGSEAGARK**KIRLY GSECIGYDPGVRK**VLRLY GF*QQKGGRGRRK***LRLY FF*QQKGGRGRRK***LRLY RGVTVKREKGVGKRGKTRLY 280 NYGKTGEVKKVKKKLTYQFS NYGKTGEVKKVKKKLTYQFS NYGKTGEVKKVKKKLTYQFS	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRNGDMKDSIW QFLLDLLRNGDMKDSIW QFLLGLLRGDMRECVW QFLLGLLRGDMRECVW QFLLGLLLRGDMRECVW QFLLGLLQRGDMQECVW KFLLEILLNGDMRDCIW EYLFESLCNSEMVSCIQ QFLMEILQDQRMCHCIW 300 GEVLGRGGLAERRHPPH GEVLGRGGLAERRHPPH GEVMGRG*VADRKHYPH GEVMGRA*SSDRKHYPH DEVLGKSH*********	C 240
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B Caiman Spi-B Toad Spi-B Mouse Spi-C Fish Spi-C Lamprey Spi Human Spi-1 Fowl Spi-1 Fish Spi-1 Human Spi-B	B SDEEG*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDM*ERQS** PPLE SDEEDM*ERQS** PPLE SDEEDM*GARS** PPLE SEEDI*PLDS** PALE SEEDI*MLDS** PALE SEEDFRHAES** PPLE SEEDFRHAES** PPLE SEEDF	180 VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSDS*ESDEALVA VSDS*ESDEALVA VSDS*ESDEALLA V	200 PG*LLPGET GSKK**KIRLY PG*LHGET GSKK**KIRLY PG*IMHGEPGSKK**KIRLY SY*IPTGELSNK**KIRLY GPEGKGSEAGTK**KIRLY GSEGRGSEAGARK**KIRLY GSEGRGSEAGARK**KIRLY GE*CIGYDPGVRK**KVRLY FF*QQKGGRGRRK***LRLY FF*QQKGGRGRKK***LRLY RGVTVKREKGVGKRGKTRLY 280 NYGKTGEVKKVKKLTYQFS NYGKTGEVKKVKKLTYQFS NYGKTGEVKKVKKLTYQFS NYGKTGEVKKVKKLTYQFS	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRNGDMKDSIW QFLLGLLRGDMKDSIW QFLLGLLRGDMRECVW QFLLGLLLRGDMRECVW QFLLGLLQRGDMQECVW KFLLEILLNGDMRDCIW EYLFESLCNSEMVSCIQ QFLMEILQDQRMCHCIW 300 GEVLGRGGLAERRHPPH GEVLGRGGLAERRHPPH GEVMGRG*VADRKHYPH GEVMGRA*SSDRKHYPH DEVLGKSH********	C 240
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B Toad Spi-B Mouse Spi-C Fish Spi-C Lamprey Spi Human Spi-1 Fowl Spi-1 Fowl Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B	B SDEEEG*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*RQS** PPLE SDEEDI*RLDS** PALE SEEDI*RLDS** PALE SEEDF*PTDA** PALE SEEDF*PTDA** PALE SEEDF*HQS****** IPDVAL**SHS****** GDGQQQQQQQGRHRHHQ KHKEALAHRWGIQKGNF KHKEALAHRWGIQKGNF KHKEALAHRWGIQKGNF KHKEALAHRWGIQKGNF KHKEALAHRWGIQKGNF KHKEALAHRWGIQKGNF KHKEALAHRWGIQKGNF KHKEALAHRWGIQKGNF	180 VSDG*EADGLEPG VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSDS*ESDEALVA VSDS*ESDEALVA VSDS*ESDEALLA V	200 PG*LLPGET GSKK**KIRLY PG*LLHGET GSKK**KIRLY PG*IMHGET GSKK**KIRLY PG*IMHGEP GSKK**KIRLY SY*IPTGELCNK**KIRLY GPEGKGSEAGARK**KIRLY GSEGRGSEAGARK**KIRLY GSEGRGSEAGARK**KIRLY GE*CIGYDPGVRK**KVRLY FF*QQKGGRGRRK***LRLY FF*QQKGGRGRRK***LRLY RGVTVKREKGVGKRGKTRLY 280 NYGKTGEVKKVKKKLTYQFS NYGKTGEVKKVKKKLTYQFS NYGKTGEVKKVKKKLTYQFS NYGKTGEVKKVKKKLTYQFS NYGKTGEVKKVKKKLTYQFS	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRNGDMKDSIW QFLLGLLRNGDMKDSIW QFLLGLLRGDMREGVW QFLLGLLQRGDMQEGVW QFLLGLLQRGDMQEGVW KFLLGLLQRGMQEGVW QFLMEILQDQRMCHCIW 300 GEVLGRGGLAERRHPPH GEVMGRG*VADRKHYPH GEVMGRA*SSDRKHYPH DEVLGKSH******* SALLPASRHV******	C 240
