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Gene Expression of Growth and Differentiation Factors-5, -6, and -7 in Developing Bovine Tooth at the Root Forming Stage

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Growth and differentiation factors (GDF)-5, -6, and -7 are members of the bone morphogenetic protein (BMP) family. Previous studies suggest their importance in bone development and in tendon/ligament morphogenesis. The cells of the dental attachment apparatus, cementum, periodontal ligament, and alveolar bone proper are derived from the dental follicle proper. In this study, we investigated the expression of GDF-5, -6, and -7 genes in tissues of the bovine incisor tooth germ at the root forming stage. The results demonstrate distinct expression of GDFs in both the dental follicle and the odontoblast layer. While GDF-5 and -6 mRNAs were expressed in both the dental follicle and the odontoblast layer, GDF-7 mRNA expression was detected only in the dental follicle. These results indicate that GDFs, expressed in the bovine tooth germ including the dental follicle, may be potent regulatory molecules in the development of the dental attachment apparatus.

Growth and differentiation factors-5 (GDF-5/CDMP-1), -6 (GDF-6/CDMP-2/BMP-13) and -7 (GDF-7/BMP-12) are members of the bone morphogenetic protein (BMP) family that constitute a part of the transforming growth factor-β (TGF-β) superfamily (1, 2). TGF-β superfamily members are important regulators of growth and differentiation of many types of cells and organs (1, 3). Known for their capacity to induce ectopic bone formation when subcutaneously implanted in rats (4), BMPs are thought to play not only skeletal but also extraskeletal roles in cell growth, differentiation and embryonic development (5-8).

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the following accession numbers: bovine mRNA for growth and differentiation factor-5, partial cds, 202 bp: AB004301; bovine mRNA for growth and differentiation factor-7, partial cds, 202 bp: AB004302.

A function for TGF-β superfamily molecules is also implicated in tooth development. BMP-4 was identified as a secondary induction signal between epithelial and mesenchymal tissues in tooth germ (9). BMP-2 has effects on the differentiation of odontoblasts *in vitro* and *in vivo* (10, 11) and its mRNA is detected in dental epithelium, dental papilla and secretory odontoblasts (12). In addition, TGF-β1 mRNA is expressed in the dental epithelium and its underlying mesenchyme during the tooth development (13) and TGF-β2 may contribute to epithelial-mesenchymal interactions in tooth morphogenesis (14).

GDF-5, -6 and -7 were identified by polymerase chain reaction using degenerate primers (2) and share high homology with each other in their C-terminal regions. GDF-5 and GDF-6 are thought to be involved in the development of skeletal structures. mRNAs encoding GDF-5 and GDF-6 are expressed predominantly in cartilaginous tissues and a mutation in the GDF-5 gene results in shortened limb skeletal elements, i.e., *brachypodism* in mouse and Hunter-Thompson type chondrodysplasia in human (2, 15, 16). Though there are only a few studies focused on the roles of GDF-7, according to Wolfman *et al.* (17), GDF-5, -6 and -7 induce neotendon/ligament formation when implanted subcutaneously and *in situ* localizations of these GDFs mRNA suggest that they are important regulatory molecules in joint morphogenesis.

The dental attachment apparatus is a unique structure that consists of the periodontal ligament, rich in cellular and fibrous connective tissue, anchoring the root cementum to the alveolar bone proper. This joint-like connection formed by the periodontal ligament is referred to as the syndesmosis. Cementum, periodontal ligament, and alveolar bone proper form a developmental, structural, and functional unit. From a developmental point of view, cells of these tissues are derived from the ectomesenchymal cells of the dental follicle proper (18). In other words, in dental follicle

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proper, there are cells with ability to differentiate into cells of cementum, periodontal ligament, and alveolar bone proper. However, little is known about the mechanisms involved in the differentiation of these tissues. Elucidation of these mechanisms may facilitate the development of a clinical approach in periodontal regenerative therapy.

