ORIGINAL ARTICLE

Identification and characterization of cryptic *SHOX* intragenic deletions in three Japanese patients with Léri–Weill dyschondrosteosis

**Maki Fukami · Sumito Dateki · Fumiko Kato ·
Yukihiro Hasegawa · Hiroshi Mochizuki ·
Reiko Horikawa · Tsutomu Ogata**

Abstract Although short-stature homeobox-containing gene (*SHOX*) haploinsufficiency is responsible for Léri–Weill dyschondrosteosis (LWD), the molecular defect has not been identified in ~20% of Japanese LWD patients. Furthermore, although high prevalence of microdeletions affecting *SHOX* is primarily ascribed to the presence of repeat sequences such as *Alu* elements around *SHOX*, it remains to be determined whether microdeletions are actually mediated by repeat sequences. We performed multiple ligation probe amplification (MLPA) assay in six Japanese LWD patients with apparently normal *SHOX*, followed by fluorescent in situ hybridization (FISH) analysis and sequencing for polymerase chain reaction (PCR) products encompassing the deletion junctions in patients with abnormal MLPA patterns. Consequently, heterozygous intragenic deletions were identified in three cases, i.e., a 5,906-bp deletion involving exons 4–5 in case 1, a 5,594-bp deletion involving exons 4–6a in case 2, and a 50,199-bp deletion involving exons 4–6b in case 3. The deletion breakpoints of cases 1 and 2 were present in nonrepeat sequences, whereas those of case 3 resided within *Alu* elements. The results suggest that cryptic *SHOX* intragenic deletions account for a small fraction of LWD and that microdeletions affecting *SHOX* can be generated by repeat-sequence-mediated aberrant recombinations and by nonhomologous end joining.

Keywords *SHOX* · Intragenic deletion · MLPA · Léri–Weill dyschondrosteosis · Repeat sequence

M. Fukami (✉) · S. Dateki · F. Kato · T. Ogata
Department of Endocrinology and Metabolism,
National Research Institute for Child Health and Development,
2-10-1 Ohkura, Setagaya, Tokyo 157-8535, Japan
e-mail: mfukami@nch.go.jp

S. Dateki
Department of Pediatrics, Nagasaki University
School of Medicine, Nagasaki, Japan

Y. Hasegawa
Endocrinology and Metabolism Unit, Tokyo Metropolitan
Kiyose Children's Hospital, Tokyo, Japan

H. Mochizuki
Department of Endocrinology and Metabolism,
Saitama Children's Medical Center, Saitama, Japan

R. Horikawa
Division of Endocrinology and Metabolism,
National Center for Child Health and Development,
Tokyo, Japan

Introduction

Léri–Weill dyschondrosteosis (LWD) is a dominantly inherited skeletal dysplasia characterized by Madelung deformity and mesomelic short stature (Langer 1965). It is caused by haploinsufficiency of the short-stature homeobox-containing gene (*SHOX*) on the short arm pseudoautosomal region (PAR1) of the human sex chromosomes (Ogata 2002; Blaschke and Rappold 2006). To date, extensive studies have been performed, identifying multiple intragenic mutations (Niesler et al. 2007) and various submicroscopic deletions encompassing the entire *SHOX* coding region and/or the putative downstream enhancer region(s) (Kosho et al. 1999; Ogata 2002; Benito-Sanz et al. 2005, 2006a, b; Fukami et al. 2006; Huber et al. 2006; Sabherwal et al. 2007). Submicroscopic deletions are more frequent than intragenic mutations (Ogata 2002), and this would be consistent with repeat sequences being

abundantly present around *SHOX*, because aberrant intrachromosomal or interchromosomal recombinations are prone to occur between such sequences (Ogata 2002; Blaschke and Rappold 2006). Indeed, *Alu* and L1 elements are abundant on the X chromosome, with *Alu* elements being more frequent and L1 elements being less frequent on the PAR1 than on the rest of the X chromosome (Lyon 2000; Blaschke and Rappold 2006). However, *SHOX* abnormalities have not been identified in a substantial fraction of LWD patients (Benito-Sanz et al. 2006a; Blaschke and Rappold 2006), and we have also failed to reveal *SHOX* abnormalities in ~20% of Japanese LWD patients (Ogata 2002). Furthermore, it remains to be determined whether microdeletions around *SHOX* are directly mediated by repeat sequences.