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B Toad Spi-B Mouse Spi-C Fish Spi-C Lamprey Spi Human Spi-1 Fowl Spi-1 Fowl Spi-1 Caiman Spi-1 Human Spi-1 Human Spi-B Mouse Spi-B Caiman Spi-B	B SDEEEG*ERQS** PPLE SDEED1*ERQS** PPLE SDEED1*ERQS** PPLE SDEED1*ERQS** PPLE SDEED1*ERQS** PPLE SDEED1*LSARS** PPLE SEED1*MLDS** PALE SEED1*MLDS** PALE SEEDFFHAES** PPLE SEEDFFHAES** PPLE SEE	180 VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSDS*ESDEALVA VSDS*ESDEALVA VSDS*ESDEALLA VSDS*ESDEALLA VSDS*ESDENLSP VQNIAESQLVQPP VQN*ESPHFYSI QCHHH*QQQQQQLP 260 KKMTYQKMARALR KKMTYQKMARALR KKMTYQKMARALR KKMTYQKMARALR KKMTYQKMARALR KKMTYQKLARALR KKMTYQKLARALR	200 PG*LLPGETGSKK**KIRLY PG*LHHGETGSKK**KIRLY PG*IMHGEPSSKK**KIRLY PG*IMHGEPSSKK**KIRLY GPEGKGSEAGTRK**KIRLY GPEGKGSEAGTRK**KIRLY GS*SLDLDSGSSR**KIRLY GG*SLDLDSGSRR**KIRLY GG*CLGYDPGVRK**KIRLY GE*CLGYDPGVRK**KIRLY GE*CLGYDPGVRK**KIRLY GE*CLGYDFGVRK**KIRLY GE*CLGYDFGVKKVKKLTYQFS NYGKTGEVKKVKKLTYQFS NYGKTGEVKKVKKLTYQFS NYGKTGEVKKVKKLTYQFS NYGKTGEIRKVKKKLTYQFG NYAKTGEIRKVKKKLTYQFG	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLRSGDMKDSIW QFLLDLLRNGDMKDSIW QFLLGLLRGDMKDSIW QFLLGLLRGDMRECVW QFLLGLLLRGDMRECVW QFLLGLLLRGDMRECVW QFLLGLLLRGDMRECVW QFLLGLLLNGDMRCVW QFLLGLLQRGMCHCIW SAUGAGGLAERRHPPH GEVLGRGGLAERRHPPH GEVLGRGGLAERRHPPH GEVMGRA*SSDRKHYPH GEVMGRA*SSDRKHYPH DEVLGKSH******* SALIPASRHV******	C 240
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B Mouse Spi-C Fish Spi-C Lamprey Spi Human Spi-1 Fowl Spi-1 Caiman Spi-1 Human Sp	B SDEEG*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*RQARS** PPLE SEEEDI*PLDS** PALE SEEDI*PLDS** PALE SEEDFRAES** PPLE SEEDFRAES**	180 VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSDG*ETDGVDPT VSDS*ESDEALLA VSDS*ESDEALLA VSDS*ESDENLSP VQDIAESQLVQPP VQN*ESPHFYSI 260 KKMTYQKMARALR KKMTYQKMARALR KKMTYQKMARALR KKMTYQKMARALR KKMTYQKMARALR KKMTYQKMARALR KKMTYQKMARALR KKMTYQKLARALR KKMTYQKMARALR	200 PG*LLPGET GSKK**KIRLY PG*IHHGET GSKK**KIRLY PG*IHHGET GSKK**KIRLY PG*IHHGEP SSKK**KIRLY SY*IPTGELGNKK**KIRLY GPEGKGSEAGTRK**KIRLY GSEGRGSEAGTRK**KIRLY GG*CIGYDPGVRK**KIRLY GG*CIGYDPGVRK**VIRLY FF*QQKGGRGRRK**LRLY FF*QQKGGRGRKK***LRLY RGVTVKREKGVGKRGKTRLY 280 NYGKTGEVKKVKKLTYQFS NYGKTGEVKKVKKLTYQFS NYGKTGEVKKVKKLTYQFS NYGKTGEVKKVKKLTYQFS NYGKTGEIRKVKKKLTYQFD NYGKTGEIRKVKKLTYQFD NYGKTGEIRKVKKLTYQFD	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLRSGDMKDSIW QFLLGLLRGDMKDSIW QFLLGLLRGDMRECVW QFLLGLLLRGDMRECVW QFLLGLLQRGMQECVW KFLLEILLNGDMRCOIW KFLLEILLNGDMRCOIW GEVLGRGGLAERRHPPH GEVLGRGGLAERRHPPH GEVLGRGGLAERRHPPH GEVMGRA*SSDRKHYPH DEVLGKSH******** SALLPAVRRA****** SALLPAVRRA******	C 240
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B Caiman Spi-B Mouse Spi-C Fish Spi-C Lamprey Spi Human Spi-1 Fowl Spi-1 Fowl Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B Caiman Spi-B Toad Spi-B Mouse Spi-C	B SDEEG*ERQS** PPLE SDEECI*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*ERQS** PPLE SDEEDI*PLDS** PALE SEECI*MLDS** PALE SEECI*MLDS** PALE SEECI*MLDS** PLE SEECI*MLDS**	180 VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSDG*ETDGVDPT VSDS*ESDEALLA V	200 PG*LLPGET GSKK**KIRLY PG*ILHGET GSKK**KIRLY PG*IMHGET GSKK**KIRLY PG*IMHGEPSSKK**KIRLY