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# A single-stranded telomere binding protein in the nematode *Caenorhabditis elegans*

Soo Young Yi, Kyu Sang Joeng, Jai Uk Kweon, Jin Won Cho, In Kwon Chung, Junho Lee*

*Department of Biology, Yonsei University, 134 Shinchon-dong, Seodaemun-ku, Seoul 120-749, South Korea*

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**Abstract** **We identified and characterized a protein (STB-1) from the nuclear extract of *Caenorhabditis elegans* that specifically binds single-stranded telomere DNA sequences, but not the corresponding RNA sequences. STB-1 binding activity is specific to the nematode telomere, but not to the human or plant telomere. STB-1 requires the core nucleotides of GCTTAGG and three spacer nucleotides in front of them for binding. While any single nucleotide change in the core sequence abolishes binding, the spacer nucleotides tolerate substitution. STB-1 was determined to be a basic protein of 45 kDa by Southwestern analyses. STB-1 forms a stable complex with DNA once bound to the telomere. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

*Key words:* Single-stranded telomere binding protein; Gel mobility shift assay; Core sequence; Two-dimensional gel electrophoresis; Southwestern analysis; *Caenorhabditis elegans*

## 1. Introduction

Telomeres are nucleoprotein complexes at the ends of linear eukaryotic chromosomes that are essential for chromosome stability and integrity [1–3]. Telomeres in most species contain repeated short sequence elements, which are five to eight nucleotides long [4]. The G-rich strands of these repeated sequences form a single-stranded 3′ overhang [5,6]. It is known that the mammalian double-stranded telomere DNA region forms a large telomere loop (t-loop), and the 3′ G-rich single-stranded overhang produces a displacement loop (d-loop), serving to protect the telomere termini [7,8].

Many previous reports have shown that telomere functions are achieved through association with specific binding proteins. Telomere binding proteins can be grouped into two subgroups. The first subgroup comprises proteins that bind double-stranded telomeric repeats. One example is RAP1 in the yeast *Saccharomyces cerevisiae*, which has been shown to contain a homeobox-like domain and to be involved in telomere length regulation by studies on temperature-sensitive RAP1 mutants [9–11]. In mammals, TRF1 and TRF2 were identified as proteins that bind human telomere $(TTAGGG)_n$ [12]. These proteins contain Myb-type DNA binding domains. TRF1 is involved in telomere length regulation, and TRF2 in chromosome stability [13,14]. Proteins in the second subgroup are proteins that bind single-stranded telomere sequences. GBP (G-strand binding protein) identified in *Chlamydomonas* is one example. GBP was shown to contain an RNA binding motif, but to specifically bind DNA, not the cognate RNA sequence [15]. CDC13 in the yeast *S. cerevisiae* is another such protein [16]. CDC13 interacts with the catalytic subunit of DNA polymerase α (POL1) and a telomere RNA-associated protein, EST1, thereby contributing to proper telomere replication [17]. Mutations in CDC13 cause decreased interaction with POL1, reducing telomere length. Although single-stranded telomere binding proteins have been identified in other species such as *Chlamydomonas reinhardtii*, rice, and *Vigna radiata* (mung bean) [15,18,19], not much is known about the biological functions of these proteins. To address this issue, we decided to study telomere binding proteins in the model organism *Caenorhabditis elegans*, in which molecular genetic techniques are well established.

*C. elegans* has 4–9 kb of $(TTAGGC)_n$ telomeric repeats at the ends of each chromosome [20]. The telomeres of the nematode are different from those of other species in two aspects. First, the telomere sequence is unique among the animal and plant kingdom in that the nematode has repeats of TTAGGC while all vertebrates have repeats of TTAGGG. Second, the subtelomeric repeats that are usually found in mammals do not exist in the nematode [20]. These differences imply that the *C. elegans* telomere structure and function may be different from those of other species. However, there has been no direct evidence that shows the existence of telomere binding proteins in this model organism. In this study, we wished to characterize protein(s) that bind single-stranded telomere in the nematode. In this study, we have identified activity in *C. elegans* embryonic nuclear extract that binds single-stranded telomere sequences, and have characterized this protein. This report is the first to show straightforward evidence of the existence of a telomere binding protein in the nematode.

