Molecular Characterization of a TIA-1-Like RNA-Binding Protein in Cells Derived from the Fall Armyworm Spodoptera frugiperda (Lepidoptera: Noctuidae)

Sayaka MUTO, Toru TANABE, Emi MATSUMOTO, Hajime MORI, and Eiji KOTANI

Graduate School of Science and Technology, Kyoto Institute of Technology, Matsugasaki, Sakyo, Kyoto 606-8585, Japan

Received October 6, 2008; Accepted December 4, 2008; Online Publication, March 7, 2009

A complementary DNA encoding a TIA-1-type RNA-binding protein (SfTRN-1) was isolated from cultured cells of the fall armyworm, Spodoptera frugiperda (Lepidoptera: Noctuidae), to characterize its function. The deduced 388-amino acid sequence of SfTRN-1, which possessed three RNA recognition motifs (RRMs) followed by a C-terminal auxiliary domain, showed significant homology with mammalian TIA-1/TIAR and silkworm BmTRN-1, factors important in the metabolism of transcripts. It was found that inhibition of SfTRN-1 gene expression by a transfected oligonucleotide encoding the antisense sequence led to a marked increase in the production of a reporter protein and the amount of reporter transcript in the cultured cells. In addition, overexpression of the recombinant full-length SfTRN-1 open reading frame in the cultured cells led to a decrease in reporter protein production, but the truncated RRM1-3 domain lacking the C-terminal auxiliary domain lost its activity. Analysis using a GFP-fused recombinant protein revealed that, unlike mammalian TIA-1/TIAR, SfTRN-1, most likely shuttling between the nucleus and cytoplasm, had the characteristic of being largely distributed in the cytoplasm, where it perhaps acts to reduce the amount of transcripts, and that RRM1 and RRM3 were related to its nuclear accumulation, but RRM2 to its nuclear export. Furthermore, the posterior half of the auxiliary domain was also found to be related to its nuclear export. This study indicates that respective RRM subdomains of SfTRN-1 play distinct roles important to its subcellular distribution, and it identified unique systems for the distribution and functional regulation of the TIA-1 family in insect cells, ones which are clearly different from those in mammalian cells.

Key words: regulation of transcripts; RNA-binding protein; TIA-1; Spodoptera frugiperda

RNA-binding proteins possessing RNA recognition motifs (RRMs) have been found in various species. The TIA-1 family has been identified as U-rich RNA-binding proteins involved in multiple aspects of RNA metabolism. In mammalian cytoplasm, TIA-1 family proteins participate in the cellular response to severe environ-
Lepidopterous insects have been utilized in the production of proteins. Hence, baculovirus vector systems in insect cells and individuals have been widely applied in the production of certain recombinant proteins.12 Although efforts have focused mainly on the improvement of viral vectors or plasmids with a strong promoter for transcription, less is known about how the host’s cellular mechanisms regulate and affect the flow of protein synthesis. Hence, it is important to elucidate transcriptional regulation in the host cells, including the RNA-binding host protein function.

In order to determine the function of these RNA-binding proteins, we also isolated the cDNA of a TIA-1-like gene using PCR with Rox8 and BmTRN-1 sequence information, and analyzed the complete nucleotide and deduced amino acid sequences of TRN-1 (SITRN-1) from cells of another lepidopterous insect, Spodoptera frugiperda (Lepidoptera: Noctuidae). The present study indicates that inhibition of the SITRN-1 gene in a Spodoptera cell line using an antisense oligonucleotide increased the production of the reporter protein and transcripts derived from co-transfected vectors, whereas overexpression of SITRN-1 induced a reduction in foreign reporter protein production. In addition, analysis using GFP-fused recombinant proteins revealed that SITRN-1 is largely distributed in the cytoplasm of cultured Spodoptera cells and that its RRM subdomains play different roles in its nuclear accumulation and export. Based on these results, we discuss the involvement of SITRN-1 in post-transcriptional processes in insect cells under normal conditions with no environmental stress.