Multiple ligation probe amplification (MLPA) is a recently developed method for relative quantification of single-copy sequences in the human genome (Schouten et al. 2002). It has been demonstrated as a powerful tool in the detection of deletions affecting several genes, including *SHOX* (Benito-Sanz et al. 2005, 2006a, b; Gatta et al. 2007). However, except for a single patient with a tiny deletion encompassing exons 4–6a of *SHOX* that could be revealed only by MLPA analysis (Benito-Sanz et al. 2006b), this method has been performed in patients with sex chromosomal abnormalities or relatively large deletions involving the entire *SHOX* coding region and/or the downstream enhancer region(s) that can be identified by other methods such as fluorescent in situ hybridization (FISH) analysis and microsatellite genotyping (Kosho et al. 1999; Benito-Sanz et al. 2006a, b; Gatta et al. 2007).

Here we report three cryptic *SHOX* intragenic deletions that were first identified by MLPA analysis. Characterization of deletions implies that microdeletions affecting *SHOX* can be generated by homologous and nonhomologous rearrangements.

Patients and methods

Patients

We studied six unrelated Japanese female patients (cases 1–6) with definitive LWD phenotype [Madelung deformity and mesomelic short stature ranging from −4.4 standard deviation (SD) to −2.0 SD] in whom *SHOX* abnormality was not demonstrated by direct sequencing of coding exons 2–6b, by FISH analysis with an ~18-kb cosmid probe spanning from intron 2 to intron 6a (Kosho et al. 1999), and by microsatellite and single nucleotide polymorphism (SNP) genotyping for previously described multiple loci utilized for localizing downstream

enhancer(s) (Benito-Sanz et al. 2005; Fukami et al. 2006; Huber et al. 2006).

MLPA analysis

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development. After taking informed consent, MLPA was performed for cases 1–6 and three control female subjects using a SALSA MLPA Kit P018B (MRC-Holland, Amsterdam, the Netherlands) that contains probes for various parts of *SHOX* (*SHOX*-specific probes) (Fig. 1a) and multiple other loci (reference probes). The protocol was as described in the manufacturer's instructions (Schouten et al. 2002). In brief, 50 ng of leukocyte genomic DNA was hybridized with the probe mix, and the hybridization mixture was subjected to ligase reaction and polymerase chain rection (PCR) amplification. Subsequently, the PCR products amplified from ligated probes were visualized on a 310 ABI PRISM genetic analyzer (ABI Prisms, Foster City, CA, USA). For each *SHOX*-specific probe, a relative peak area was calculated by dividing each measured peak area by the sum of peak areas of the reference probes. The relative areas were compared between cases and controls, and relative peak areas less than 65% of those of controls were assessed to be indicative of heterozygous deletions (Schouten et al. 2002; Kozak et al. 2006).

FISH analysis

FISH was performed with probes detecting the presumably deleted regions indicated by MLPA. The probes were obtained by long PCR using LA taq polymerase (Takara, Ohtsu) and were labeled with digoxigenin and detected by rhodamine antidigoxigenin. A SpectrumGreen-labeled probe for *DXZ1* (Abbott, Abbott Park, IL, USA) was utilized as an internal control. For comparison, FISH was also performed with the cosmid probe (Fig. 1a) using previously described methods (Kosho et al. 1999).

