**INTRODUCTION**

Nucleopolyhedroviruses belong to the family Baculoviridae and possess a single large dsDNA genome within a pathogenic virion (Rohrmann, 2011). Amongst these, *Bombyx mori* nucleopolyhedrovirus (BmNPV) is a well-studied and important species that poses a threat to sericulture. In addition, the viral replication mechanisms of BmNPV in particular have been examined in numerous investigations as it is a popular and versatile expression vector for foreign proteins in both cultured *B. mori* cells and larvae.

There is a noticeable decline in cellular protein synthesis and a commensurate increase in the production of baculovirus proteins following BmNPV infection of permissive cells. This is partly attributable to baculovirus strategies, including the substantial downregulation of cellular mRNA levels (Nobiron et al., 2003) and dominant expression of viral mRNA through the actions of late viral RNA polymerases and virus gene-specific promoters (Acharya & Gopinathan, 2002; Guarino et al., 1998). However, although some baculovirus genes appear to regulate protein synthesis, the details of the underlying mechanisms remain obscure.

We have examined the relationships between baculovirus proliferation and the function of cellular endogenous T-cell intracellular antigen 1 (TIA-1) homologues in cultured *B. mori* BM-N cells (Muto et al., 2009; Kotani et al., 2003). TIA-1 is a nucleo-cytoplasmic shuttling RNA binding protein with an auxiliary domain for protein–protein interaction and is involved in multiple aspects of RNA metabolism in mammalian cells (Zhang et al., 2005). These include the intranuclear regulation of RNA splicing for alternatively spliced pre-mRNAs (Izquierdo & Valcárcel, 2007a, b) and the formation of cytoplasmic stress granules, which act as reservoirs of untranslated and sequestered translation.
initiation complexes that are induced by environmental stress (Anderson & Kedersha, 2006, 2008; Gilks et al., 2004). BmTRN-1 was found to shuttle between the nucleus and cytoplasm (Muto et al., 2009), and in the present study we revealed the formation of amorphous conspicuous cytoplasmic foci containing BmTRN-1 in BmNPV-infected BM-N cells (Fig. 1). These observations suggested that BmTRN-1 is recruited to cytoplasmic foci and acts as a cellular response protein during BmNPV infection.

Previous studies demonstrated that TIA-1 binds flavivirus genomic RNA within the replication complex during viral infection and interferes with stress granule formation to facilitate viral replication (Emara & Brinton, 2007). However, TIA-1 also participates in the formation of antiviral cellular granules during infection with vaccinia virus and poliovirus in the absence of environmental stresses (Piotrowska et al., 2010; Simpson-Holley et al., 2011). Taken together, these observations indicate that cellular proteins of the TIA-1 family have multiple translation-silencing roles that facilitate both virus replication and cell survival. In the cytoplasmic foci, BmTRN-1 may associate with other substances via protein–protein interactions, particularly in the cytoplasmic regions that are central to BmTRN-1 responses to BmNPV infection. However, little is known about BmTRN-1 function during BmNPV infection.

In the present study, we screened BmTRN-1-associated proteins during BmNPV infection and identified the BmNPV nucleic acid binding proteins BRO-B and BRO-E (Kang et al., 2006; Zemskov et al., 2000). BRO-B/E are both expressed during the early stage of infection. Accordingly, we showed the colocalization of BmTRN-1 and BRO-B/E in amorphous conspicuous cytoplasmic foci, suggesting the formation of a complex that regulates protein synthesis. Subsequent experiments showed that a complex including BmTRN-1 and BRO-B/E regulated an artificial transcript carrying the 5′ untranslated region (UTR) from a cellular mRNA at the post-transcriptional level. These experiments demonstrated a novel baculovirus strategy that utilizes a host TIA-1 homologue and exerts control over protein synthesis during infection.

