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A hydrophilic polymer grafted with a histone tail peptide as an artificial gene regulator

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ABSTRACT

In chromatin, gene transcription is regulated through posttranslational modifications on the histone *N*-terminal tail sequences, typically an acetyl group modification on lysine residues. To realize a simple model of the gene regulation of chromatin, we designed a hydrophilic polymer grafted with histone H3 tail peptides. The polyplex formed from the polymer and DNA suppressed the gene expression effectively although the polyplex was weaker than the polyplex of poly-L-lysine and DNA. This weaker polyplex afforded the acetylation of the lysine residue of the grafted peptides by histone acetyltransferase. Subsequently, the gene expression was activated due to the relaxation of the polyplex which was brought by a cationic charge decrease in the grafted peptides. This molecular system is the first functional model of the gene regulation of the chromatin.

1. Introduction

In eukaryote cells, genomic DNA forms a stable complex with a histone octamer, which is composed of four histone proteins (H2A, H2B, H3, H4), mainly through an electrostatic interaction.[1] This supramolecular structure, called chromatin, suppresses gene transcription by protecting DNA from the access of transcriptional factors.[2–4] This gene suppression is recovered by posttranslational modifications on a cationic region of the amino terminal of histone, called histone tail.[5,6] Especially, acetylation of particular lysine residue on the histone tail with histone acetyltransferase (HAT) has been clarified as a typical activation signal of gene transcription.[7,8] The acetylation changes the electrostatic interactions between DNA and histone by decreasing the positive charge of lysine residues. Thereafter, effecter proteins, which recognize acetylated lysine residue, leads eviction of histone proteins from the chromatin.[9,10] The exposed promoter region of DNA recruits the transcription–initiation complex then transcription proceeds.

Several groups have succeeded in modeling the chromatin structure by using artificial cationic nanoparticles and dendrimers, in which the transcription of DNA is suppressed by complex formation.[11–13] However, to our knowledge, a chromatin model which turns on the gene expression responding to the posttranslational modifications have not been reported so far.

We have recently reported artificial gene regulation systems by using novel peptide–polymer conjugates.[14–17] The conjugates consist of a hydrophilic polymer backbone and side chains of cationic peptides, which are designed as specific substrates for protein kinases which are one of the typical posttranslational modification enzymes. The conjugate forms polyplex with DNA through an electrostatic interaction, and suppresses the gene transcription very efficiently. However, when the grafted peptides are phosphorylated by target kinases, the net positive charges of the peptide are decreased, and then the gene transcription is dramatically activated. This system is the first artificial system that regulates gene transcription with posttranslational modification.

In this study, we designed a gene regulation system that can respond to HAT activity (Fig. 1). Then, we synthesized a gene regulating polymers, which includes histone tail peptide of H3 protein as a side chain. Polyplex between the polymer and DNA will turn on the gene expression through acetylation of the grafted peptide.

Abbreviations: PLL, poly-L-lysine; HAT, histone acetyltransferase.

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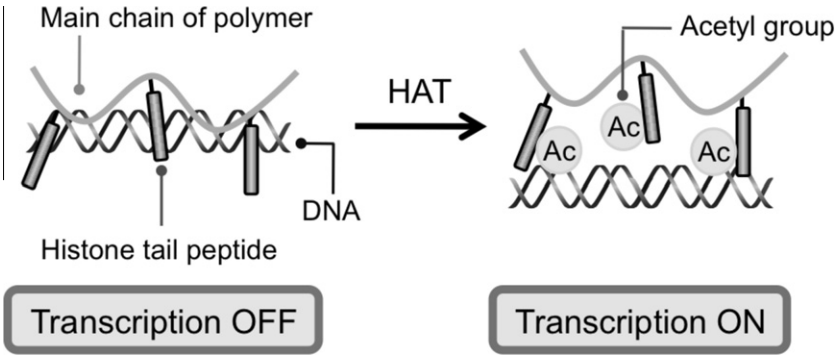

Figure 1. Schematic illustration of gene regulation in response to HAT activity. When grafted-histone tail peptides are acetylated by HAT, the electrostatic interaction between the DNA and cationic polymer weakens, thereby leading to activation of gene transcription.