Characterization of the deletions

Long PCR was performed with multiple primer pairs flanking the deleted regions. When long PCR products were obtained, they were subjected to direct sequencing using serial primers. The deletion size and the junction structure were determined by comparing the obtained sequences with the BX004827 and AL683871 sequences [National Center for Biotechnology Information (NCBI) database]. The presence or absence of repeat sequences around the breakpoints was examined with Repeatmasker (http://www.repeatmasker.org).
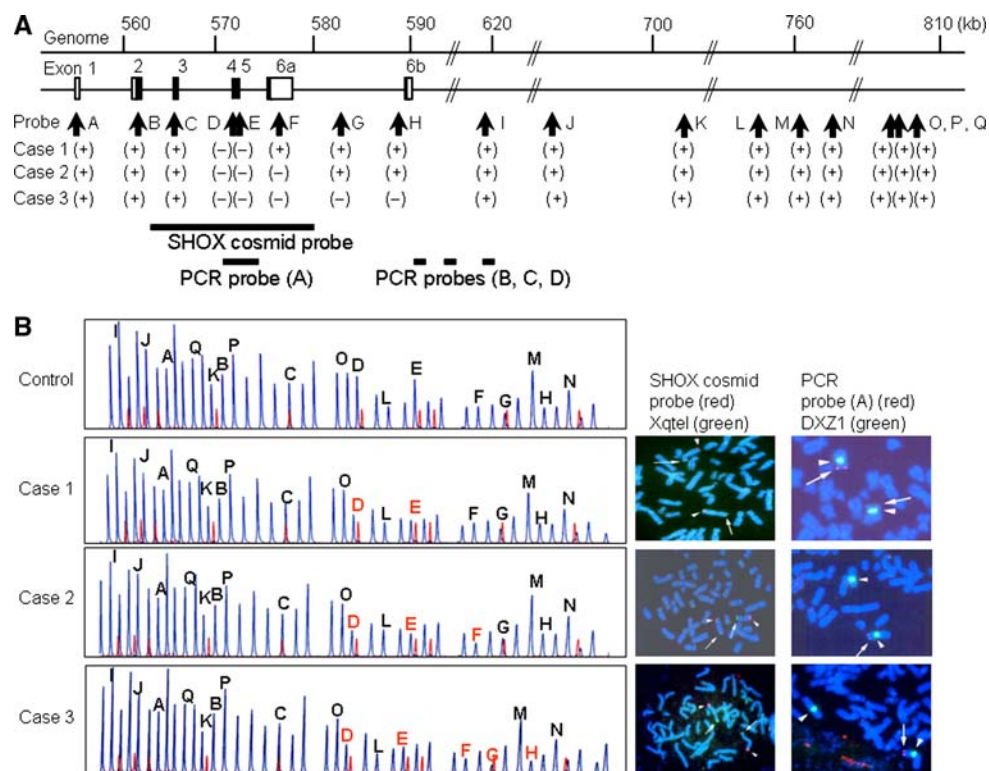
Fig. 1 Identification of cryptic intragenic short-stature homeobox-containing gene (*SHOX*) deletions in cases 1–3. **a** Summary of the multiple ligation probe amplification (MLPA) analyses and the fluorescent in situ hybridization (FISH) probes utilized in this study. The *upper horizontal line* indicates the physical distance from the Xp/Yp telomere. The *rectangles* represent *SHOX* exons (1–6b); the *black* and *white areas* denote the coding regions and the untranslated regions, respectively. The sites examined by MLPA probes (*A–Q*) are indicated by *arrows*; of these, the *A–F* sites reside on exons 1–6a, the *H* site lies just upstream of exon 6b, and thr *I–Q* sites reside around the enhancer region postulated at a position 30–250 kb downstream of *SHOX* coding region. The *plus* and the *minus symbols* represent the presence of two copies and a single copy of the corresponding sites, respectively. The *lower thick lines* represent the regions detected by FISH probes. The previously reported *SHOX* cosmid probe (Kosho et al. 1999) detects a ∼18-kb region from intron 2 to intron 6a, polymerase chain reaction (PCR) probe *A* detects a ∼4.0-kb region from intron 3 to intron 5, PCR probe *B* detects a ∼5.2-kb region just proximal to exon 6b, PCR probe *C* detects a ∼5.6-kb region at a position ∼19-kb proximal to exon 6b, and PCR probe *D* detects a ∼5.3-kb region at a position ∼32-kb proximal to exon 6b. The primers utilized were 5′-TCTCTCTCTGCTTCTCCCCA-3′ and