GDF-5, -6, and -7 molecules are likely to be involved in the development of the tooth germ, including the dental follicle proper, the source of tooth supporting tissues. Since they are important factors for tendon/ligament morphogenesis, GDFs might play significant roles in the differentiation of dental follicular tissues into periodontal ligaments. In order to establish possible functions for GDF-5, -6 and -7 molecules during development of the dental attachment apparatus, the expression of GDF-5, -6 and -7 mRNAs was measured in bovine incisor tooth germs at the stage of root formation.

MATERIALS AND METHODS

Isolation of tissues. Fresh tissues were isolated from approximately two year-old calves obtained from a local slaughterhouse. Incisor tooth germs of secondary dentition were dissected from the mandibles. All tooth germs used in this study were at the same stage of development consisting of an enamel organ with completely shaped crowns and developing roots 4~6 mm in length (Fig. 1).

Five tooth germs were used in each set of experiment. Dental papillae and dental follicles were removed carefully from permanent tooth germs and some dental follicles were dissected to obtain those tissues surrounding the root portion. After the removal of dental papillae, odontoblasts were recovered from the inside of the dentinal organ by the method of Oida *et al.* (19). Skeletal muscle and subcutaneous connective tissue from the same animal were isolated for comparative purposes. The animal experimental protocol used in this study is in agreement with the standards of the Tokyo Medical and Dental University.

RNA preparation. Total RNA was isolated from tissues by a single-step method using acid guanidium thiocyanate/phenol/chloroform extraction (20).

Reverse transcription-polymerase chain reaction (RT-PCR). Single-strand cDNA was prepared from 2 μg of each total RNA sample using random hexamers as primers and 200 U of reverse transcriptase (Super Script, GIBCO BRL, Gaithersburg, USA). A set of primers was designed on the basis of the conserved regions of the mouse GDF-5, -6, -7 and the human GDF-5 (Storm *et al.*, 1994; Chang *et al.*, 1994) as follows: sense primer 5′-TGGGACGACTGGATCATCGC-3′, antisense primer 5′-CACCATGTCCTCATACTGCTT-3′. One microliter of each cDNA preparation was added to a reaction mixture containing 25 pmol of each primer set, 5 μl of 10 × PCR buffer, 5 μl of dNTP mixture (0.25 mM for each deoxynucleotide), 35 μl of autoclaved, distilled water, and 1.25 U of AmpliTaq polymerase (TAKARA SHUZO Co., Ltd., Ohtsu, Japan). Amplification was performed using a 2 step protocol on a Perkin-Elmer/GeneAmp PCR system 9600: *step1* 5 cycles at 94°C (1 min), with a ramp from 50°C (1 min) to 72°C (1 min); *step2* 25 cycles at 94°C (30 sec), 55°C (30 sec), and then 72°C (30 sec). PCR products were analyzed by polyacrylamide gel (5%) electrophoresis in TBE buffer (pH 8.0). Gels were stained with ethidium bromide.

cDNA cloning and nucleotide sequencing. The PCR products (243 bp) were inserted into a pBluescript SK(+) plasmid vector (Stratagene, La Jolla, USA) to which dTTP was added at the 3′-end after digestion by EcoRV. DNA inserts were obtained from these clones by an alkaline lysis kit (Wizard Minipreps, Promega, Madison, USA), and then sequenced by a double-stranded dideoxy method using thermostable DNA polymerase (BcaBEST, TAKARA SHUZO Co., Ltd., Ohtsu, Japan).

