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Depletion of *Bacillus subtilis* Histone-like Protein, HBsu, Causes Defective Protein Translocation and Induces Upregulation of Small Cytoplasmic RNA

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Small cytoplasmic RNA (scRNA) is a metabolically stable homologue of mammalian SRP RNA that contains an *Alu*-like domain. The *Bacillus subtilis* histone-like protein HBsu can bind this domain. We demonstrate here that repressing the level of HBsu results in slow growth and the accumulation of precursor of β-lactamase fusion proteins having the signal sequence of alkaline protease, penicillin binding protein 5* (PBP5*) or CGTase. The degree of the translocation defect varied among the various signal sequences tested. A pulse-chase experiment showed that processing the α-amylase signal sequence is significantly inhibited in HBsu-depleted cells. Northern blot analysis indicated that repressing the HBsu gene induces scRNA upregulation, indicating that the defective translocation of presecretory proteins is not due to a reduced scRNA level. The data presented here suggest that HBsu plays a pivotal role in SRP function rather than simply stabilizing the other SRP components such as scRNA.

Signal recognition particle (SRP) purified from canine pancreas is composed of a 7SL RNA that consists of 300 nucleotides and six proteins (9, 14, 19, 54, 68 and 72 kDa proteins)(1). Among these, SRP54 is the best characterized and the most important component, since it interacts with both 7SL RNA and signal sequences and binds GTP (2). Mammalian 7SL RNA consists of *Alu*- and S-domains, both of which are functional (3). The *Alu*-domain comprises the sequence at the 5′ and 3′ ends of 7SL RNA that are homologous to the *Alu* family of repetitive sequences (4). In contrast, the S-domain comprises a central region of 7SL RNA and is not homologous to any *Alu* sequence. SRP54 associates with the S-domain and the polypeptides SRP9 and SRP14 bind the *Alu*-domain (5). *E. coli* 4.5S RNA consists of 114 nucleotides that can fold into a single hairpin corresponding to an S-domain (6). Almost all SRP RNA from Gram-negative bacteria have a secondary structure similar to that of *E. coli.* On the other hand, the secondary structure of SRP RNAs from Gram-positive bacteria differs from that of *E. coli. B. subtilis* scRNA is predicted to fold into a structure strikingly similar to that of eukaryotic SRP RNAs (7) and it contains an *Alu*-domain. Like mammalian SRP54, Ffh can bind the S-domain of scRNA (8). We recently discovered that HBsu is an integral component of *B. subtilis* SRP-like particle that can bind the *Alu*-domain of scRNA (9). However, direct evidence showing HBsu function in protein translocation has not yet been obtained.

Here, we describe that HBsu depletion led to defects in the translocation of tested secretory proteins. In addition, the loss of HBsu caused upregulation of the intracellular scRNA level.

MATERIALS AND METHODS

Plasmids. Plasmids pTUBE1234, pTUBE1235, and pTUBE1236 encode β-lactamase fusion proteins with the signal sequences of *B. subtilis* alkaline protease (AprE-Bla), PBP5* (PBP5*-Bla) and Bacillus sp#1011 CGTase (CGTase-Bla), respectively (10).

Western blotting. Proteins were separated by SDS-PAGE, then electroblotted onto polyvinylidene difluoride membranes (Millipore) that were then blocked with 5% skim milk in buffer A (20 mM Tris-HCl (pH 7.5), 154 mM NaCl, 0.2% Tween 80), probed with a rabbit anti- (*E. coli* β-lactamase) serum (5′-3′ Inco. Co.) (1:1,000 dilution in 5% BSA/buffer A) for 1h, rinsed with buffer A, then incubated with secondary antibody for 1 h. After washing, bound antibody was detected using enhanced chemiluminescence (Amersham).

Pulse chase experiment. To determine whether or not HBsu depletion affects the translocation of secretory proteins, signal cleavage of α-amylase was examined in a pulse-chase experiment using *B. subtilis* UT1682 harboring plasmid pTUB256 (11), which directs the synthesis of extracellular α-amylase. Cells were incubated at 37°C in M9 medium supplemented with 18 amino acids (20 mg/ml each, excluding methionine and cysteine), in the presence or absence of 1 mM IPTG. When cultures reached a Klett colorimeter reading of 150 to 200, the cells were pulse-labeled with 44 μCi of [^{35}S]methionine

