Endoplasmic Reticulum Stress Protein Expression in Selected Organs of *Limanda yokohamae* from Masan-Jinhae Bay, Korea

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Abstract – Changes in stress-associated biomolecules can be used as an important criterion for assessing the levels of environmental pollution because living organisms demonstrate contamination-stimulated stress responses. This study was conducted to determine the environmental status of Masan-Jinhae Bay, Korea, and its effects on marine organisms by investigating the endoplasmic reticulum (ER) dysfunction in the organs of the flat fish, *Limanda yokohamae*. ER dysfunction was evaluated via Western blot analysis of the ER stress proteins, immunoglobulin heavy chain binding protein (BiP) and C/EBP-homologous protein (CHOP), and the ER stress-associated protein caspase-12. The results showed that the amount of BiP and CHOP immunoreactivity in the flat fish from the bay area was much greater than that from the Gangneung, as a reference site. Similar to the ER stress proteins, the immunoreactivity of caspase-12 was also found to be elevated in the bay area when compared with that of Gangneung. These data suggest that the environmental status of Masan-Jinhae Bay induces the ER stress response, which is able to lead to phenotypic changes in marine organisms including fish.

Key words : BiP, CHOP, Environmental pollution, ER stress, *Limanda yokohamae*

INTRODUCTION

The endoplasmic reticulum (ER) regulates the correct folding and assembly of proteins during posttranslational modification (Ellgaard *et al.* 1999; Yu *et al.* 1999; Hitomi *et al.* 2004). The ER chaperones such as immunoglobulin heavy chain binding protein (BiP) and C/EBP-homologous protein (CHOP) play critical roles in the unfolded protein response (UPR)-mediated cell survival and cell death (Kim *et al.* 2006). Protein folding and assembly are monitored by these chaperones and folding enzymes, which are collectively referred to as the ER quality control system (Hammond and Helenius 1995). Disturbances such as Ca^{2+} reduction, glucose deprivation and the accumulation of unfolded proteins cause ER stress (Groenendyk and Michalak 2005; Xu *et al.* 2005), which is known to increase the expression of the ER chaperones (or stress protein markers) such as BiP, inositol-requiring enzyme-1 (Ire1) and CHOP (Marciniak *et al.* 2004; Momoi 2004). Stressful cellular stimuli in animals induce apoptosis through complicated cell signaling pathways (Federica *et al.* 2006). A previous study showed that the expression of caspase-12 directly correlates with the expression of ER stress proteins in tumor cells (Kim *et al.* 2006).

The Masan-Jinhae Bay is located in an industrial area of

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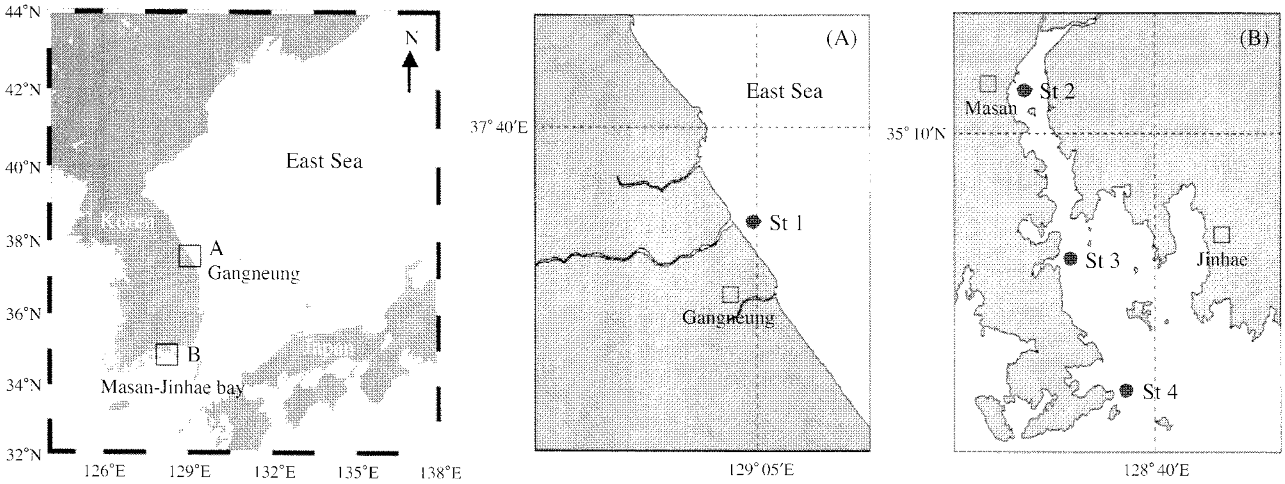

