

# Coexpression of the Partial Androgen Receptor Enhances the Efficacy of Prostate-specific Antigen Promoter-driven Suicide Gene Therapy for Prostate Cancer Cells at Low Testosterone Concentrations

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## Abstract

The prostate specific antigen (PSA) promoter/enhancer has been clearly demonstrated to be tissue specific, and has been applied to prostate-specific gene therapy. However, the transcription of the PSA gene is strictly androgen dependent, and its promoter activity is very weak at low concentrations of testosterone, which are generally observed in prostatic cancer patients treated with androgen deprivation. In this study, we used a partial androgen receptor (ARf) containing amino acids 232–429 and 481–657 to transactivate the PSA gene without androgens. We made two expression vectors, ARfPPLUC and ARfPPTK. They contained ARf cDNA driven by cytomegalovirus promoter and cDNAs of either firefly luciferase (LUC) or herpes simplex virus thymidine kinase (TK) driven by PSA promoter/enhancer (PP). The expressed ARf enhanced the PP activity by about 110-fold in the PSA-producing prostate cancer cell line, LNCaP, under low testosterone concentrations. Moreover, in a PSA-nonproducing prostate cancer cell line, DU145, ARf also enhanced the PP activity by about 60-fold in an androgen-independent manner. In a growth inhibition assay, ARfPPTK treated with ganciclovir was found to inhibit the cell growth of LNCaP cells much more effectively than PPTK. Furthermore, in contrast to PPTK, ARfPPTK also had an inhibitory effect on DU145 cells. This system is thus considered to provide a useful therapeutic option in patients with prostate cancer who are receiving hormonal therapy.

## Introduction

Prostate cancer is one of the most prevalent malignant diseases among men over 50 years of age in most western countries (1). At present, hormonal therapy is the accepted treatment of advanced cancers, and tumor regression is observed in most patients with this treatment. However, after a period of time, virtually all cancers progress to a state refractory to androgen ablation (2). As a result, the development of new therapeutic modalities including gene therapy is eagerly awaited.

PSA<sup>2</sup> is expressed in the normal and hyperplastic prostate as well as in prostate cancer, and the transcriptional regulatory region is considered to be suitable for tissue specific gene therapy trials (3–5). However, PSA is also well known to be strictly regulated by androgens. The 5′-flanking region of the PSA gene contains multiple AREs within 6 kb, and these AREs bind AR cooperatively and act syner-

gistically to stimulate transcription (6). The proximal and distal ARE-rich regions are called the proximal promoter and distal enhancer. Therefore, a serious problem regarding the introduction of PP in gene therapy is that, in most patients, the androgen concentration is extremely low because of the androgen ablation therapy and that some tumor cells have already become unresponsive to androgen when the gene therapy is started.

AR comprises about 910 amino acids and is a cytosolic receptor of androgens. It mainly consists of three functional domains including the NH<sub>2</sub>-terminal domain, DNA binding domain, and LBD. After binding androgens with LBD, AR changes to an activating form, moves to the nucleus, binds with ARE in androgen target genes, and thereby enhances their transcriptions. Interestingly AR was found to become androgen independent when the first 201 amino acids and the LBD have been removed (7–9).

In this report, we cloned a gene for the ARf, which codes for AR amino acids 232–657. This ARf cDNA driven by CMV promoter was incorporated into the plasmid that expresses herpes simplex TK under PP. We examined this plasmid to determine: (a) whether the combination of TK/GCV could exert its cell-growth inhibitory effect more efficiently in AR-positive LNCaP cells at low concentrations of testosterone; and (b) whether the ARfPPTK system could work even on DU145 cells, which are androgen-unresponsive because of the loss of AR.