2. Results and discussion

2.1. Design of HAT responsive gene regulating polymer

To design gene regulating polymer responding to HAT, we took histone tail sequence of H3 from 1 to 17 residue and synthesized H3-peptide possessing additional triptophane and lysine at C-terminus. The triptophane and lysine residues are used for UV quantification and modification of polymerizable methacryloyloxy group, respectively. The H3-peptide includes three lysine residues which are potential acetylation site of HAT. It has been reported that recombinant human GCN5 (hGCN5) which is a representative HAT which acetylates the lysine 14 of H3 histone.[18,19] Thus, we confirmed that the lysine 14 residue of H3-peptide is still acetylated by hGCN5 selectively. MALDI-TOF MS analysis of the H3-peptide after reaction with hGCN5 showed an increase of 42 in mass number, showing the acetylation of single lysine residue. In order to confirm the acetylation site, the acetylated H3-peptide was then digested with Lysyl Endopeptidase®, which cleaves the peptides specifically on the carboxy-terminal of lysine residues.[20] The peptide fragments resulting from the digestion at lysine 4 and 9 residues were not detected. These results showed that H3-peptide is acetylated by hGCN5 on lysine 14 residue similar to native H3 protein.

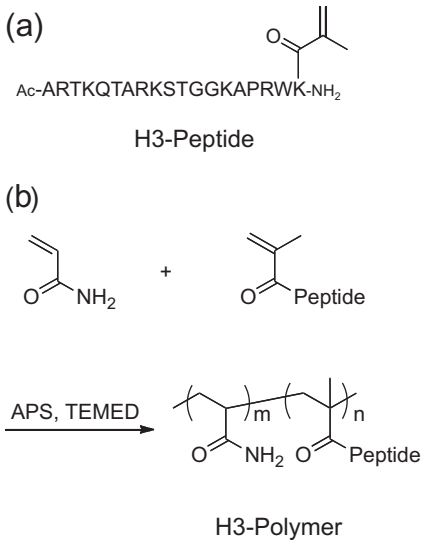

Figure 2. (a) Amino acid sequence of the H3-peptide. (b) Synthetic scheme of the H3-polymer.

Then, H3-peptide was successfully grafted onto the hydrophilic polyacrylamide main chain by radical polymerization (Fig. 2). M_w and M_w/M_n values of the H3-polymer determined by GPC were 6.9×10^4 and 1.8, respectively. The content of H3-peptide was determined from the UV absorbance of tryptophan residue as 1.1 mol %.

2.2. Binding mode of H3-polymer to DNA

Since the H3-peptide grafted onto the H3-polymer has +6 charge at physiological pH, H3-polymer forms a polyplex with polyanionic DNA through an electrostatic interaction. Agarose gel electrophoresis assay of the polyplexes is shown in Figure 3. Poly-L-lysine (PLL, MW $3–7 \times 10^4$) was used as a reference. Two states, free DNA and polyplex, were observed in the case of PLL, indicating the all-or-none-type stoichiometric binding of PLL to DNA. On the other hand, in the case of H3-polymer, increasing amino groups/phos-

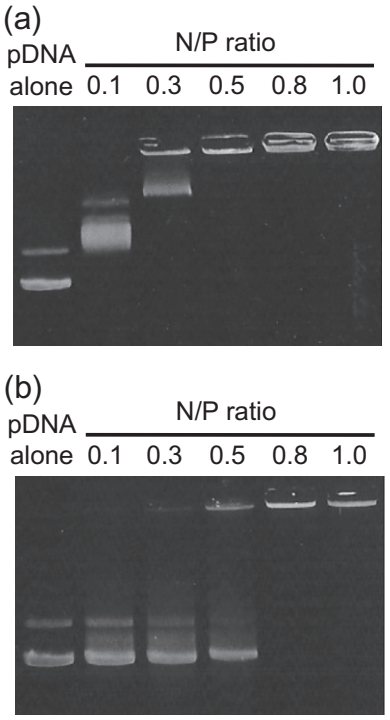

Figure 3. Agarose gel electrophoresis showing the N/P ratio dependent formation of the H3-polymer/DNA complex (a) and PLL/DNA complex (b). The pictures presented are from a single experiment representative of three independent analyses.