## 2. Materials and methods

### 2.1. Culture of C. elegans

The *C. elegans* N2 strain was used as the wild-type strain throughout this study [21]. In order to obtain large amounts of embryos sufficient for preparing nuclear extract, NGM lite agar plates were used. NGM lite medium contains 0.3% sodium chloride, 2% bacto-peptone (Difco), 2% bacto-agar (Difco), 20 µg/l cholesterol, 1 mM calcium chloride, 1 mM magnesium sulfate, 25 mM potassium phosphate (pH 6.0), and 1% agarose. After the plates were dried, the

*Corresponding author. Fax: (82)-2-312 5657.
*E-mail address:* leej@yonsei.ac.kr (J. Lee).

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streptomycin-resistant *Escherichia coli* strain OP50-1 was inoculated on the plates, and the nematodes were cultured at 20°C.

*2.2. Preparation of the C. elegans embryonic nuclear extract*

In order to collect embryos, freshly starved synchronized worms were collected using phosphate-buffered saline (PBS), and harvested by centrifugation at 1600×*g* for 5 min at 4°C. Embryos that remained on the plates after washing off the worms were harvested and frozen immediately in liquid nitrogen until use. The collected worms were washed three times with PBS. Adults were separated by flowing the worms through a 30 μm mesh. Adult worms on the mesh were harvested and treated with bleaching solution (NaClO 3 ml, 5 M KOH 2.5 ml, DW 19.5 ml) for 10 min. After centrifugation at 2000 rpm for 5 min, embryos released from the adult worms were collected and washed three times with PBS, then were frozen in the liquid nitrogen and kept at −70°C until use.

The embryonic nuclear extract was prepared using the method previously described [22]. In brief, homogenization buffer (15 mM HEPES–KOH/pH 7.6, 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM EGTA, 44 mM sucrose, 1 mM dithiothreitol (DTT), and 1×protease inhibitors) was added to 15 g of embryos, and the mixture was homogenized with a motor-driven Teflon pestle. After flowing through miracloth, the debris was rinsed with 30 ml of homogenization buffer. The resulting homogenate was centrifuged in a JA-14 rotor (Beckman) at 8000 rpm for 15 min. The nuclear pellet was washed with 30 ml of homogenization buffer. The net weight of the nuclear pellet was measured and an equal amount of 2×lysis buffer (100 mM HEPES–KOH/pH 7.6, 500 mM potassium acetate, 2 mM EDTA, 2 mM DTT, 20% glycerol, 2×protease inhibitors) was added. The resulting nuclear suspension was transferred to a beaker, and the genomic DNA was removed by stirring with a glass rod for 30 min. Ultracentrifugation was performed in an SW55Ti rotor (Beckman) at 30 000 rpm for 45 min, and the supernatant was carefully transferred to a new tube. The concentration of proteins in the supernatant was measured and the concentration of the *C. elegans* embryonic nuclear extract adjusted to 1 mg/ml.

*2.3. Electrophoretic mobility shift assay (EMSA)*

DNA probes used for EMSA were ordered from BIONEER and end-labeled using [$\gamma$-$^{32}$P]ATP (Amersham) and T4 polynucleotide kinase (USB). The probes were eluted and purified on 15% polyacrylamide gels. DNA competitors used in the assays were also ordered from BIONEER. The RNA $(UUAGGC)_4$ competitor was in vitro transcribed from a DNA construct that contains the T7 RNA polymerase promoter sequence and $(TTAGGC)_4$, and was purified by phenol/chloroform extraction. For the gel shift assay, 4 μg of the nuclear extract in 20 μl of binding buffer (10 mM Tris–HCl/pH 8.0, 1 mM EDTA, 1 mM DTT, 50 mM NaCl, 5% glycerol) was pre-incubated with 500 ng of poly(dI-dC) for 10 min on ice. 0.25 ng of end-labeled DNA probe was added to this pre-incubated mixture, and incubated for 15 min at room temperature. The binding mixture was loaded on an 8% non-denaturing polyacrylamide gel. After drying, the gel was exposed to X-ray film. Competitor DNA or RNA was added to the nuclear extract before adding the radioactive probes. For the proteinase K treatment experiment, proteinase K was added either before or after the probe was added to the nuclear extract.