5′-GTGCAGGACGCGCGGT-3′ for PCR probe A, 5′-GTTAATGC TGAGAAGCTCTCCAAGCTAC-3′ and 5′-GTCCCTTACAAGGAC ACCTGTTATTGGAT-3′ for PCR probe B, 5′-GCTTGGTAGGAA GAGCCACAACTGTTCA-3′ and 5′-CTAGACGTCCACGGACCT ATGTTGTAAC-3′ for PCR probe C, and 5′-TGAGTAATTAAT CCCCACCAGTGAGGTC-3′ and 5′-CGTAGTTGGCCAAGACTC CACCATATTG-3′ for PCR probe D. **b** Results of MLPA assays (*left part*) and FISH analyses (*right part*). The *peaks* corresponding to sites around *SHOX* are indicated (*A–Q*); of these, the *A–F* sites reside on exons 1–6a, the *H* site lies just upstream of exon 6b, and *I–Q* sites reside around the enhancer region postulated at a position 30–250 kb downstream of *SHOX* coding region. MLPA assays indicate that compared with the relative peak areas in control female subjects, the relative peak areas for *D* and *E* in case 1, those for *D–F* in case 2, and those for *D–H* in case 3 are significantly reduced (shown in *red*). Relative peak areas for other loci (not specifically indicated) are similar between control female subjects and cases 1–3. The *red peaks* indicate the internal size markers. FISH analysis demonstrates that the ∼4-kb PCR probe A detects one faint and one clear signal in case 1 and only a single signal in cases 2 and 3, whereas the ∼18-kb cosmid probe detects two signals in cases 1–3

Results

MLPA analysis

Cryptic *SHOX* intragenic deletions were detected in cases 1–3 (Fig. 1b). Comparisons of relative peak areas indicated heterozygous deletion involving exons 4 and 5 in case 1, that involving exons 4–6a in case 2, and that involving exons 4–6b in case 3. No deletion was identified in cases

4–6. The results were reproduced in two independent experiments.

FISH analysis

FISH was performed with a PCR probe (A) for a region from intron 3 to intron 5 (Fig. 1a), detecting two signals with a marked difference in intensity (apparently one normal and one faint signal) in case 1 and only a single
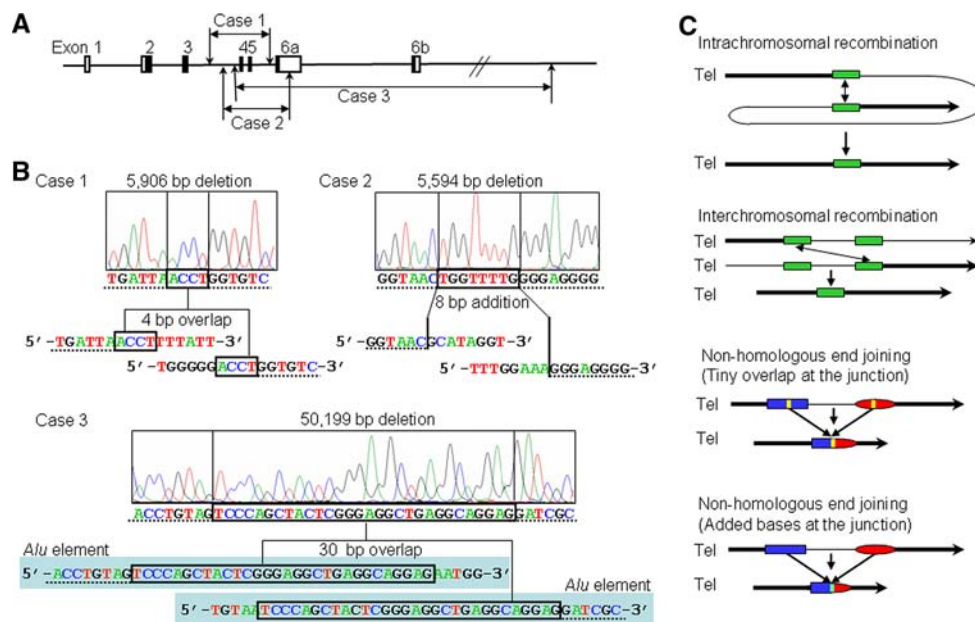
Fig. 2 Deletion sizes and junction structures. **a** Schematic representation of the intragenic deletions in cases 1–3. **b** Electrochromatograms showing the fusion points of the intragenic deletions. In case 1, a 4-bp segment surrounded by a *rectangle* is shared by the distal and the proximal breakpoint sequences. In case 2, an 8-bp segment of unknown origin surrounded by a *rectangle* is added to the deletion junction. In case 3, a 30-bp segment surrounded by a *rectangle* is shared by the distal and proximal breakpoint sequences. The breakpoints in cases 1 and 2 reside in nonrepeat sequences, whereas those in case 3 are present within *Alu* elements (shaded in *light blue*). Polymerase chain reaction (PCR) products encompassing the deletion junctions were obtained with the following primers: 5′-AAATTGGTTGTGGGGGTGTGT-3′ and 5′-GTGCAGGACGCGCGG-3′ in case 1, 5′-TGGATCGTGAATCACTCCAA-3′ and 5′-GCCATCTCTACACCCGTGAT-3′ in case 2, and 5′-AACAGA