SY*IPTGELGNKK**KIRLY GPEGKGSEAGTRK**KIRLY GSEGRGSEAGARK**KIRLY GG*SLDLDSGSRR**KIRLY GG*SLDLDSGSRR**KIRLY GG*CIGYDPQVRK**VRLY FF*QQKGGRGRRK**LRLY GG*CIGYDPQVRK**VRLY FF*QQKGGRGRK**LRLY PPQRNGKGRKK**LRLY RGVTVKREKGVGKRGKTRLY 280 NYGKTGEVKKVKKLTYQFS NYGKTGEVKKVKKLTYQFS NYGKTGEVKKVKKLTYQFS NYGKTGEVKKVKKLTYQFS NYGKTGEIKKVKKLTYQFG NYAKTGEIRKVKKLTYQFG NYGKTGEIRKVKKLTYQFT NYGKTGEIRKVKKLTYQFT NYGKTGEIRKVKKLTYQFT NYGKTGEIRKVKKLTYQFT NYGKTGEIRKVKKLTYQFT	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLRNGDMKDSIW QFLLGLLRNGDMKDSIW QFLLGLLRGDMRECVW QFLLGLLRGDMRECVW QFLLGLLQRGDMQECVW KFLLEILLNGDMRDCIW EYLFESLCNSEMVSCIQ QFLMEILQDQRMCHCIW 300 GEVLGRGGLAERRHPPH GEVLGRGGLAERRHPPH GEVLGRGGLAERRHPPH GEVLGRGGLAERRHPPH GEVLGRGGLAERRHPH GEVLGRGGLAERRHPH GEVLGRGGLAERRHPH HENGRA*SSDRKHYPH SALLPASRHV****** SALLPASRHV******	C 240
Human Spi-1 Mouse Spi-1 Fowl Spi-1 Caiman Spi-1 Fish Spi-1 Human Spi-B Mouse Spi-B Toad Spi-B Mouse Spi-C Fish Spi-C Lamprey Spi Human Spi-1 Fowl Spi-1 Caiman Spi-1 Human Spi-1 Human Spi-1 Human Spi-B Mouse Spi-B Caiman Spi-B Toad Spi-B Mouse Spi-C Fish Spi-C	B SDEEG*ERQS**PPLE SDEEDI*ERQS**PPLE SDEEDI*ERQS**PPLE SDEEDM*ERQS**PPLE SDEEDM*ERQS**PPLE SDEEDI*PLDS**PALE SEEDI*PLDS**PALE SEEDI*PLDS**PALE SEEDFRHAES**PLE YLGGGF**HQS****** GDGQQQQQQQGRHRHHQ KHKEALAHRWGIQKGNF KHKEALAHRWGIQKGNF KHKEALAHRWGIQKGNF KHKEALAHRWGIQKGNF KHKEALAHRWGIQKGNF KHKEALAHRWGQKGNF KHKEALAHRWGQCKGNF KHKELLARRWGQCKGNF KHKELLAHRWGQCKGNF KHKELLAHRWGQCKGNF KHKELLAHRWGQCKGNF KHKELLAHRWGQCKGNF KHKELLAHRWGQCKGNF KHKELLAHRWGQCKGNF KHKELLAHRWGQCKGNF KHKELLAHRWGQCKGNF KHKELLAHRWGQCKGNF KHKELLAHRWGQCKGNF KHKELLAHRWGQCKGNF KHKELLAHRWGQCKGNF KHKELLAHRWGQCKGNF KHKELLAHRWGQCKGNF KHKELLAHRWGQCKGNF KHKELLAHRWGQCKGNF KHKELLAHRWGQCKGNF KHKELLAHRWGQCKGNF KHKELLAHRWGQCKGNF	180 VSDG*EADGLEPG VSDG*ETDGVDPG VSDG*ETDGVDPT VSDG*ETDGVDPT VSDS*EDEALVA VSDS*ESDEALVA VSDS*ESDEALLA VSDS*ESDENLSP VQDIAESQLVQPP VQDIAESQLVQPP VQDI*ESPHFYSI QCHHH*QQQQQQLP 260 CKKMTYQKMARALR KKMTYQKMARALR KKMTYQKMARALR KKMTYQKMARALR KKMTYQKMARALR KKMTYQKMARALR KKMTYQKMARALR KKMTYQKMARALR KKMTYQKMARALR	200 PG*LLPGET GSKK**KIRLY PG*INHGET GSKK**KIRLY PG*INHGET GSKK**KIRLY PG*INHGEP GSKK**KIRLY SY*IPTGELGNKK**KIRLY GPEGKGSEAGTRK**KIRLY GG*SLDLDS GSRR**KLRLY GG*SLDLDS GSRR**KLRLY GG*SLDLDS GSRR**KLRLY GG*SLDLDS GSRR**KLRLY GG*SLDLDS GSRRK**LRLY GG*SLDLDS GSRRK**LRLY GG*SLDLDS GSRRK**LRLY GE*CIGYDPGVRK**VRLY FF*QQKGGRGRRK**LRLY GE*CIGYDPGVRK**LRLY GE*CIGYDPGVRK**LRLY GE*CIGYDPGVRKKKLTYQFS NYGKTGEVKKVKKKLTYQFS NYGKTGEIRKVKKKLTYQFD NYAKTGEIRKVKKKLTYQFD NYAKTGEIRKVKKKLTYQFD NYAKTGEIRKVKKKLTYQFD NYAKTGEIRKVKKKLTYQFD NYAKTGEIRKVKKKLTYQFD NYARTGEIIKIRKLTYQFS NYARTGEIIKRKTYQFS	220 QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRSGDMKDSIW QFLLDLLRNGDMKDSIW QFLLGLLRGDMRECVW QFLLGLLLRGDMRECVW QFLLGLLQRGDMQECVW KFLLEILLNGDMRCVW QFLGLQLQRGDMQECVW KFLEILLNGDMRCVW QFLGGLQRGDMQECVW SUUGRGSVCCCC GEVLGRGGLAERRHPH GEVMGRG*VADRKHYPH GEVMGRG*VADRKHYPH GEVMGRG*VADRKHYPH GEVMGRA*SSDRKHYPH SALIPASRHV****** SALIPASRHV****** SLLGESGRAPS****	C 240