Materials and Methods

Insect cell line. Spodoptera frugiperda (Lepidoptera: Noctuidae)-derived Sf21 cells were cultured at 27°C in ESF921 medium (Expression Systems, Woodland, CA) supplemented with 14% (v/v) fetal calf serum (Gibco BRL, Grand Island, NY). Sf9 cells derived from the same insect were cultured at 27°C in Grace’s insect medium (Gibco) supplemented with 10% (v/v) fetal calf serum (Gibco).

Cloning of the complete cDNA for SITRN-1. Cloning of the cDNA for a TRN-1-related gene from the cultured cells was carried out using a combination of primers encoding partial cDNA sequences of Bombyx mori BmTRN-113 especially homologous to the corresponding sequence of Drosophila Rox8.14 Single-stranded cDNA was obtained using a ThermoScript™ RT-PCR System (Invitrogen, Carlsbad, CA) with 0.1 μg of heat-denatured total RNA and the oligo-dT primer for 60 min at 65°C. Thirty-five cycles of PCR with 0.2 μl of primer (sense primer, 5′-CTGGCCGCGCATGAA-3′; antisense primer, 5′-CGGTTT-GACCGATT-3′) and recombinant Taq polymerase (Toyobo Biochemical, Osaka), were performed at 57°C for annealing. An approximately 0.3-kbp fragment electrophoretically detectable in the PCR mixture was found by sequencing to encode a significantly homologous nucleotide sequence of BmTRN-1. The full-length sequence was analyzed with a Gene Racer 5′ RACE and 3′ RACE kit (Invitrogen). In 3′ RACE, PCR was performed using a gene-specific primer (5′-GAAGGCCTGACGCGAGAGGCTATT-3′), the kit’s 3′ primer (5′-GGCAGCGCCGCTAGTATG-3′), and oligo-dT-primed cDNAs at an annealing temperature of 65°C. In 5′ RACE, PCR was performed using a gene-specific primer (5′-GGCTTCGTGACGGCTTCTTGCAGAAAGA-3′) and the kit’s 5′ primer (5′-GGCACTGACAGTGACTGAAGGATG-3′) at 63°C for annealing.

Preparation of antibody using recombinant SITRN-1. PCR-amplified DNA encoding the complete ORF of SITRN-1 plus 6-his-polystyline with an Nhe I site and a BamH I site was inserted into the Nhe I-BamH I site of the pET11 vector (Novagen, Madison, WI). A bacterial culture of Escherichia coli BL21(DE3) transformed with the recombinant plasmid was grown in LB medium to an OD600 of 0.6 and adjusted to contain 1 mM IPTG. After incubation with shaking for 5 h at 37°C, bacterial cells were lysed with a 6 M urea-containing buffer (20 mM sodium phosphate, 0.5 M NaCl, and 5 mM imidazole, pH 7.4). The recombinant SfTRN-1 was purified with an Ni2+-chelating resin column (Novagen). Both the non-denatured recombinant protein and the recombinant protein eluted from the SDS–PAGE gel were used to immunize female mice by intramuscular injection 3 times at 10-d intervals, followed by preparation of the anti-serum. An IgG fraction was prepared by affinity chromatography using a protein A-sepharose column (GE Healthcare).

Knock-down of SfTRN-1 protein expression in Spodoptera cells using antisense oligonucleotide. The plasmid pLEX4 (Novagen) encoding a fusion protein comprising chloramphenicol acetyl trans- ferase plus the paramyxovirus SV5 epitope and a His6-tag at the C-terminus as a reporter (CAT/SV5-His6) under the control of the IE1 promoter was used to investigate the effect of the antisense oligonucleotide of SfTRN-1. An antisense oligonucleotide encoding 5′-GGCTGCGGCAGACTCCGACATT-3′ at nt. 370–391 of SfTRN-1 with a phosphortioate modification at its 5′ end was used as an antisense inhibitor, since it was the most effective of the pre-tested oligonucleotides for different portions of the ORF. Phosphothioated oligonucleotides encoding the sense strand sequence of the same portion of SfTRN-1, and a partial EGFP sequence, 5′-CATCTCGTGC- GAGCTGACG-3′, were used as a sense strand control and as a mock control respectively unrelated to the Spodoptera frugiperda gene sequence. After 0.5 μg of plasmid DNA, 0.5 μg of the oligonucleotide DNA to be assessed, and 3 μl of FuGENE™ HD transfection reagent (Roche Diagnostics, Mannheim) were mixed in 100 μl of ESF921 medium, each mixture was incubated for 25 min at 25°C and then added to a 1.5–ml culture of 0.7 × 10⁷ monolayer cells on plastic plates (35 mm in diameter). Following 5 h of incubation at 25°C, the culture medium in each plate was replaced with 1.5 ml of ESF921 containing 14% fetal calf medium. A high level of efficiency (approximately 97% of insect cells evenly took up the modified oligonucleotide with a biotin-labeled 3′ end under the conditions), was verified by in situ streptavidin-HRP staining, as described by Kotani et al.10

