No Apparent Damage in the Thyroid of Transgenic Mice Expressing Antiapoptotic FLIP

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FLIP is an antiapoptotic protein that has been demonstrated to play an important role in inflammation, cancer, and autoimmune diseases. However, it is not known whether increased expression of FLIP (FLICE inhibitory protein) in thyrocytes would alter the development of the thyroid and/or pathogenesis of thyroiditis. To examine the effects of overexpression of this antiapoptotic molecule on the thyroid, we have developed transgenic mouse lines that specifically express FLIP in thyrocytes. A DNA construct designed with an in-frame coding sequence for the E8 protein, a viral FLIP, was put under the control of the thyroglobulin (Tg) promoter (the Tg-FLIP transgene). In 8 of 12 resultant transgenic mouse lines, FLIP expression in thyrocytes driven by the Tg promoter was documented, and confirmed at RNA and protein levels. These Tg-FLIP transgenic mice were monitored for 1 year. Throughout the entire observation period, the transgenic mice remained alive and healthy without evidence of thyroid dysfunction. Adult mice were able to breed. Histologic examination of thyroids obtained at various time points did not reveal significant differences between transgenic mice and their control littermates. Therefore, transgenic mice with thyrocyte-specific expression of FLIP have normal thyroid development with no significant changes in thyroid cell death or proliferation.

Introduction

Disruption of the normal regulation of cell death pathways has been implicated in a number of pathologic conditions such as autoimmunity and cancer. There is increasing evidence showing that signal transduction through death receptors, such as Fas or TRAIL, contributes to the development of autoimmune thyroiditis. Recent studies have attempted to define the regulation of receptor-mediated cell death pathways in both normal and diseased thyroid in vitro; however the function of these pathways in vivo is more complex.

Thyroid cells are known to express Fas (1), but the expression of Fas does not necessarily render thyrocytes susceptible to Fas ligand-induced apoptosis because of the existence of certain cellular inhibitors (2). One of these regulators of death receptor-mediated apoptosis is FLIP (FLICE inhibitory protein) (3,4). Cross-linking of Fas ligand induces apoptosis through procaspase-8 recruitment to the Fas-mediated death-inducing signaling complex (DISC), where procaspase-8 is then cleaved to initiate apoptosis. The recruitment of the caspase-8 inhibitor FLIP into the DISC prevents the cleavage of procaspase-8, resulting in reduced apoptotic activity (3,4).

There are several publications indicating that altered FLIP concentrations are found in inflammation and autoimmune diseases, including rheumatoid arthritis and experimental autoimmune thyroiditis (5–9). A study by Wei et al. (8) demonstrated that the upregulation of FLIP by inflammatory cells blocks Fas-mediated apoptosis, contributing to chronic inflammation. In contrast, increased FLIP expression by thyrocytes in resolving granulomatous experimental autoimmune thyroiditis (G-EAT) protects thyrocytes from apoptosis. This study further suggests that increased FLIP and decreased Fas ligand expression by inflammatory cells might block apoptosis of CD4+ T cells, resulting in chronic granulomatous thyroiditis (9). Overexpression of FLIP has also been found to exacerbate experimental autoimmune encephalomyelitis and multiple sclerosis (10–13). This suggests an important immunoregulatory role for FLIP in autoimmunity.

To determine whether the overexpression of FLIP in thyrocytes would have any impact on thyroid development or the pathogenesis of thyroid diseases, we developed transgenic mice that specifically expressed FLIP in the thyroid. This was accomplished by a transgene in which the promoter for thyroglobulin (Tg), a thyroid-specific protein, drove the expression of the E8 protein, a viral FLIP. The expression of E8 has been shown to block apoptosis induced through several death domain-containing receptors, including TNF-R, Fas, and TRAIL receptors (14,15). In this report we describe the construction of this transgene and the initial characterization of Tg-FLIP transgenic mice.
Materials and Methods

