

# Adenoviral Gene Transfer Restores Lysyl Hydroxylase Activity in Type VI Ehlers–Danlos Syndrome

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**Type VI Ehlers–Danlos syndrome is a disease characterized by disturbed lysine hydroxylation of collagen. The disease is caused by mutations in lysyl hydroxylase 1 gene and it affects several organs including the cardiovascular system, the joint and musculoskeletal system, and the skin. The skin of type VI Ehlers–Danlos syndrome patients is hyperelastic, scars easily, and heals slowly and poorly. We hypothesized that providing functional lysyl hydroxylase 1 gene to the fibroblasts in and around wounds in these patients would improve healing. In this study we tested the feasibility of transfer of the lysyl hydroxylase 1 gene into fibroblasts derived from rats and a type VI Ehlers–Danlos syndrome patient (*in vitro*) and into rat skin (*in vivo*). We first cloned human lysyl hydroxylase 1 cDNA into a recombinant adenoviral vector**

**(Ad5RSV-LH). Transfection of human type VI Ehlers–Danlos syndrome fibroblasts (about 20% of normal lysyl hydroxylase 1 activity) with the vector increased lysyl hydroxylase 1 activity in these cells to near or greater levels than that of wild type, unaffected fibroblasts. The adenoviral vector successfully transfected rat fibroblasts producing both  $\beta$ -galactosidase and lysyl hydroxylase 1 gene activity. We next expanded our studies to a rodent model. Intradermal injections of the vector to the abdominal skin of rats produced lysyl hydroxylase 1 mRNA and elevated lysyl hydroxylase 1 activity, *in vivo*. These data suggest the feasibility of gene replacement therapy to modify skin wound healing in type VI Ehlers–Danlos syndrome patients. Key words: collagen/connective tissue/inherited disease. *J Invest Dermatol* 116:602–605, 2001**

**T**ype VI Ehlers–Danlos syndrome (EDS VI) is a recessively inherited disease of connective tissues caused by mutations in the gene for lysyl hydroxylase 1 (LH) (Hyland *et al*, 1992; Hautala *et al*, 1993; Heikkinen *et al*, 1994; 1997; Pousi *et al*, 1994; 1998; Pajunen *et al*, 1998). LH catalyzes formation of hydroxylysine residues that are required for cross-link formation and O-linked glycosylation in collagens (Kivirikko and Pihlajaniemi, 1998). Human LH consists of two identical subunits of 709 amino acids, and the 40 kb gene for the enzyme contains 19 exons producing an mRNA of 3.2 kb (Hautala *et al*, 1992; Heikkinen *et al*, 1994). LH activity requires X-Lys-Gly sequence in collagenous substrates as well as ferrous iron, ascorbate, 2-oxoglutarate, and molecular oxygen as cofactors (Kivirikko and Pihlajaniemi, 1998).

Lack of LH activity in EDS VI patients yields structurally weak collagen molecules causing severe musculoskeletal, cardiovascular, and skin disease. Kyphoscoliosis, recurrent joint dislocations, ocular complications, and arterial aneurysms can occur in these patients (Wenstrup *et al*, 1989; Byers, 1994; Heim *et al*, 1998). The skin of EDS VI patients may be hyperelastic and easily bruised. Poor wound healing with subsequent scarring leads to secondary complications. Unfortunately, little treatment is available for the

primary defect in these patients. Large doses of ascorbate, a cofactor for the LH enzyme, has been shown to be beneficial for some patients, improving wound healing, bleeding time, and muscular strength (Elsas *et al*, 1978; Dembure *et al*, 1987).

Gene replacement therapy may be feasible and beneficial for some of the cutaneous manifestations of EDS VI. Skin is easily accessible and wound healing may and should benefit from locally administered LH activity. Heterozygous carriers of the defective recessive gene who have diminished LH activity are healthy; this suggests that even partial correction of the LH defect in EDS VI patients may be beneficial. In this study, we determined that an adenoviral vector expressing human LH cDNA restores LH enzyme activity in EDS VI patient skin fibroblasts, *in vitro*. We also determined that this vector drives human LH mRNA expression and enzymatic activity following intradermal injection in rats, an animal with low endogenous skin LH activity (Armstrong and Last, 1995).