Protein from each cell plate was dissolved in 200 μl of sample buffer. Ten micrograms of protein in 3 μl of each sample was electrophoresed on a 12% SDS–PAGE gel and electroblotted to a PVDF membrane (Hybond™-P, GE Healthcare). For detection of CAT/SV5-His6, Western blotting using a mouse-anti V5 antibody (Invitrogen) that specifically reacts with the SV5 epitope was carried out in antibody buffer (antibody:buffer = 1:5,000). The result obtained using secondary antibody conjugated with HRP was visualized with an ECL detection system (GE Healthcare).

Analysis of reporter mRNA in Spodoptera cells. Total RNA was extracted from the transfected Spodoptera cells with the SV Total RNA Isolation System (Promega, Madison, WI), which almost completely removed the transfected plasmid DNA using DNase I during handling. The isolated total RNA was heated for 10 min at 65°C and then placed on ice for 2 min. Synthesis of cDNA from total RNA was carried out with a First-Strand cDNA Synthesis Kit (GE Healthcare). Seven microliters of the kit’s bulk first-strand cDNA reaction mixture, 1 μl of 200 mM DTT solution, 1 μl of pd(N)6 primer (0.2 μg/μl mixture), and 1.0 μg of heat-denatured RNA were added to a sterile 1.5-ml microcentrifuge tube to synthesize the first-strand cDNA, and this was incubated for 60 min at 37°C. Triplicate cDNA samples were subjected to real-time PCR analysis using sense primer 5′-CACC- TTGTACACGATTTCC-3′ and antisense primer 5′-CACCGTAACA- GCCCATATGTG-3′ specifically to detect the CAT reporter gene. Real-time PCR was carried out in 96-well plates with a 50-μl reaction volume containing 25 μl of 2× SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 10 ng of cDNA, and 0.2 μl of each forward and reverse primer. Forty cycles of amplification (94°C for 15 s and 60°C for 60 s) were performed using an ABI Prism 7000 sequence detection system (Applied Biosystems). Sequence-specific amplification was detected as an increase in the fluorescence of SYBR Green during the cycles. Expression was normalized against 28S
ribosomal RNA as an endogenous reference. Specific amplification of the 28S ribosomal RNA was performed using sense primer 5'-ACC-C CCCGATTCAAGGATCAGC-3' and antisense primer 5'-GTGTGTT-CAAGACGGTGCTCCG-3', synthesized based on the nucleotide sequence of Spodoptera 28S rRNA as reported by Abraham et al.\(^{(13)}\)

