The ubiquitous transcription factor NF-Y positively regulates the transcription of human p27^{Kip1} through a CCAAT box located in the 5′-upstream region of the p27^{Kip1} gene

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Abstract Abnormally low levels of the cyclin-dependent kinase inhibitor p27^{Kip1} are found frequently in human carcinomas, and these levels correlate directly with both histological aggressiveness and patient mortality. p27^{Kip1} is haplo-insufficient for tumor suppression. Thus, p27^{Kip1} may be a useful molecule for the development of cancer therapies. To know the possible mechanisms underlying transcriptional control, we previously cloned the promoter region of human p27^{Kip1} gene. We report here the characterization of the 5′-regulatory region of the human p27^{Kip1} gene. Promoter analysis using 5′-deletion mutants revealed that a 39-bp region between −549 and −511 was required for maximal promoter activity. Point mutation analysis revealed that a CCAAT box within this region was essential for promoter activity. Gel shift assays and cotransfection experiments using a dominant negative form of the NF-Y transcription factor showed that NF-Y directly regulates p27^{Kip1} transcription through this CCAAT box. This finding might provide a clue to approach the mechanism of tumorigenesis.

Key words: p27^{Kip1}; Transcription; Promoter; NF-Y

1. Introduction

p27^{Kip1} is an inhibitor of multiple cyclin-dependent kinases, and is thought to be an essential participant in the strict regulation of the cell cycle both in vitro and in vivo. Although mutations in p27^{Kip1} are found rarely in human tumors [1], abnormally low levels of the p27^{Kip1} protein are found frequently in human tumors, and reduced expression of the protein correlates well with poor survival among patients with breast [2,3], lung [4], prostate [5], and gastric carcinomas [6]. Furthermore p27^{Kip1} was recently shown to be haplo-insufficient for tumor suppression [7]. That is, inactivation of only one allele, or moderate decrease in protein expression, is sufficient to predispose to tumorigenesis. A potentially important strategy for cancer therapeutics will be to restore expression of tumor suppressor proteins. For this purpose, p27^{Kip1} may be a useful molecule. Recent work has shown an increase in p27^{Kip1} mRNA levels after incubation of neuroblastoma cells with thyroid hormone, embryonal carcinoma cells with retinoic acid, or gastric carcinoma cells with interferon-β [8–10]. In these experimental systems, the increase in p27^{Kip1} transcripts augments the p27^{Kip1} protein level as well as stabilization of the p27^{Kip1} protein, suggesting that transcriptional regulation of p27^{Kip1} is more generally important. Nevertheless, little is known about the regulatory mechanisms of p27^{Kip1} transcription. For this purpose, we previously cloned the human p27^{Kip1} gene promoter and reported that nucleotide position −774 to −435 relative to the translation start site is essential to its promoter activity [11].

To improve our knowledge of the transcriptional regulation of the human p27^{Kip1} gene, the next step would involve the characterization of the elements found in the promoter region. We describe here the *cis*-elements and *trans*-acting factors that regulate p27^{Kip1} gene expression at the basal level.

2. Materials and methods

2.1. Cell culture

The human cervical carcinoma cell line C33A, the human osteosarcoma cell line U2OS cells and the human osteosarcoma cell line MG63 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere of 5% CO_2.

2.2. Plasmid construction and mutagenesis

The human p27^{Kip1}-luciferase fusion plasmid, p27PF, and some deletion mutants, p27*Apa*I, p27*Afl*II, and p27*Sac*II, were previously generated [11]. Other deletion mutants, p27No. 1, p27No. 2, and p27No. 12 were generated using a Mungbeans-Exonuclease III system, the Kilo-sequence Deletion Kit (Takara, Tokyo, Japan).

Plasmids with point mutations in p27PF, p27mSp1-1, p27mSp1-2, and p27mCTF, were generated by site-directed mutagenesis [12] using the Quick Change Site-Directed Mutagenesis Kit (Stratagene). The top strands of the oligonucleotides were as follows, with mutations indicated by underlines: p27mSp1-1, 5′-CAGCCTCGGCGGGATG-GCTCCCGCCG-3′; p27mSp1-2, 5′-CGGGGCGGCTCCTACCGC-CGCAACCAATG-3′; p27mCTF, 5′-GCCGCCGCAACCTTTGGA-TCTCCTCC-3′. To avoid undesirable mutations, each *Apa*I-*Hin*dIII fragment corresponding to −774/−12 of these three mutants was subcloned back into *Apa*I and *Hin*dIII digested p27PF. The integrity of all generated constructs were confirmed by restriction analysis and sequencing.