*2.4. Protein gel electrophoresis of the nuclear extract*

For one-dimensional SDS–PAGE, 10 μg of the *C. elegans* embryonic nuclear extract was used. Before loading on the gel, the mixture was incubated in 2×gel loading buffer (100 mM Tris–HCl/pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol) at 95°C for 5 min. The samples were run on a 12% SDS–PAGE gel at 30–35 mA for 5–6 h. For two-dimensional gel electrophoresis, SDS/DTT lysis buffer (10 mM SDS, 200 mM DTT) was added to 600 μg of the nuclear extract, and incubated at 37°C for 30 min. ES solution (Enhanced Solubilizing solution) (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris, 0.002% BpB dye, 2 mM TBP) was added to the mixture, the mixture was centrifuged at 4°C at 17 000 rpm for 15 min. Carrier ampholytes were added to the supernatant, and a 12 cm IPG strip (pH 3–10, Bio-Rad) was soaked in this mixture overnight. The strip was isoelectrofocused using Multiphore II (Pharmacia Biotech) in the following sequence: 100 V (1 h), 300 V (1 h), 600 V (1 h), 1000 V (1 h), 3000 V (1 h), 5000 V (1 h), and 8000 V (9 h). The second dimensional separation was performed on a 12% SDS–PAGE gel for 5–6 h.