Fig. 1. Sampling sites (St) of *L. yokohamae* from Gangneung (A) (St 1) and Masan-Jinhae Bay (B) (St 2, Gapo; St 3, Dukdong; St 4, Okgye), Korea.

the southern part of Korea. Increasing industrial development and population in the bay area over the past decades have increased the risks for pollution in the bay and deterioration of the water quality. Accumulated evidence demonstrated that pollutants such as heavy metals (Lee *et al.* 1990), polycyclic aromatic hydrocarbons (PAHs) (Yim *et al.* 2005), polychlorinated biphenyls (PCBs), chlorinated pesticides (Hong *et al.* 2003), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) (Im *et al.* 2002; Kannan *et al.* 2007) in the sediments of the bay are detected at high levels and are found to affect biota including fish (Kong *et al.* 1998). It was therefore hypothesized that fish in the bay may experience an ER stress response evoked by their contaminated environment. In order to determine the environmental status of the bay and its effects on marine organisms, the level of ER dysfunction in fish was investigated by visualizing the immunoreactivity of the ER stress proteins, such as BiP and CHOP, and the ER stress-associated protein caspase-12. Marbled sole, *Limanda yokohamae* (*L. yokohamae*), was used in this study because this flat fish is a resident species of the bay.

MATERIALS AND METHODS

1. Study area and sampling

L. yokohamae adults weighing 90 ~ 120 g each were collected from Gapo, Dukdong and Okgye in the Masan-Jinhae Bay and from Gangneung, which served as a reference site, during the period from April to June 2005 (Fig. 1) (n=7 ~ 8 per site). The selected organs of *L. yokohamae*, such as the brain, gill, muscle, kidney and gonad were immediately isolated, dissected and frozen in liquid nitrogen and then stored at −70°C for Western immunoblot analysis.

2. Western immunoblot analysis

Tissue samples were lysed in sodium dodecyl sulfate (SDS) sample buffer for 5 min at 95°C. The samples were then sonicated for 30 sec on ice and centrifuged at 700 g for 10 min at 4°C. After discarding the pellet containing mainly nuclei and large debris, the supernatant was centrifuged again at 12,000 g for 30 min at 4°C and was resolved using 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were then transferred to a nitrocellulose membrane. The membrane was blocked with blocking buffer containing 5% skim milk and was probed with rabbit primary antiserum against BiP (1 : 2,000), CHOP (1 : 1,000) or caspase-12 (1 : 1,000) overnight at 4°C on a shaker. The membrane was then incubated with the appropriate secondary antiserum for 1 hr at room temperature. Immunoreactive protein bands were detected by enhanced chemiluminescence reagents (ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA) on X-ray films. All of blots were probed for BiP, CHOP, and caspase-12, after which they were striped and reprobed with antiserum against actin (1 : 1,000) for normalization. The results are presented as a percentage of