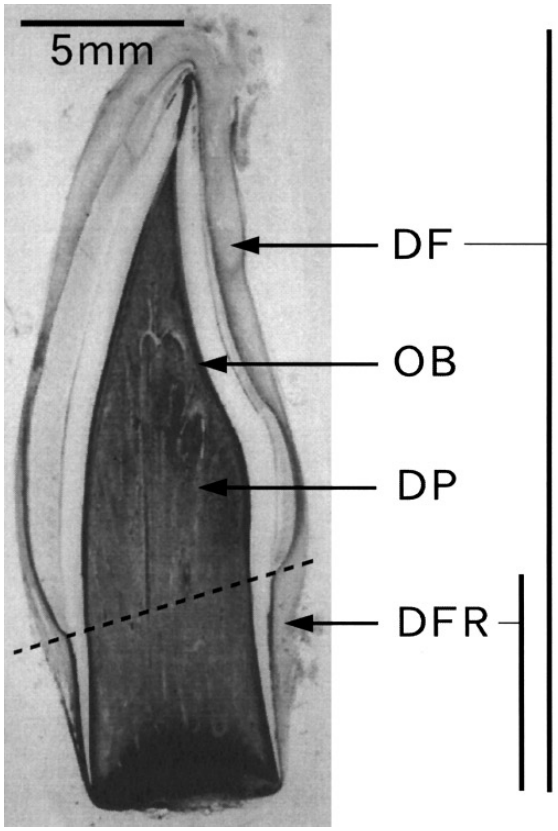

FIG. 1. A representative bovine incisor tooth germ at the root forming stage used in this study. DF, dental follicle; OB, odontoblast layer; DP, dental papilla; DFR, dental follicle surrounding the developing root. Doted line indicates the boundary between the crown and the root. DFR was removed from the portion of the tooth germ located under the line delineating the future CEJ. Stained with toluidine blue.

Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP). One half of a microliter of each cDNA preparation was subjected to a PCR reaction (10 μl) comprising 15 pmol of each primer set, 1 μl of 10 × PCR buffer, 1 μl of dNTP mixture (0.25 mM for each deoxynucleotide), 6 μl of autoclaved, distilled water, 5 μCi[α-^{32}P] dCTP (3000 Ci/mmol, ICN Pharmaceuticals, Inc., Irvine, USA), and 0.25 U of AmpliTaq DNA polymerase. The cycle conditions were the same as described above. A sample of each PCR product (2 μl) was diluted to 10 μl with formamide buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol), heated at 90°C for 4 min and chilled on ice. Aliquots (1 μl) were electrophoresed at 20 W for 5 hrs at room temperature in 6% nondenaturing polyacrylamide gels (acrylamide : bisacrylamide = 49: 1, cast with 10% glycerol and 0.6 × TBE) that were cooled by fans. The gel was dried on 3MM filter paper and exposed to an Imaging Plate (Fuji Photo Film Co., Ashigara, Japan), which was analyzed by a Fuji Imaging Analyzer (Fuji Photo Film Co., Ashigara, Japan). To obtain standard electrophoresis patterns of amplified GDFs, 0.1 ng of each plasmid containing either a GDF-5, -6, or -7 fragment (determined by DNA sequencing) was also subjected to PCR and analyzed by the same gel.

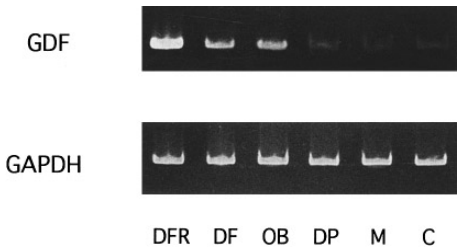

FIG. 2. Detection of mRNAs encoding GDFs and GAPDH by RT-PCR in 5% polyacrylamide gel electrophoresis. DNA was stained with ethidium bromide. High levels of GDF mRNAs were seen in samples from dental follicle surrounding the developing root (DFR), dental follicle (DF), and odontoblast layer (OB), while low levels were detected in samples from dental papilla (DP), skeletal muscle (M), and subcutaneous connective tissue (C).

RESULTS

We performed two sets of separate experiments where similar results were obtained. Expression of GDF (GDF-5, -6 and -7) mRNAs in tissues from tooth germ at the stage of root formation was detected by means of RT-PCR (Fig. 2). For comparison, skeletal muscle (M) and subcutaneous connective tissues (C) were also evaluated. Because the primer set was designed from the conserved regions of mouse GDF-5, -6, -7 and human GDF-5, PCR products were potentially a mixture of amplified GDF-5, -6 and -7 cDNA fragments. Polyacrylamide gel electrophoretic analysis of the PCR products from dental follicle (DF), dental follicle surrounding the developing root (DFR) and the tissue from the odontoblast layer (OB) showed a band of expected cDNA size (243 bp). In contrast, only a faint band was found in the PCR products of dental papilla (DP), skeletal muscle and subcutaneous connective tissue samples.