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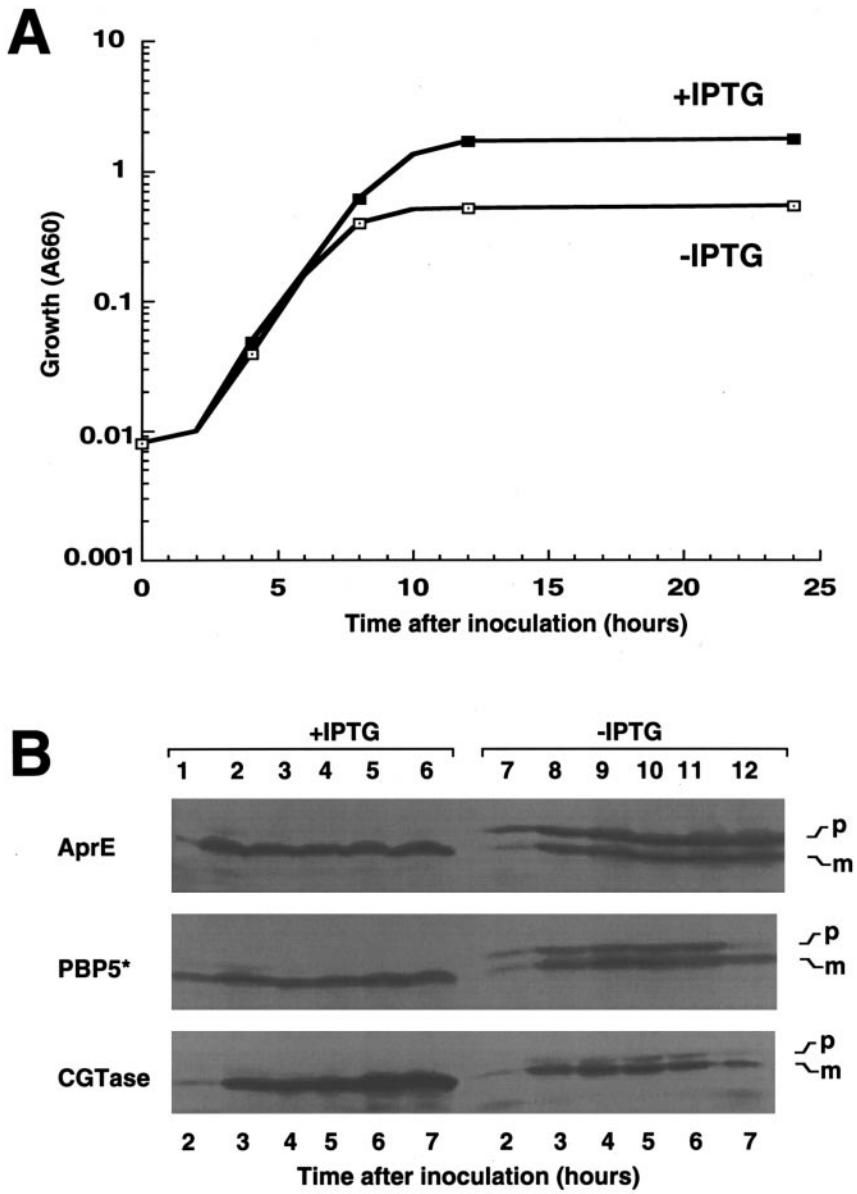

FIG. 1. Growth curves and accumulation of precursor proteins during HBsu depletion. (A) Growth curves of UT1682 harboring plasmid pTUE1338 in the presence or absence of 2 mM IPTG. (B) Accumulation of precursor proteins during HBsu depletion in *B. subtilis* UT1682 (containing HBsu gene under control of the *Pspac-1* promoter) harboring pTUBE1234, pTUBE1235, and pTUBE1236 cultured in the presence or absence of IPTG. Cell extracts were prepared at various times after inoculation (time = 2-7 h) as described in Materials and Methods. Total proteins (3 mg) from each cell lysate were resolved by SDS-PAGE and the indicated fusion proteins were detected by Western blotting using anti-β-lactamase antibody. m, mature; p, precursors of fusion proteins.

(Amersham) per ml for 5 min, then chased with 200 μg of unlabeled L-methionine per ml. The labeled cells were incubated with 10 mg of egg white lysozyme per ml at 37°C for 10 min, boiled for 1 min in the presence of SDS, then immunoprecipitated using antiserum against *B. subtilis* α-amylase. The precipitates were then separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

Northern blotting. Total RNAs in cell extracts were recovered using ISOGEN (Nippon gene, Japan). Denatured RNAs were resolved by electrophoresis on an agarose gel and blotted onto a Gene Screen Plus membrane (DuPont NEN). A 271-bp ^{32}P-labeled DNA fragment encoding mature scRNA served as the probe for DNA-RNA hybridization.

RESULTS AND DISCUSSION

HBsu Is Required for Efficient Translocation of Several Presecretory Proteins

HBsu is essential for cell growth (12), since all efforts to obtain null mutants of HBsu failed. To understand the function of HBsu in protein translocation, we constructed strain UT1682, in which the gene expression of HBsu is regulated by IPTG (9). The transformants proliferated as well as wild type cells in the presence of IPTG. In contrast, the level of HBsu decreased in the