## Materials and Methods

**PCR Cloning and Construction of Plasmids.** The genomic DNA for PCR template was extracted from the peripheral blood cells of a normal Japanese male. The DNA fragments for PSAR, located between –4757 and –3928, and for PSAP, located between –633 and +12 were obtained by PCR amplification. To obtain a part of the AR, RNA was extracted from LNCaP cells using an RNeasy Mini Kit (Qiagen, Valencia, CA). Next, reverse transcription was done with the oligo(dT) primer (Life Technologies, Inc., Rockville, MD) and SuperScript II RNaseH<sup>-</sup> Reverse Transcriptase (Life Technologies). The PCR primers, 5′-gaaggatccGAGTGTGTAAGGCAGTGT-3′ and 5′-tctagagCT-TCTGGGTTGTCTCCTCAGT-3′, were designed to amplify sequences coding amino acids from 232 to 657 of AR. The amplified PCR fragment, ~1.3 kb in length was cloned into pCR2.1 (Invitrogen, Carlsbad, CA). After restriction enzyme digestion with *KpnI* and *XbaI*, this fragment was subcloned into pcDNA4HisMax2 (Invitrogen) to make ARf expression vector CMVARf (Fig. 1A). The positive and negative control plasmids used for the LUC assay were pGL3Control and pGL3Basic (Promega, Madison, WI). The plasmid, PPLUC was made by inserting PSARPSAP in the multicloning site of pGL3Basic. We also made two expression vectors, ARfPPLUC and ARfPPTK, which contained CMVARf fused to cDNAs of the firefly LUC or herpes simplex virus TK driven by PP. The plasmid CAGTK was made by inserting TK cDNA into the plasmid CAGGS, which contains the CAG promoter. All of the plasmids used were confirmed by restriction enzyme digestion and sequencing with ABI PRISM 310 Gene Analyser by the BigDye Termination Method (PE Applied Biosystems, Branchburg, NJ).

**Cell Culture and Maintenance.** Human prostate cancer cell lines, LNCaP, PC-3, and DU145, and a bladder cancer cell line, T24, were obtained from

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<sup>2</sup> The abbreviations used are: PSA, prostate-specific antigen; PSAR, PSA enhancer; PSAP, PSA promoter; PP, PSAP/PSAR; FBS, fetal bovine serum; DHT, dihydrotestosterone; AR, androgen receptor; ARE, androgen responsive element; LBD, ligand binding domain; LUC, luciferase (gene), GCV, ganciclovir; CMV, cytomegalovirus; CAG, chicken  $\beta$ -actin promoter/rabbit  $\beta$ -globin poly(A); ARf, partial AR; TK, thymidine kinase.

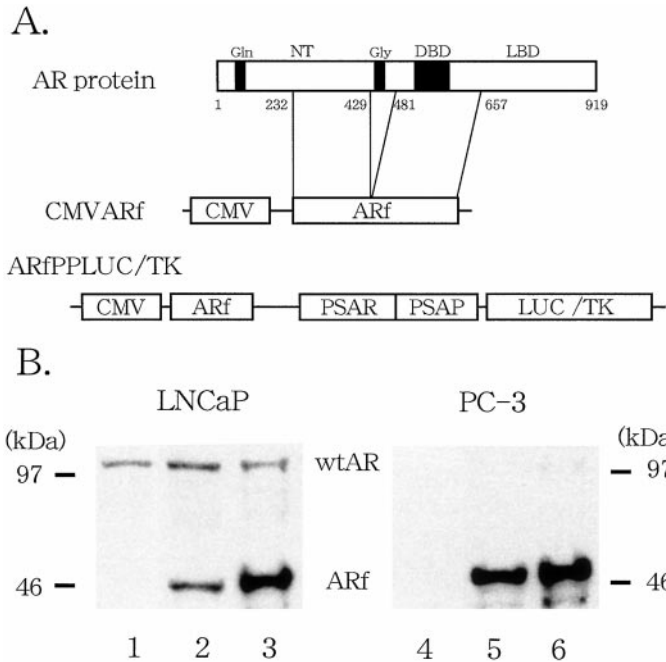


Fig. 1. Expression of endogenous AR and exogenous ARf in LNCaP and PC-3 cells transfected with ARf expression vector. **A**, structure of a ARf expression vector. The plasmid, CMVARf is CMV-promoter-driven ARf coding amino acids 232–429 and 481–657. The plasmid, ARfPPLUC/TK is the expression vector, which has the ARf driven by CMV promoter and the *LUC* gene (*LUC*) or *TK* gene (*TK*) driven by PP (*PSAR* and *PSAP*). *NT*, NH<sub>2</sub>-terminal domain, *DBD*, DNA binding domain. **B**, expression of endogenous AR (*wtAR*) and exogenous ARf. The LNCaP (Lanes 1–3) and PC-3 (Lanes 4–6) cells were transfected CMVARf. At 0 (Lanes 1 and 4), 48 (Lanes 2 and 5), and 96 (Lanes 3 and 6) h after transfection, the cells were harvested, and the lysates were electrophoresed with 7.5% SDS-PAGE. After the transferring of proteins to the membrane, immunoblotting was performed with AR411 followed by goat antimouse immunoglobulin-horseradish peroxidase. The detection was performed by ECL detection kit. *kDa*, *M<sub>r</sub>* in thousands.

