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H. 知的財産権の出願・登録状況

なし

研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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VERSITA

RESEARCH NOTE

Isolation of the cDNAs encoding secreted and membrane binding proteins from egg of *Schistosoma japonicum* (Chinese strain)

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Abstract

The granulomatous reaction which occurs around egg trapped in the intrahepatic venules ultimately may lead to fibrosis, which is the main pathogenesis of schistosomiasis. The excreted proteins from eggs play an important role during this process, and they may be a target for developing new strategies to control the hepatic pathogenesis caused by schistosome infection. In this study, fifteen genes encoding secreted or membrane binding protein were identified with the signal sequence trapping method by retrovirus mediated expression screening (SST-REX) of cDNAs from the egg of *Schistosoma japonicum* (Chinese strain).

Keywords

Schistosoma japonicum, secretory or membrane binding protein, signal sequence trapping (SST)

Schistosomiasis is spreading throughout South America, Africa and Asia with the implementation of irrigation programs, and thus hundreds of millions are exposed to infection (Chitsulo *et al.* 2000). Severe disease is associated with high rates of infection and represents an important health problem for a number of subtropical countries. In persons infected by schistosomes, severe clinical forms are mainly characterized by hepatosplenomegaly, high portal hypertension and ascites, which are the consequences of a chronic inflammation caused by the egg products (Phillips and Lammie 1986). Proteins excreted from the egg surface of schistosomes are exposed to the host liver tissue, which may play a critical role in the process of egg granuloma and fibrosis formation through mediating inflammation; they thus present as potential targets for developing new methods to prevent liver granulomatous hypersensitivity, and are of additional interest as candidate vaccine antigens of anti-pathogenesis.

Although there are about 100 000 *Schistosoma japonicum* (Chinese strain) expressed sequence tags (ESTs) and 17 000 *Schistosoma mansoni* expressed sequence tags (ESTs) in the dbEST database (LoVerde *et al.* 2004, Liu *et al.* 2006), recent research shown that some important genes are not included (Smyth *et al.* 2003, Wuhrer *et al.* 2006). Moreover, many schistosome mRNAs do not share sequence identity with proteins of known function only about 20–25% of the total schistosome genes have been discovered, and there is no effective technique available to identity target genes for developing an anti-pathogenesis vaccine for schistosomiasis.

Schistosome egg excreted proteins posses N-terminal hydrophobic signal peptides that direct the traffic of these proteins through the secretory pathway to the cell surface. The secretory signal sequences of proteins from different organisms are functionally interchangeable, highlighting the conserved nature of the secretory apparatus. These observations led to the development of many signal sequence trapping (SST) methods of identifying secretory molecules by expression screens in heterologous expressing systems (Tashiro *et al.* 1993, Klein *et al.* 1996, Kojima and Kitamura 1999, Moffatt *et al.* 2002, Smyth *et al.* 2003).

This is the first report of using signal sequence trapping method to identify the secreted proteins from *S. japonicum* (Chinese strain) egg cDNAs possessing a signal sequence

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Table I. Genes harbouring signal sequence isolated from egg of *Schistosoma japonicum* (Chinese strain)