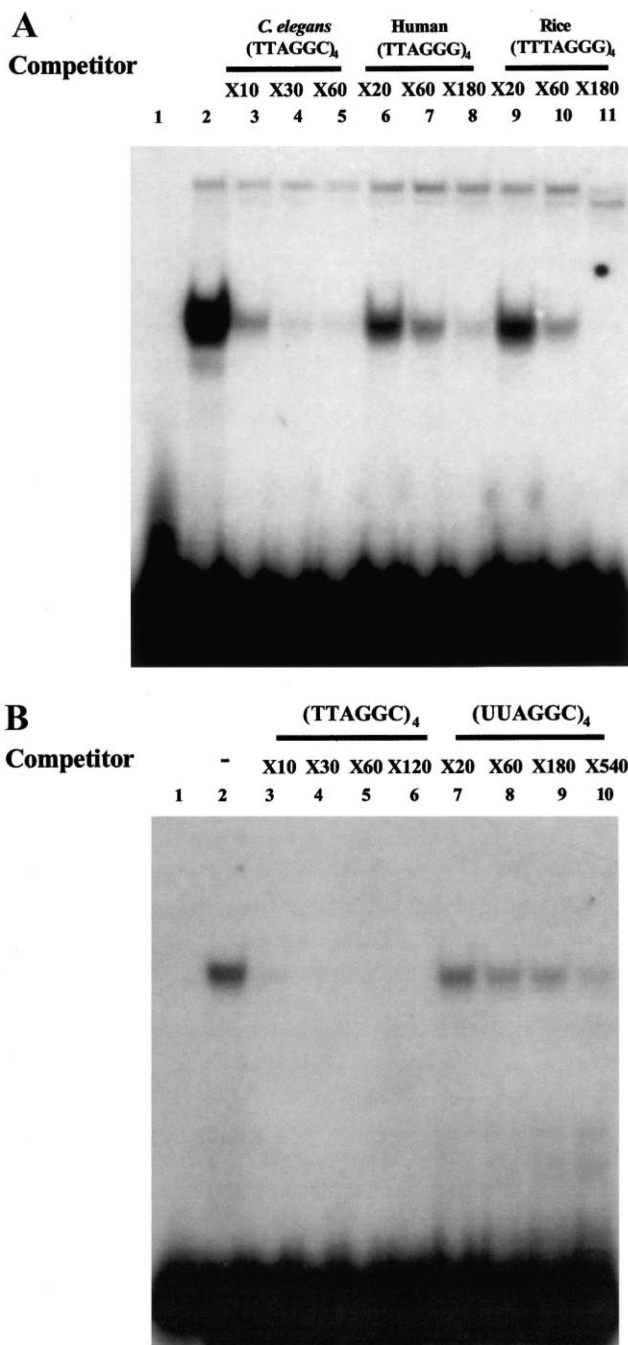


Fig. 1. The *C. elegans* embryonic nuclear extract contains single-stranded telomere binding activity. A: Specificity of STB-1 binding to the nematode telomere sequence. 0.2 ng of $^{32}$P-end-labeled $(TTAGGC)_4$ probe was incubated either with 4 μg of the nuclear extract (lanes 2–11) or without (lane 1). Lane 2, no competitor; lanes 3–5, the *C. elegans* $(TTAGGC)_4$ as competitors; lanes 6–8, human telomere $(TTAGGG)_4$ as competitor; and lanes 9–11, rice telomere $(TTTAGGG)_4$ as competitor. The excess molar amounts of the competitors are indicated above the lanes. Poly(dI-dC) (500 ng) was contained in all reactions as non-specific competitor. The efficiency of the human or rice telomere as competitors of the STB-1 activity was approximately six-fold lower than that of the *C. elegans* telomere (lanes 3, 7 and 10). B: Specificity of STB-1 binding to DNA, but not to RNA. End-labeled $(TTAGGC)_4$ probe was incubated with the nuclear extract in the presence of either unlabeled $(TTAGGC)_4$ competitors (lanes 3–6) or $(UUAGGC)_4$ competitors (lanes 7–10). The excess molar amounts of the competitors are indicated above the lanes. The RNA repeats can be seen to be highly inefficient competitors compared to the DNA repeats.

*2.5. Southwestern analysis*

The protein gels were electroblotted on a nylon membrane and the membrane was incubated in soaking buffer (1% Triton X-100, 20 mM Tris/pH 7.5, 1 mM EDTA, 100 mM NaCl, 5 mg/ml bovine serum albumin, 2 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) at 4°C overnight for protein renaturation. The membrane containing renatured proteins was prehybridized with binding buffer (10 mM Tris–HCl/pH 8.0, 1 mM EDTA, 1 mM DTT, 50 mM NaCl, 5% glycerol, 1% Triton X-100, 0.1 mM PMSF, 5% non-fat milk) at 4°C for 30 min. 1 ng of radio-labeled $(TTAGGC)_4$ probe was added to the binding buffer and incubated at 4°C for 8 h. After washing three times with the binding buffer, the membrane was dried at room temperature, and exposed to X-ray film.

## 3. Results and discussion

*3.1. C. elegans embryonic nuclear extract shows G-rich single-stranded telomere binding activity*

In order to study the functions of telomeres in the nematode, we decided to characterize telomere binding proteins in *C. elegans*, a representative species of the nematode. In this study, we pursued the biochemical characterization of a single-stranded telomere binding protein in *C. elegans*. We first examined whether the nematode nuclear extract contains protein(s) that specifically bind single-stranded telomere sequences. Nuclear extract was prepared from embryos and gel shift assays were performed as described in Section 2. As shown in Fig. 1, the embryonic nuclear extract contained binding activity specific to the *C. elegans* telomere sequence. The nuclear extract showed strong binding activity that was significantly reduced by 10-fold excess of cold competitor consisting of the *C. elegans* telomere sequence. In contrast, human and rice telomere sequences showed much lower competition efficiency (Fig. 1A, lanes 6–11). In order to examine the possibility that this binding activity contains RNA as a working component, we treated the nuclear extract with RNase and examined the binding activity. We found that RNase treatment did not affect the binding activity at all (data not shown).

In some species, single-stranded telomere binding proteins were shown to contain RNA recognition motifs with little RNA binding activity [23]. We examined whether STB-1 shares this biochemical property. The efficiency of the cold RNA competitor sequence was much lower than that of the cold DNA competitor (Fig. 1B). Even a 540-fold excess of cold RNA was not able to compete completely with the binding activity of STB-1. Therefore, we concluded that the nematode STB-1 binds specifically to DNA, but not to RNA.

It has been reported that some single-stranded telomere binding proteins identified in other species were resistant to salt or heat [18,24]. In order to examine the properties of STB-1 in *C. elegans*, we examined its biochemical characteristics. STB-1 was shown to be sensitive to the salt concentration (data not shown). Addition of 0.1 M of LiCl abolished the binding activity in the nuclear extract. Other salts such as NaCl and $MgCl_2$ were similar in this effect (data not shown). STB-1 was shown to be resistant to temperature since binding activity was preserved even after treating the extract at 95°C for 5 min (data not shown). We concluded that the *C. elegans* nuclei contain a nematode-specific single-stranded telomere DNA binding protein that is salt-sensitive and heat-resistant. We named this activity STB-1 (single-stranded telomere binding protein-1).