2.3. Transient transfection and luciferase assay

C33A cells (3×10^5 cells) and MG63 cells (3×10^5 cells) were seeded into 6-cm diameter tissue culture dishes. After 24 h, 2 μg of reporter plasmid and 1 μg of pACTβ-gal, a plasmid containing the β-galactosidase driven by actin promoter, were cotransfected into cells using the calcium phosphate co-precipitation method [13]. Cells were harvested after 48 h or 24 h and preparation of extracts and luciferase assays were performed as described previously [14]. The luciferase

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Abbreviations: DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; CDK, cyclin-dependent kinase; CTF, CCAAT box binding transcriptional factor

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activity of each cell lysate was measured and was normalized by β-galactosidase or protein activities in the cell lysates. All the transfection assays were carried out in triplicate. Each experiment was repeated at least twice. A dominant negative NF-YA expression plasmid (pNF-YA29) is a kind gift from Dr. R. Mantovani at the University of Milano.

2.4. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to the method of Andrews and Faller [15]. The reaction mixture for the gel shift assay (20 μl final volume) contained 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% glycerol, 0.05% Nonidet P-40, 5 μg of bovine serum albumin (BSA), 2 μg of poly(dI-dC), and 5 μg of nuclear extract. After preincubation for 5 min at room temperature, probe DNA (approximately 2 ng, 20 000 cpm) was added to the mixture, and the binding reaction was allowed to proceed at room temperature for 20 min. The product was then resolved by electrophoresis on 5% acrylamide-bisacrylamide (29:1), 0.5×TBE non-denaturing gel at 10 V/cm for 120 min. The sequences of the top strands of oligonucleotides used in these studies are as follows, with mutations indicated by underlines: WT−530/−510, 5′-CTAGCGC-CGCAACCAATGGATCTCC-3′; MUT−530/−510, 5′-CTAGCGC-CGCAACCTTTGGATCTCC-3′; NF-I, 5′-CTAGTTTTGGATTG-AAGCCAATATGATAA-3′ [16]; NF-Y, 5′-ACTTTTAACCAATC-AGAAAAATCTAG-3′ [17]; CP2, 5′-CTAGTGACCAGTTCCAG-CCACTCTTTA-3′ [18]. Competition analyses were performed by mixing an indicated amount of the appropriate competitor DNA to the binding reaction prior to addition of nuclear extracts. In supershift experiments, antibody (IgG fraction) against NF-YB or antibody against C/EBP-α (sc-7204X; Santa Cruz) was added to the incubation mixture containing nuclear protein before addition of probe DNA. Antibody against NF-YB was kindly provided by Dr. R. Mantovani.

3. Results and discussion

We have reported previously that a 339-bp region between −774 and −435 relative to the translation start site is essential for human $p27^{Kip1}$ promoter activity [11]. To further define *cis*-elements that are required for the human $p27^{Kip1}$ promoter activity, several additional deletion mutants between −774 and −435 were generated and transiently transfected into C33A, MG63, and U2OS cells. We used C33A and U2OS cells to represent cells whose p53 and RB genes are mutated or intact, respectively, and MG63 cells to represent cells that carry rearrangements in the p53 gene but contain an intact RB gene. As shown in Fig. 1, deletions up to position −549 (p27No. 2) did not result in significant changes in the promoter activity, whereas the promoter activity was decreased markedly using a deletion up to −511 (p27No. 12) in C33A cells. A similar result was obtained in U2OS and MG63 cells (data not shown). These results showed that potential regulatory elements appeared to be located downstream from −549, and a drastic decrease in promoter activity using a deletion up to −511 suggested that the region between −549 and −511 (−112 and −74 relative to the transcription start site) contained the elements required for basal promoter activity.