Name	Accession no.	Kozak seq[a]	Putative signal sequence[b]	SignalP-NN				Signal P-HNN	No. of internal transmembrane domain
				C	Y	S	s		
SjP4001	AY570756	GAA_	MFKMRIINLVNISTVLLLINLLQTKSQ GH···	N	Y	Y	Y	SP	1
SjP1531	AY570742	TAA_	MFKMRIINLVNISTVLLLINLLQTKSQ VN···	N	Y	Y	Y	SP	2
SjP3842	AY570748	GAA_	MFKMRIINLVNISTVLLLINLLQTKSR LV···	N	Y	Y	Y	SP	3
SjP3611	AY570744	AAA_	MRIIILGIISTVLLLINLLQTKSQ TQ···	Y	Y	Y	Y	SP	2
SjP1084	AY570737	AAA_	MRIINLVIISTALLLINLLQTKSQ AN···	Y	Y	Y	Y	SP	2
SjP3742	AY570746	AAA_	MQMLNFVKIPTLLLLLHVISINA QL···	N	Y	Y	Y	SP	1
SjP1183	AY570783	AAA_	MQMLNFVKISTMLLLPQLISTNT QH···	N	Y	Y	Y	SP	1
SjP171	AY570743	TCA_	MIPTIKLLSLVCLLSYVKA GV···	Y	Y	Y	Y	SP	1
SjP1412	AY570741	GAA_	MYPLLICLIVLSSMITLKSQS VQ···	Y	Y	Y	Y	SP	1
SjP391B	AY570749	GAT_	MKMRGSANQLVTCILTLVIFTGTTLTQS SD···	Y	Y	Y	Y	SP	1
SjP4071	AY596288	CAA_	MLYLFSVLFVVNITLA VT···	Y	Y	Y	Y	SP	1
SjP3782	AY570747	AGA_	MIMFDPTILMKQFLVVFVSFSIF IC···	N	Y	Y	Y	SP	1
SjP422	AY570752	GTG_	MHECMIVFFIFAVVSIYYADA ES···	Y	Y	Y	Y	SP	1
SjP122	AY570740	ATA_	MMNKLWLIVAIFTMIVVTNQTNA QA···	Y	Y	Y	Y	SP	1
SjP3811	AY596287	GCG_	MSFNLNQMNSLLYWLIPSIYFNILYLDNSVA LF···	Y	Y	Y	Y	SP	4

[a]The Kozak consensus sequence for eukaryotes is RCC_, where R is A or G_, represent initial codon ATG; [b]the putative cleavage point of each signal peptide is denoted by a space followed by the first two N-terminal residues of the processed protein.

were isolated with a signal sequence trap by retrovirus-mediated expression screening (SST-REX). In this SST-REX system, the vector pMX-SST was constructed by Kojima and Kitamura (1999), a truncated MPL^M (ΔMPL^M, which belongs to the cytokine receptor family and is known to transmit a proliferation signal in interleukin-3 dependent cell lines, including the mouse pro-B cell line Ba/F3) which is used as a reporter. When a foreign cDNA possessing a signal sequence is inserted upstream of ΔMPL^M to replace the ΔG^M sequence in the vector pMX-SST, the fusion expressing ΔMPL^M will be transported onto the cell membrane and transmits a proliferation signal, which permits Ba/F3 cell growth in the IL-3 free medium otherwise, the Ba/F3 cells die as they lack a proliferation signal. Then the foreign cDNA fragments containing a signal sequence can be recovered from the genomic DNA of the factor independent growth Ba/F3 cells by PCR.

The life cycle of *S. japonicum* (Chinese strain) was maintained in *Oncomelania hupensis* snails and New Zealand white rabbits in the Department of Schistosomiasis Control Jiangsu Institute of Parasitic Diseases. Eggs were collected from the liver tissue of New Zealand rabbits infected with cercariae of *S. japonicum* (1500 per rabbit) after 45 days (Dalton *et al.* 1997) method. Egg mRNA extracted and purified by oligo (dT)-cellulose chromatography using a QuickPrep@ mRNA Purification Kit (Phamarcia) following the manufacturer's instructions was transcribed into double cDNA with random primers by SuperScript™ Choice System (Invitrogen). cDNA fragments (>400 bp) were excised from electrophoresis gels purified by digesting the low melting agarose gel with β-agarase I (NEB) and precipitated with cold ethanol then the purified cDNAs were added to the BstX1 adapter at its 3', 5' end and cloned into the BstX1 digested pMX-SST vector (Kojima and Kitamura 1999). The resultant *S. japonicum* (Sj) egg cDNA-ΔMPL^M SST library was electroporated into Ecoli.DH10B competent cells and plated onto LB plates containing ampicillin (100 μg/ml). A 0.66×10^6 cfu of the Sj egg cDNA-ΔMPL^M SST library was obtained inferred from restriction analysis of the plasmid DNA of 20 colonies selected randomly by EcoR1 digestion. High titer retroviruses representing the Sj cDNA-ΔMPL^M SST library were produced using the packaging cell line 293T by co-transfecting the Sj cDNA-ΔMPL library plasmids into 293T cells together with plasmid pE-eco expressing Ecotropic env protein and plasmid pGP expressing gag-pol protein (TaKaRa Retrovirus Packaging Kit Eco) using the Fugene 6 reagent (Roche) (Pear *et al.* 1993).