**Overexpression of the SfTRN-1 protein and several truncated SfTRN-1-derived proteins, fused with GFP protein.**

1. **DNA constructs.** For overexpression in Sf9 cells of the artificially designed proteins, N-terminal GFP plus C-terminal SfTRN-1-derived sequences were inserted downstream of the *Orgyia pseudotsugata* NPV IE2 promoter in insect expression vector pIZ/His-V5 (Invitrogen). The recombinant vectors were constructed by inserting the full-length SfTRN-1 amino acid sequence (a.a.1–388), or a truncated sequence such as the RRM1-3 domain (a.a.1–283) or the C-terminal auxiliary domain (a.a.284–388), into the site between EcoRI and XhoI, and the full-length sequence of improved GFP from the start Met to the C-terminal codon, as described by Crameri et al., at the EcoRI site.\(^{(14)}\) Also, by PCR methods, we constructed recombinant plasmids including the designed insert encoding modified GFP-full-length SfTRN-1 fusion proteins lacking characteristic subdomains such as RRM1 at amino acid positions 6 to 90 (designated RRM1), RRM2 from 94 to 181 (ARRM2), RRM3 from 189 to 283 (ARRM3), the anterior half of the auxiliary domain from 284 to 336 (AA1), and the posterior half of the auxiliary domain from 337 to 388 (AA2). These plasmids were cloned in the bacterial cells, and purified from the bacterial culture using a Genopure Plasmid Midi Kit (Roche).

2. **Transfection method.** The DNA constructs were transfected into Sf9 cells as follows: 25 min after 1.2 μg of plasmid DNA dissolved in 100 μl of Grace’s insect medium was mixed with the Fugene\(^{TM}\)HD transfection kit, the mixtures were added to the medium without the serum in which an Sf9 cell monolayer (0.7 × 10\(^5\) cells per each 35 mm culture dish) was formed. The medium in each dish was then changed to a new one with the serum, and the cells were further cultured in the dish.

To investigate the effect of full-length and that of truncated SfTRN-1 protein overexpression on the amount of the gene transcript, dish.

iii) **Poly(U) RNA-binding of the recombinant proteins.** To investigate the effect of full-length and that of truncated SfTRN-1 protein overexpression on the amount of the gene transcript, 0.5 μg of plasmid DNA encoding full-length or truncated SfTRN-1 fused with N-terminal GFP and GFP as a control was used to co-transfect Sf9 cells with 0.1 μg of reporter CAT/SV5-His6-producing pIEX4 under transfection conditions similar to those described above. A resulting CAT/SV5-His6 protein band was detected by Western blotting using the anti-SV5 antibody, as described above.

iv) **Fluorescence microscopy.** The subcellular distribution of GFP-fused recombinant proteins was analyzed 36 h after transfection. Cells grown on coverslips were washed with PBS, fixed with 4% paraformaldehyde for 40 min at room temperature, washed 3 times with PBS for 5 min, washed 2 times with PBS containing 25 mM glycine for 5 min, permeabilized with chilled methanol, and washed with PBS containing 0.3% Triton X-100. The cells were counterstained with propidium iodide (1.0 μg/ml, Invitrogen) and mounted with VECTASHIELD (Vector Laboratories, Burlingame, CA). They were then observed by confocal fluorescence microscopy (LSM510, Carl Zeiss Micromaging, Thornwood, NY).

Quantification of cytoplasmic and nuclear fluorescence was done by integrating the signal in identical surface units taken in each subcellular compartment, under the assumption that the nuclear and cytoplasmic compartments of the Sf9 cells were of similar surface.

This analysis was performed using at least 20 cells, and the average ratio of cytoplasmic and nuclear fluorescence was plotted as a percentage.

**Results**

**Primary structure of SfTRN-1**

In order to identify the gene of the RNA-binding protein that belongs to the TIA-1 family and putatively regulates the intracellular transcripts, effective homologous cloning was performed by the PCR method using a pool of single-stranded cDNA from the *Spodoptera frugiperda* cells and primers with the BmTRN-1 nucleotide sequences encoding the amino acid sequences also conserved in *Drosophila* Rox8. An initial clone was obtained, and by employing RACE methods for both ends of mRNA, the complete cDNA sequence was determined, from which the corresponding amino acid sequence was deduced (Genbank accession no. AB331732). The cDNA sequence of SfTRN-1 had an ORF containing 1,164 nucleotides, in which a protein with an estimated molecular mass of 42.0 kDa consisting of 388 amino acids was encoded.