GGTGAAGTGGATAATTGAG-3′ and 5′-TGGTTGGTTAGGAACTTGAATAGAG-3′ in case 3. The deletion junction sequences were determined with the following primers: 5′-AAATTGGTTGTGGGGTGTGT-3′in case 1, 5′-TTCGGTTCTCCTACAGGGTCT-3′ in case 2, and 5′-GCTAGGTGTGGTGGTGTGC-3′ in case 3. **c** Schematic representation of mechanisms leading to the generation of microdeletions. An aberrant intrachromosomal or interchromosomal recombination mediated by repeat sequences (*green rectangles*) causes a microdeletion (loss of a chromosomal region depicted by *thin lines*) between the repeat sequences. Nonhomologous end joining between nonrepeat sequences (*a blue rectangle and a red ellipse*) yields a microdeletion (*thin lines*) between the nonrepeat sequences and is often associated with an addition of a short segment of unknown origin (*a green segment*) or with a tiny overlapping segment common to the nonhomologous sequences (*yellow segments*)

signal in cases 2 and 3 (Fig. 1b). In case 3 with a relatively large deletion, FISH was further carried out with three PCR probes (B, C, D), localizing the proximal breakpoint between the regions identified by PCR probes C and D (not shown). These microdeletions were not identified by the cosmid probe (Fig. 1b), as mentioned in "Patients".

Deletion characterization

After examination with multiple primer sets, PCR products harboring the deletion junctions were obtained, and the deletion junction sequence was determined in cases 1–3 (Fig. 2a, b). Deletion size was 5,906-bp in case 1, 5,594-bp in case 2, and 50,199-bp in case 3. The deletion breakpoints of cases 1 and 2 were present on nonrepeat sequences, whereas those of case 3 resided within *Alu* elements. The fusion point resided at a 4-bp segment in case 1 and at a 30-bp segment in case 3 and was associated with an addition of an 8-bp segment of unknown origin in case 2.

Discussion

MLPA analysis identified cryptic *SHOX* intragenic deletions in cases 1–3. Since the cryptic deletions were detected in three of the six LWD patients with apparently normal *SHOX*, such tiny intragenic deletions may also be hidden in a substantial fraction of LWD patients without demonstrable *SHOX* haploinsufficiency. In this context, microdeletions affecting *SHOX* are frequently observed in LWD (Ogata 2002), and MLPA can identify at once various types of microdeletions affecting *SHOX*, including those involving a single or a few exons and those involving the entire coding region and/or the downstream enhancer region(s) (Benito-Sanz et al. 2006a, b), using genomic DNA of patients only. Thus, in conjunction with its simple and easy procedure, MLPA will serve as a powerful screening method for *SHOX* molecular defects.

The deletion junction resided in nonrepeat sequences in cases 1 and 2 and within *Alu* elements in case 3. The
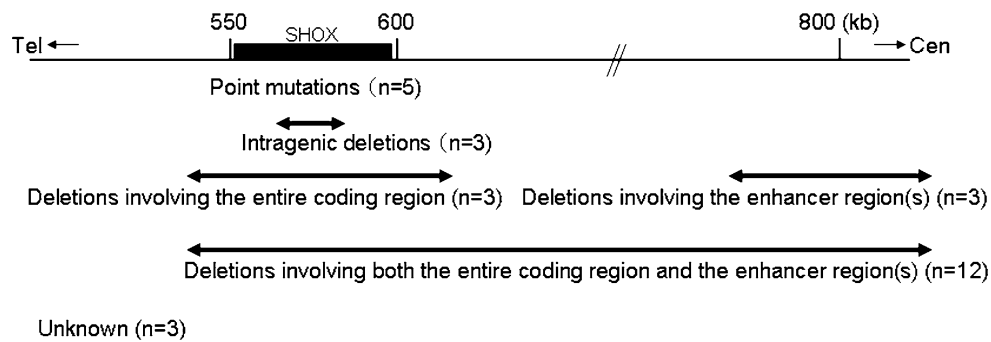
Fig. 3 Short-stature homeobox-containing gene (*SHOX*) abnormalities in 29 Japanese families with Léri–Weill dyschondrosteosis (LWD) and a normal karyotype. The *upper horizontal line* indicates the physical distance from the Xp/Yp telomere ("*Tel*"). The *rectangles* represent *SHOX* coding region. The *black arrows* denote the deleted regions. *SHOX* molecular defects were not identified in three families

