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Clonorchis sinensis tropomyosin: Cloning and sequence of partial cDNA amplified by PCR

Sung-Jong Hong

Department of Parasitology, Gyeongsang National University College of Medicine, Chinju, Kyongnam 660-280, Korea

Abstract: *C. sinensis* total RNA was containing large amount of 18S rRNA but little 28S rRNA. The size of the double-stranded cDNA synthesized from poly (A)$^+$ mRNA was 0.4-4.2 kb long with tapering upto 9.5 kb. Degenerated oligonucleotides (as 2 scnse and 3 antisense primers) were designed on the conserved regions of the known tropomyosin amino acid sequences. From one out of the PCR amplifications using total cDNA and matrix of primers, a specific gene product, 580 bp in size, was produced. Upon Southern hybridization of the PCR products with *Schistosoma mansoni* tropomyosin (SMTM) cDNA, only one signal appeared at the band of 580 bp product. This 580 bp product was considered to encode *C. sinensis* tropomyosin (CSTM) and cloned in pGEM-3Zf(-) for DNA sequencing. CSTM cDNA was 575 bp containing one open reading frame of 191 predicted amino acids, which revealed 86.3% homology with SMTM and 51.1% with *Trichostrongylus colubriformis* tropomyosin. CSTM cDNA obtained will serve as a probe in the studies of molecular cloning of CSTM.

Key words: *Clonorchis sinensis*, tropomyosin, cDNA sequence, PCR, total RNA

INTRODUCTION

Clonorchiasis is endemic at the river basins and the most common fluke infection in Korea. The infected population by *Clonorchis sinensis*, estimated about one million (KAH, 1986), is decreased remarkably when compared with previous reports. Since intradermal test using crude worm-extract was introduced, the crude saline extracts or the protein fractions purified partially from *C. sinensis* have been employed for serological diagnosis of clonorchiasis. These antigenic preparations give high sensitivity but low specificity due to their cross-reactivity with other trematode infections such as metagonimiasis and/or paragonimiasis (Lee *et al.*, 1988). The definitive diagnosis of chlonorchiasis is still made by demonstrating the egg on fecal examination.

Tropomyosin (TM) is a component of the contractile muscle and the cytoskeleton of nonmuscle cells, and mediates Ca^{2+}-dependent actin-myosin contractions (Simillie, 1979). In the adult schistosome, TM occurring as a muscle and nonmuscle form is strongly associated with the tegument (Xu *et al.*, 1989), and their membrane is turned over every 3-6 hours (McLaren, 1980). This membrane shed may provide TM as an antigenic source for the host immune system. Schistosome and chicken TM's had no immunological cross-reactivity (Xu *et al.*, 1989) in spite of the significant homology (46.8%) between the two molecules (Lau *et al.*, 1985). TM is a conserved molecule of human infecting schistosomes such as *S. mansoni*, *S. japonicum and S.*

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haematobium, but the recombinant *S. mansoni* TM shows high species-specificity in serological tests of human schistosomiases (Xu *et al.*, 1991). Therefore, *S. mansoni* TM is suggested to be potential as an immunodiagnostic reagent for schistosomiasis mansoni.

This study was carried out to clone and characterize the *C. sinensis* TM cDNA amplified by PCR, and to compare its homology of predicted amino acid sequence with other species.

MATERIALS AND METHODS

Isolation of poly (A)$^+$ mRNA and synthesis of double-stranded cDNA: Total RNA was extracted by the method of Chirgwin *et al.* (1979) and by some modifications. Briefly, about 1 g of *C. sinensis* adult flukes collected from rabbits were put in 20 ml of 4 M guanidium thiocyanate (GTC) buffer (4M GTC, 0.5% N-laurylsarcosine, 25 mM sodium citrate, pH 7.0, 0.1 M β-mercaptoethanol) and homogenized for 30-60 seconds at full speed in ice bath (Polytron, Sorval). The homogenate was centrifuged for 20 minutes at 11,000 rpm in Beckman High speed fixed angle rotor at 20° C to sediment particulate materials. The supernatant was layered onto 3 ml of 5.7 M cesium chloride and centrifuged in Beckman SW40 Ti rotor for 18 hours at 34,000 rpm and 18° C. The RNA pellet was dissolved in water and precipitated by adding sodium acetate and ethanol. Total RNA was electrophorosed on 1% formaldehyde agarose gel to examine its quality. Poly (A)$^+$ mRNA was seperated from total RNA by three cycles of applying to oligo $(dT)_{\sim 30}$-cellulose spun column (Boehringer Mannheim, Germany) and quantified by spectrophotometry (Sambrook *et al.*, 1989).