The data from protein comparison revealed that a large part of the SfTRN-1 amino acid sequence, from the N-terminus to position 283, was comprised of three repetitive sequence domains showing significant homology to the N-terminal RNA-recognition motifs, designated RRM1, 2, and 3, of TIA-1 family members such as fruit fly Rox8,\(^{(15)}\) human TIA-1,\(^{(15)}\) and TIAR,\(^{(16)}\) in addition to a 42.5-kDa isoform of the silkworm BmTRN-1,\(^{(10)}\) as shown in Fig. 1A. The entire RRM1-3 domain of SfTRN-1 showed 95.1% identity with that of BmTRN-1, 74.6% with that of Rox8, 59.0% with that of TIA-1, and 57.6% with that of TIAR. In the RRM1-3 region of SfTRN-1, there were fully conserved residues, Tyr\(^{1}\), Tyr\(^{148}\), Phe\(^{226}\), Phe\(^{360}\), Tyr\(^{364}\), Phe\(^{367}\), and Tyr\(^{380}\), which would be essential aromatic residues in RNP1 and RNP2 of the RRMs for binding RNA, exclusive to the TIA-1 family among RRM-type RNA-binding proteins. A phylogenetic tree, as shown in Fig. 1B, was constructed by the neighbor-joining method using CLUSTAL X software,\(^{(17)}\) showing that SfTRN-1 is clustered with other TIA-1 family members rather than other RRM-type RNA-binding proteins such as marmalian PABP1,\(^{(18,19)}\) and the splicing factors. The various complete primary structures presented contain C-terminal auxiliary domains which might be important to the unique functions of TIA-1 family protein, but the portion from position 284 to the C-terminus of SfTRN-1, corresponding to the putative C-terminal auxiliary domain, showed relatively weak homology with that of other TIA-1 family members as compared to the RRM1-3 region, exhibiting 81.9% identity with that of BmTRN-1, 31.4% with that of Rox8, 19.0% with that of human TIAR, and 16.2% with that of human TIA-1. Western blot analysis using an antibody raised against the SfTRN-1 ORF revealed that the authentic SfTRN-1 was a single protein with a molecular mass of 42.0 kDa (Fig. 2).

Recently, we reported in a sister paper describing TRN-1 function in *Bombyx mori* cells\(^{(20)}\) that there are two isoforms of BmTRN-1, the 44.1- and 42.5-kDa isoforms, which were almost equally expressed in every
tissue and cell from a single copy gene. These isoforms with auxiliary domains of different molecular sizes were generated through alternative splicing identified by genomic DNA analysis. Also, analyses of human and mouse TIA-1/TIAR genomes showed that they had isoforms with different RRM sequences resulting from alternative splicing, but so far, we have been able to identify only a single TRN-1 protein in Sf21 and Sf9 cells. Based on this evidence, it was suggested that different transcriptions of TIA-1 families with and without alternative splicing occur in different species.

Regulatory role of SfTRN-1 in protein production in cells

To elucidate the role of TRN-1 in Spodoptera cells, we investigated the production of a foreign chloramphenicol acetyl transferase plus SV5 antigen and a His6 tag (designated CAT/SV5-His6), as a reporter encoded in the transfected plasmids under cellular conditions in which SfTRN-1 protein expression was artificially inhibited by co-transfection of an oligonucleotide encoding the antisense SfTRN-1 cDNA sequence. As shown in Fig. 3A (upper panel), by Western blotting using the specific antibody, the protein level of authentic SfTRN-1, with a molecular mass of 42.0 kDa, was reduced due to the existence of the antisense TRN-1 nucleotide, although SfTRN-1 expression was not affected by mock-transfection or transfection with the oligonucleotide encoding the sense sequence of SfTRN-1, revealing that the antisense oligonucleotide of SfTRN-1 was effective in producing cells with impaired SfTRN-1. Consequently, a marked increase in the production of CAT/SV5-His6 protein in the cells, while authentic SfTRN-1 expression was reduced, was found as compared to the control cells transfected with the sense oligonucleotide and mock-transfected cells (Fig. 3A, lower panel). Densitometric analysis using
shown). It was confirmed, in the results shown in agarose, but not to poly(G) RNA-agarose (data not shown). SfTRN-1 had the ability to bind to poly(U) RNA-recombinant SfTRN-1 showed that authentic full-length protein of SfTRN-1 was detected using anti-SfTRN-1 IgG by the method described in “Materials and Methods.” A 14.4 to 97.4 kDa protein ladder was used as a size standard.