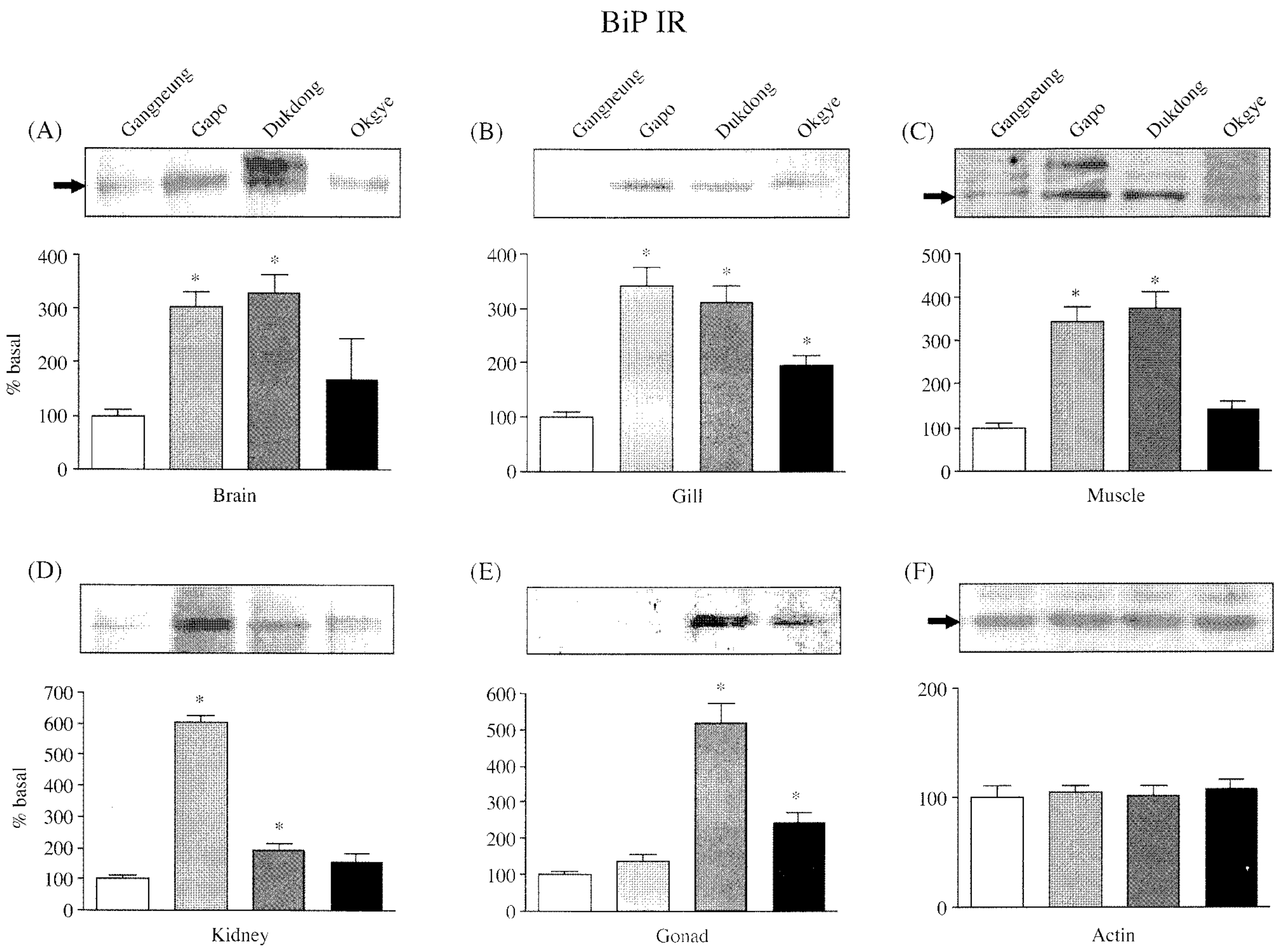

Fig. 2. Western immunoblot analysis for BiP and actin (A-F) immunoreactivity in the organs of *L. yokohamae* from Gangneung (Lane 1) and Masan-Jinhae Bay (Lane 2, Gapo; Lane 3, Dukdong; Lane 4, Okgye), Korea. The BiP immunoreactivity appears to be increased in the bay area when compared with that of Gangneung.

the control values. Antiserum for BiP, CHOP, and actin was obtained from Sigma-Aldrich (St. Louis, MO, USA) and antiserum for caspase-12 was purchased from Santa Cruz Biotech (Santa Cruz, CA, USA).

3. Quantitation of immunoreactivity

Immunoreative protein bands were semi-quantified using a digital camera and NIH Image 1.62 software. Briefly, the film background was measured and saved as a "blank field" to correct for uneven illumination. The upper limit of the density slice option was set to eliminate any background and this value was used to measure all images. The lower limit was set at the bottom of the LUT scale. The immuno-reactive protein bands were measured using a rectangle that encompassed the individual band.