Double-stranded cDNA was synthesized using TimeSaver^{TM} kit according to the mannufactor's instruction (Pharmacia P-L Biochemicals, Milwaukee, WI). Briefly, first-strand cDNA was synthesized on 8 μg poly(A)$^+$ mRNA with Moloney Murine Leukemia Virus (MMLV) reverse transcriptase, oligo$(dT)_{12-18}$ primer (0.5 μg) and dNTP's for one hour at 37° C. The reaction was transferred to the second-strand reaction mix containing RNase H and DNA polymerase I and incubated for 30 minutes at 12° C and for one hour at 22° C then heated at 65° C for 10 minutes. At room temperature, the reaction was extracted with phenol and precipitated by ethanol and dissolved in 50 μl TE. To verify the cDNA synthesis, a control reaction initiated independently by adding 0.7 μg of *C. sinensis* Poly(A)$^+$ mRNA and [α-^{32}P] dCTP (Amersham, Buckinghamshire, UK) to the first-strand reaction mix was run simultaneously with the main reaction, and electrophorosed on 1% agarose gel and autoradiographed.

PCR amplification of CSTM cDNA: The degenerated oligonucleotides (Fig. 1) used for

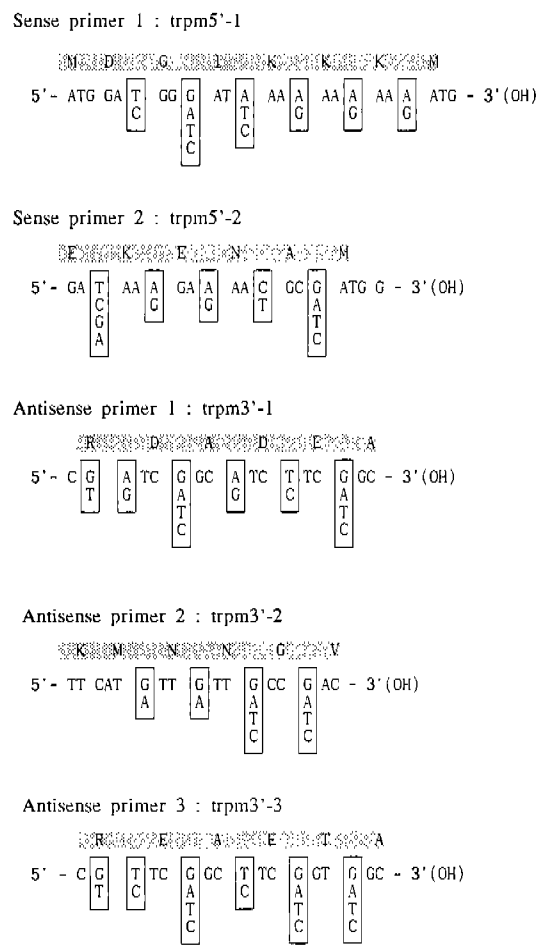

Fig. 1. Degenerated oligonucleotides used as primers in PCR amplification of *C. sinensis* tropomyosin cDNA were designed on the base of amino acid sequences of tropomyosin of *S. mansoni* and the others (Xu *et al.*, 1989).