The PCR products of DF, DFR and OB samples were subcloned into a plasmid vector and their nucleotide sequences determined. Three distinct nucleotide sequences, which were homologous to mouse GDF-5, -6 and -7 mRNAs were found and were designated as bovine GDF-5, GDF-6 and GDF-7. The nucleotide sequences of bovine GDF-5, -6 and -7 excluding the primer portions were 81%, 93% and 90% homologous to those of mouse GDF-5, GDF-6 and GDF-7, respectively. Deduced amino acid sequences from these cDNA clones are shown in Fig. 3. The amplified bovine GDF-6 cDNA sequence obtained in this study is identical to a previously reported bovine GDF-6 nucleotide sequence (15).

The PCR products of DF, DFR and OB appear as a single, strong band, and potentially contain a mixture of GDF-5, -6 and -7 amplified fragments (Fig. 2). To separate these fragments, we performed a PCR-SSCP technique. Using this technique, the mobility of single-stranded GDF-5, -6 and -7 cDNAs shift and reveal distinguishable electrophoretic patterns (Fig. 4). Plasmids, containing GDF-5, GDF-6 and GDF-7 cDNA inserts were used as controls to obtain standard patterns. Although GDF-5 and GDF-6 mRNAs were detected in all samples, expression of GDF-7 mRNA was detected only in samples from dental follicles (DF and DFR) and the level of GDF-7 mRNA was highest in the DFR sample.

DISCUSSION

The results of the present study indicate that GDFs, members of the BMP family, are present during tooth and periodontal tissue development. It has been shown that BMPs are essential for embryonic development and organogenesis (21), and it is proposed that select BMPs induce the differentiation of mesenchymal cells into various types of connective tissues such as bone, cartilage, muscle, adipose and tendon/ligament (22-26). In addition, some BMPs are involved in tooth development. For example, agarose beads containing BMP-4, when applied onto dental mesenchyme, induce a translucent zone similar to that induced by dental epithelium, and BMP-4 induces its own mesenchymal expression (9). BMP-2 stimulates odontoblastic differentiation of cells in mouse dental papillae explants cultured *in vitro* (10). These studies show that BMPs participate in the differentiation of ectomesenchymal cells during tooth development. The dental follicle is an important tissue involved in the development of tooth supporting tissues (cementum, periodontal ligament, and alveolar bone proper) (18, 27) as well as the eruption of teeth (28, 29). However, factors controlling the differentiation of dental follicle cells have not been identified. In a previous study, TGF-β1 did not increase the number of cells in cultured dental follicles, but did stimulate them to increase their secretion of extracellular matrix proteins, i.e., type I collagen and fibronectin, both of which are needed for the development of the periodontal ligament (30). These effects may suggest a role for TGF-β1 in the differentiation of dental follicle cells. Therefore, some TGF-β superfamily members, and perhaps GDFs, are possible cytokines that regulate periodontal tissue formation and its regeneration after tissue destruction by diseases such as periodontitis.

Previously reported nucleotide sequences of mouse GDF-5, -6, -7 and human GDF-5 genes indicate the presence of highly conserved regions. Thus, a set of PCR primers, which were designed on the basis of these conserved regions, might allow the amplification of unknown, new GDF members. The amplified PCR products, however, corresponded to a mixture of GDF-5, -6 and -7 fragments. In the present study, we successfully separated amplified cDNAs of GDF-5, GDF-6 and GDF-7 from this mixture by means of PCR-SSCP. Whereas the nucleotide sequence of bovine GDF-6 cDNA has been previously reported (15), those of bovine GDF-5 and GDF-7 are revealed in this study. Direct comparison of the mRNA levels of these GDFs is difficult since