About 1×10^7 Ba/F3 cells were infected by 10 ml of the retroviral supernatant containing 3×10^6 virus particles, following incubation at 24 hours with retroviruses in the presence of polybrene and IL-3. The infected Ba/F3 cells were washed with PBS (-) three times, then the cell was seeded in twelve 96 multiwell plates (1152 wells) in the absence of IL-3. The cells were cultured in a CO_2 incubator (5% CO_2) at 37°C for 1 to 2 weeks factor-independent growth was observed in 210 wells. These clones were expanded, and their genomic DNA was isolated. Integrated cDNAs were recovered by PCR with a pair of specific vector primer (GGGGG TGGACCATCCTCTA and CGCGCAGCTGTAAACGGT AG) one hundred and fifty nine cDNA fragments were produced and sequenced after being TA cloned with PCR2.1 vector (Invitrogen).

All the sequences were analyzed with the Editseq program of DNAstar (DNASTAR Inc., Madison, WI, USA) software to find the fusion open read frame (ORF) between egg cDNA and ΔMPL^M, and matched to the Genbank and dbEST databases with the blast program of the National Center for Biotechnology Information (NCBI). The signal sequence prediction was performed using the SignalP3.0 server (Nielsen *et al.* 1997). The internal transmembrane domains were predicted with the TMpred server (Hoffmann and Stoffel 1993). The results of DNA sequence analysis showed that the 159 egg cDNA fragments belonged to 35 kinds of different sequences, each with its own fusion open read frame of the egg cDNA

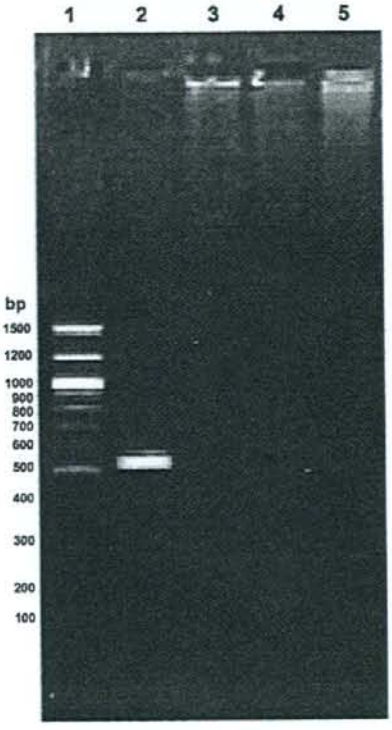

Fig. 1. The PCR product of *Schistosoma japonicum* genomic DNA amplified by gene specific primer of gene possessing signal sequence: Lane 1 – 100 bp DNA molecular weight marker. Lane 2 – the PCR product amplified from genomic DNA of *S. japonicum* by gene specific primer of SjP1183. Lane 3 – the PCR product amplified from genomic DNA of *S. japonicum* by gene specific primer of SjP3742. Lane 4 – the PCR product amplified from genomic DNA of *S. japonicum* by gene specific primer of SjP1531. Lane 5 – the PCR product amplified from genomic DNA of *S. japonicum* by gene specific primer of SjP4001

and ΔMPL^{M}, and encoding a hydrophobic domain at the N-terminal. These sequences could be attributed to two different groups: sequences homologous to schistosome ESTs (31 cDNA fragments), and sequences of genes with known function (4 sequences).

We also found that the internal hydrophobic domain of each gene could function as a signal sequence, an example being the sequence SjP110 (sequence data not published), which encodes the partial amino acid sequence of *S. japonicum* actin; translation was initiated at Met(134^{th}aa) of the actin ORF. The amino acid sequence from 134aa to 154aa produced a positive C and S score as predicted by the SignalP-NN server, implying that the internal hydrophobic domain of this protein could also be able to trapped by the signal sequence trapping method. Thus, the signal sequence of an unknown gene selected by SST must be confirmed further by checking its full length cDNA sequence. To investigate the full length cDNA sequence of each cDNA fragment trapped by SST in this study, we designed and synthesized the gene specific primer and nested gene specific primer following each egg cDNA sequence trapping by SST to amplify the 5' or 3'end sequence of each SST trapped cDNA fragment by a rapid amplification cDNA end method using GeneRacer Kit (Invitrogen). The PCR products were purified and again amplified by nested PCR with the nested gene specific primer. The specific band produced by the nested PCR was sequenced after being TA cloned. The full length cDNA sequences of egg genes were determined by overlapping the 5' end cDNA sequence, SST trapping cDNA sequence and 3' end cDNA sequence of each egg gene possessing a signal sequence. In total, fifteen full length *S. japonicum* egg cDNA sequences were acquired and their signal sequence and internal transmembrane domains again determined with SignalP3.0 and Tmpred; the results were identical to the original prediction