PCR amplifications were designed on the conserved amino acid sequences of known tropomyosins [TRPM5'-1, (1 to 8, MDGIKKM), TRPM5'-2 (4 to 21, RDADEA), TRPM3'-1 (155 to 160, AEDADR), TRPM3'-2 (200 to 205, VGNNMK) and TRPM3'-3 (239 to 244, ATEAER)] (*Xu et al.*, 1989), and synthesized by Korea Biotech, Inc. (Taejon, Korea). PCR amplification was carried out in 50 μl using 0.04 μg cDNA, standard PCR buffer (Cetus), and 100 pmoles of sense and antisense primers. The reactions overlayed with mineral oil were preheated for 10 minutes at 98° C and cooled to 4° C. After adding 2.5 units of Taq DNA polymerase (Promega Co., Madison, WI) the heat-denatured reaction was subjected to 34 cycles through the following temperature profile: 45° C for 1 minute for annealing, 72° C for 2 minutes for extension and 94° C for denaturation. The PCR products were electrophorosed on 3% low melting or 1.4% agarose gels and stained with EtBr. The PCR product in the agarose gel was denatured for 30 minutes in 1.5 M NaCl/0.5 M NaOH, rinsed in deionized water, and neutralized for 30 minutes in 2 M NaCl/0.1M Tris, pH 7.4 twice then dried on gel dryer at 60° C. The gel dried was probed at 50° C overnight with the cDNA fragment of *S. mansoni* tropomyosin (SMTM) (Xu *et al.*, 1989) radiolabeled by nick translation (Boehringer Mannheim) using [α-^{32}P] dCTP. DNA fractions showing positive signal were recovered from the gel (GeneClean™, BIO 101 Inc., La Jolla, CA), and amplified by secondary PCR with the same primer combination and thermal cycles, and probed again with SMTM cDNA.

Cloning and sequencing of CSTM cDNA: A positive DNA fraction of secondary PCR product was flushed with T4 DNA polymerase, and phosphorylated with T4 polynucleotide kinase, and ligated into plasmid vector pGEM-3Zf(-) (Promega). *E. coli* MV1184 transformed with the ligation reaction containing recombinent plasmid by heat-shock method was plated on LB/amp medium and transferred to nitrocellulose filter, and hybridized with SMTM cDNA at 60° C overnight, and washed under high-stringency condition (Sambrook *et al.*, 1989). The plasmid DNA extracted from positive colonies was double restricted with EcoR I and Pst I endonucleases and fractionated by agarose gel electrophoresis, and probed with radiolabeled SMTM cDNA.

DNA sequencing on both strands was performed by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using Sequenase™ kit according to manufacturer's instruction (USB, Cleveland, OH) with modification. Double-stranded template DNA was prepared by alkali denaturation method (Hattori and Sakaki, 1986). Briefly, 4 μg of template DNA and 40 ng of sequencing primers (T7 and SP6) were used. Sequenase was diluted 1:4 instead of 1:8. To get further sequense, a part of CSTM cDNA was cut out from recombinent plasmid with EcoR I endonuclease and recloned into another pGEM-3Zf(-). The remainder cDNA insert after deletion with EcoR I restriction was religated and subjected to further DNA sequencing. DNA and amio acid sequences were analyzed using program DNAStar (Madison, WI).

Northern hybridization of CSTM cDNA to *C. sinensis* RNA: *C. sinensis* total RNA, 10 μg, was electrophorosed on 1% formaldehyde agarose gel and soaked for 2 hours in water. The gel dried was hybridized with the radiolabeled CSTM cDNA insert at 65° C overnight, washed within 2X SSC/0.1% SDS for 20 minutes at 65° C twice, and dried again for autoradiograhpy.

RESULTS

Poly (A)$^+$ mRNA and double-stranded cDNA: *C. sinensis* total RNA appeared to contain a large amount of 18S rRNA but very small amount of 28S rRNA in formaldehyde gel electrophoresis (Fig. 2). Large amount of poly (A)$^+$ mRNA was eluted from the first application of total RNA to oligo(dT)-cellulose column, and small amount from the second, and undetectible from the third. These fractions were pooled, precipitated with ethanol, and used in cDNA synthesis. Double-stranded (ds) cDNA synthesized was ranged upto 9.5 Kb with a majority between 0.4 Kb and 4.2 Kb in size (Fig. 3).