Computer-aided imaging showed the relative CAT/SV5-His6 protein signal strengths in equal amounts of sample proteins of mock-transfected, SfTRN-1 sense oligonucleotide-transfected, and SfTRN-1 antisense oligonucleotide-transfected cells to be 7661.91 ± 41.57, 5452.72 ± 34.13, and 21997.87 ± 82.63 respectively. Further, we investigated the level of CAT/SV5-His6 transcript by real-time PCR during inhibition of SfTRN-1 (Fig. 3B). The relative amount of transcript increased approximately 4-fold in the cells transfected with the SfTRN-1 antisense oligonucleotide as compared to the control cells. This indicates that the level of CAT/SV5-His6 transcript was increased by the knockdown of SfTRN-1.

In addition, we investigated the effects of overexpressed recombinant proteins such as GFP-fused full-length SfTRN-1 (GFP-SfTRN-1), truncated RRM1-3 (GFP-SfTRN-1-RRM1-3 domain), and the C-terminal auxiliary domain (GFP-SfTRN-1-auxiliary domain) (Fig. 3C) in cells on the production of CAT/SV5-His6 protein. Observation of the fluorescence of these transfected cells by fluorescence microscopy and Western blotting employing the antibody specific for the GFP sequence showed that all the recombinant proteins were expressed at levels similar to the GFP mock protein.

An assay for poly(U) RNA-binding of all recombinant full-length SfTRN-1 had the ability to bind to poly(U) RNA-agarose, but not to poly(G) RNA-agarose (data not shown). It was confirmed, in the results shown in Fig. 3C, that no poly(G)-binding but significant poly(U)-binding of the recombinant full-length protein and truncated protein, GFP-SfTRN-1-RRM1-3 domain, in which the RRM1-3 domain had no sequential deficit, can be detected although they are fused to GFP, indicating no inhibitory influence of their fusion with GFP on the original RNA-binding activity of the RRM1-3 domain. Therefore, investigations using recombinant proteins should be effective at elucidating the cellular function of SfTRN-1.

A marked decrease in CAT/SV5-His6 protein production was noted in cells overexpressing the GFP-SfTRN-1 as compared to cells expressing the mock protein, the GFP-SfTRN-1-RRM1-3 domain, and the GFP-SfTRN-1-auxiliary domain (Fig. 3D). Densitometric analysis using computer-aided imaging showed the relative CAT/SV5-His6 protein signal strengths in equal amounts of sample proteins of mock protein-, the GFP-SfTRN-1-, the GFP-SfTRN-1-RRM1-3 domain-, and the GFP-SfTRN-1-auxiliary domain-expressing cells to be 7830.68 ± 28.70, 4657.54 ± 21.36, 7564.19 ± 30.02, and 6420.46 ± 47.39 respectively. CAT/SV5-His6 protein expression was found to be slightly decreased in cells overexpressing only the auxiliary domain, probably due to some other function of the domain, which participates in protein expression. Taking these findings together, it was found that the amount of foreign gene transcript used here was reduced by the cellular functions, including the activity of SfTRN-1.

Subcellular distribution of SfTRN-1 in cultured cells

In order to determine the subcellular distribution of SfTRN-1 in cells by confocal microscopy, several DNA constructs expressing the GFP-SfTRN-1 or truncated mutants of SfTRN-1, such as the GFP-SfTRN-1-RRM1-3 and the GFP-SfTRN-1-auxiliary domain, were generated and introduced into Sf9 cells to observe the subcellular distribution of these fusion proteins (Fig. 4A). On observation of the cells and a comparison of intensity between the nucleus and cytoplasm, as shown in Fig. 4B and C, the mock GFP was always detected at slightly higher levels in the nucleus than in the cytoplasm, probably due to its general properties, since similar results were obtained for mammalian cos-7 cells.11 According to some reports, GFP-fused TIA-1 family proteins are distributed similarly to the authentic proteins in mammalian cells, and GFP-fusion recombinants have been verified to be effective tools to investigate the subcellular distribution of the TIA-1 family.21,11 It was found that the truncated mutants, the GFP-SfTRN-1-RRM1-3 and the GFP-SfTRN-1-auxiliary domains, were distributed largely in the nucleus (Fig. 4B and C), and that the GFP-SfTRN-1 was distributed in both the nucleus and the cytoplasm, but a larger proportion of the expressed GFP-SfTRN-1 was distributed in the cytoplasm than the nucleus. Based on these results, it was concluded that SfTRN-1 differs distinctly from mammalian TIA-1 and TIAR, which are mainly distributed in the nucleus of cos-7 cells in the absence of environmental stress.11 Also, it has been found that Bombyx mori BmTRN-1 was distributed in both the nucleus and the cytoplasm.20