## MATERIALS AND METHODS

**Cell culture** Hydroxylysine-deficient human skin fibroblasts (CRL1195) (Sussman *et al*, 1974) were obtained from the American Type Culture Collection (Manassas, VA). These cells and locally established rat and human skin fibroblasts were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Rockville, MD) supplemented with 10% fetal bovine serum, 28  $\mu$ M ascorbate, 2 mM glutamate, 100 U per ml penicillin, and 100  $\mu$ g per ml streptomycin.

**Construction of adenoviruses** A 3040 nucleotide (nt) BamHI-EcoRI fragment of human LH cDNA LH3A clone (Hautala *et al*, 1992) containing polyadenylation signal was subcloned into adenovirus shuttle

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Abbreviations: EDS VI, type VI Ehlers–Danlos syndrome; LH, lysyl hydroxylase 1.

plasmid. This shuttle plasmid and the Ad5 backbone, sub360, were cotransfected into HEK293 cells. Ad5RSV-LH was purified using conventional techniques (Jones and Shenk, 1979; Rowe *et al.*, 1983) by the University of Iowa Gene Transfer Vector Core. Cell lysates from infected cells were evaluated for LH activity as described below. Recombinant Ad5RSV-LH was plaque-purified and concentrated using CsCl centrifugation. Ad5RSV $\beta$ gal has been described in detail elsewhere (Davidson *et al.*, 1994).

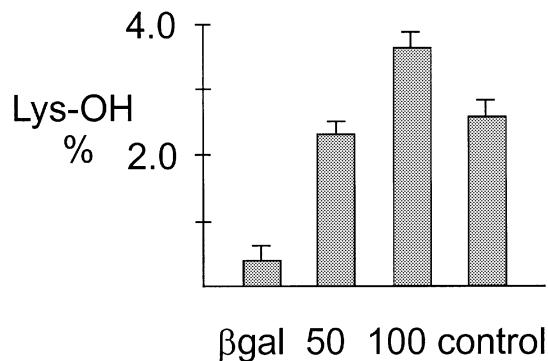
**LH activity assay**  $5 \times 10^6$  cultured human skin fibroblasts or rat skin samples (50–100 mg) were homogenized in a buffer containing NaCl 0.2 M, glycine 0.1 M, Triton X-100 0.1%, Tris-HCl (pH 7.5) 0.02 M at 4°C and centrifuged at 12,000g for 10 min. A 50  $\mu$ l fraction of soluble protein of cell lysates or tissue samples was mixed with collagenous peptide substrate [ $^{14}$ C]lysine-labeled procollagen prepared in cultures of tendon cells (120,000 cpm) or nonradioactive GLKGEPGRKGEK-peptide in final concentration of 0.5 mg per ml and brought up to 1 ml volume with reaction buffer [Tris-HCl (pH 7.8) 50 mM, bovine serum albumin 1 mg per ml, catalase 0.1 mg per ml, dithiothreitol 0.1 mM, ascorbate 1 mM, FeSO<sub>4</sub> 0.05 mM, 2-oxo-glutarate 0.5 mM]. The mixture was incubated for 30 min at 37°C and then for 5 min at 95°C (Kivirikko and Myllylä, 1982). A fraction of reaction mixture was hydrolyzed with 6 M HCl and analyzed for amino acid composition using Applied Biosystems 421 AminoAcidAnalyzer. In experiments with [ $^{14}$ C]-procollagen substrate, the amino acid analyzer was connected to a fraction collector and fractions corresponding to lysine and hydroxylysine residues were measured for radioactivity. The protein concentration in each sample was determined (Bio-Rad Laboratories, Hercules, CA) and LH activity is expressed as percentage of hydroxylated lysine residues normalized for sample protein concentration.