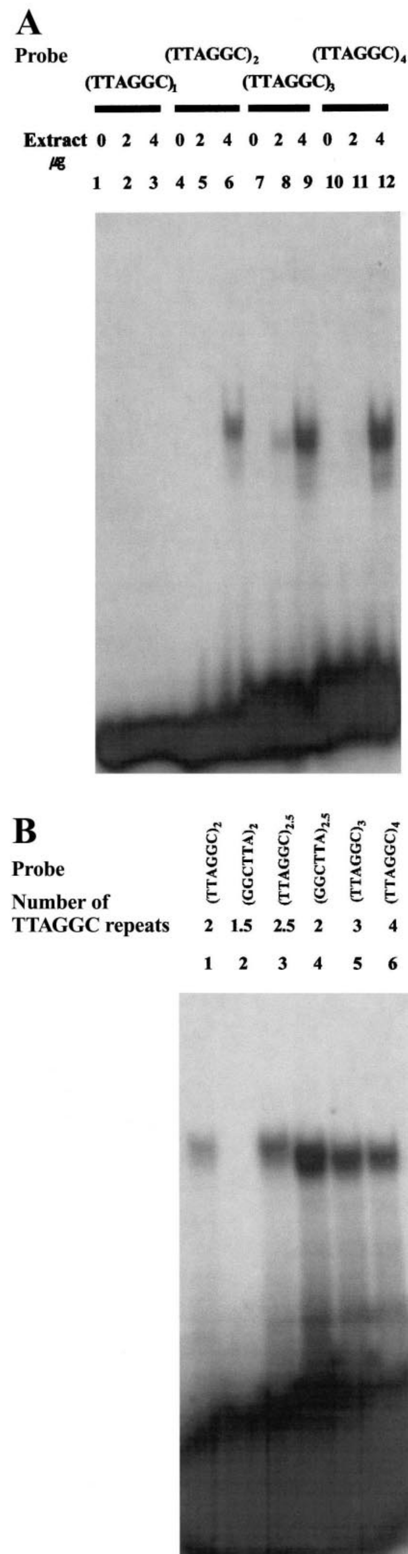


Fig. 2. The minimal copy number of telomere repeats required for STB-1 binding is two copies of TTAGGC. A: Gel shift assays on different copy numbers of the TTAGGC repeat. The amounts of nuclear extract used in the reactions are indicated above the lanes. B: Gel shift assays on different copy numbers of the GGCTTA repeats. The numbers of TTAGGC repeats included in the probes are indicated above the lanes.

### 3.2. STB-1 requires at least two repeats of TTAGGC for efficient binding

The minimal copy number of the telomere repeats needed for binding was determined by gel shift assays using oligonucleotides containing different copy numbers of the telomere repeat. STB-1 did not bind a single copy of TTAGGC, but it efficiently bound two copies of TTAGGC (Fig. 2A), although the binding affinity is somewhat lower than three or four copies of TTAGGC. In order to define more precisely the minimal requirement of the telomeric sequence, we examined the binding ability of oligonucleotides containing different copy numbers of GGCTTA. We found that STB-1 efficiently bound two copies of TTAGGC, but did not bind two copies of GGCTTA, which contain only one and half copies of TTAGGC (Fig. 2B). Therefore, we concluded that two copies of TTAGGC is the minimal requirement for STB-1 protein binding.

### 3.3. Determination of the core sequence for STB-1 binding

We next determined which nucleotides in the two repeats of TTAGGC are essential for STB-1 binding. We designed 12 different oligonucleotides consisting of two repeats of TTAGGC, with mutations of one nucleotide to another in each corresponding position. We found that mutations in any single nucleotide from the fifth through the 11th abolished STB-1 binding ability, indicating that these nucleotides are the core sequence of STB-1 binding within which the STB-1 binding site resides (Fig. 3A). It is worth noting that the mutation of C, which is a unique nucleotide that exists only in the telomere of the nematode, also abolishes STB-1 binding completely. This fact may imply that the nematode telomere binding protein has a different structure from those in other species.

One apparent conflict in our results described above was that although two repeats of GGATTC also had an intact core binding sequence, it did not have any STB-1 binding ability. This implies that the core sequence may be required for STB-1 binding activity, but is not sufficient. One possible explanation for this apparent conflict is that the core sequence may need spacer nucleotides, which do not have to be specific. To examine this hypothesis, we designed and examined several oligonucleotides containing spacer nucleotides in front of or behind the core sequence for binding capability (Fig. 3B). All the oligonucleotides used in this assay contained 12 nucleotides and the core sequence GCTTAGG. Placing up to five extra nucleotides behind the core sequence did not rescue the binding capacity of the core sequence (Fig. 3B, lanes 1–3), and one or two extra nucleotides in front of the core sequence were also not efficient. In contrast, adding three extra nucleotides in front of the core sequence, regardless of whether they are identical to the telomere sequence, restored the binding capability of the core sequence (Fig. 3B, lanes 4 and 5). This result clearly shows that the core sequence is not sufficient for STB-1 binding, but needs at least three extra spacer nucleotides in front of it. It was also noted that the original telomere spacer sequences conferred more efficient binding capability than random nucleotide sequences (in this case, CGA instead of TAG). The reason for this is not clear at this point, but a possibility is that the original telomere sequence works better at stabilizing the DNA–protein complex.