The sequence of this 39-bp region carried two consensus Sp1 binding sites and one CCAAT box (CTF site). In this study, we termed the two Sp1 sites in the upstream region Sp1-1 and Sp1-2 (Fig. 2). To determine whether the two Sp1 sites and the CCAAT box were involved in the regulation of $p27^{Kip1}$ transcription, a series of mutations of the full-size promoter construct, p27PF, having mutations in each Sp1 site or the CCAAT box were generated, and were termed p27mSp1-1, p27mSp1-2, and p27mCTF, respectively (Fig. 2). These constructs were transiently transfected into C33A, MG63, and U2OS cells, and their luciferase activities were analyzed. As shown in Fig. 2, a 2-bp mutation in the CCAAT box significantly reduced the promoter activity of p27PF in all cell lines. Furthermore, a mutation in the Sp1-1 site but not the Sp1-2 also significantly reduced the promoter activity of p27PF in U2OS cells (Fig. 2, lower panel), whereas the reduction of promoter activity caused by a mutation in the Sp1-1 site was smaller in C33A and MG63 cells than in U2OS cells (Fig. 2, MG63 data not shown). This result indicated that the CCAAT box was indispensable for the maximum basal promoter activity of the $p27^{Kip1}$ gene and the requirement for the Sp1-1 site for promoter activity depended on the cell line.

To identify *trans*-acting factor(s) that interacts with the

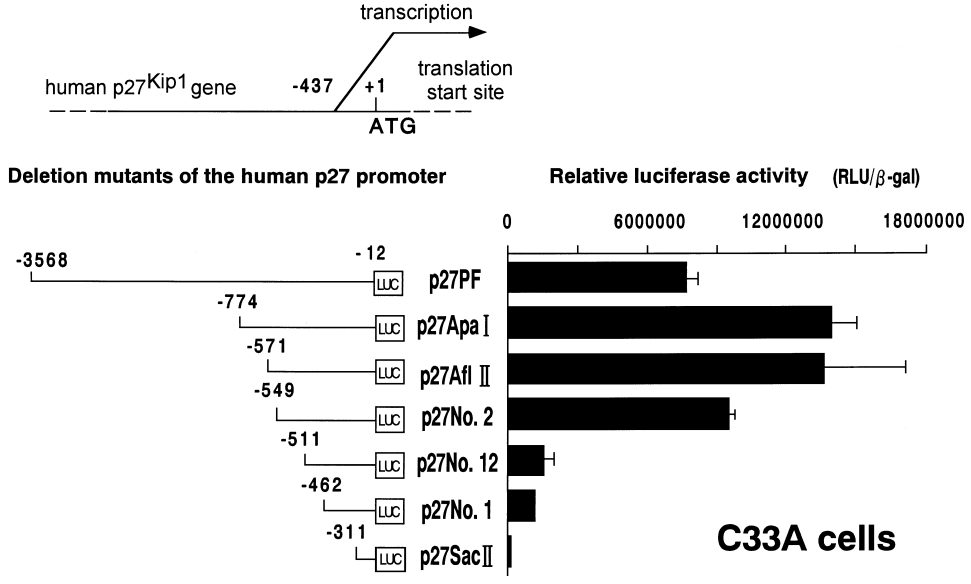

Fig. 1. Effects of 5′-deletion mutations on the promoter activity of the human $p27^{Kip1}$ gene in C33A cells. The 5′-deletion mutants of the promoter region of the $p27^{Kip1}$ gene were fused to the luciferase gene. Two μg of each plasmid was transiently transfected into C33A cells with 1 μg of pACTβ-gal, and luciferase activities were analyzed after 48 h. The luciferase activity in the cell extracts was determined as described in Section 2. Each construct is shown schematically on the left. Data are shown as means (bars, standard error) ($n=3$).