intragenic deletion in case 3 would be ascribed to an aberrant intrachromosomal or interchromosomal recombination mediated by repeat sequences (Fig. 2c) (Ogata 2002; Blaschke and Rappold 2006). By contrast, the intragenic deletions in cases 1 and 2 would be due to nonhomologous end joining (NHEJ), i.e., an aberrant breakage and re-union between nonhomologous sequences (Fig. 2c) (Shaw et al. 2004). In particular, the presence of a short segment of unknown origin at the deletion junction in case 2 is characteristic of NHEJ (Shaw et al. 2004). Furthermore, while a short segment common to distal and proximal breakpoint sequences was identified at the deletion junction in case 1, the segment appears to be too short to permit an aberrant recombination, and NHEJ associated with such a tiny overlapping segment has been reported previously (Kozak et al. 2006). In addition, the scattered distribution of the microdeletion breakpoints around *SHOX* (Kosho et al. 1999; Benito-Sanz et al. 2005, 2006a, b; Fukami et al. 2006; Huber et al. 2006; Sabherwal et al. 2007) may primarily reflect genomic rearrangements caused by NHEJ, and NHEJ may be facilitated by the high recombination frequency in the PAR1 and by the abundant presence of repeat sequences (e.g., *Alu* elements) (Shaw et al. 2004; Blaschke and Rappold 2006). Collectively, the present study implies that the microdeletions affecting *SHOX* can be caused by both homologous and nonhomologous rearrangements.

To date, we have examined a total of 29 families containing at least one patient with LWD and a normal karyotype (total 50 patients). Consequently, we identified various types of *SHOX* abnormalities in 26 of the 29 families (~90%), i.e., 12 microdeletions involving the entire coding region and the putative downstream enhancer region(s), three microdeletions involving the entire coding region alone, three microdeletions involving the enhancer region(s) alone, three intragenic deletions, and five intragenic mutations (Kosho et al. 1999; Ogata 2002; Fukami et al. 2006; unpublished data) (Fig. 3). The frequency of *SHOX* abnormalities is higher than that reported by other groups (50–90%) (Blaschke and Rappold 2006). In particular, Benito-Sanz et al. (2006a) detected *SHOX* abnormalities only in 16 of 26 Spanish probands after performing extensive analysis, including MLPA analysis. Although the cause of the difference in the frequency of *SHOX* abnormalities (especially deletions) remains to be examined, this could be due to ethnic differences. Indeed, deletions encompassing *NSD1* for Sotos syndrome are also much more frequently identified in Japanese than in other ethnic groups (Kurotaki et al. 2003).

SHOX molecular defects has not been demonstrated so far in cases 4–6. There are two possible explanations for this. First, there still may be a hidden abnormality impairing *SHOX*. For example, a mutation or a tiny deletion may exist in the promoter region, the nonexamined exonic sequences (MLPA examines only a part of exonic sequences), the intronic sequences, or the enhancer sequences. Second, there may be a mutation in some gene(s) other than *SHOX*. In this regard, one possible locus may reside near the *HOXD* gene cluster. An association between LWD-like skeletal abnormality and a balanced translocation t(2;8)(q31;p21) has been found in a father and his three children, with the 2q31 breakpoint being mapped near the *HOXD* gene cluster (Spitz et al. 2002), and chromosomal breakage around the *HOXD* cluster is known to result in various limb malformations (Dlugaszewska et al. 2006).

In summary, the results suggest that MLPA analysis is a highly useful method to identify microdeletions affecting *SHOX*, including cryptic intragenic deletions, and that such microdeletions can be caused by homologous sequence-mediated aberrant recombinations and by nonhomologous end joining. Further studies will permit re-evaluation of the prevalence of *SHOX* molecular defects and the mechanisms leading to microdeletions affecting *SHOX*.

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