American Type Culture Collection (Rockville, MD). LNCaP and T24 cells were maintained in RPMI 1640 supplemented with 10% FBS (Hyclone, Logan, UT), 50 units/ml penicillin and 50  $\mu$ g/ml of streptomycin. DU145 and PC-3 cells were maintained in modified Eagle's medium with FBS and the antibiotics as above. DHT and GCV were purchased from the Sigma (St. Louis, MO) and the Tokyo Tanabe Co. Ltd. (Tokyo, Japan), respectively.

**Western Blot Analysis.** LNCaP and PC-3 cells were transfected with 0.75  $\mu$ g of CMVARf using LipofectAMINE Plus (Life Technologies) according to the manufacturer's protocol. These cells were harvested at the indicated hours after transfection, and were lysed in lysis buffer [20 mM Tris/HCl (pH 8), 1 mM phenylmethylsulfonyl fluoride, and 1% (v/v) Triton X-100]. The lysates were centrifuged and the supernatants were recovered and placed on a 10% SDS-PAGE gel. After electrophoresis, the separated proteins were transferred to nitrocellulose membranes. The membranes were blotted by the monoclonal antibody AR411 (Santa Cruz Biotechnology, Santa Cruz, CA), which recognizes the epitope corresponding to amino acids 299–315 of human AR, followed by goat antimouse immunoglobulin-horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ). After washing, the membranes were developed using ECL Western Detection Reagent (Amersham Pharmacia Biotech).

**LUC Expression Assay.** LNCaP, PC-3, DU145, and T24 cells were plated at  $1 \times 10^5$  cells/well of 24-well-plate in adequate media containing 10% charcoal-stripped FBS (Hyclone) with various concentrations of DHT. DNA plasmid (0.75  $\mu$ g) and 2.5  $\mu$ l of Plus Reagent (Life Technologies) in 25  $\mu$ l of OPTI-MEM (Life Technologies) and 3  $\mu$ l of LipofectAMINE in 25  $\mu$ l of OPTI-MEM were mixed gently and were incubated at room temperature to form the complexes. The mixture was then poured into each well. Forty-eight h after incubation in 5% CO<sub>2</sub> at 37°C, the cells were washed once with PBS and were lysed with LUC cell culture lysis reagent (Promega). The supernatant recovered from each well was the LUC activity measured using the LUC assay system (Promega) by Tropix TR717 Microplate Luminometer (PE Applied

Biosystems). The 100% relative LUC activity was obtained based on the value of the pGL3Control in the absence of DHT.

**GCV-mediated Cell Growth Inhibition.** LNCaP cells ( $4 \times 10^4$ ) or  $1 \times 10^4$  DU145 and T24 cells per well were seeded into a 24-well plate in the complete medium containing dialyzed FBS. The plasmids were transfected into the cells after coupling with LipofectAMINE Plus. The cells were incubated with the medium containing 10  $\mu$ g/ml of GCV after transfection. The number of recovered viable cells in each well was counted on days 1, 3, 5, and 6 or 7 of cultivation, and the results were expressed as the mean cell number of three wells.

## Results

**The Plasmid CMVARf Could Express the ARf in Cultured Cells.** The LNCaP and PC-3 cells were transfected with CMVARf and the cells were harvested at 0, 48, and 96 h after transfection. An immunoblot analysis of transfected cell lysates using AR411 showed that the exogenous ARf could be detected at *M<sub>r</sub>* ~50,000 48 h after transfection, and the expression of ARf increased at 96 h (Fig. 1B). The endogenous AR (wild-type AR) could also be detected at *M<sub>r</sub>* ~110,000 in LNCaP cells, and its expression decreased at 96 h. The scant band seen at *M<sub>r</sub>* ~100,000 in the PC-3 cell lysate was thought to be a dimer of exogenous ARf.