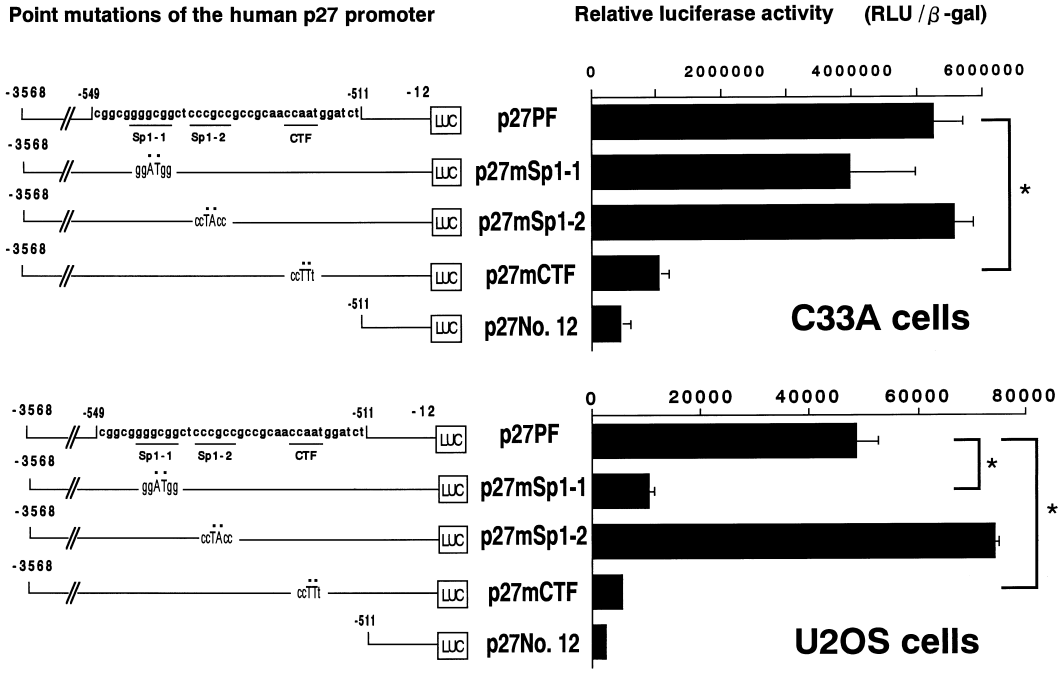

Fig. 2. Mutation analysis to identify the *cis*-elements required for the basal promoter activity of $p27^{Kip1}$ gene in C33A and U2OS cells. Three different mutants, shown on the left, were identical to the wild-type p27PF except for the mutation in capital letters. Two μg of each constructed plasmid was transiently transfected into C33A or U2OS cells with 1 μg of pACTβ-gal, and luciferase activities were analyzed after 48 h. Data are shown as means (bars, standard error) ($n=3$). *$P<0.05$.

CCAAT box, we performed EMSA using ^{32}P-labeled oligonucleotides spanning −530 to −510 of the human $p27^{Kip1}$ promoter (WT−530/−510) that contained the CCAAT box. Three shifted bands were observed when the probe was incubated with the C33A cell nuclear extract. Only the slowest migrating complex was specifically competed out by excess unlabeled WT−530/−510 but not MUT−530/−510 in which the CCAAT had been mutated to CCTTT (data not shown), suggesting that nuclear protein(s) can bind specifically to the CCAAT sequence in the WT−530/−510 probe.

It has been shown that CCAAT sequences can interact with various nuclear proteins including NF-Y(CP1), NF1(CTF), CP2, and C/EBP [17–21]. To elucidate which nuclear proteins bind to the −524/−519 CCAAT box, we performed competitive EMSAs using oligonucleotides carrying CCAAT sequences that had been reported to bind to each of these transcription factors in a specific manner [16–18,22]. As shown in Fig. 3, binding of the factor to the WT−530/−510 probe was effectively competed by a molar excess of oligonucleotides containing an NF-Y binding site (NF-Y, lanes 7 and 8) as well as by the WT−530/−510 probe (lanes 3 and 4). On the other hand, oligonucleotides containing NF1 or CP2 binding sites did not efficiently compete with the binding (Fig. 3, lanes 5, 6, 9, and 10). In addition, when the gel shift band was generated using the NF-Y probe, it was competed with excess amounts of the WT−530/−510 and the NF-Y probes, but not the MUT−530/−510 probe (data not shown). These results suggested that the ubiquitous transcription factor NF-Y can bind to the CCAAT box of the $p27^{Kip1}$ promoter.