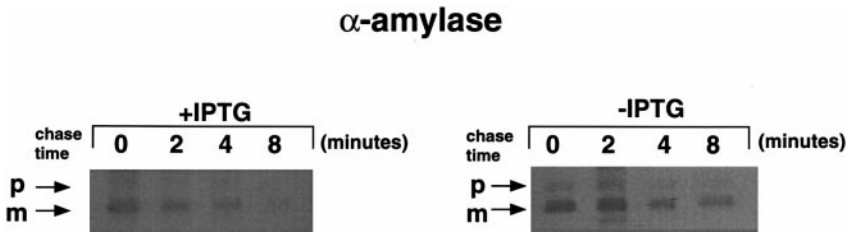

FIG. 2. Effect of HBsu depletion on α-amylase processing. Cleavage of the α-amylase signal sequence was monitored by pulse-chase experiments as described in Materials and Methods. p, precursor of α-amylase; m, mature forms of α-amylase.

absence of IPTG and after 5 h, was reduced to approximately 60% of that in wild-type cells (data not shown). At this time, growth defects were not apparent in UT1682 cells. By 8-9 h after inoculation in the absence of IPTG, the rate of UT1682 cell growth began to diminish (Fig. 1A). The time lag before the cell growth rate declined may have been associated with the time required for the complete disappearance of HbBsu that is stable and abundant. Previous analyses indicate that *B. subtilis* Ffh and scRNA are important for cell growth and essential for the translocation of a subset of presecretory proteins (13, 14). In addition, Bunai et al. (10) used a chemical cross-linker to demonstrate that Ffh can bind the signal sequence of *B. subtilis* AprE and penicillin binding protein 5* (PBP5*) as well as *E. coli* OmpA. Therefore, translocation should be disrupted in the absence of HBsu. To directly determine whether or not HBsu functions in protein translocation, we examined the presence of several precursors in the absence of IPTG. Plasmids pTUBE1234, pTUBE1235, and pTUBE1236 were transformed into *B. subtilis* UT1682. Proteins in the cell extract and medium were collected at hourly intervals between 2-7 h after depleting IPTG (Fig. 1A). Precursor and mature fusion proteins were detected by Western blotting after SDS-PAGE.

The translocation of all proteins examined was defective upon HBsu depletion in the absence of IPTG even at 3 h, when growth irregularities were not evident (Fig. 1B). The degree of the defect varied among the tested translocation substrates. The processing of CGTase-Bla was partially inhibited compared with that of PBP5*-Bla and AprE-Bla. Densitometric measurements of each autoradiographic band indicated that 50% of PBP5*-Bla and AprE-Bla remained in the precursor form. The results for the Ffh-disrupted mutant were the same using the same presecretory protein expression system (15). CGTase-Bla processing was partially disrupted in Ffh-depleted cells.

We further investigated the effect of HBsu depletion on protein translocation by performing pulse-chase experiment to monitor cleavage of the α-amylase signal sequence. Almost all of the precursor of α-amylase was converted into its mature form after a 2 min chase when cells were cultured in the presence of IPTG (Fig. 2, +IPTG). In the absence of IPTG, the enzyme precursor accumulated in the cells and about 20% of protein remained in precursor form after a 2 min chase (Fig. 2, −IPTG). These data demonstrated that HBsu depletion primarily affects the translocation of extracellular proteins.

HBsu Depletion Upregulated scRNA Gene Expression

Brown et al. (16) have demonstrated that the stable assembly of yeast SRP *in vivo* relies on the presence of all subunits. The levels of scR1 RNA and other SRP proteins were significantly reduced in strains lacking any one of Srp14p, Srp21p, Srp68p or Srp72p. To determine the effects of HBsu depletion on the expression level of the scRNA gene, total RNA was isolated from the UT1682 strain cultured in the presence or absence of IPTG. Figure 3 shows the result of a Northern blot where the level of the scRNA transcript is noticeably upregulated at 6–8 h. Northern blotting showed that the level of scRNA expression was increased 10 fold compared with that in the presence of IPTG at 3 h after HBsu expression was repressed and that it continuously increased for 8 h (Fig. 3B). These effects of HBsu depletion were specific for scRNA since the level of ribosomal RNA was not changed (Fig. 3, rRNA). These results indicated that the defective translocation of

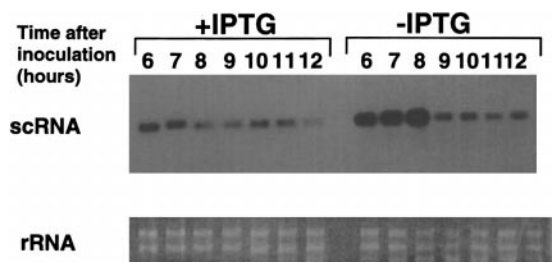

FIG. 3. Effects of HBsu depletion on the scRNA expression level. Total RNA (200 μg) obtained at different times was loaded onto each lane. Membranes were hybridized with a ^{32}P-labeled DNA fragment encoding mature scRNA and washed at high stringency. The bottom panel represents ribosomal RNA resolved on 0.8% agarose and stained with ethidium bromide.

presecretory proteins is not due to the reduced level of scRNA. These data suggest that HBsu plays a pivotal role in SRP function rather than simply stabilizing other components of SRP, such as scRNA.

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