**Detection of mRNAs for LH** Total RNA from cultured cells or tissue samples was extracted using guanidium thiocyanate followed by CsCl gradient centrifugation. RNA was treated with Dnase (Pharmacia Biotech) for 1 h in order to remove contaminating DNA, after which 1  $\mu$ g of RNA was reverse transcribed according to the manufacturer's instructions (First-Strand cDNA Synthesis Kit, Pharmacia Biotech). LH sequences were amplified using oligonucleotide primers 5'-GGCATTGGGGATGAAGCTCT and 5'-GGTTGGTCAGGAAC-ATGAAC. This primer sequence is identical in rat and human cDNA and both generate a 661 nt polymerase chain reaction (PCR) product. The PCR mixture consists of 1  $\mu$ M of each primer, 20  $\mu$ M of deoxynucleoside triphosphates, 1 IU DNA polymerase (DynaZymeII, Finnzymes, Helsinki, Finland), and buffer [1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1% Triton X-100, 10 mM Tris-HCl (pH 9.0)]. Amplification was performed at 94°C for 60 s, 55°C for 60 s, and 72°C for 60 s for a total of 30 cycles in a thermocycler (Minicycler, MJ Research, Waltham, MA). The rat cDNA sequence has a unique HindIII site; the human has none. To distinguish between rat and human cDNA, we incubated the PCR products at 37°C with HindIII (Pharmacia Biotech) for 2 h and separated the products on 1.5% agarose gel. The 661 nt product was considered human sequence and 155 and 506 nt fragments were considered rat sequence. The amplification protocol was not optimized for quantitative analysis.

**Virus injection into rat skin** Sprague-Dawley rats were anesthetized with intraperitoneal injection of fentanyl citrate 0.315 mg per ml, midazolam 5 mg per ml, and fluanisone 10 mg per ml (1:1:1).  $5 \times 10^8$  infectious units of Ad5RSV-LH virus in 50  $\mu$ l volume were injected into the abdominal dermis of rats. The rats were sacrificed at indicated times after the injection and skin samples were collected. The samples were used for LH activity assay, mRNA preparation, or amino acid analysis.

**Rat skin amino acid analysis** Rat skin samples were analyzed for amino acid composition on days 0, 7, 14, or 28 after injection of  $5 \times 10^8$  infectious units of Ad5RSV-LH or Ad5RSV $\beta$ gal. The skin biopsies were weighed and cut in small pieces, and the tissues were sealed in tubes under a nitrogen atmosphere for hydrolysis with 6 M HCl and 1 mM  $\beta$ -mercaptoethanol at 110°C for 24 h. Subsequently, the samples were filtered with disposable 0.2  $\mu$ m filter (Schleicher & Schuell), lyophilized, and analyzed with Applied Biosystems 421 AminoAcidAnalyzer. The data are expressed as number of hydroxylated lysine residues per 1000 amino acids.

**Gene transfer efficiency in rat skin** Five  $\times 10^8$  infectious units of Ad5RSV $\beta$ gal in 50  $\mu$ l volume were injected into the dermis of rat abdominal skin in order to demonstrate gene transfer efficiency. The skin samples were removed 48 h later, fixed in 4% paraformaldehyde in 0.15 M sodium phosphate (pH 7.2), and incubated with X-gal (1 mg per



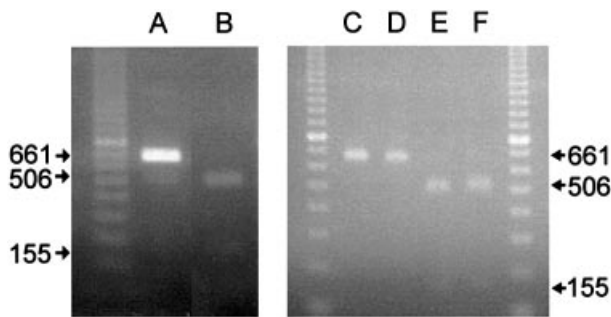
**Figure 1. Correction of LH activity *in vitro*.** LH activity in cultured skin fibroblasts from an EDS VI patient and control human skin fibroblasts was determined using [ $^{14}$ C]lysine labeled procollagen. The EDS cells were incubated with 50 pfu per cell of Ad5RSV $\beta$ gal ( $\beta$ gal) or 50 or 100 pfu per cell of Ad5RSV-LH and the LH activity was measured 48 h later. The enzyme activity was also determined in skin fibroblasts from a healthy control individual (control). The LH activity is expressed as percentage of hydroxylated lysine residues in procollagen substrate and is normalized for cell lysate protein concentration. Each condition was repeated at least three times. Error bars indicate standard deviation (SD).

ml) in reaction buffer (2 mM MgCl<sub>2</sub>, 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O, 1  $\times$  phosphate-buffered saline) at 37°C for 3 h. The tissue samples were sectioned and analyzed with a microscope for  $\beta$ -galactosidase expression.