**RESULTS**

**Cytoplasmic localization of BmTRN-1 in BmNPV-infected BM-N cells**

Previous studies have shown that the TIA-1 homologue BmTRN-1 in *B. mori* has alternatively spliced isoforms of 42.5 and 44.1 kDa, and that BmTRN-1 shuttles between the nucleus and cytoplasm in BM-N cells, possibly through intracellular RNA trafficking (Muto et al., 2009). We examined the subcellular localization of BmTRN-1 at 18 and 24 h after BmNPV infection using a recombinant 42.5 kDa BmTRN-1 labelled with N-terminal GFP (GFP-42.5 kDa BmTRN-1; Muto et al., 2009). GFP-42.5 kDa BmTRN-1 was distributed in the nucleus and cytoplasm (Fig. 1, upper panel) of uninfected BM-N cells, and was observed in amorphous conspicuous cytoplasmic foci at 18 and 24 h after viral infection (Fig. 1, middle and lower panels). This result differed from the previous results of the nuclear accumulation of BmTRN-1 in BM-N cells during abortive infection by Autographa california multiple nucleopolyhedrovirus (Muto et al., 2009). This discrepancy suggested that the subcellular distribution of BmTRN-1 was affected by the difference of viral infectivity. After heat-shock treatment of uninfected BM-N cells at 46°C for 2 h, recombinant 42.5 kDa BmTRN-1-FLAG was recruited into numerous fine structures in the cytoplasm (Fig. S1, available in the online Supplementary Material). These were shown to be typical stress granules, indicating properties similar to TIA-1 family members (Fig. S1) (Anderson & Kedersha, 2006, 2008; Gilks et al., 2004). However, the apparent sizes of heat-shock-induced stress granules (Fig. S1) differed from those of foci containing recombinant BmTRN-1 in BmNPV-infected BM-N cells (Fig. 1). TIA-1 family proteins localize to cytoplasmic structures through associations with TIA-1 and other proteins, which are also induced by other virus infections under physiological conditions (Piotrowska et al., 2010; Simpson-Holley et al., 2011). Thus, the localization of BmTRN-1 to cytoplasmic foci...
suggested associations with other cellular and/or viral molecules during BmNPV infection.

**Coprecipitation of BmTRN-1 with BmNPV BRO-B/E**

In order to identify proteins that associated with BmTRN-1, GFP-42.5 kDa BmTRN-1 was expressed in BmNPV-infected cells and proteins were coimmunoprecipitated by targeting the GFP-tag. These experiments revealed a protein of ~28 kDa in the protein fraction that coimmunoprecipitated with GFP-42.5 kDa BmTRN-1. This protein fraction was soluble in buffer containing 15% urea, but was not efficiently immunoprecipitated in urea-free buffer. Subsequent amino acid analyses revealed the sequence YVLQTEPTVHHT, which is identical to sequences of the BmNPV nucleic acid binding proteins BRO-B (239 aa) and BRO-E (241 aa) (Zemskov et al., 2000). Coimmunoprecipitation and amino acid sequence analyses further identified two other cellular proteins, *B. mori* ribosomal protein L23A (GenBank accession number PTZ00191) and a protein with partial sequence identity to *Bombyx* actin proteins, in the protein fraction coimmunoprecipitated with GFP-42.5 kDa BmTRN-1 from BmNPV-infected cells. Given the formation of protein complexes from BmTRN-1-localized foci, it follows that proteins colocalized with BmTRN-1 in BmNPV-infected cells were relatively insoluble in immunoprecipitation experiments and were collected in urea-containing buffer. These observations suggested that BmTRN-1 was recruited to foci during BmNPV infection, where BmTRN-1 and BmNPV BRO-B/E formed complexes with other cellular proteins involved in ribosomal function.

We next examined the association of BmTRN-1 with BRO-B/E in cells expressing recombinant proteins carrying V5–His8-tags. BRO-B-V5–His8 and BRO-E-V5–His8 were precipitated from lysates of BM-N cells coexpressing the 42.5 kDa BmTRN-1-FLAG (estimated mass ~43.5 kDa; Fig. 2, left panel) using anti-V5-tag beads, which were analysed by immunoblotting with anti-V5 and anti-FLAG antibodies (Fig. 2, right panel). The expression of 42.5 kDa BmTRN-1-FLAG and BRO-E-V5–His8 had a tendency to be depressed after cotransfection for unknown reasons. The calculated molecular masses of BRO-B-V5–His8 (30.8 kDa) and BRO-E-V5–His8 (31.2 kDa) differed from those estimated using SDS-PAGE (~28 kDa for BRO-B-V5–His8 and ~25 kDa for BRO-E-V5–His8), potentially reflecting biochemical properties of the recombinant proteins. The 42.5 kDa BmTRN-1-FLAG coprecipitated with BRO-B-V5–His8 and BRO-E-V5–His8, but did not coprecipitate in the lysates of the cells transfected with pIZ/V5–His (empty vector; Fig. 2). Thus, these data clearly indicated an association between BmTRN-1 and BRO-B/E in virus-permissive cells.