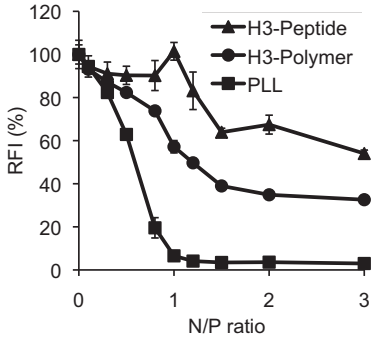

Figure 4. Condensation of the DNA with cationic molecules assessed by the EtBr exclusion assay. The results are presented as the relative fluorescence intensity (RFI). Data are the means ± SEM of three independent experiments.

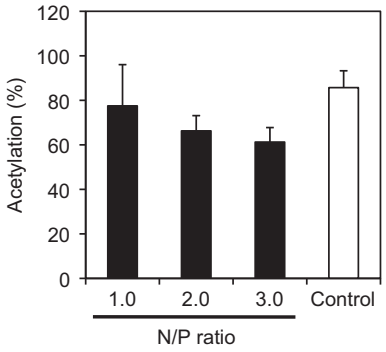

Figure 5. Quantitative estimation of the acetylation reaction of the H3-polymer/DNA polyplex (*closed bars*) at varying N/P ratios. The acetylation ratio of the free H3-peptide in the absence of DNA was also examined (*open bar*; the concentration of the H3-peptide was identical to that of the H3-polymer polyplex, i.e., N/P = 3.0). The fluorescence intensity was converted to the concentration of CoA that was produced by the acetylation reaction. Data are the means ± SEM of six independent experiments.

phate groups (N/P) ratio gradually retarded the migrations of DNA and free DNA was not detected even at N/P = 0.1, suggesting the high capacity of H3-polymer in DNA binding.

We then carried out ethidium bromide (EtBr) exclusion assay to evaluate a DNA compaction capability of H3-polymer. EtBr intercalates into DNA, leading an increase of a fluorescence intensity of EtBr. When DNA forms polyplex, EtBr dissociate from DNA causing the decrease of fluorescence intensity. Thus, the strength of the polyplex is evaluated by monitoring the fluorescence intensity.[21,22]

Results of the EtBr exclusion assay are summarized in Figure 4. All three samples exhibited N/P ratio-dependent decrease of fluorescence intensity, indicating the polyplex formation. PLL highly condensed DNA due to the high density of cationic charges along the polymer chain. In contrast, in the case of H3-polymer and H3-peptide, the polyplex was much weaker than that with PLL. It is noteworthy that H3-polymer showed stronger binding ability than H3-peptide due to entropic advantage in the multivalent binding of the grafted peptides. H3-polymer and H3-peptide also needed excess cationic charges against the anionic charges of DNA (N/P >1.0) to reach saturation of DNA binding.

Consequently, here we found that the binding mode of H3-polymer to DNA is different from the simple polycation, PLL. H3-polymer "softly" binds to DNA not to condense it, thereby H3-polymer has high capacity in DNA binding. This unique binding mode is important for HAT-responsive gene regulation, which will be discussed later.

2.3. Regulation of gene expression via hGCN5 acetylation

First, we examined the reactivity of grafted H3-peptide when H3-polymer forms polyplex. To quantitate the degree of acetylation, we used fluorescence assay developed by Trievel et al., which detects CoA resulting from the reaction of the acetyl group transfer from Acetyl-CoA to H3-peptide.[23] As shown in Figure 5, the degree of acetylation was almost constant irrespective of the N/P ratio in both polyplex from H3-polymer and free H3-peptide. Surprisingly, the difference in the degree of acetylation between the polyplex and free H3-peptide was small, indicating that the steric hindrance resulting from the polyplex formation was not serious to impede the access of hGCN5 the grafted H3-peptides.