Fig. 3. Amino acid alignment (GenBank accession codes) of human (X66079), mouse (U87614-20), caiman (AF247364), and toad (AF247365) Spi-B sequences, human (X52056), mouse (M32370), fowl (Y12225), caiman (AF247363), and fish (AF247366) Spi-1 sequences, as well as the sea lamprey (AF247362) Spi, and the mouse (AF098863) and fish (AF247367) Spi-C sequences. An asterisk indicates an alignment gap. The mouse and fish Spi-C sequences have an extended carboxyl terminus; the final AAR (numbering 22 and 45, respectively) are not shown. The coloring groups AAR conserved in Spi-1 (blue), Spi-B (red), or all sequences (yellow). Numbers indicate alignment positions. The relationship found in the conserved final exon was used as a guide tree for the clustal alignment of the complete sequence. The location of known domains within the mouse Spi-1 sequence are indicated with solid horizontal lines: A is the glutamine-rich transactivation domain; B is the PEST domain; and C is the Ets domain.

thostome Spi-1 and Spi-B domains. Its similarity to the human Spi-1 (12) and Spi-B (32) sequences is 62.2% and 64.3%, respectively. Similarly, phylogenetic analysis places the lamprey sequence in a cluster of the gnathostome Spi-1 and Spi-B sequences apart from all of the other members of the Ets family (Fig. 4). In the cluster, the lamprey sequence assumes an outgroup position.