**Subcellular localization of BmTRN-1 and BRO-B/E**

The subcellular localization of 42.5 and 44.1 kDa BmTRN-1-FLAG, BRO-B-V5–His8 and BRO-E-V5–His8 was investigated in BM-N cells using immunofluorescence and

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**Fig. 2.** Coprecipitation of BmTRN-1 and BRO-B/E in BM-N cells. The plasmid pIZ-42.5 kDa BmTRN-1-FLAG was transfected into BM-N cells with pIZ/V5–His (empty vector), pIZ-BRO-B-V5–His8 to express BRO-B-V5–His8 or pIZ-BRO-E-V5–His8 to express BRO-E-V5–His8. BRO-B-V5–His8 and BRO-E-V5–His8 were precipitated from cell lysates using anti-V5-tag beads. Cell lysate proteins before immunoprecipitation (IP) (left panel) and proteins prepared by immunoprecipitation with beads (right) were analysed by immunoblotting with anti-V5 (for the detection of BRO-B-V5–His8 or BRO-E-V5–His8) and anti-FLAG (for 42.5 kDa BmTRN-1-FLAG) antibodies.
confocal microscopy (Figs 3a and S2). Although GFP-42.5 kDa BmTRN-1 was detected in the nucleus (Fig. 1) (Muto et al., 2009), 42.5 and 44.1 kDa BmTRN-1-FLAG were distributed weakly in the nucleus and strongly in the cytoplasm of BM-N cells (Figs 3a and S2). This discrepancy could be attributable to the difference of properties between these two BmTRN-1 recombinant proteins with the different tags at the different ends. At 24 h post-transfection, both BRO-B-V5–His6 and BRO-E-V5–His6 were predominantly observed in the cytoplasm (Fig. 3a), and were observed in amorphous conspicuous cytoplasmic foci at 36 h after transfection (Fig. 3a, middle and lower panels).

Subsequently, colocalization of BmTRN-1 and BRO-B/E was examined in BM-N cells (Fig. 3b). Colocalization of 42.5 kDa BmTRN-1-FLAG with BRO-B-V5–His6 (Fig. 3b, upper panels) and BRO-E-V5–His6 (Fig. 3b, lower panels) was observed in amorphous conspicuous cytoplasmic foci.

![Fig. 3](image_url)

**Fig. 3.** Subcellular localization of 42.5 kDa BmTRN-1 and BRO-B/E. (a) Fluorescent immunostaining of BM-N cells expressing 42.5 kDa BmTRN-1-FLAG (upper panel), BRO-B-V5–His6 (middle panel) and BRO-E-V5–His6 (lower panel). BM-N cells expressing each recombinant protein were fixed at 24 and 36 h post-transfection, and were stained with Cy3-conjugated anti-FLAG antibody to detect 42.5 kDa BmTRN-1-FLAG and with FITC-conjugated anti-V5 antibody to detect BRO-B-V5–His6 or BRO-E-V5–His6. Nuclei were stained with TOTO-3. Fluorescence images were acquired and generated using merged confocal plane sections with a confocal microscope. Bars, 5 μm. (b) BM-N cells expressing 42.5 kDa BmTRN-1-FLAG plus BRO-B-V5–His6 (upper panel) or 42.5 kDa BmTRN-1-FLAG plus BRO-E-V5–His6 (lower panel) were analyzed at 36 h post-transfection using a confocal microscope as described in (a). (c) Intensity analyses of BmTRN-1-FLAG (Cy3; red) and BRO-B-V5–His6 or BRO-E-V5–His6 (FITC; green) in the regions of interest (r.o.i.; dashed lines) in the areas indicated by the boxes in the merged images of (b).
and these were similar to the cytoplasmic structures that included GFP-42.5 kDa BmTRN-1 in BmNPV-infected BM-N cells (Fig. 1). Intensity analyses showed that the distribution patterns of 42.5 kDa BmTRN-1-FLAG and BRO-B-V5–His6/BRO-E-V5–His6 substantially overlapped (Fig. 3c). Moreover, 44.1 kDa BmTRN-1-FLAG and BRO-B-V5–His6/BRO-E-V5–His6 were observed in similar foci, indicating that the properties of BmTRN-1 isoforms did not differ (Fig. S2). These results indicated that both BmTRN-1 isoforms and BRO-B/E were colocalized in the cytoplasmic foci.