We demonstrated the gene expression regulated by acetylation of the polyplex in cell-free system (Fig. 6). The H3-polymer suppressed the expression as effectively as PLL with increasing N/P ratio. However, as mentioned before, the condensation of DNA by H3-polymer is much weaker than PLL, indicating a unique mechanism of gene suppression works in H3-polymer. The hydrophilic mainchain of H3-polymer should be the origin of the gene suppression, by which the access or the sliding motion of

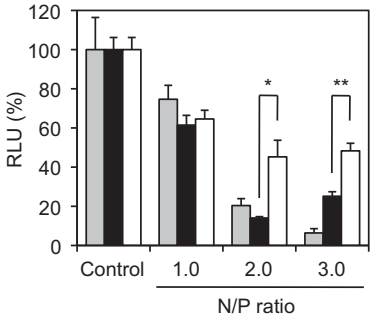

Figure 6. Luciferase expression from the DNA polyplex in a cell-free system. Polyplexes were prepared by mixing DNA with PLL (*gray bars*) or the H3-polymer (*closed bars*), and incubated with a cocktail of acetyl-CoA, hGCN5 and anacardic acid. The polyplex of the H3-polymer was also acetylated by HAT (*open bars*), and the acetylation reaction was terminated by the addition of anacardic acid. The relative luminescence units (RLU) were calculated by normalizing the level of each complex to that of DNA containing acetyl-CoA, hGCN5 and anacardic acid. Data are the means ± SEM of six independent experiments. $^{*}P$ <0.05; $^{**}P$ <0.01.

the transcription–initiation complex will be impeded. In fact, H3-peptide did not suppress the gene expression (see Supplementary data Fig. S1). It is noteworthy that H3-polymer suppressed the transcription of DNA while it afforded the acetylation of the side chain H3-peptide.

After acetylation of the polyplex by hGCN5, a significant recovery of the gene expression was observed at N/P 2.0 and 3.0 (Fig. 6). It is interesting to note that hGCN5 decreased only one positive charge of H3-peptide from 6 to 5 while resulting in such significant expression. Therefore, the number of positive charges of the peptide seems to determine the gene expression level. In fact, it was reported that the binding ability of the oligocationic peptides to DNA depends strongly on the length of the peptides.[24,25]

2.4. Effect of acetylation on strength of the polyplex

Acetylation of the peptide side chains of H3-polymer by hGCN5 successfully regulated the gene expression of the polyplex. Then, we tried to observe the changes in the strength of the polyplex after acetylation. Gel electrophoresis analysis of the H3-polymer/DNA polyplex showed no detectable difference of DNA migration between before and after the acetylation of the polyplex at any N/P ratios (Fig. 7).

Next, we monitored the changes in the polyplex diameter during the acetylation by hGCN5 (Fig. 8). The addition of the

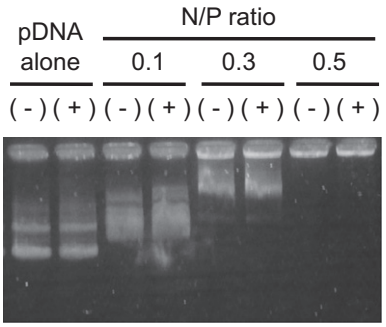

Figure 7. Agarose gel electrophoresis analysis of the H3-polymer/DNA complex in the presence of inactivated hGCN5 (−) and active hGCN5 (+). The figure presented is from a single experiment representative of three independent analyses.

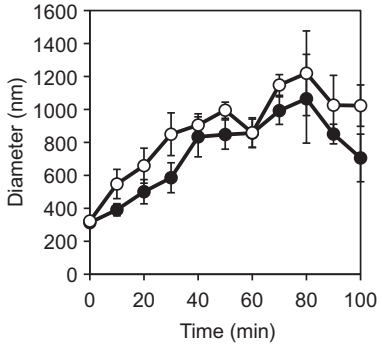

Figure 8. Monitoring of the complex size in the presence (*open circles*) and absence (*closed circles*) of hGCN5. The enzymatic reaction was started at 0 min by adding hGCN5 to the reaction solution containing the complexes (N/P = 2.0). Data are the means ± SEM of three independent experiments.

heat-inactivated hGCN5 resulted in the increase of the diameter due to the aggregation of the polyplex induced by components included in hGCN5 solution. The addition of active hGCN5 showed the same change in the diameter, and no significant difference was observed.

These two results indicate that the structural change in the polyplex induced by acetylation was not significant. Thus the polyplex seems to be formed even after acetylation, while affords the expression of DNA.