**The ARf Enhanced PP Activity in Prostate Cancer Cell Lines.** We examined the effect of ARf transactivation on the prostatic tissue specific activity of PP promoter. The plasmids ARfPPLUC and PPLUC were transfected into the PSA-producing prostate cancer cell line, LNCaP; PSA nonproducing prostate cancer cell lines, PC-3 and DU145; and bladder cancer cell line, T24. The amount of the expression plasmids for transfection was adjusted to the same mole by supplementing it with pcDNA4HisMax2. Forty-eight h after transfection, we prepared cell lysates for the LUC assay. In LNCaP cells, the presence of ARf enhanced the LUC activity 110-fold compared with that of PP alone. However, by the addition of  $10^{-8}$  M DHT, the LUC activity greatly increased in the cells with PPLUC, and no big differences were observed in the activity between the cells with and without ARf (Fig. 2). In contrast, in the DU145 cells, the LUC activity remained at background levels in the presence or absence of DHT, and the activity was remarkably enhanced when the *Arf* gene was expressed in the same cells. In comparison with the prostate cells, an increase in the PP activity in the presence of ARf was not so obvious

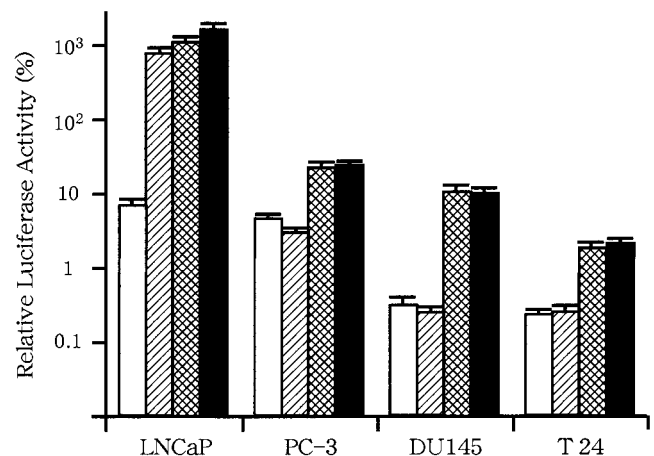


Fig. 2. The tissue-selective enhancement of the PP promoter activities by ARf in various cell lines (LNCaP, PC-3, DU145, and T24) in the absence or presence of  $10^{-8}$  M DHT. Cells were transfected with PPLUC in the absence (□) or presence (▨) of DHT and with ARfPPLUC in the absence (▧) or presence (■) of DHT. The 100% of relative LUC activity denoted the value of pGL3Control. The actual 100% mean LUC activities were  $8.5 \times 10^2$  in LNCaP,  $7.8 \times 10^2$  in PC-3,  $3.4 \times 10^2$  in DU145, and  $1.7 \times 10^2$  in T24. The results were expressed as the mean value  $\pm$  SD of three independent wells.