a

bovine GDF-5

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        10         20         30         40         50         60
tgggacgactggatcatcgcACCCCTGGAATACGAAGCGTATCACTGCGAAGGTCTGTGT
W  D  D  W  I  I  A  P  L  E  Y  E  A  Y  H  C  E  G  L  C
        70         80         90        100        110        120
GAGTTCCCCCTGCGATCCCACCTGGAGCCCACCAATCACGCCGTTATCCAAACTTTAATG
E  F  P  L  R  S  H  L  E  P  T  N  H  A  V  I  Q  T  L  M
       130        140        150        160        170        180
AACTCAATGGACCCCGAATCAACGCCTCCAACTTGCTGTGTCCCAACCAGGCTGAGTCCT
N  S  M  D  P  E  S  T  P  P  T  C  C  V  P  T  R  L  S  P
       190        200        210        220        230        240
ATCAGCATTCTCTTCATTGACTCTGCAAACAATGTGGTCTACaagcagtatgaggacatg
I  S  I  L  F  I  D  S  A  N  N  V  V  Y  K  Q  Y  E  D  M
gtg
V
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bovine GDF-7

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        10         20         30         40         50         60
tgggacgactggatcatcgcGCCCCTGGACTACGAGGCATACCACTGCGAGGGCGTGTGC
W  D  D  W  I  I  A  P  L  D  Y  E  A  Y  H  C  E  G  V  C
        70         80         90        100        110        120
GACTTCCCGCTGCGCTCGCACCTTGAGCCCACCAACCACGCCATCATTCAGACGCTGCTG
D  F  P  L  R  S  H  L  E  P  T  N  H  A  I  I  Q  T  L  L
       130        140        150        160        170        180
AACTCCATGGCCCCGGATGCGGCGCCAGCCTCCTGCTGCGTGCCCGCGCGCCTCAGCCCG
N  S  M  A  P  D  A  A  P  A  S  C  C  V  P  A  R  L  S  P
       190        200        210        220        230        240
ATCAGCATCCTCTACATCGACGCCGCCAACAACGTGGTCTACaagcagtatgaggacatg
I  S  I  L  Y  I  D  A  A  N  N  V  V  Y  K  Q  Y  E  D  M
gtg
V
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b

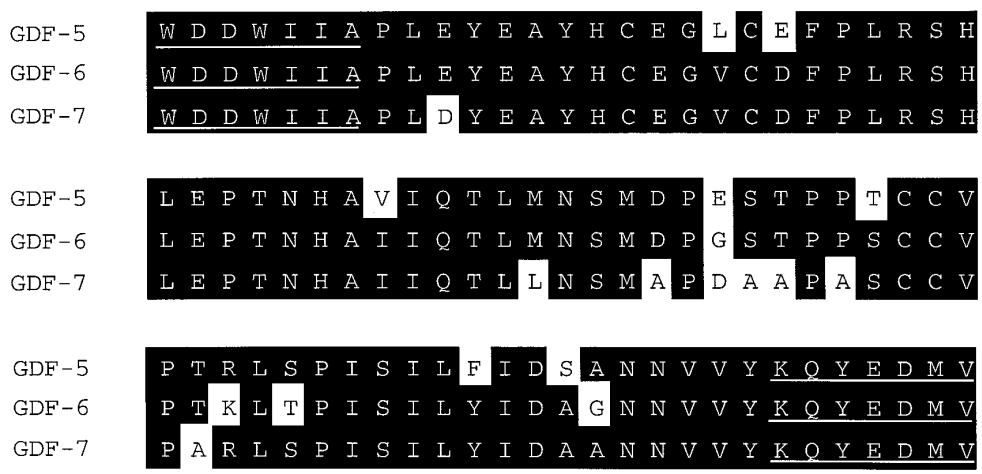

FIG. 3. (a) The partial nucleotide and deduced amino acid sequences of bovine GDF-5 and GDF-7. Primers used for PCR are written in lower case letters. (b) Alignment of bovine GDF-5, -6, and -7. Identical amino acids are shaded.

the PCR primers might have varying affinities to different GDF cDNAs and the efficiency of PCR amplification of each cDNA may be different. However, PCR-SSCP analysis of bovine genomic DNA yielded bands of similar intensity corresponding to amplified GDF-5, GDF-6 and GDF-7 DNA fragments, suggesting that the af-