Although the structural change in the polyplex was not detectable, enzymatic reactions such as DNA degradation with DNase was similarly activated by acetylation (see Supplementary data Fig. S2). These results suggest that decrease of only one positive charge on the peptide has a great meaning for gene regulation. The detail of the acetylation-induced acceleration in these enzymatic reactions has not been cleared yet, but these accelerations would result from undetectable slight loosening of the polyplex.

3. Conclusion

Here we succeeded in modeling the HAT responsive gene expression system by using hydrophilic polymer grafted with H3 tail peptides as an artificial regulator. The polymer binds to DNA in a unique manner comparing with the simple polycations: The polymer binds to excess DNA and does not condense DNA strongly. This loose structure of polyplex affords the acetylation of the peptide side chain, while it is enough to suppress the transcription. After the acetylation, the polyplex affords the transcription of the DNA without dynamic structural transition of the polyplex. To our knowledge, this is the first functional model of the chromatin responding to HAT acetylation.

4. Experimental

4.1. Synthesis of histone H3 N-terminal peptides

The H3 histone tail peptide (H3-peptide: Ac-ARTKQTARKSTGGKAPRWK ($COC(CH_3)=CH_2$)–NH_2) was synthesized as reported,[26] using the corresponding Fmoc amino acids and *N*α-Fmoc-*N*ε-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl-L-lysine (Fmoc-Lys(ivDde)-OH) for the residue to modify the vinyl group. N-Terminal was capped by acetic acid anhydride. Vinyl-group modification of the peptide was conducted by selective deprotection of ivDde from Lys, by incubating the peptide-loaded resin for 30 min with DMF/2% hydrazine twice, and subsequently coupling with methacrylic acid in DIEA/NMP (0.90 M) and HBTU, HOBt/DMF (0.45 M) for 20 min. The obtained peptides were purified using a LaChrom Elite reverse phase HPLC (Hitachi High-Technologies Corporation, Tokyo, Japan) under a linear gradient, at flow-rate of 3 mL/min, where eluent A was 0.1% TFA in water and eluent B was 0.1% TFA in acetonitrile.

4.2. Synthesis of peptide-grafted polymer

The H3-peptide grafted polymer (H3-polymer) was synthesized as described.[14,17] Briefly, acrylamide (15.8 mg, 223 μmol) and H3-peptide (5.04 mg, 2.25 μmol) were dissolved in water, degassed with nitrogen bubbling for 5 min, and then polymerized using ammonium persulfate (1.49 mg, 6.53 μmol) and *N*,*N*,*N*′,*N*′-tetramethylenthylenediamine (19.5 μL, 13.1 μmol) as a redox couple at room temperature for 90 min. The reaction mixture was dialyzed against water overnight dialysis membrane bag (with a molecular cutoff of 25,000), followed by lyophilization to obtain a white powder. The peptide content of polymers was determined from UV-absorbance of Tryptophan at 280 nm in water ($\varepsilon = 5630\ M^{-1}\ cm^{-1}$).

4.3. Gel permeation chromatography (GPC) analysis of polymers

A molecular weight of the H3-polymer was determined by GPC (Shimazu Company Limited, Japan) with tandem connected-columns of TSKgel G5000PWXL and G6000PWXL (Tosoh Corporation, Japan) at ambient temperature using 0.5 M acetate buffer (pH 4.1) containing 0.1 M $NaNO_3$ at a flow rate of 0.45 mL/min. Polyethylene oxides were used as standard samples.

4.4. Agarose gel electrophoresis of polymer/DNA polyplexes

0.2 μg of pCMV-luc, which has a cytomegalovirus promoter and codes firefly luciferase, dissolved in distilled water. Various concentrations of polymers were then added to the solution, and allowed to stand for 15 min at room temperature. All the solutions were diluted to 10 μL with HEPES buffer (pH 7.5) at a final concentration of 10 mM. Then analyzed by 1% agarose gel electrophoresis in Tris–Acetate–EDTA buffer (pH 8.3).