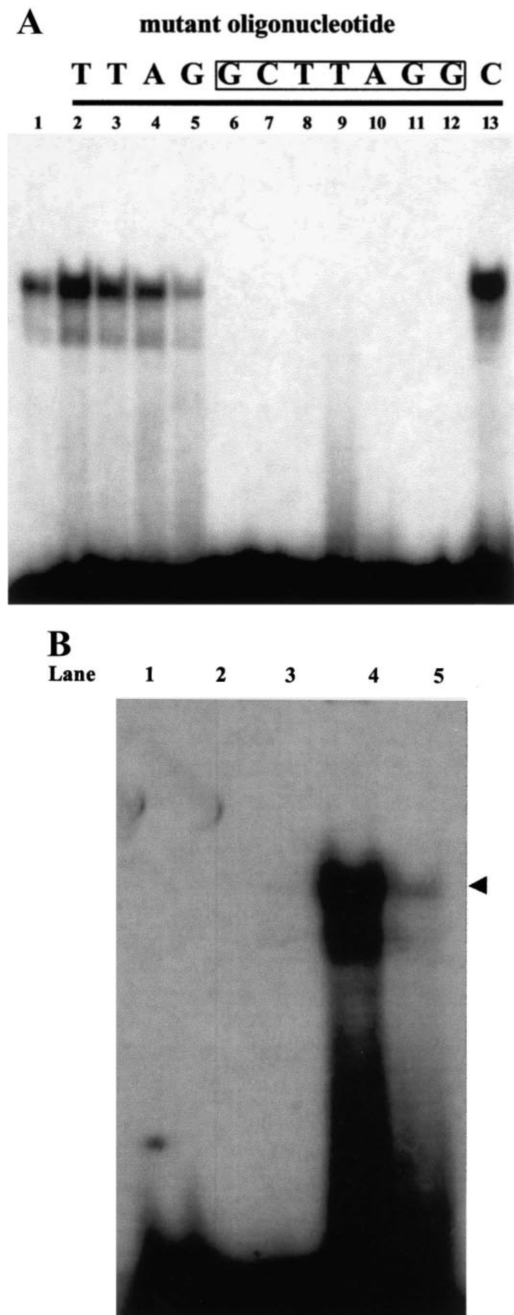


Fig. 3. The core sequence required for STB-1 binding. A: Mutation analysis of each nucleotide within two repeats of TTAGGC. The probe used in lane 1 contains the wild-type sequence of TTAGGCTTAGGC. The probes in lanes 2–13 contain single nucleotide changes from the first to the 12th nucleotide respectively. In each mutated position, T was changed to G, A to C, and G to T. For example, the probe in lane 6 contains the sequence TTAGtCTTAGGC, in which the fifth nucleotide was mutated from G to T. B: Requirement of spacer nucleotides in front of the core sequence for efficient binding. The following are the sequences of the probes used. Lane 1, GCTTAGGCTTAG. This probe, which has five spacer nucleotides behind, but no spacer in front of the core, does not show any binding capability. Lane 2, GGCTTAGGCTTA; lane 3, AGGCTTAGGCTT; each probe has one or two spacers in the front, and four or three in the back, respectively. These probes do not show any binding capability. Lane 4, TAGGCTTAGGCT; lane 5, CGAGCTTAGGCT. These probes can bind the nuclear extract.