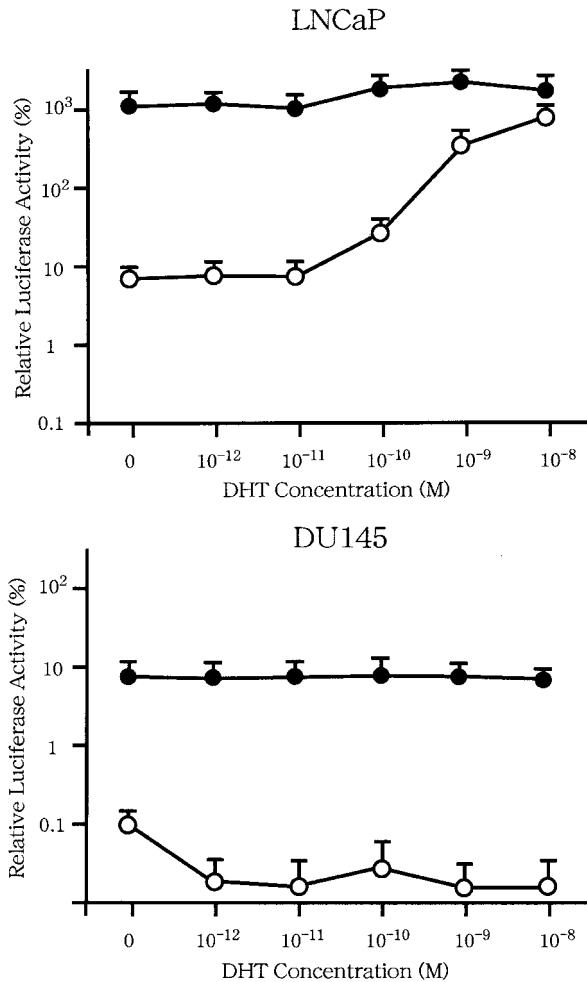


Fig. 3. The effect of DHT concentration on the enhancement of PP promoter activity by ARf. The LNCaP and DU145 cells were transfected with ARfPPLUC (●) and PPLUC (○), and were incubated for 48 h in the presence of various concentrations of DHT. The cells were harvested and a LUC assay was performed as described in "Materials and Methods." The 100% relative LUC activity was set to the value of pGL3Control in the absence of DHT. The actual 100% mean LUC activities were  $8.5 \times 10^5$  in LNCaP and  $1.7 \times 10^6$  in DU145. The results were expressed as the mean value  $\pm$  SD of three independent wells.

in T24 cells, thus indicating that the enhancing effect of ARf was preferentially observed in prostatic cells.

**The ARf Enhanced PP Activity in an Androgen-independent Manner.** To demonstrate the effect of androgens on enhanced PP activity, we performed a LUC assay on LNCaP and DU145 cells

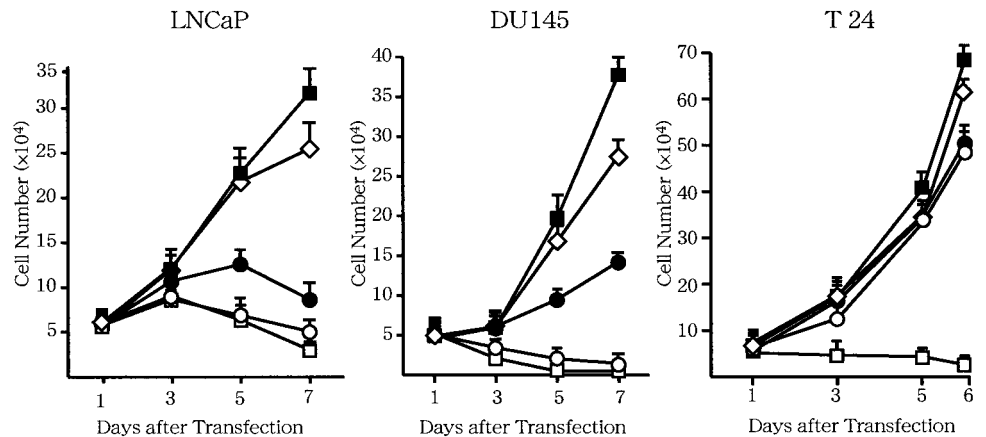
transfected with ARfPPLUC and PPLUC. In LNCaP cells, the enhanced PP activity by ARf seemed to be almost constant despite the DHT concentration, whereas the PP activity without ARf increased its activity in an androgen-dependent manner (Fig. 3). The ARf enhanced the PP activity by about 110-fold at low testosterone concentrations, and the transactivated PP activity was more than the activity of PP observed in the presence of  $10^{-8}$  M DHT. In the PSA-nonproducing prostate cancer cells DU145, ARf also increased more than 60 times in an androgen-independent manner.

**The ARfPPTK Could Inhibit the Cell Growth of Transfected Cells More Effectively than the PPTK in Prostatic Cancer Cell Lines.** We showed that ARf-enhanced PP had not only tissue specific activity, but also a high promoter activity. To determine the application of this enhanced promoter activity for suicide gene therapy, we transfected the cells with ARfPPTK, PSARPSAPTK, CMVARf, and CAGTK for positive control and CAGGS for negative control. The media used in this experiment were supplemented with 10% dialyzed FBS and 10  $\mu$ g/ml of GCV without DHT. In LNCaP cells, ARfPPTK inhibited the cell growth more effectively than did PPTK (Fig. 4). In DU145 cells, ARfPPTK was able to inhibit cell growth, whereas PSARPSAP could not (Fig. 4). However, in T24 cells, no differences in the cell growth among the plasmids were observed (Fig. 4).