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ATG CAA ATG CTC AAC TTT GTG AAG ATT TCA ACA ATG CTA CTA CTG CCG CAG TTG ATT TCA ACC AAT ACT CAA    72
 M  Q  M  L  N  F  V  K  I  S  T  M  L  L  L  P  Q  L  I  S  T  N  T  Q    24

CAC AAT AAT CCA TAC ACT G GTAAGTTCCTTGATTATTTCAGTGTTTATATAACACAAGTCCCACATTTTGTATATTGAGACCATTAA    158
 H  N  N  P  Y  T                                                          30
TGATATTTCTCATTGTCTCATCAATATCGAATCTAAAAATGCATCAATTAACTTAAATAGGCACATTCATTCATTCGTCAACATGTGACTGGTA    252
AATTAAATTAAGCTGTGGTTATTTTAGAAACACAAACACTTTAATTAATTTGCCTAGTAAGACGGAAATCACAATTACTTTCATGCTAACACTC    346
TGTGAGTTTGAATACAGAGTAGAATGTGATAGTTGTTAAAGAAAAAGTCAATGGCATTGTTTCAATTGATTCTATTTGTTATACATAAGTAGAT    440
TGTCATGTATTAAACAATTTACTTAACATTTTCCAATTTTTCAG TC GTC AGA ATT AAC GTC AAA AGT ATT CGA CTC AAT AAT    519
                                              V  V  R  I  N  V  K  S  I  R  L  N  N    45

AAT CTA GCA AAT ATT GAT GGA AAT AGT AGA CCA GTC TAC CAA TAT GAT AAT GCA GGC GAA ATA AAG GAA CAA    591
 N  L  A  N  I  D  G  N  S  R  P  V  Y  Q  Y  D  N  A  G  E  I  K  E  Q    67

ATA CCC TAC TGT GAA GTA TTT ACG CAA TGG CCG ATG GAT TTA CTT CCA CCA AAC CCA CAA GGG ACT GGA TGT    663
 I  P  Y  C  E  V  F  T  Q  W  P  M  D  L  L  P  P  N  P  Q  G  T  G  C    91

ACG GAT AAT AAC TTT GAA CCA GCA GAA AAA TAT TAT CAC GAA TAT GGA TTA AGA GTA TAT TCA GAA TTG AAA    735
 T  D  N  N  F  E  P  A  E  K  Y  Y  H  E  Y  G  L  R  V  Y  S  E  L  K    115

TAT ATT TAC CCG GAA ATA GTG GAA GTA GAT ATC AAT AAT TTC ATT AAA GAG ATG CGG AAA AGG ATT GAA AAT    807
 Y  I  Y  P  E  I  V  E  V  D  I  N  N  F  I  K  E  M  R  K  R  I  E  N    139

AAA GCA GAT AAC TTA CAA ATG GTT CGA AAT TTG ATA GAG GTT GAA TAT ATG AAT ATT TCG GTA GAA ACA ATA    879
 K  A  D  N  L  Q  M  V  R  N  L  I  E  V  E  Y  M  N  I  S  V  E  T  I    163

ACA CCT GAT GTT CCC TCG AGC AAA CCA ACC AGC ACT CCT GTT ACA AAT GGA AAA ACT GTC TTA ACA ATT TTC    951
 T  P  D  V  P  S  S  K  P  T  S  T  P  V  T  N  G  K  T  V  L  T  I  F    187

GTC AAA ACT GTT AGT TTC AGA GCG AAA GAT GAG AAA GGT GAT GTT CAT CGC GGT GAA TTT TTA GCT AAA TCG    1023
 V  K  T  V  S  F  R  A  K  D  E  K  G  D  V  H  R  G  E  F  L  A  K  S    211