4. Statistics

The statistical significance of the immunoreactive pixels per measured area between each group was determined using one-way ANOVA on ranked data followed by a Tukey's HSD (honestly significant difference) test in GraphPad Prism4 (GraphPad Software Incorporation, San Diego, CA, USA). The level of statistical significance was set at $P<0.05$.

RESULTS AND DISCUSSION

Since living organisms have been shown to demonstrate contamination-stimulated stress responses, changes in stress -associated biomolecules can be used as an important cri-

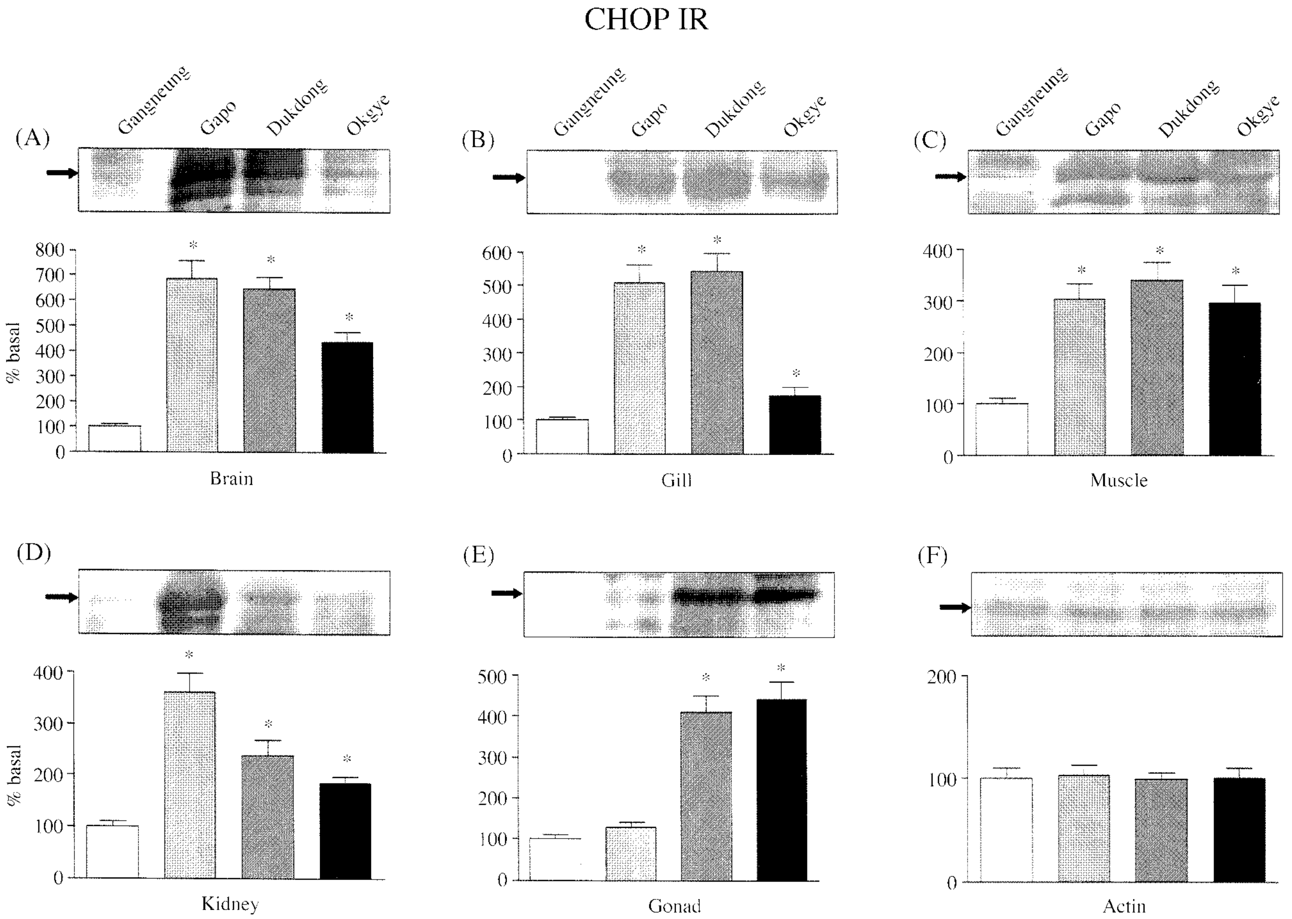

Fig. 3. Western immunoblot analysis for CHOP and actin (A-F) immunoreactivity in the organs of *L. yokohamae* from Gangneung (Lane 1) and Masan-Jinhae Bay (Lane 2, Gapo; Lane 3, Dukdong; Lane 4, Okgye), Korea. The CHOP immunoreactivity appears to be increased in the bay area when compared with that of Gangneung.

terion for assessing the status of environmental pollution. In this study, the immunoreactivity of the ER stress proteins and the ER stress-associated protein caspase-12 were determined in the selected organs of *L. yokohamae* acquired from three sites (Gapo, Dukdong and Okgye) in Masan-Jinhae Bay area and from a site in Gangneung, which served as a reference.