**BRO-B/E expression impacts protein synthesis**

BmNPV carries five related genes that encode the putative nucleic acid binding proteins BRO-A, BRO-B, BRO-C, BRO-D and BRO-E (Zemskov et al., 2000; Kang et al., 2006). Amongst these, only BRO-D has been shown to be involved with the nuclear export of mRNA (Kang et al., 2006); however, the nucleic acid binding capabilities of the other BmNPV BRO proteins have not been characterized. Although BRO-B and BRO-E have 87% amino acid identity, both proteins commonly contain the amino acid sequence determined in this study and were indistinguishable in protein sequence analyses. Moreover, the presence of ribosomal proteins in the protein fraction coimmunoprecipitated with BmTRN-1 suggested a role for the protein complexes containing BmTRN-1 and BRO-B/E in regulation of protein synthesis. We next sought to investigate the impact of both BRO-B and BRO-E on protein synthesis. Although it is important to investigate the impact of recombinant BRO-B/E on protein synthesis in virus-infected cells, our preliminary experiments showed the difficulty in controlling DNA transfection efficiency in the BmNPV-infected cells. Thus, the functional consequences of BRO-B/E on protein synthesis were examined using assay systems in uninfected BM-N cells expressing reporter transcripts. In these experiments, a series of reporter transcripts was used for interpretation of BRO-B/E function, as illustrated in Fig. 4(a). These assay transcripts all carried the luciferase ORF and an *Orgyia pseudotsugata* nucleopolyhedrovirus *ie*-2-derived 3′ UTR (vector-derived sequence) expressed under the control of the *Bombyx* A3 actin promoter (Mounier & Prudhomme, 1986), but had differing 5′ UTR sequences. Assay transcripts included actin-5′ UTR (+intron)-luci, which carried the 5′ UTR with the first intron from A3 actin; actin-5′ UTR (−intron)-luci, which carried the A3 actin 5′ UTR lacking the first intron; ie-1-5′ UTR-luci, which carried the BmNPV *ie*-1 5′ UTR; and lef-8-5′ UTR-luci, which carried the BmNPV *lef*-8 5′ UTR (Acharya & Gopinathan, 2002; Kamita & Maeda, 1997). Equivalent expression of BRO-B-V5–His6 and BRO-E-V5–His6 in BM-N cells was confirmed by immunoblotting (Fig. 4b), and the effects of BRO-B/E on protein synthesis were assessed by comparing relative luciferase activity from coexpressed assay transcripts at 36 h post-transfection (Fig. 4c). BRO-B-V5–His6 and BRO-E-V5–His6 did not strongly affect luciferase activity from transcripts carrying actin-5′ UTR (−intron)-luci, ie-1-5′ UTR-luci or lef-8-5′ UTR-luci, with weak reductions of ~5–12% compared with pIZ/V5–His-transfected cells (Fig. 4c, panels ii–iv). Luciferase activity from transcripts with actin-5′ UTR (−intron)-luci, ie-1-5′ UTR-luci and lef-8-5′ UTR-luci were slightly reduced by 14.37 ± 7.12, 15.22 ± 6.35 and 11.67 ± 4.83%, respectively, in cells expressing both BRO-B-V5–His6 and BRO-E-V5–His6. However, the expression of BRO-B-V5–His6 and BRO-E-V5–His6 resulted in remarkable decreases in luciferase activity from actin-5′ UTR (−intron)-luci; luciferase activity was 51.76 ± 5.14 and 49.97 ± 5.01% of the control, respectively (*P* < 0.01, *n* = 5; Fig. 4c, panel i). In addition, luciferase activity from actin-5′ UTR (−intron)-luci was only 33.75 ± 5.14% of the control in cells expressing both BRO-B-V5–His6 and BRO-E-V5–His6 (*P* < 0.01; Fig. 4d, panel i). Real-time (RT)-PCR analysis of the cells expressing BRO-B-V5–His6 and BRO-E-V5–His6 showed that there were no significant differences between the transcription levels of actin-5′ UTR (−intron)-luci or lef-8-5′ UTR-luci (Table S1), indicating that differing luciferase activities reflected levels of protein synthesis and that BRO-B/E synergistically regulated actin-5′ UTR (−intron)-luci at the post-transcriptional level.

In order to further examine the correlations between BmTRN-1 function and the effects of BRO-B/E on the protein synthesis from certain transcripts, RNA interference (RNAi) plasmids to deplete endogenous BmTRN-1 expression were introduced into control cells and cells expressing both BRO-B-V5–His6 and BRO-E-V5–His6 as in the luciferase assays with actin-5′ UTR (−intron)-luci and lef-8-5′ UTR-luci. The production of both authentic BmTRN-1 isoforms, which could be detected as separated bands by SDS-PAGE, was decreased by ~72.5% following the introduction of BmTRN-1 RNAi (Fig. 5a). However, the introduction of BmTRN-1 RNAi significantly rescued luciferase activity from actin-5′ UTR (−intron)-luci, to 64.99%, in cells expressing both BRO-B-V5–His6 and BRO-E-V5–His6 (*P* < 0.05, *n* = 5; Fig. 5b), suggesting that association of BmTRN-1 with BRO-B/E was responsible for the post-transcriptional regulation of actin-5′ UTR (−intron)-luci. After the introduction of expression plasmids for 42.5 and 44.1 kDa BmTRN-1–FLAG, no significant changes in luciferase action from actin-5′ UTR (−intron)-luci and lef-8-5′ UTR-luci transcripts were detected in the presence of overexpressed BRO-B-V5–His6 and BRO-E-V5–His6 (data not shown), suggesting that the expression of endogenous BmTRN-1 in BM-N cells was sufficient for the BmTRN-1–BRO-B/E association-dependent inhibition of protein synthesis. Taken together, the present data demonstrated that the association of BmTRN-1 with BRO-B/E was responsible for the inhibitory regulation of certain mRNAs at the post-transcriptional level.