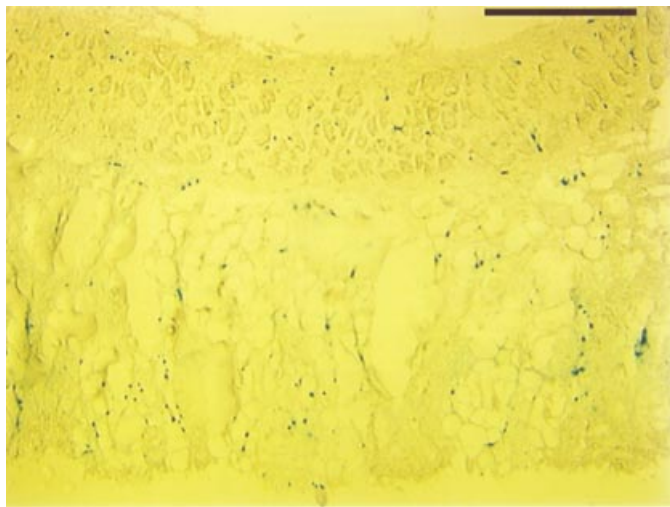
## RESULTS

**Correction of LH activity *in vitro*** We first demonstrated that the recombinant adenovirus carrying human LH cDNA (Ad5RSV-LH) corrects the LH activity in EDS VI skin fibroblast cultures. **Figure 1** shows that cells transfected with the control virus produced 0.4%  $\pm$  0.2% lysine hydroxylation (10%–15% of normal skin fibroblasts), which equals previously reported LH activity levels (Sussman *et al.*, 1974). The fibroblasts transfected with 50 pfu per cell Ad5RSV-LH produced 2.3%  $\pm$  0.1% hydroxylation (85% of normal skin fibroblasts), and 100 pfu per cell further elevated the hydroxylation level to 3.4%  $\pm$  0.2% (128% of normal skin fibroblasts). The LH activities shown in **Fig 1** were obtained using [ $^{14}$ C]lysine-labeled procollagen as a substrate, explaining the low level of lysine hydroxylation. This is due to the fact that not all lysine residues of type I collagen can be hydroxylated by LH. The hydroxylation rate analyzed with collagenous peptide substrate optimal for LH activity was significantly higher (3.7% for control virus, 18.6% for 50 pfu per cell, and 24.0% for 100 pfu per cell Ad5RSV-LH). The data demonstrate that the Ad5RSV-LH significantly increased the LH activity *in vitro* in EDS VI skin fibroblasts.

**Detection of LH mRNA** We incubated cultured rat skin fibroblasts with Ad5RSV-LH and demonstrated human LH mRNA in cell lysates 48 h later using PCR as described in *Materials and Methods*. Our protocol amplified a 661 nt band from both rat and human sequences. The two species were distinguished with a unique HindIII restriction site; the rat sequence was cut into 506 and 155 fragments whereas the human sequence was undigested. **Figure 2 (lane A)** demonstrates that the 661 nt band for human LH mRNA dominates in Ad5RSV-LH transfected rat skin fibroblast cultures. The 661 nt band is clearly more intense than the 506 nt band, suggesting strong human LH mRNA expression from the gene transferred with Ad5RSV-LH. Control cells that received equal amounts of Ad5RSV $\beta$ gal showed fragments corresponding to rat sequences (**Fig 2, lane B**). For *lane B*, the amount of cDNA template was doubled in order to produce the bands shown.