The results from this study showed that the level of BiP immunoreactivity in the brain of fish studied at Gapo and Dukdong was significantly elevated in comparison to that of the control group at Gangneung (Fig. 2A). Similarly, the level of BiP immunoreactivity in the gill, muscle, kidney, and gonad was also significantly elevated in fish from the bay area when compared to that of fish from Gangneung (Fig. 2B-E). In Masan-Jinhae Bay, the level of overall BiP immunoreactivity was relatively higher at Gapo and Dukdong compared to that of Okgye. However, alteration of actin immunoreactivity was not observed in the bay area or Gangneung (Fig. 2F). Although direct evidence for the regulated expression of BiP in fish is not currently available, a previous study demonstrated that the expression of the BiP gene in the early development of *Xenopus* is increased in response to stress (Miskovic and Heikkila 1999). These data suggest that BiP is expressed and accumulated in response to stressful stimuli. Similar to the immunoreactivity of BiP, the immunoreactivity of CHOP was also significantly elevated in the bay area in comparison to that of Gangneung, while the alteration of actin immunoreactivity was not observed in any location, as shown in Fig. 3A-F. A previous study showed that CHOP is activated by ER stress in order to protect cells against various inducers of the ER stress response (Marciniak *et al.* 2004). In addition, chemicals caus-