**Discussion**

We herein demonstrated that the transactivation of PP by ARf markedly enhanced the PP-regulated LUC activity in the prostate cancer cells LNCaP, PC-3, and DU145. Furthermore, cell growth inhibition was significantly reinforced in LNCaP cells when they were cultured in the presence of low doses of testosterone. The ARf lacks the LBD of AR and transactivates ARE-regulated gene transcription in an androgen-independent manner. Fig. 3 clearly demonstrated that the level of PP activity with ARf remained constant regardless of the increase in the DHT dose. Because antiandrogen drugs work as a competitor in the binding between androgens and AR at the LBD, ARf is highly expected to exert its effect even in patients with a maximal androgen blockade. Actually, in our preliminary experiments with PC-3 and DU145 cells, the PPLUC activity enhanced by ARf did not change in the presence of hydroxyflutamide (data not shown). Furthermore, it was interesting to note in this study that, in the presence of ARf, cell growth inhibition was observed in the DU145 cells, whereas the PPTK/GCV system did not work because of the lack of AR expression in DU145 cells. In contrast, in the bladder cancer cell line T24, the enhancement of PSAP activity was not so great, and the cell growth inhibition by TK/GCV was also not observed in the presence of ARf. These results indicate that the enhancing effect of ARf is preferentially observed in the prostate-derived cells. This may

Fig. 4. Selective reinforcement of cell growth inhibition by ARf in prostate cell lines LNCaP, DU145, and T24 cells were transfected with CAGGS (■), which contains only CAG; CAGTK (□), which contains herpes simplex virus TK cDNA driven by CAG; CMVARf (◇), which contains ARf cDNA driven by CMV promoter; PPTK (●), which contains TK driven by PP; or ARfPPTK (○), which contains both ARf driven by CMV promoter and TK driven by PP. On days 1, 3, 5, and 6 or 7 of cultivation in the presence of 10  $\mu$ g/ml of GCV, the cells were harvested, and the number of recovered viable cells was counted. The results were expressed as the mean cell number  $\pm$  SD of three independent wells.





be attributable to a large degree to the relative availability of accessory cofactor proteins that may interact with specific promoters and/or different regions of the AR to modulate AR-induced transcription (10–13).

One of the weak points of using tissue-specific promoters in gene therapy is that the promoter activity is generally low in comparison to the universal promoters such as CMV, CAG, and so forth. This may be particularly serious when a PSAP is used for patients who receive antiandrogen therapy. To overcome this, Cre-loxP or GAL-VP16 (14) systems could be applicable to the prostate-specific amplification of the target gene expression. However, these systems still require the expression of the PSA gene and are not applicable to the PSA nonproducing prostatic cancer cells. In contrast, in our system, ARf was able to transactivate the androgen target gene regardless of the concentration of androgens. Therefore, our gene therapy system is applicable not only to inhibit the cell growth of the tumor in the patients who were treated with androgen deprivation therapy, but also those treated with AR-mutant cells such as DU145, which do not produce PSA because of the loss of AR expression.

In addition to the incorporation of the ARf gene, we used cationic liposomes as a gene vector in this study. In most of experiments thus far reported (3, 15, 16), adenoviral vectors have been used for the gene therapy of prostate cancer cells. Adenovirus can transduce the target genes into replicating and nonreplicating cells, and its transduction efficiency is also relatively high. However, adenovirus can evoke nonspecific inflammation, and strong antitumor immune responses are also easily induced. Therefore, the repeated administration will not be practical, especially when delivered systemically. Liposome-mediated gene therapy has many advantages over viral vectors in clinical application, but the transfection efficiency is low, especially for prostate cancer cells (10–15% at LNCaP cells in our experiments). One of the advantages of using liposomes, however, is that such usage allows for the conjugation of monoclonal antibodies. In addition to PSA, prostate cancer cells express other tissue-specific antigens such as prostate-specific membrane antigens. Monoclonal antibodies have been established to this membrane antigen (17), and we expect the conjugation of these monoclonal antibodies to liposomes to promote both targeting and transfection efficiency. These trials are now in progress.

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