### 3.4. STB-1 is a protein of about 45 kDa

As a first step towards the molecular characterization of STB-1, we determined the molecular weight of STB-1 by Southwestern analysis. Southwestern analysis was first performed using the embryonic nuclear extract. By probing a one-dimensional separation of the embryonic nuclear extract with a radioactive telomere sequence, we identified a single band of about 45 kDa (Fig. 4A). We next performed two-dimensional gel electrophoresis followed by hybridization with the telomere probe. Three spots of the same size with different p*I* values were found to hybridize with the probe (Fig. 4B). The p*I* values of the proteins ranged from pH 8 to 10. These results of one- and two-dimensional gel electrophoresis and Southwestern analysis prove the existence of one major single-stranded telomere binding protein in *C. elegans*, which is a basic protein and may be subject to posttranslational modification such as phosphorylation. Our two-dimensional results can be interpreted as an indication that the spots on the two-dimensional gel with identical or similar molecular weight but different p*I* values represent identical proteins with different degrees of modification. It will be interesting to examine changes, if any, in the states of posttranslational modification of this protein in different cell cycles or in different developmental stages. We obtained identical Southwestern results using extract from mixed-stage animals, indicating that the same protein works in both the embryonic and postembryonic stages (data not shown).

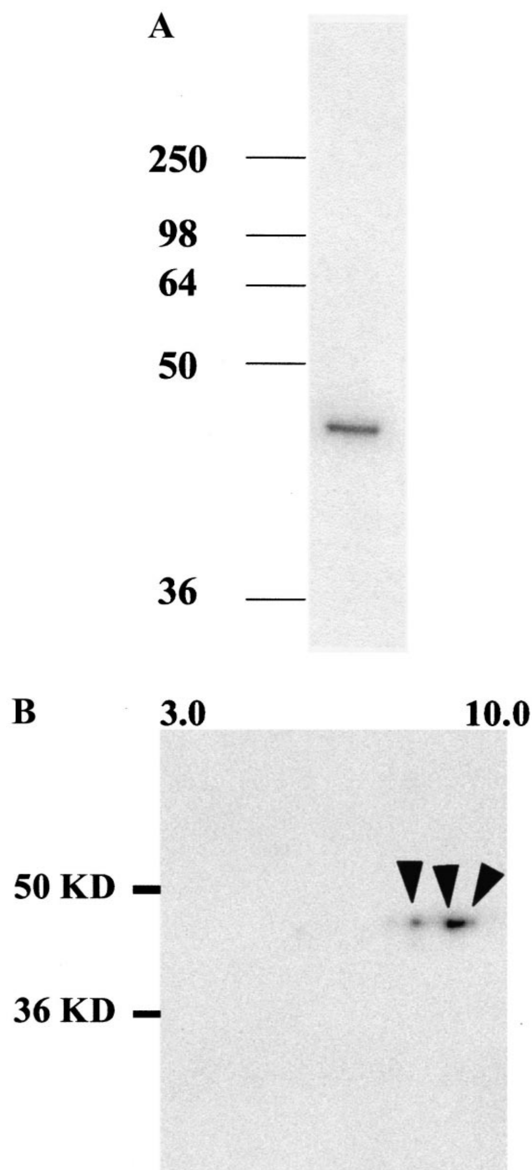


Fig. 4. STB-1 is a basic protein of 45 kDa. A: Southwestern analysis of a one-dimensional gel. A single band of approximately 45 kDa hybridized to the radioactive telomere probe. The sizes of the molecular weight markers are indicated on the left of the blot. B: Southwestern analysis of a two-dimensional gel. Three spots of approximately 45 kDa, which are indicated by arrowheads, hybridized to the radioactive telomere probe. These three spots are thought to represent an identical protein with different degrees of posttranslational modification.

Fig. 5. STB-1 forms a stable complex with DNA. The nuclear extract was treated with proteinase K either after adding the radioactive telomere probe (lane 3), or before adding the probe (lane 4). Lane 1, probe only; lane 2, without proteinase K treatment.

### 3.5. STB-1 forms a stable complex with DNA

Since binding by STB-1 to telomere was highly sensitive to the salt concentration, we wanted to know whether binding by STB-1 per se is weak. We treated the nuclear extract with proteinase K either before or after adding the telomere probe and examined the binding activity of the extract. When treated with proteinase K after adding the telomere probe, the extract still contained a protein that binds the telomere, while the extract treated with proteinase K before adding the probe did not. The size of the binding protein after proteinase K treatment was smaller than that of the protein without proteinase K treatment (Fig. 5). This result indicates that STB-1 can form a rather strong DNA–protein complex and that only specific regions of the protein participate in this binding. It is notable that the DNA–protein complex is strong enough so that even proteinase K is inaccessible to STB-1 once the telomere binds to the protein.

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