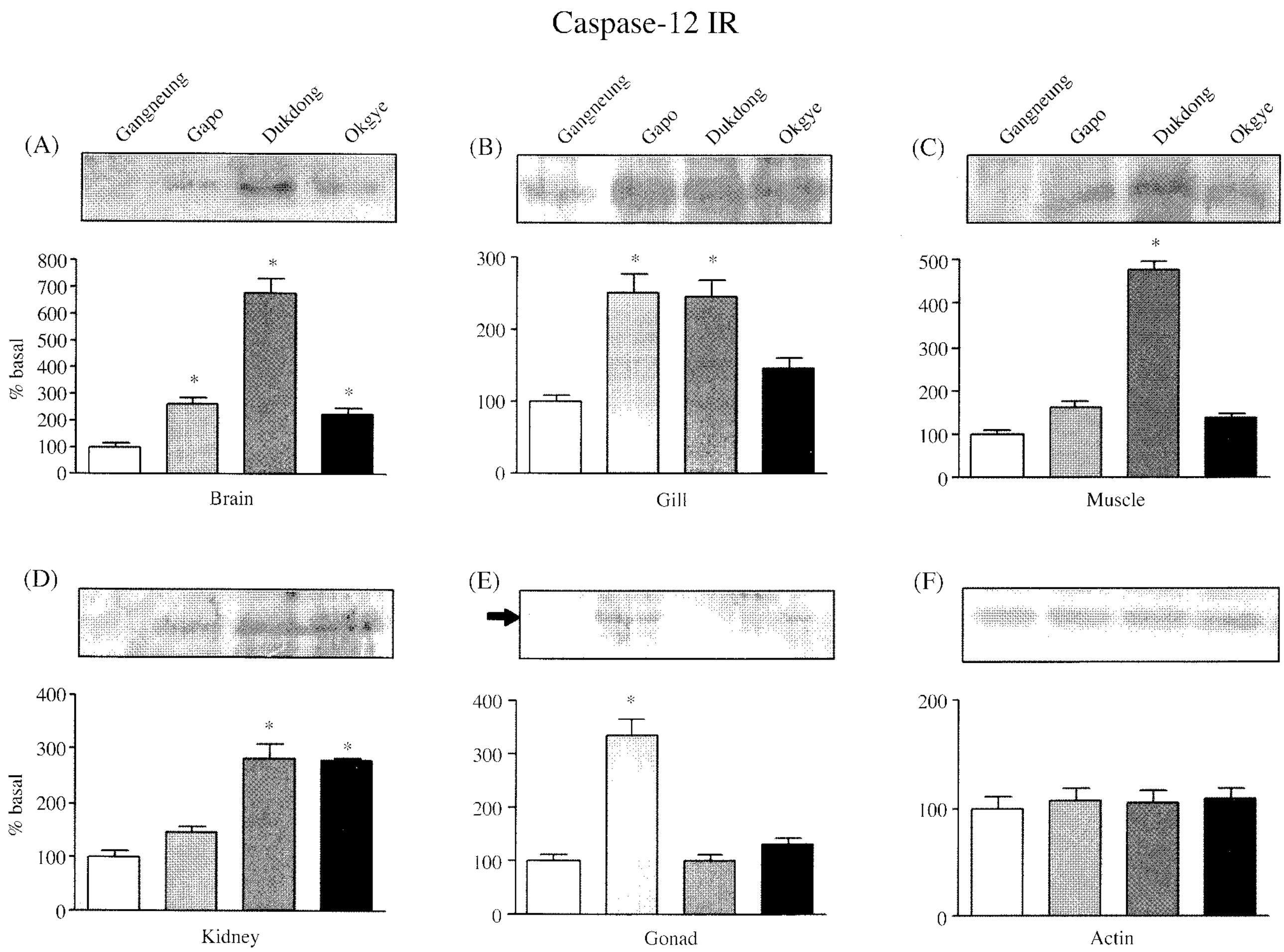

Fig. 4. Western immunoblot analysis for caspase-12 and actin (A-F) immunoreactivity in the organs of the *L. yokohamae* from Gangneung (Lane 1) and Masan-Jinhae Bay (Lane 2, Gapo; Lane 3, Dukdong; Lane 4, Okgye), Korea. The caspase-12 immunoreactivity appears to be increased in the bay area when compared with that of Gangneung.

ing toxicity, such as heavy metals, PAHs, PCBs, PCDDs, and PCDFs, are detected in the sediments of the bay and found to be at a high enough concentrations to influence the phenotypes of fish (Lee *et al.* 1990; Kong *et al.* 1998; Im *et al.* 2002; Hong *et al.* 2003; Yim *et al.* 2005; Kannan *et al.* 2007). These results suggest that the environmental status of Masan-Jinhae Bay is stressful enough to produce an ER stress response, which may lead to a dysfunction in protein synthesis in the organs of *L. yokohamae*. In order to determine the probability of cell death related to the ER stress response, caspase-12 immunoreactivity was observed in the organs of *L. yokohamae* and the results are shown in Fig. 4. As in BiP and CHOP immunoreactivity, the level of caspase-12 immunoreactivity was significantly increased in Gapo and Dukdong in comparison to that of Gangneung. These findings suggest that the environmental status of Masan-Jinhae Bay is worse than Okgye in terms of the ER stress-mediated cell death, even though there is some variability in caspase-12 immunoreactivity in the bay area. This prediction is supported by the fact that apoptosis in neuronal cells is caused by ER stress factors, such as BiP and CHOP (Luo *et al.* 2006; Tajiri *et al.* 2006).

In this study, the immunoreactivity of the ER stress proteins, BiP and CHOP, and the ER stress-associated protein caspase-12 was consistently increased in the organs of *L. yokohamae* in Masan-Jinhae Bay when compared with that fish from Gangneung. This suggest that the environmental status of Masan-Jinhae Bay can induce the ER stress response, which may lead to atypical phenotypes in these flat fish. However, the cellular mechanisms underlying this process still remain to be investigated.

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