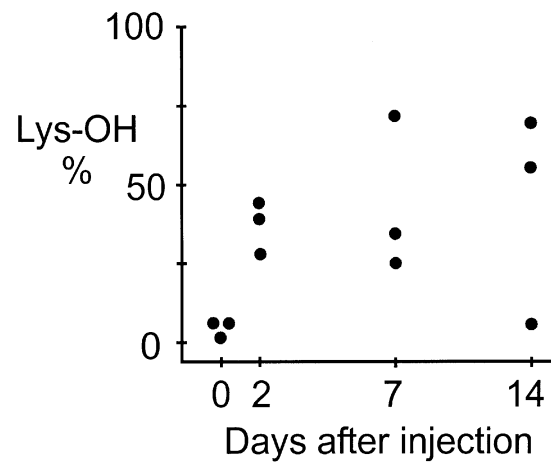
**Figure 2. Detection of LH mRNA.** Cultured rat skin fibroblasts were transfected with 50 pfu per cell of Ad5RSV-LH (lane A) or Ad5RSV $\beta$ gal (lane B) and analyzed 48 h later. A 661 nt fragment of human and rat LH was amplified from cDNA template as described in *Materials and Methods*. HindIII digestion cuts the rat sequence into 155 and 506 nt fragments whereas the 661 nt human sequence amplification product remains undigested. On lane A, the 661 nt band for human LH mRNA is significantly stronger than the 506 nt product for rat sequences, demonstrating strong expression from Ad5RSV-LH. Rat skin samples injected with Ad5RSV-LH (lanes C and D) demonstrate human LH mRNA (661 nt fragment) and a very faint 506 nt band for rat sequences. Several amplification reactions were pooled in order to demonstrate the 506 nt band (lanes E and F) in control samples that received Ad5RSV $\beta$ gal. The arrows indicate positions of the 661, 506, or 155 nt markers.



**Figure 3. Adenoviral  $\beta$ -galactosidase gene transfer in rat skin.** Ad5RSV $\beta$ gal ( $5 \times 10^8$  pfu in 50  $\mu$ l volume) was injected into rat abdominal skin in order to demonstrate adenoviral gene transfer. Tissue samples were stained for  $\beta$ -galactosidase expression 48 h later.

Rat skin samples were analyzed for LH mRNA after Ad5RSV-LH or Ad5RSV $\beta$ gal injection. Lanes C and D in **Fig 2** show a 661 nt band for human and a very faint 506 nt band for rat LH mRNA after Ad5RSV-LH injection. Products of several amplifications were pooled to demonstrate the rat LH mRNA in control (Ad5RSV $\beta$ gal) skin samples (lanes E and F). The data clearly demonstrate that Ad5RSV-LH gene transfer produces specific human LH expression *in vivo*. The amplification protocol, however, was not optimized for quantitative analysis.

**Adenoviral  $\beta$ -galactosidase gene transfer in rat skin** In order to demonstrate the recombinant adenovirus gene transfer into rat skin, we injected  $5 \times 10^8$  pfu of Ad5RSV $\beta$ gal into abdominal skin and the skin samples were stained for  $\beta$ -galactosidase expression 48 h later. The skin sections that received Ad5RSV $\beta$ gal had blue nuclei demonstrating successful adenoviral



**Figure 4. LH activity in rat skin.** LH activity in rat skin samples 0, 2, 7, or 14 d after gene transfer with Ad5RSV-LH ( $5 \times 10^8$  pfu in 50  $\mu$ l volume). The tissue samples were homogenized and LH activity was analyzed from skin homogenate supernatants using optimal collagenous peptide substrate on days 0, 2, 7, or 14 as described in *Materials and Methods*. The data are expressed as percentage of hydroxylated lysine residues in collagenous peptide substrate and normalized for protein concentration in the tissue sample.

**Table I. Skin amino acid composition was analyzed 0, 7, 14, or 28 d after Ad5RSV-LH or Ad5RSV $\beta$ gal gene transfer<sup>a</sup>**

Day	Ad5RSV-LH	Ad5RSV $\beta$ gal
0		7.90 $\pm$ 0.77
7	5.20 $\pm$ 0.71	4.70 $\pm$ 0.14
14	6.98 $\pm$ 1.52	4.60 $\pm$ 1.13
28	4.80 $\pm$ 2.26	6.25 $\pm$ 0.21

<sup>a</sup>The table shows the number of hydroxylysine residues per 1000 amino acids  $\pm$  SD. Amino acid analysis was repeated at least three times in each condition. Statistical difference is seen between the LH and control virus groups on day 14 ( $p < 0.05$ ); all other time points show no statistically significant difference. There is no significant difference in lysine hydroxylation on day 14 compared with the initial value on day 0.

gene transfer. **Figure 3** shows typical nuclear intensively blue  $\beta$ -galactosidase staining.