To identify the main sites of expression of the lamprey *Spi* gene, a series of paraffin sections was prepared from different

body parts of premetamorphosing ammocoetes in stage III (sea lamprey) or stage IV (freshwater lamprey) of development in the classification of Ardavin *et al.* (39). The sections were then subjected to *in situ* hybridization with a lamprey antisense *Spi* probe. Strong expression of the *Spi* gene was observed in the gut epithelium, particularly in the parts overlaying and opposite to the typhlosole ("spiral valve," Fig. 5), and in the ovary (data not shown). Weaker expression was detected in the skin epithelium and the opisthonephros. The signal in the gut epithelium was



Fig. 4. Phylogenetic tree of sequences shown in Fig. 3. The tree is based on the alignment of the conserved DNA-binding domain encoded by the last exon of the Spi sequences. Genetic distances were measured from the proportion of amino acid identity of the aligned sequences and the tree was drawn by using the neighbor-joining method of Saitou and Nei (30). The human ELF5 sequence is taken as an outgroup. Numbers above each node indicate the percentage recovery of that node in 500 bootstrap replications.

particularly strong in the pharyngeal region (the epi- and hypopharyngeal folds), the esophagus, and the intestinal region. It emanated from cells which on hematoxylin/eosin-stained preparations had lymphocyte morphology (Fig. 5). They presumably represent lymphocyte-like cells invading the epithelia. In the ovary, the signal appears to originate in the cytoplasm of the oocytes and in some preparations also from the surrounding cells. Shared expression between hemopoietic tissue and gonads has been reported for several genes (e.g., refs. 40-42); its significance is obscure.

The Ets is a large family of genes of which only three members (Spi-1, Spi-B, and Spi-C) are known to be involved in lymphopoiesis (8, 9, 14). For the purpose of the present study, it is therefore critical to establish that the lamprey gene is Spi rather than some other member of the family. Four observations support the Spi identity of the lamprey gene. First, exon 6, which is the only part of the gene conserved among the Ets family members, has the highest sequence similarity with the mammalian Spi-1, Spi-B pair, and on phylogenetic analysis, the exon 6-encoded domain of the lamprey protein clusters with the corresponding gnathostome Spi-encoded domains (Fig. 4). Second, the exon-intron organization of the lamprey gene resembles most closely that of the Spi-1 and Spi-B genes of all of the Ets members (Fig. 2). (The other members generally have eight, nine, or more exons.) Third, the conserved exon 6 has a size within the variation found in Spi-1 and Spi-B; more marked variation (up to 20 residues) in exon 6 length occurs among the other Ets family members. (It can also be argued that the



Fig. 5. Transverse section through the esophageal region of *P. marinus* larva. Strong hybridization signals (asterisks) are seen inside the esophageal epithelium (e) in the area of lamina propria (l) folds. Weaker hybridization signals (arrow) are seen in other areas of the esophageal epithelium. (A) Dark-field (original magnification $\times 100$). (B) Dark-field, negative control (original magnification $\times 100$). (C and D) Bright-field illumination (original magnifications $\times 100$ and $\times 400$, respectively). Dark spots represent hybridization signals. Areas of strong hybridization signals under bright-field illumination (*E*) colocalize with areas of high lymphocyte densities under hematoxylin and eosin staining (*F* and upper rectangle in *D*). Areas of weak hybridization signals (G) colocalize with areas of low lymphocyte densities (*H* and lower rectangle in *D*). (Original magnification: *E*–*H*, $\times 1000$.)

expression pattern of the lamprey *Spi* gene is additional evidence for its identity, but there would be certain circularity in such a reasoning.) Fourth, Southern blot analysis indicates that the lamprey *Ets* domain-encoding segment hybridizes with only two or three restriction fragments and hence that it is not promiscuously associated with multiple loci (data not shown).

The expression of the lamprey *Spi* gene in the lymphocyte-like cells supports the notion that these cells, previously identified only by their morphological appearance, are indeed related to the lymphocytes of jawed vertebrates ontogenetically. Further support for this notion is provided by the observation that two other transcription factors, members of the Ikaros family pri-

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marily involved in lymphopoiesis (43), are also present in the lamprey and show a similar expression pattern to the *Spi* gene (W. E. Mayer, J.T., C.O., and M.S.-B., in preparation). These observations reopen the issue of the presence or absence of adaptive immune response in jawless vertebrates.

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