**DISCUSSION**

In the present study, the TIA-1 homologue BmTRN-1 in *Bombyx* cells and BmNPV-derived nucleic acid binding
Fig. 4. Luciferase activity assays in BM-N cells expressing BRO-B-V5–His$_6$ and BRO-E-V5–His$_6$. (a) Schematic representation of reporter plasmids for luciferase assays. Luciferase ORF and $i e$-2 3'UTR, which are all common, are presented with white boxes. The 5'UTR sequences of $i e$-1 and $l e f$-8 from the BmNPV gene are indicated by grey and striped boxes, respectively. The $B. m o r i$ A3 actin promoter and the 5'UTR sequence are indicated by black boxes. The plasmid plZ-actin-pro-5'UTR(+intron)-luci carries the A3 actin promoter and the 5'UTR with the first intron from A3 actin; plZ-actin-pro-5'UTR(-intron)-luci carries the A3 actin 5'UTR lacking the first intron; plZ-actin-pro-iej-1-5'UTR-luci and plZ-actin-pro-lef-8-5'UTR-luci carry the A3 actin promoter. Numbers of nucleotides are indicated for each gene. Arrows indicate transcription.
proteins BRO-B/E were clearly shown to colocalize by coimmunoprecipitation assays and fluorescence microscopy (Figs 2 and 3). Previous reports identified five related genes in the BmNPV genome that encode BRO-A, BRO-B, BRO-C, BRO-D and BRO-E, all of which carry conserved putative N-terminal nucleic acid binding sites (Kang et al., 2006; Zemskov et al., 2000). Bideshi et al. (2003) previously performed wide-ranging DNA searches and showed that BRO-like sequences could not be identified in whole eukaryotic genes, yet they were widely distributed amongst dsDNA viruses of invertebrates and bacteriophages, and in several transposons. These observations suggest that the BRO-B/E genes have specifically evolved through insect–dsDNA virus relationships.

These BRO genes carry promoter regions that are common amongst baculovirus early genes; their expression was initiated at 4 h and reached maximum levels at 8–12 h after infection, indicating that BRO-B/E is an early gene involved in the infection process (Kang et al., 1999). As endogenous mechanisms for protein synthesis, including BmTRN-1 activity, are notably unchanged during the early stages of BmNPV infection, the colocalization of BmTRN-1 and BRO-B/E in uninfected cells (Fig. 3), and subsequent inhibitory effects on protein synthesis (Figs 4 and 5), seem to reflect the practical biochemical functions of these proteins during the early stages of BmNPV infection.

It is noteworthy that both recombinant BmTRN-1 and BRO-B/E were colocalized in amorphous conspicuous cytoplasmic foci that differed considerably in size from those of heat-shock-induced stress granules (Figs 3 and S1). Coimmunoprecipitation of proteins from BmNPV-infected cells identified other BmTRN-1-associated proteins, including ribosomal protein L23A and actin-like proteins, suggesting that the virus-induced cytoplasmic foci consist of protein complexes of several cellular proteins in addition to BmTRN-1 and BRO-B/E. Ribosomal proteins and actin are both potential stress granule components, and under conditions of environmental stress, such as heat shock, stress granules formed from prion-like aggregates of TIA-1 C-terminal auxiliary domains harbour components of translation pre-initiation complexes, several types of chaperones and microtubules that facilitate the transport of granule components (Anderson & Kedersha, 2006, 2008; Ivanov et al., 2003). Thus, BmNPV-induced foci consisting of BmTRN-1 and BRO-B/E likely have features in common with stress granules, such as proteins that relate to translation, but contain viral BRO-B/E. Furthermore, limited local formation of BmNPV-induced foci and foci consisting of BmTRN-1 and BRO-B/E in the cytoplasm was observed in fluorescence microscopy experiments (Figs 1 and 3), whereas stress granule formation extends to the whole cytoplasm (Fig. S1) (Anderson & Kedersha, 2006, 2008). Formation of the amorphous conspicuous cytoplasmic foci was observed in the cells solely expressing the recombinant BRO-B/E, but not expressing the recombinant BmTRN-1 (Fig. 3a). Together, these observations suggest that BRO-B and BRO-E are key factors responsible for limited local formation of the BmNPV-induced foci consisting of BmTRN-1 and other proteins. Although no regions homologous with the sequences responsible for the protein–protein interaction to form these cytoplasmic structures have thus far been found in the amino acid sequences of BRO-B/E, there may be novel properties of their C-terminal amino acid sequences necessary for formation of the foci.