To confirm that the factor binding to the WT−530/−510 probe was NF-Y, we performed supershift experiments using antibodies. NF-Y is composed of three subunits, NF-YA, NF-YB, and NF-YC and we used antibodies raised against the B subunit of NF-Y [23–25], or against C/EBP-α[26] as a

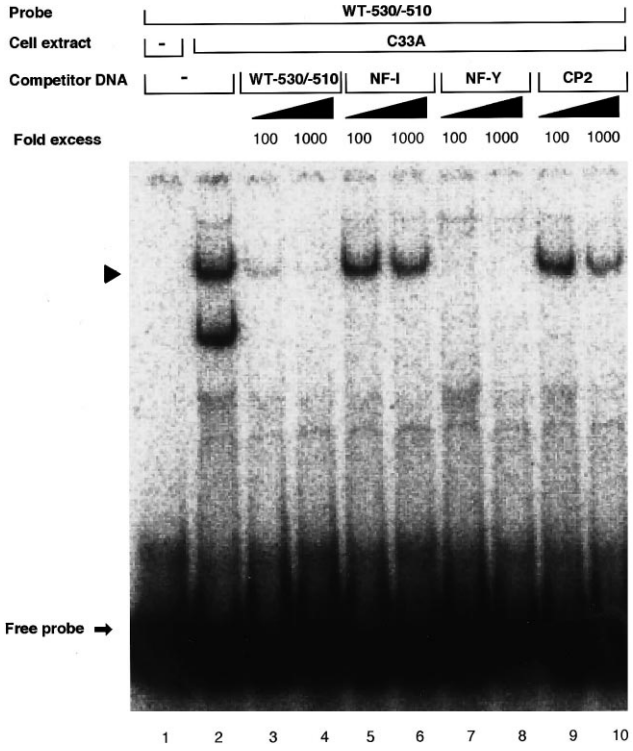

Fig. 3. Binding of nuclear proteins from C33A cells to the CCAAT box of the $p27^{Kip1}$ gene and competition analysis. Electrophoretic mobility shift assay was carried out with nuclear extracts prepared from C33A cells. A set of oligonucleotides containing the CCAAT box between −530 and −510 (WT−530/−510) was used as a probe. Nuclear extracts were incubated with ^{32}P-labeled WT−530/−510 in the absence (−) (lanes 1 and 2) or in the presence of 100- or 1000-fold amounts of various unlabeled oligonucleotides that can bind to each CCAAT binding protein. The specific retarded band containing CCAAT box binding protein(s) is indicated on the left.

negative control. As shown in Fig. 4, in the presence of anti-NF-YB antibody but not anti-C/EBP-α antibody, the complex was supershifted (lanes 2–5). This demonstrated that NF-Y binds to the CCAAT box of the $p27^{Kip1}$ promoter.

To further confirm the involvement of NF-Y in the regulation of $p27^{Kip1}$ transcription, we cotransfected p27PF or p27mCTF with a dominant negative NF-YA mutant expression plasmid (pNF-YA29) [25,27,28] or the parental expression vector (pSG5). As shown in Fig. 5, the luciferase activity from p27PF was reduced to about 70% when cotransfected with pNF-YA29. This result suggested that NF-Y at least partially regulates $p27^{Kip1}$ transcription via the CCAAT box within its promoter.

The results presented here suggest that the transcription factor, NF-Y, plays a central role in regulating the basal level of the $p27^{Kip1}$ promoter activity by direct binding to the CCAAT box. Currently, there is no precise report about the relationship between NF-Y expression and the level of $p27^{Kip1}$ in tumor cells. Future investigation of mutations in the CCAAT box of the $p27^{Kip1}$ gene promoter and the expression of NF-Y in various malignancies, whose level of $p27^{Kip1}$ is low, should reveal the precise role of $p27^{Kip1}$ and NF-Y in malignant tumors. From the standpoint of cancer therapeutics, $p27^{Kip1}$ may be a possible target for the development of strategies for cancer therapeutics, by means of restoring expression of tumor suppressor proteins [7]. On the other hand, NF-Y is a ubiquitously expressed multi-component factor that