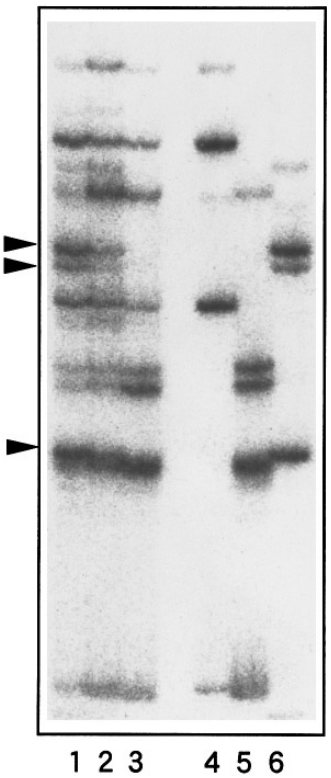

FIG. 4. SSCP analysis of amplified PCR products. Amplified products from DFR, DF, and OB cDNA were separated into GDF-5, -6, and -7 by a PCR- SSCP technique. Lane 1, DFR; lane 2, DF; lane 3, OB; lanes 4, 5, and 6, plasmid vector in which GDF-5, -6, and -7 fragments were subcloned, respectively (standard patterns). Note the bands which reflect GDF-7 (arrowheads) are detected in DFR and DF, but not in OB.

finities of these primers for each GDF DNA sequence may not be significantly different (data not shown). Providing the primers anneal with equal frequency to the GDF cDNAs, the expression level of GDF-5, -6 and -7 mRNAs could be compared.

We hypothesize that growth factors which are important for the development of dental attachment apparatus may be expressed during the early stage of root formation. Our results demonstrate that the expression of GDF mRNAs is higher in the tooth germ at the root forming stage, particularly in the dental follicle (DF and DFR) and in the odontoblast layer, as compared with that in skeletal muscle and subcutaneous connective tissues. The expression levels of GDF mRNAs are different among dental follicle, odontoblast layer and dental papilla samples despite the fact that they are derived from a common origin developmentally. Interestingly, different GDF types are expressed in dental follicle and odontoblast layer samples at this developmental stage. PCR-SSCP analysis demonstrates that the GDF-7 gene is not expressed in the odontoblast layer. However, a novel observation in this study demonstrates that GDF-7 is significantly expressed in dental follicle tissue, particularly in the portion surrounding the root. As GDF-7 is thought to have the potential of inducing tendon/ligament differentiation in mesenchyme, the GDF-7 gene expression in dental follicle encapsulating the developing root, suggests the involvement of this molecule in the development of root and periodontal tissue such as periodontal ligament. GDF-5 and GDF-6 mRNAs, which are thought to be involved in the development of hard tissues as well as in the tendon/ligament morphogenesis, were both expressed in the odontoblast layer and also in dental follicle tissue. While GDF-5 and GDF-6 may contribute to the development of dental and periodontal hard tissues such as dentin, cementum and alveolar bone proper, there have been no reports regarding tooth abnormalities in *brachypod* mice, in which GDF-5 is lacking (2). This may be the result of redundancy by the presence of other BMP-related factors.

We also detected the expression of GDF mRNAs by method of RT-PCR in whole tooth germs of 2 Wister rats which were 1day of age, however, GDF mRNAs were not found in the periodontal ligament tissue and the dental pulp tissue from 6 Wistar rats 9 weeks of age(data not shown). This result suggest that these GDFs have some functions during the development of the tooth germ, including the dental follicle. According to Osborn *et al.* (27), cells in the dental follicle of the root forming zone (DFR) show active division and differentiation from the very beginning of root development in mouse molar tooth germ. In the DFR region of the developing tooth used in the present study, we expect that many types of growth factors including GDFs are required for cell division and differentiation. The remarkable specificity in the types and expression levels of GDF in DFR suggest that these growth factors are involved in the differentiation of cells in DFR region in a specific manner. However, it is difficult to determine the cell types which express GDF mRNAs in the DFR region. Besides the cells of dental follicle proper, Hertwig's epithelial sheath is also contained in the DFR tissue samples obtained in this study. Although the induction mechanism of root formation by this epithelial tissue has not been clearly identified, the epithelial root sheath is suspected to play significant roles (31-34). Further studies are underway using such as *in situ* hybridization to further investigate the temporospatial expression pattern of GDFs in the development of the tooth and the tooth supporting tissues.

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