More noteworthy in the present results is the finding that expression of recombinant BRO-B/E impairs the protein synthesis from an artificial transcript containing a certain 5’UTR. Thus, multi-protein complexes of BmTRN-1, BRO-B/E and other cellular proteins are likely relevant to the post-transcriptional regulation of certain mRNAs. Also, the introduction of BmTRN-1 RNAi significantly inhibited the effects of BRO-B/E on protein synthesis (Fig. 5). As documented previously, TIA-1 has multiple functions, including the decay of specific mRNAs with TIA-1 bound at 3’UTRs through enzyme-associated major mRNA degradation pathways and polysome disassembly in non-stressed macrophages (Yamasaki et al., 2007). In contrast, our results showed that BRO-B/E expression impaired protein synthesis from an artificial transcript with no significant change of transcript abundance in the cells (Figs 4 and 5, Table S1). The impaired protein synthesis induced by BRO-B/E expression seemed not to be caused by mRNA decay, changes in mRNA expression levels or influences on mRNA nuclear export, as these events should lead to remarkable changes in the amounts of mRNA. Thus, these observations suggest that BmTRN-1 and BRO-B/E complexes likely harbour polysomes with
impaired translational activity, and that BmNPV BRO-B/E enables the control of protein synthesis by exploiting the *Bombyx* TIA-1 homologue. This mechanism differs from that of flaviviruses, which exploit cellular TIA-1 to facilitate replication of their RNA genome and to prevent the formation of stress granules (Emara & Brinton, 2007).

In a recent study, the expression of intronless mRNAs by Kaposi’s sarcoma-associated herpesvirus (KSHV; human herpesvirus 8) was demonstrated to use an unusual mechanism for translation (Boyne et al., 2010). In this mechanism, the KSHV protein ORF57 interacts with cellular PYM, which only binds intronless mRNA in the presence of ORF57, recruits 40S ribosomes to intronless mRNA without exon junction complexes and enhances the translation of intronless KSHV mRNA (Boyne et al., 2010). In contrast, the present study is an unprecedented demonstration of a baculovirus strategy that involves the association of viral BRO-B/E with a cellular translational regulator and relates to the inhibitory post-transcriptional regulation of certain mRNA species. Thus, it is possible that various DNA viruses commonly exploit host mechanisms to recognize certain types of mRNAs, control protein synthesis systems with specificity and enhance viral proliferation.

Intriguingly, the present experiments also indicated that BmTRN-1–BRO-B/E association impairs protein synthesis from an artificial transcript carrying the A3 actin 5’UTR with the first intron, as opposed to transcripts with different 5’UTRs (Figs 4 and 5). The specific structure in the A3 actin 5’UTR including the first intron remains unidentified. Further exploration of the BmTRN-1–BRO-B/E complex-mediated decrease in protein synthesis may identify the specific structures of certain mRNAs, leading to the differential post-transcriptional regulation of certain mRNAs during viral infection. Accordingly, wide-range searches for target mRNAs of the BmTRN-1–BRO-B/E complex-mediated decrease in protein synthesis are required to further investigate the specificity of the association between BmTRN-1 and BRO-B/E for some types of mRNAs, and to characterize the mechanisms by which BmNPV controls protein synthesis. Such analyses will comprehensively elucidate baculovirus strategies for regulating protein synthesis, and offer critical contributions to the development of improved protein production systems that employ cultured insect cells and viral vectors.

**METHODS**

**Insect cell lines and viruses.** *B. mori*-derived BM-N cells were cultured at 27 °C in Grace’s medium (Gibco-BRL) supplemented with 10% FBS (MP Biomedicals). To investigate viral proteins in BM-N cells, 1 × 10⁵ cells were infected with BmNPV T3 (Kamita & Maeda, 1997) at m.o.i. 10.