In addition, whereas removal of the RRM1 and RRM3 subdomains from the GFP-fused full-length
SfTRN-1 (Fig. 4B panel GFP-SfTRN-1/C1 RRM1 and C1 RRM3 respectively and C) led to greater distribution in the cytoplasm, removal of the RRM2 subdomain led to clear accumulation in the nucleus (Fig. 4B panel GFP-SfTRN-1/C1 RRM2 and C), suggesting that RRM1 and RRM3 are related to nuclear accumulation, and that RRM2 has a role related to nuclear export.

Furthermore, as shown in Fig. 5, confocal analysis of the cells expressing the truncated mutant lacking the posterior half of the auxiliary domain (GFP-SfTRN-1 ΔA2) revealed the GFP-fused recombinant protein to be distributed in the nucleus more than the full-length SfTRN-1. The truncated mutant lacking the anterior half of the auxiliary domain (GFP-SfTRN-1 ΔA1) was found to be distributed in the cytoplasm, although the truncated mutant in the cytoplasm appeared to be aggregated, for some unknown reason. Based on this evidence, it was suggested that not only the RRM2 subdomain but also the posterior half of the auxiliary domain is essential for the nuclear export of SfTRN-1.

Since the truncated domain, such as the SfTRN-1-RRM1-3 domain and the SfTRN-1-auxiliary domain, was more likely to be distributed in the nucleus, full-length SfTRN-1 was distributed largely in the cytoplasm, and the RRM subdomains were found to have potential roles related to both nuclear accumulation and nuclear export, it was suggested that SfTRN-1 newly synthesized in the cytoplasm is transported into the nucleus by its domain function and then a large amount of SfTRN-1 is moved into the cytoplasm due to the role of RRM2 and the anterior half of the auxiliary domains. Based on these findings, it is most likely that full-length SfTRN-1 shuttles between the nucleus and the cytoplasm.

Discussion

The present study indicates that a Spodoptera insect, S. frugiperta, has an RNA-binding protein, SfTRN-1, with a molecular mass of approximately 42.0 kDa, that is...
related to the cellular function of reducing the amount of reporter transcripts in cultured cells. Subcellular distribution analysis using GFP-fused recombinant proteins indicated that SfTRN-1 most likely shuttles between the nucleus and the cytoplasm, that the functions of subdomains RRM1 and RRM3 are related to nuclear accumulation, and that the function of subdomain RRM2 is related to nuclear export. It is possible that the RRM subdomains of SfTRN-1 possess their own RNA-recognition and RNA-binding properties, since the motifs, RNP1 and RNP2, important for interaction with RNA were fully conserved in each RRM, and the conserved poly(U)-binding site of the recombinant RRM1-3 was detectable (Fig. 3). Hence, it was suggested that the roles of RRM in nuclear accumulation and nuclear export are directly linked with properties such as RNA-recognition and RNA-binding.

Our recent results regarding BmTRN-1 from Bombyx mori cells, the RRM1-3 domain of which shares 95% homology with that of SfTRN-1, indicated that RRM1 was not associated with the subcellular distribution of BmTRN-1, while RRM2 and RRM3 of TRN-1 from S. frugiperda and B. mori shared similar properties regarding their subcellular distribution.20) Also, it has been reported that RRM2 and RRM3 of mammalian TIA-1 and TIAR have roles related to nuclear accumulation and nuclear export respectively.11) Based on this evidence, it was suggested that respective species have different cellular systems for the transport and the accumulation of TRN-1, and that TRN-1s from different sources have their own characteristic RRMs carrying out different roles in subcellular distribution.