TTA TCA GGC AGA TTT TAT TTA ACC GAA GAA TTC TGT GAT ATC ITT ATG CTA TTC GCT CAA AAG TAT GTT CCC    1095
 L  S  G  R  F  Y  L  T  E  E  F  C  D  I  F  M  L  F  A  Q  K  Y  V  P    235

GTA AAT TGG GGA GAA TTA AGA TGC AAC AAT GTT AGT TCT AAA ATA CTC GGG ACC CGT TCT AAT GAT CTT ATT    1167
 V  N  W  G  E  L  R  C  N  N  V  S  S  K  I  L  G  T  R  S  N  D  L  I    259

TTA CAA AAA GTG TCA CAA TTA CAA TTC ATA TAC ATG AAT GAA GAA AAT TTA GAT AAA GCG AAG TTA GCT GCA 1239
 L  Q  K  V  S  Q  L  Q  F  I  Y  M  N  E  E  N  L  D  K  A  K  L  A  A    283

ACA TTA TAT CAA AAT TAC CGA GAA GGC TAT TTG AAT CAT GAA CGT TCA TTA AAT GAT ATT GAC GAT GTC GAA 1311
 T  L  Y  Q  N  Y  R  E  G  Y  L  N  H  E  R  S  L  N  D  I  D  D  V  E    307

TTC GAA GTT TTA GTT CAA CAT ACT ATT TAG                                    1341
 F  E  V  L  V  Q  H  T  I  *                                              316
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Fig. 2. The genomic DNA sequence graphic of gene SjP1183. The underlined letters present an intron sequence

(Table I). Among them, 11 genes expressed excreted proteins, while 4 were membrane binding proteins.

By aligning the deduced amino acid sequences of these fifteen genes trapped by SST, we found that the signal peptide sequence of gene SjP1531 was the same as gene SjP4001 and similar to genes SjP3842, 3611, and 1084, and the signal peptide sequence of gene SjP3811 was very similar to gene SjP3742 (Table I). To provide insight into the relationship between the signal peptide sequence and mature peptide sequence of these genes, 4 pairs of gene specific primers were designed and synthesized following to flanking sequence of the signal peptide cleavage site of these genes (primer SjP 1183 forward: 5'-TGCTACTACTGCTGCAGTTGATTTC-3', primer SjP1183 reverse: 5'-CGCCTGCATTATCATATTG GTAGA-3', primer SjP3742 forward: 5'-ATAATTGAGATT TTCCTGTGAACGTTTACTC-3', primer SjP3742 reverse: 5'-CAATCGAGCGGAGAAATCACTT-3', primer SjP4001 forward: 5'-TTCTGTGAACGTTTTCGTATCTGAG-3', primer SjP4001 reverse: 5'-ACTGACCCTTTAGGAATCTGA GATT-3') and primer SjP1531 forward: 5'-ACTTTGTGA ATATTTCAACTGTGCTACTT-3', primer SjP1531 reverse: 5'-GGATTCCACTCGACCAAAACTTC-3'). The forward primer was located upstream of the cleavage site of the gene, and the reverse primer was located downstream of the cleavage site of the gene. The genomic DNA of *S. japonicum* (Chinese strain) was amplified with these gene specific primers, a 542 bp specific DNA band was produced when the genomic DNA was amplified with the gene specific primer of SjP1183 (Fig. 1) this band is longer than the expected cDNA length (149 bp) between the forward primer and the reverse primer on the cDNA fragment, and the DNA sequencing data showed that there was an intron of 393 bp between the signal sequence and the mature portion of the gene SjP1183. No DNA band was produced when the genomic DNA were amplified by the gene specific primers of gene SjP1531, SjP4001 or SjP3742 (Fig. 1). These results suggest that the splicing model between the signal sequence and mature portion of gene SjP1183 involves alternative splicing, whereas the signal sequence of gene SjP3742, SjP1531 and SjP4001 might come from independent transcription of mRNA and their splicing model might be due to *trans* splicing (Sutton and Boothroyd 1986), but this requires confirmation. If true, this means that both an alternative splicing model and a *trans* splicing model exist during the transcribing process of egg genes of *S. japonicum* (Chinese strain) simultaneously. As the signal sequence of SjP4001 is same to the one of SjP1531 completely, this phenomenon confirmed further that different gene could share same signal sequence and a same signal sequence could also be spliced with different gene through alternative splicing model or *trans* splicing model.

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