**LH activity in rat skin** We injected  $5 \times 10^8$  pfu of Ad5RSV-LH in 50  $\mu$ l volume into the abdominal skin of healthy Sprague-Dawley rats. The first rats were analyzed immediately for basal LH activity using collagenous peptide substrate optimal for lysine hydroxylation reaction. The activity was nearly undetectable with the method used on day 0. On days 2 and 7 the LH activity was clearly elevated, but on day 14 the LH activity was down to the initial level in one animal whereas two rats continued to show increased activity (**Fig 4**). The data demonstrate successful adenoviral LH gene transfer although there was significant variation within each group of animals.

**Hydroxylysine content in rat skin**  $5 \times 10^8$  pfu of Ad5RSV-LH was injected into rat abdominal skin as described in *Materials and Methods*. The skin samples were collected on days 0, 7, 14, or 28 after the injection and analyzed for amino acid composition. The hydroxylysine content in skin samples was not significantly increased after the gene transfer (**Table I**) demonstrating that endogenous LH activity is sufficient to produce fully hydroxylated skin collagen.

## DISCUSSION

The most common mutation causing EDS VI is a large duplication in the LH gene caused by homologous recombination between Alu sequences in the introns 9 and 16 (Hautala *et al*, 1993; Pousi *et al*, 1994; Heikkinen *et al*, 1997). This duplication rearrangement is estimated to account for 19% of the diseased LH alleles and it is also found in the patient of this study (Pousi *et al*, 1994). The basal LH activity in the skin fibroblasts of the patient is about 20% compared to a healthy control individual (Sussman *et al*, 1974). Ad5RSV-LH gene transfer elevates LH activity up to the levels found in normal skin fibroblasts. It is probable that restoration of the LH function leads to correction of mechanical properties of collagens that may correlate with the severity of the disease symptoms in EDS VI.

There is no genetically defective animal model available for LH deficiency. This prevents us from addressing the question of the real biologic effect on collagen function in LH-deficient tissues *in vivo*. Ad5RSV-LH gene transfer, however, produced human LH mRNA and an increased LH activity in rat abdominal skin. Analysis of skin collagen showed no significant increase in lysine hydroxylation after Ad5RSV-LH gene transfer. It should be noticed, however, that major skin collagens (types I and III) have a very low number of hydroxylated lysine residues compared with other collagen types (Kivirikko *et al*, 1992). Our experiment demonstrates very clearly that endogenous LH activity is not a rate-limiting component in lysine hydroxylation reaction in healthy skin tissues.

Harvey *et al* demonstrated that first generation adenoviral vector persisted in human skin for at least 28 d with mild/moderate local cellular inflammation (Harvey *et al*, 1999). In our study, the elevated LH activity was still evident 2 wk after the virus injection in two animals. This should allow reasonable time for initiation of successful wound healing. Studies on adenoviral gene transfer have demonstrated loss of transgene expression due to immune response against the vector or the transgene product. It is probable that LH overexpression will gradually diminish and it may not be possible to achieve permanent correction with the vector used in this study. Adenoviral vector can be used to test the principle of LH enzyme replacement, but other vectors, such as AAV or recombinant retroviruses, may provide lasting levels of expression.

Gene therapy for inherited diseases of connective tissue has proved to be difficult, although there are some examples of successful strategies (Vailly *et al*, 1998; Hengge *et al*, 1999; Seitz *et al*, 1999; Somani *et al*, 1999). The majority of diseases of collagen metabolism exhibit a dominant inheritance pattern and in general the defective structural protein disturbs the function of a product of healthy allele. In contrast to most inherited collagen diseases, heterozygous relatives of EDS VI patients demonstrate that products of defective LH allele should not interfere with the wild type enzyme. Therefore, EDS VI is a good candidate disease to consider for therapeutic gene transfer. Unfortunately, the major symptoms of EDS VI as well as other inherited connective tissue diseases arise during embryonic development and curative treatment may no longer be possible in a newborn. Our data support the possibility, however, that gene transfer may offer alleviation for local symptoms of EDS VI.

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