**Plasmid constructs.** All constructs were confirmed using DNA sequencing and oligonucleotide sequences are summarized in Table S2. The plasmids pIZ-GFP-42.5 kDa BmTRN-1 and pIZ-GFP-44.1 kDa BmTRN-1 were used for the transient expression of N-terminal GFP-labelled 42.5 and 44.1 kDa isoforms of BmTRN-1 (GenBank accession...
number AB092510), respectively, as described previously (Muto et al., 2009). The plasmids pIZ-42.5 kDa BmTRN-1-FLAG and pIZ-44.1 kDa BmTRN-1-FLAG were used to express 42.5 and 44.1 kDa BmTRN-1 proteins with C-terminal FLAG-tags, respectively, and were constructed by inserting PCR-amplified sequences from each template into the EcoRI/Xhol site of the plasmid pIZ/V5-His (Invitrogen). The plasmids pIZ-BRO-B-V5–His8, pIZ-BRO-E-V5–His8, were constructed to express the recombinant protein BRO-B with the vector-derived V5–His8 and BRO-E-V5–His8, respectively, by inserting PCR-amplified BmNPV BRO-B (GenBank accession number L31380-84) and BRO-E (GenBank accession number L33180-140), respectively, from the template BmNPV T3 DNA into the EcoRI/Xhol site. Plasmids pIZ-actin-pro-5′UTR (+-intron)-luc and pIZ-actin-pro-5′UTR (–intron)-luc (Fig. 4a) were generated by inserting the luciferase gene into the BamHI/Xhol site as described previously (Mori et al., 1992), and then inserting the PCR-amplified fragments —657 to —1 and —657 to —460 from the region upstream of the B. mori cytoplasmic actin A3 (GenBank accession number AF422795) from the genome of BM-N cells, respectively, into the HindIII/BamHI site (Moumier & Prudhomme, 1986). The plasmid pIZ-actin-pro-ie-1.5′-UTR-luc (Fig. 4a) was generated by inserting the ligated fragment of ie-1.5′-UTR plus the luciferase ORF (ligated at the EcoRI site) and then inserting the fragment downstream (BamHI/Xhol) of the actin promoter region (nt —657 to —460). Two complementary oligonucleotides were annealed to each other to produce the lef-8-5′UTR fragment with EcoRI/BamHI restriction sites, and the plasmid pIZ-actin-pro-lef-8-5′-UTR-luc (Fig. 4a) was generated by inserting the ligated fragment of lef-8-5′UTR and the luciferase ORF and then inserting the fragment downstream of the actin promoter region. To knock-down BmTRN-1 in BM-N cells, the plasmid pIZ-BmTRN-TRNAi was constructed by inserting the universal sequence of the +1 to +750 positions into the BamHI/EcoRI site and the reverse sequence of +1 to +630 from BmTRN-1 into the EcoRI/Xhol site.

Plasmid DNAs were purified using Midl-Plasmid kits (Qiagen) according to the manufacturer’s instructions.

Analyses of subcellular localization of BmTRN-1 during BmNPV infection. BM-N cells (0.2 × 10^6 cells in 500 μl medium) were first cultured on glass dishes (Iwaki) and then transfected with 0.5 μg pIZ-GFP-42.5 kDa BmTRN-1 to express the GFP-42.5 kDa BmTRN-1 fusion protein using X-tremeGene HP reagent (Roche Diagnostics) according to the manufacturer’s instructions. The cells were infected with BmNPV at 48 h post-transfection. At 24 h post-infection, cells were washed twice with calcium-free PBS and fixed in 4% paraformaldehyde at room temperature for 15 min. After washing three times for 5 min with PBS, cells were permeabilized for 5 min with 0.5% Triton X-100 at 4°C, treated with 1 μg RNAse A ml^-1 and stained with 1.0 ng propidium iodide ml^-1 (Molecular Probes) to stain nuclei. Subsequently, coverslips were mounted on dishes using Vectashield H-1000 medium (Vector Laboratories), and dishes were examined by fluorescence microscopy with an Olympus Fluoview FV1000-IDX81 confocal microscope (Olympus) and a ×100 objective lens (oil immersion). Fluorescence images were acquired and generated from merged confocal plane sections using Olympus FV10-ASW software (version 1.7). More than 20 cells were analysed for each experiment and representative cells are presented to show typical features of cells in each treatment group.

Identification of the BmTRN-1-associated protein. Cells expressing GFP-42.5 kDa BmTRN-1 were infected with BmNPV, washed twice in PBS, and lysed in PBS containing 0.1% NP-40 and 15% urea (IP buffer) at 36 h post-infection. Lysates were centrifuged at 5000 g for 5 min, the supernatants were mixed with Dynabeads Protein G (Invitrogen) pre-incubated with anti-GFP antibody (Roche Diagnostics) and the mixture was incubated for 1 h at 4°C. Beads were washed three times in IP buffer, bound proteins were denatured in the sample buffer and proteins were then separated on 12.5% SDS-PAGE gels. Protein bands were excised from the gel and peptides were prepared using in-gel tryptic digestion. Amino acid sequences of peptides were determined using a Procise 494HT Protein Sequencing System (Applied Biosystems) and searched against the whole-sequence database (http://www.ddbj.nig.ac.jp) using BLAST software.

Coprecipitation and immunoblotting. Equal amounts (2.5 μg) of plasmids pIZ/V5–His (empty vector), pIZ-BRO-B-V5–His8, or pIZ-BRO-E-V5–His8 were transfected into monolayers of 1 × 10^6 cells in 4 ml culture medium with pIZ-42.5 kDa BmTRN-1-FLAG (2.5 μg). After 48 h, cells were lysed in 0.5 ml of IP buffer. BRO-B-V5–His8 and BRO-E-V5–His8 were coprecipitated with associated proteins by incubating proteins with 20 μl anti-V5-tag beads from a V5-tagged Protein Purification kit (MBL) at 4°C for 1 h. Proteins bound to the beads were then denatured in sample buffer, separated on 12.5% SDS-PAGE gels and transferred onto PVDF membranes. Proteins on membranes were then incubated overnight with 1: 3000 HRP-conjugated anti-V5-tag antibody (Abcam) and mouse monoclonal anti-FLAG antibody conjugated to HRP (Sigma-Aldrich), and antibody-specific bands were developed using ECL Select (Life Technologies).