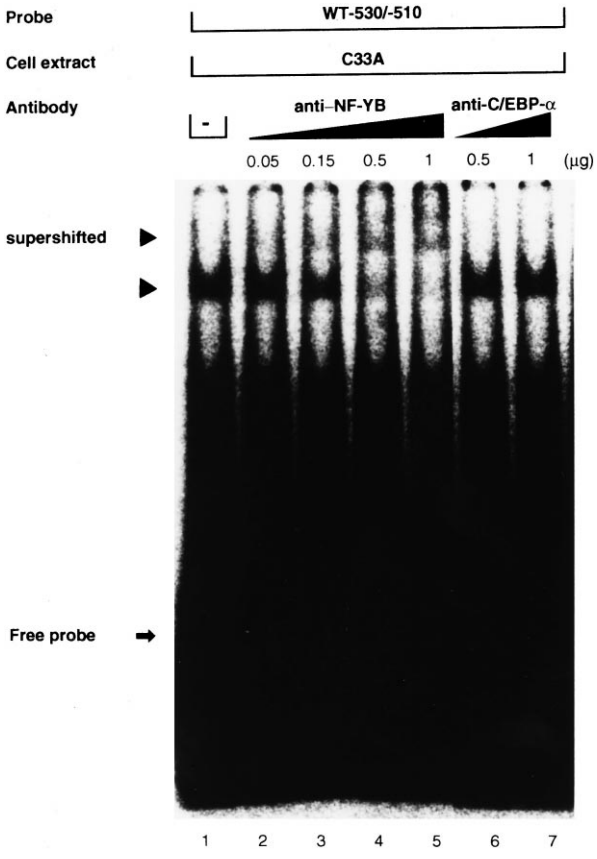

Fig. 4. Antibody supershift assay. WT−530/−510 was used as a probe. Nuclear extracts were incubated with ^{32}P-labeled WT−530/−510 in the absence (−) (lane 1) or in the presence of anti-NF-YB antibody (lanes 2–5) or anti-C/EBP-α antibody (lanes 6 and 7). The retarded and supershifted bands are indicated on the left.

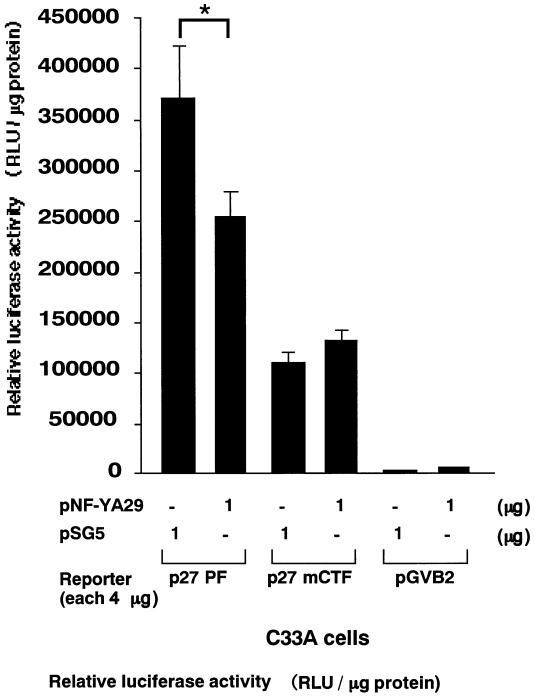

Fig. 5. A dominant negative NF-YA expression plasmid (pNF-YA29) suppresses $p27^{Kip1}$ promoter activity through the CCAAT box. Four μg of p27PF or p27mCTF was cotransfected into C33A cells with 1 μg of a dominant negative NF-YA expression plasmid (pNF-YA29) or the parental expression vector (pSG5). Luciferase activities were analyzed after 24 h. Data are shown as means (bars, standard deviation) ($n = 3$). *$P < 0.05$.

interacts with the CCAAT box, a common element present in various mammalian gene promoters. NF-Y appears to cooperate functionally with different transcription factors or tissue specific factors [29–31], but the mechanism is not yet understood. Investigation of this mechanism might provide useful information for the development of agents that promote transcription of the $p27^{Kip1}$ gene in a therapeutic or chemopreventive context, which we have termed gene-regulating chemotherapy or chemoprevention [32].

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