CSTM cDNA: A specific band, 580 bp in size, was appeared in the PCR product amplified using TRPM5'-2 and TRPM3'-2 oligonucleotides

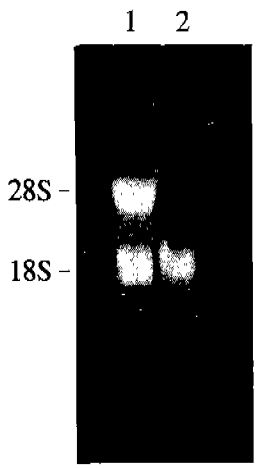

Fig. 2. Total RNA 10 μg each electrophorosed in 1% formaldehyde gel. *C. sinensis* total RNA (lane 2) revealed a thick 18S rRNA band but not 28S r RNA. Rat liver total RNA (lane 1) extracted simultaneously as an RNA extraction control was containing thick and undegraded 18S and 28S rRNA bands.

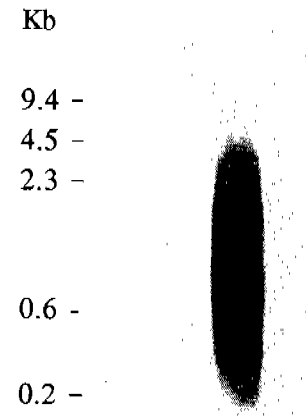

Fig. 3. Autoradiograph of double-standed cDNA synthesized (see MATERIALS AND METHODS) was showing major product ranged in 0.4-4.2 Kb in size. Lambda phage DNA digested with Hind III endonuclease was end-labeled by using Klenow fragment, dATP, dGTP and [α-^{32}P] dCTP, and run as DNA size marker.

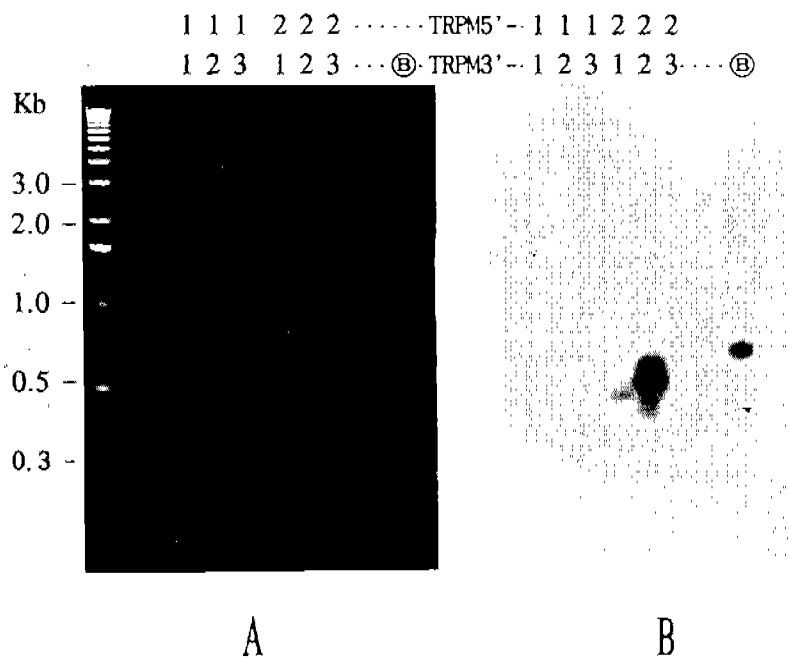

Fig. 4. Panel A; PCR amplification was carried out on *C. sinensis* total double-stranded cDNA using degenerate oligonucleotide primers and the products were electrophorosed on 1.4% agarose gel. A specific band, 580 bp in size, appeared in a lane where sense primer #2 and antisense primer #2 were used. On lane Ⓑ, cDNA fragment of *S. mansoni* tropomyosin (SMTM), 730 bp in size, was run as a positive control. Panel B; The gel was dried and hybridized with radiolabeled SMTM cDNA. A strong signal appeared at 580 bp band.