Fluorescence microscopy of BM-N cells expressing BmTRN-1 and BmNPV BRO proteins. For protein expression, 0.5 μg pIZ-42.5 kDa BmTRN-1-FLAG, pIZ-44.1 kDa BmTRN-1-FLAG, pIZ-BRO-B-V5–His8, pIZ-BRO-E-V5–His8, or the combinations of plasmids (0.25 μg of each plasmid) presented in Figs 3 and S2 was transfected into cultures of 0.2 × 10^6 cells on glass dishes. After 24 or 36 h, cells were fixed, blocked using RNase A-containing Blocking One reagent (Nacalai Tesque) for 1 h at 4°C, incubated with labelled antibodies for 1 h at 4°C and washed three times in PBS. Cy3-conjugated anti-FLAG (Sigma-Aldrich) and FITC-conjugated anti-V5 (Invitrogen) were used for detection with 20 μl anti-V5-tag and V5-tagged proteins, respectively. Nuclei were stained with 1 μM TOTO-3 (Molecular Probes). Confocal microscopy and image acquisition were performed as described above. Analyses for the intensities of FITC and Cy3 were performed using Olympus FV10-ASW software (version 1.7).

For stress granule assessment, cells expressing 42.5 kDa BmTRN-1-FLAG at 24 h post-transfection were incubated for 2 h at 46°C and then cultured for an additional 10 h. Stress granules containing 42.5 kDa BmTRN-1-FLAG were observed by immunofluorescence using Cy3-conjugated anti-FLAG as described above.

Analysis of luciferase production in BM-N cells expressing recombinant BRO-B/E. The luciferase reporter plasmids pIZ-actin-pro-5′UTR (+ intron)-luc, pIZ-actin-pro-5′UTR (–intron)-luc, pIZ-actin-pro-ie-1.5′-UTR-luc or pIZ-actin-pro-lef-8.5′-UTR-luc (0.05 μg; Fig. 4a) were cotransfected with 0.1 μg pIZ/V5–His, pIZ-BRO-B-V5–His8, pIZ-BRO-E-V5–His8, or a combination of pIZ-BRO-B-V5–His8, pIZ-BRO-E-V5–His8, and pIZ-BRO-E-V5–His8 (0.05 μg) and pIZ-BRO-E-V5–His8 (0.05 μg) into 2 × 10^6 cells per well on 24-well plates (Iwaki). After 36 h, cells were lysed in 180 μl Cell Lysis Solution (Toyo Ink). Protein expression was verified by immuno blotting for V5-tagged proteins in pIZ-actin-pro-5′UTR (+ intron)-luc-transfected cells and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; gel loading control) was then detected using an anti-Drosophila GAPDH antibody (Novus Biologicals) that reacts with Bombyx GAPDH. Separate cells were transfected with pIZ-BmTRN-1 RNAi (0.1 μg) to evaluate the effects of endogenous BmTRN-1 knock-down on the consequences of BRO-B/E expression. The effects of BmTRN-1 RNAi were evaluated by verifying endogenous BmTRN-1 expression by immunoblotting with a specific antibody,
as described by Kotani et al. (2003). BmTRN-1 amounts were compared using densitometric analyses with Image software (http://rsb.info.nih.gov/nih-image/).

Cell lysates (4 µl containing 1 µg protein µl⁻¹) were mixed with 20 µl of the substrate mixture Steady Glo (Promega) and the luciferase activity of each lysate was measured using a GloMax 20/20 luminometer (Promega). Each assay was performed five times. Mean ± SD normalized relative activities were calculated for all experimental samples and significance was tested using a one-way ANOVA followed by Tukey’s test.

To verify quantities of luciferase transcripts, quantitative RT-PCR was performed as follows. Total RNA (125 ng) from cells at 36 h post-transfection was treated with 1 U RNase-free DNase I (Promega) to remove DNA contamination and analysed using a SuperScript III Platinum SYBR Green One-Step RT-PCR kit (Life Technologies) with standard reagents according to the manufacturer’s instructions. Luciferase primer sequences are listed in Table S2. Amplification of DNAs (n = 3) was determined using the LightCycler Nano System (Roche Diagnostics). The expression of luciferase transcripts was normalized to that of 28S rRNA (GenBank accession number X58442), which was amplified using the primers listed in Table S2.

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