Based on our data indicating that the SfTRN-1-RRM1-3 domain which lacked the auxiliary domain, the SfTRN-1 \( \Delta \)RRM2 which contained the complete auxiliary domain but lacked RRM2 subdomain, and the SfTRN-1 \( \Delta \)A2 which contained the complete RRM1-3 domain but lacked the posterior half of the auxiliary domain largely distributed in the nucleus (Figs. 4 and 5), it was suggested that RRM2 and the posterior half of the auxiliary domain have a synergetic role linked to nuclear export of SfTRN-1. So far, it is not clear whether the insect system for the nuclear import and export of SfTRN-1 associates with CRM1- and Ran-GTP-dependent nucleo-cytoplasmic trafficking,23,24) because exposure of cells to a physiological concentration of leptomycin B and 2-deoxyglucose/sodium azide for more than 1 h did not affect the distribution of SfTRN-1 (data not shown). Since the auxiliary domain but not the RRMs can interact with other proteins, it might be that the posterior half of the sequence of the auxiliary domain has a function closely related to protein factors essential for organizing unknown types of nucleo-cytoplasmic traffic systems in insects.

In addition, the function of SfTRN-1’s auxiliary domain appeared to be essential not only for nuclear export but also to reduce the amount of transcript, since the full-length SfTRN-1 lowered reporter protein production, while truncated SfTRN-1-RRM1-3 domain by itself did not affect reporter productivity (Fig. 3). Full-length recombinant SfTRN-1 was detected largely in the cytoplasm. Given these findings, the subcellular distribution of SfTRN-1 might be associated with its interaction with certain cellular transcripts destined to be decomposed, and SfTRN-1 might act to eliminate transcripts in the cytoplasm. However, it remains to be clarified whether SfTRN-1 can specifically recognize non-self transcripts derived from introduced foreign artificial genes or aberrant transcripts resulting from abnormal processing during post-transcription processes.

![Fig. 4. Continued](image-url)
Confocal analysis has indicated that numerous stress granule-like structures with overexpressed GFP-fused TRN-1 in the cytoplasm of *S. frugiperda* and *Bombyx mori* cells were induced by oxidative stress (Muto and Kotani, unpublished results), but not by extensive production of reporter gene transcripts, indicating that the putative SfTRN-1 function of reducing the amount of reporter transcript in the cytoplasm does not involve the translational silencing process through marked stress-induced eIF2α phosphorylation and stress granule assembly, but possibly involves translational regulation by binding to AU-rich elements located in mRNA 3′ untranslated regions, like mammalian TIA-1 in the cytoplasm, which has been found to regulate mRNAs encoding TNF-α, Cox-2, HMMP-13, and the β2-adrenergic receptor in the absence of oxidative stress. 4–7)

Technologies for foreign protein production in lepidopterous insect cells have been applied in a wide range of industrial and research fields using transient expression plasmids, permanent expression vectors, and baculoviruses carrying a strong promoter, and improved transgenic technologies should expand the applications of lepidopterous insects. 12,25,26) Notably, *Spodoptera* insect cells and individuals are versatile for these purposes worldwide, compared to *Bombyx mori*, which is utilized in limited areas in Asia and has a long history of sericulture, because *Spodoptera* insects exhibit attributes such as rapid growth (of both cultured cells and individuals) and a non-diapausing life cycle. Under present conditions, it is worth investigating the cellular and developmental functions of lepidopterous TRN-1 and further establishing a procedure for the constant operation of TRN-1 in order to improve the situation regarding unsuccessful foreign protein production, which can be due to the elimination of transcripts.

**Acknowledgments**

This work was supported in part by Grants-in-Aid for Scientific Research (nos. 18580054 and 20580051) from the Japan Society for the Promotion of Science, which is administered by the Ministry of Education, Culture, Sports, Science, and Technology, and by a grant from the Kato Memorial Bioscience Foundation.
References