as sense and antisense primers (Fig. 4A). On hybridization of the PCR products with the radiolabeled SMTM cDNA, 580 bp band produced a strong signal (Fig. 4B) and the cDNA was cloned into pGEM-3Zf(-). Among a large number of positives in colony hybridzation, 5 colonies (CSTMP-1, 2, 3, 4 & 5) producing stronger signal after prolonged exposure were selected for the extraction of plasmid DNA. Of the 5 CSTMP's, cDNA inserts of three clones (CSTMP-1, 2 & 3) were revealed to be positive by double restriction and hybridization with SMTM cDNA (data not shown). These clones were subjected to DNA analysis on both strands by dideoxynucleotide chain termination method with SP6 and T7 sequencing primers.

A sequence of 575 bp obtained from these clones contained 2 internal restriction sequences of EcoR I and Xho I endonucleases (data not shown). The DNA sequences of the 3 clones were identical throughout the insert except for the sense primer region, where CSTMP-2 and CSTMP-3 having C, C and G at nucleotide 3, 12 and 15 instead of G, T and A of CSTMP-1. In DNA sequence of sense primer, clone CSTMP-1 was more close to SMTM cDNA. The DNA sequence and deduced amino acid sequence are shown in Figure 5. According to computer analysis of the DNA sequence, there was only one open reading frame containing 191 amino acids starting at the 14th amino acid of the known tropomyosins (Xu *et al.*, 1989). The predicted amino acid sequence of CSTMP was found to be highly homologous with amino acid sequence of SMTM by 86.3% (Xu *et al.*, 1989) and with that of *Trichostrongylus colubriformis* by 51.1% (Frenkel *et al.*, 1989) (see Fig. 6).

When *C. sinensis* total RNA was hybridized with CSTMP-1 cDNA probe corresponding to encoding residues 14-204 (Fig. 6), a single hybridizing RNA band appeared at about 1.3 Kb in size (Fig. 7).

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GAGAAGGAGAATGCAATGGAAAGGGCTGTTCAGTACGAGGCACAATTGAAGCAGAAA
E K E N A M E R A V Q Y E A Q L K Q K

GAAGAGGAACAGGAGAAGAAAGAAACTGAGATTGGTGAGCTGAACAACCGAATGAAA
E E E Q E K K E T E I G E L N N R M K

CAAGCGCAAACCGAGTTGGATGAGATTCAGGAGGCACTCCAGGACTCGTTGAATAAG
Q A Q T E L D E I Q E A L Q D S L N K

ATGGAAGAGACGGACAAACGGGCGACAAACGCtGAAGCTGAGGTTGCTCGAATGACT
M E E T D K R A T N A E A E V A R M T

CGACGAATTCGGTTGCTCGAGGAAGATTTAGAAGTCTCTTCAAGTCGTCTGACGGAA
R R I R L L E E D L E V S S S R L T E

ACCTTGGCGAAGTTCGATGAAGCTAGTAAGACCGCAGAGGAAAGTGAACGAGGCCGA
T L A K F D E A S K T A E E S E R G R

AAAGAACTCGAAATTCGGTCCATTGCTGATGACGAACGCCTCAGTCAGCTTGAAGAT
K E L E I R S I A D D E R L S Q L E D

CAACAGAAGGAAGCAAAGTATATTGCTGAGGATGCCGATCGAAAATACGATGAGGCT
Q Q K E A K Y I A E D A D R K Y D E A

GCACGTAAACTGGCTATCGTCGAGGTTGATTTCGAACGAGCTGAGTCTCGTCTAGAA
A R K L A I V E V D F E R A E S R L E

GCTGCTGAAAGTAAAATCGTGGAACTTGAAGAGGAACTCCGAGTTGTCGGCAACAACATGAA
A A E S K I V E L E E E L R V V G N N M
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Fig. 5. DNA sequence and deduced amino acid sequence of PCR-amplified *C. sinensis* tropomyosin cDNA (CSTMP-1).