4.5. Ethidium bromide (EtBr) exclusion assay

Polymer/DNA polyplexes were prepared by mixing at various N/P ratios in the presence of EtBr in distilled water. The solutions were incubated at room temperature for 15 min, after which the final concentration of DNA and EtBr were adjusted to 50 μg/mL and 12.5 μg/mL with 10 mM HEPES buffer (pH 7.5), respectively. Fluorescence measurements of sample solutions were carried out at room temperature using the multilabel counter ARVO (Wallac Incorporated, Turku, Finland). Excitation and emission wavelength were 531 and 590 nm, respectively.

The relative fluorescence intensity was determined using the following equation:

$$\text{Relative fluorescence intensity [RFI (\%)]} = (F_{\text{sample}} - F_0)/(F_{100} - F_0) \times 100$$

where F_{sample} is the fluorescence intensity of the polymer/DNA complexes at each N/P ratio, F_0 is EtBr without DNA, and F_{100} is EtBr/DNA complex before the addition of cationic polymer.

4.6. HAT assay

In this study, hGCN5 (Enzo Life Sciences, USA) which is a kind of HAT was used to enzymatic acetylation reaction. The polymer/DNA polyplexes were prepared by mixing pT7-luc DNA encoding firefly luciferase with T7 promotor, and various concentrations of the H3-polymer. The reaction mixture was allowed to stand for 15 min at room temperature. The resulting polyplexes were then incubated in 10 μL of 10 mM HEPES buffer (pH 7.5) containing 100 μM acetyl-CoA and 2.5 ng/μL hGCN5. The final concentration of DNA was adjusted to be 30 μg/mL. The reaction was initiated by addition of a cocktail of the HEPES buffer containing acetyl-CoA and hGCN5 to the polyplex solutions, and incubated for 60 min at room temperature.

4.7. Measurement of acetylation of polymer/DNA polyplexes by fluorescence detection assay

Acetylation ratios of the polymer/DNA polyplexes determined by a fluorescence detection assay using pT7-luc as a DNA. After the HAT assay was complete, the reaction solutions were quenched with equal volume of 2-propanol. Then, each solution was mixed with 80 μL of 12.5 μM 7-dietylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) and transfer the sample to 96 well white plate, then allowed to react in darkness for 10 min. The plate was then measured in multilabel counter ARVO with emission and excitation filters for 405 nm and 460 nm, respectively. Obtained each fluorescence intensity was subtracted by the background fluorescence intensity at reaction time of zero due to the reaction of CPM with cysteins in hGCN5. Standard curves were obtained using each concentration of β-mercaptoethanol solutions concurrently with the assays. The fluorescence values were then converted to the concentration of CoA the mean conversion factor calculated from the calibration curves. The acetylation ratio was defined by concentration of CoA dividing by concentration of peptide in reaction mixture.

4.8. Luciferase assay in cell-free system

All experiments were performed by using an in vitro expression system (TNT® T7 Quick Coupled Transcription/Translation System) (Promega, USA). After the acetylation reaction of polymer/DNA complex by HAT assay, 1 nmol of anacardic acid, which is an hGCN5 inhibitor, was added to the samples. Detection of luciferase expression was according to manufacture's protocols. pT7-luc DNA containing acetyl-CoA, hGCN5 and anacardic acid was used as the control. One hundred microliters of luciferase assay solution (Promega, USA) were added to 5 μL of the reaction mixture, and the chemiluminescence intensity was measured by the multilabel counter ARVO.

4.9. Gel electrophoresis analysis

H3-polymer/DNA complex was prepared by mixing pCMV-luc and various concentrations of the H3-polymer and allowed to stand for 15 min at room temperature. After the acetylation by HAT assay, each reaction solutions were analyzed by 1% agarose gel electrophoresis as described above. As a control, inactivated hGCN5 by heating at 97 °C for 3 min was used in place of hGCN5.

4.10. Dynamic light scattering (DLS) measurement of polymer/DNA polyplexes

Changes of diameter of the polymer/DNA polyplexes were monitored by using the Zetasizer nanoseries (Malvern Instruments Limited, UK) at the detection angle of 173° and temperature of 25 °C. A He/Ne laser ($\lambda_0 = 633$ nm) provided the incident beam. Preparation of polyplexes was identical to that of HAT assay.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.05.011.

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