DISCUSSION

Total RNA containing small amount of 28S RNA was obeserved from several parasites such as *Trichinella spiralis*, *Leishmania donovani* and *Plasmodium berghei*, and its integrity was demonstrated to be normal by *in vitro* translation and cDNA synthesis of the selected poly $(A)^+$ mRNA (Grausz *et al.*, 1983; Zarlenga and Gamble, 1987). From the size of ds-cDNA synthesized (Fig. 3) with poly $(A)^+$ mRNA, it is considered that the quality of *C. sinensis* total rRNA containing very small quantity of 28S RNA (Fig. 2) is fairly good.

On PCR amplification with degenerated oligonucleotides, a lower annealing temperature than usual (55° C) was adopted in order to anneal any possible primers which might have some mismatching bases in the middle. As a result, predicted amino acid sequences in primer regions of CSTMP clones were deduced to be same but DNA sequences were different each other at the third position of the genetic codes. Tropomyosin cDNA sequence cloned by using CSTMP as a probe from *C. sinensis* cDNA library, therefore, could be variable in primer regions.

The tropomyosin is a major molecule which regulates muscle contraction through actin-myosin interaction together with troponin complex in muscle and an important

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                1         10         20         30         40         50
SMTM    MDGIKKKMIAMKLEKENAMERAVQYEELLKKKEEEREKRESEIAELNNKMKQAQI
                          ************  ** **** ** * ** **** *****
CSTM    ------------EKENAMERAVQYEAQLKQKEEEQEKKETEIGELNNRMKQAQT
                          ** **  **   *    *  *  *  * *        * *
TCTM    MDAIKKKMQAMKIEKDNALDRADAAEEKVRQITEKLERVEEELRDTQKKMMQTEN


                  60         70         80         90        100        110
SMTM    DCDEAQETLQEQMNKLEETDKRATNAEAEVAAMTRRIRLLEEDLEVSSSRLTETL
                  ** ** **   ** *************** ************************
CSTM    ELDEIQEALQDSLNKMEETDKRATNAEAEVARMTRRIRLLEEDLEVSSSRLTETL
                  **  ** *       **  *    ******   **  **** **    **
TCTM    DLDKAQEDLAAATSQLEEKEKKVQEAEAEVAALNRRMTLLEEELERAEERLKIAT


                 120        130        140        150        160
SMTM    TKLEEASKTAEESERGRKDLEIRSIADDERLNQLEDQQKEAKYIAEDADRKYDEA
                 *  *************** ************ ************************
CSTM    AKFDEASKTAEESERGRKELEIRSIADDERLSQLEDQQKEAKYIAEDADRKYDEA
                 *  **     **** **  *  *  * **    * * ***   ** ********
TCTM    EKLEEATANVDESERVRKVMENRSFQDEERANTIEAQLKEAQMLAEEADRKYDEV


                 170        180        190        200
SMTM    ARKLAIAEVDFERAEARLEAAESKIVELEEELRVVGNNM
                ****** ******** ************************
CSTM    ARKLAIVEVDFERAESRLEAAESKIVELEEELRVVGNNM
                ***** ** * **** * ** * ****************
TCTM    ARKLAMVEADLERAEERAEAGENKIVELEEELRVVGNNL
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Fig. 6. Comparison of homologies between the amino acid sequences of tropomyosin from *C. sinensis* (CSTM), *S. mansoni* (SMTM) and *T. colubriformis* (TCTM). Identities between the two species are indicated by (*).

component of the cytoskeleton in nonmuscle cells. The muscle isoforms consist of 284 amino acids with a molecular weight of 35 kDa and have a highly conserved amino terminal region from the first to the 22nd amino acid (Basi and Storti, 1986). Comparison of predicted amino acid sequence of CSTMP with known tropomyosins shows that it is significantly homologous with SMTM, a muscle isoform, where the sequence from 72 through 204 reveals all but seven to be identical between the two molecules (Fig. 6).

In skeletal muscles, α-tropomyosins have one cysteine residue at position 190 and β-tropomyosins usually at positions 36 and 190. The residue 190 appears to be in the troponin T binding region (Stewart, 1975). There was none of cysteine residue in CSTMP nor *T. colubriformis* tropomyosin (Frenkel *et al.*, 1989), but one cysteine residue was located at unusual position 57 in SMTM molecule. In the tropomyosins of these three parasites, isoleucine substitutes for cysteine at position 190 (Fig. 6). The interaction between troponin T and its binding region missing cysteine in these parasites is unclear.

The mRNA's encoding muscle tropomyosins of invertebrates are 1.25-1.55 kb long including 5'-leader sequence and 3'-untranslated sequence (Hanke and Storti,

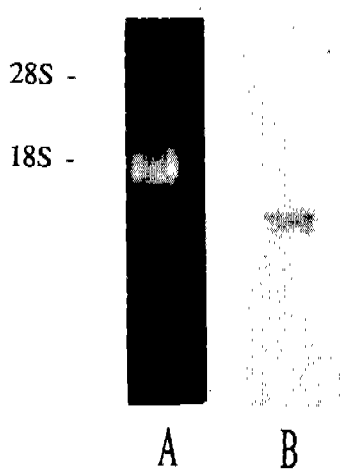

Fig. 7. Northern hybridization of *C. sinensis* total RNA (A) probed with radiolabeled CSTMP-1 cDNA showing a signaling band at approximately 1.3 kb in size (B).

1986; Frenkel *et al.*, 1989; Xu *et al.*, 1989). When hybridized to *C. sinensis* total RNA at high stringency, CSTMP cDNA probe recognized only one RNA band having about 1.3 kilobase nucleotides. From the molecular size, the mRNA is suggested to encode tropomyosin isoform.

CSTMP cDNA clone obtained in this study can be used as a possible homologous probe in the experiments to clone complete cDNA encoding muscle tropomyosins by screening *C. sinensis* cDNA library and as a partial cDNA to expressCSTM peptide.

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=국문초록=

간흡충 tropomyosin: PCR로 일부분 증폭된 cDNA의 cloning 및 염기서열

경상대학교 의과대학 기생충학교실

홍 성 종

간흡충 total RNA에는 많은 량의 18S rRNA가 함유되어 있었지만 28S rRNA는 그 양이 매우 적었다. 약 8 μg의 poly (A)$^+$ mRNA로부터 합성된 double-stranded cDNA는 대부분이 0.4-4.2 kb 크기이었으며 9.5 kb에 달하는 것도 있었다. 이미 보고되어 있는 tropomyosin의 amino산 서열을 기준하여 5개의 degenerated oligonucleotide (sense primer 2개와 antisense primer 3개)를 합성하였다. Total cDNA를 template로 하고 sense primer와 antisense primer를 조합하여 실시한 PCR 산물 중에서 580 bp 크기의 특이 유전자가 나타났다. 만손주혈흡충의 tropomyosin cDNA를 탐색자로 써서 Southern hybridization했을 때 이 유전자만이 검출되어서, 이 유전자는 간흡충 tropomyosin (CSTM) cDNA의 일부분일 가능성이 높다고 생각되어 sequencing vector인 pGEM-3Zf(-)에 cloning한 다음 염기서열을 결정하였다. PCR로 증폭된 CSTM cDNA는 크기가 575 bp이었으며 191개의 predicted amino산 서열은 한 개의 open reading frame을 갖고 있었다. CSTM cDNA의 amino산 서열은 만손주혈흡충 tropomyosin과 86.3%, *Trichostrongylus colubriformis* tropomyosin과 51.1% 의 유사성을 갖고 있었다. 이 CSTM cDNA fragment는 앞으로 간흡충 cDNA library를 screening하여 완전한 CSTM cDNA를 cloning하기에 좋은 probe로 쓰일 것으로 예상된다.

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