The transgene construct

The plasmid Tg-FLIP-myc was constructed from a CMV-FLIP plasmid containing a 519-bp insert of the viral FLIP in pCDNA3.1(-)/Myc-HisA (16) as a generous gift from Dr. Claudius Vincenz (University of Michigan, Ann Arbor, MI). The rat Tg promoter region (17) was generated by polymerase chain reaction (PCR) from rat tail DNA using XbaI and Xhol restriction enzyme sites: the 5′ primer: 5′-ATATAC TTATCT AGACTG CAGACA AGCAGG CATGC-3′ and the 3′ primer: 5′-TAACT ATATCT GAGTAC TCAAAT GATGGG GTAGGA G-3′. The resulting 889-bp PCR product was cut with XbaI and Xhol to create an 869-bp insert. The insert was then purified and cloned in-frame at the 5′ end of the open reading frame of the FLIP sequence in CMV-FLIP. The distance between the start of the TATA box and the start of transcription (+1) is 30 bp, and between +1 and the ATG of FLIP is 39 bp. Both intervals are the same distances as those in the natural rat Tg gene (Fig. 1). The correct sequence of the Tg promoter and its insertion site were verified with DNA sequencing by the Core Facility at the University of Michigan.

Transgenic mice and breeding

To generate Tg-FLIP transgenic mice, the plasmid Tg-FLIP was digested with XhoI and DntIII to create a 2014-bp fragment of transgene, which was gel purified for microinjection (Fig. 1). Microinjection of the Tg-FLIP transgene into eggs from (C57BL/6 × SJL)F1 × (C57BL/6 × SJL)F1 females was performed by the University of Michigan Transgenic Animal Core. Of 81 resultant pups, 12 were identified as positive for Tg-FLIP by PCR (described below). These founder lines were housed in SPF facilities at the University of Michigan and maintained according to UCUCA protocols. Transgenic positive mice are currently being crossed into a CBA/J (Jackson Laboratory, Bar Harbor, ME) background for use in mouse models of thyroiditis. Tg-FLIP-positive and Tg-FLIP-negative mice produced from these crosses were used to characterize expression of the transgene further in these lines.

PCR screening

DNA for transgene screening was performed on tail biopsies of 3-week-old mice. Tail sections were digested overnight with proteinase K, and DNA was then extracted with phenol and chloroform followed by precipitation with ethanol and reconstitution in 200 μL of TE buffer. DNA samples from transgenic mice were screened for the presence of the Tg-FLIP transgene by PCR using primers that amplify a 1285bp sequence between position +600 in the Tg promoter and the myc tag (Fig. 1): TGP1: 5′-ATATTC TTGCCA CTTCCT GCCC-3′ and TGP4: 5′-ATGGTC GACGCG GCTTATT CAG-3′. Two microliters of tail DNA were used in a PCR reaction with 1× AmpliTag buffer containing 1.5 mM MgCl2, 0.2 mM dNTPs, 0.3 mM each primer, and 2.5 units AmpliTag DNA polymerase in a volume of 50 μL. PCR was performed using a Perkin-Elmer 2400 thermocycler with the following program: 94°C for 5 minutes; first 10 cycles of denaturation at 94°C for 30 seconds, annealing at 64°C for 30 seconds, and extension at 72°C for 90 seconds; then 25 similar cycles with an extension time of 120 seconds; followed by 72°C for 7 minutes and finishing at 4°C. PCR reaction products were analyzed by electrophoresis on 1.5% agarose gel. DNA quality was confirmed using primers for rat β-actin or mouse β-globin: β-actin forward: 5′-ACCAAC TGCTCA CACAGG-3′ and β-actin reverse: 5′-TCTCAG CTGTGG TG-3′; β-globin 1: 5′-CCAATC TGCTCA CACAGG ATAGAG AGGGCA GG-3′, and β-globin 2: 5′-CCCTTG AGCTGT CCAAGT GATTCA GGCCAT CG-3′. PCR for β-actin yielded a 402-bp product that was obtained using the same protocol as PCR using the Tg-FLIP primers except for annealing at 62°C and extension times of 75 seconds in the first 10 cycles followed by 90 seconds during the final 25 cycles. PCR for β-globin yielded a 494-bp fragment and was run at 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 90 seconds. This was followed by 72°C for 7 minutes and the process was finishing at 4°C.

Southern blot

Ten micrograms of tail DNA of transgenic mice was digested with 30 units of SalI for 6 hours at 37°C. Tail DNA of negative mouse and tail DNA of negative mouse spiked with 100 copies of the transgene Tg-FLIP plasmid were also included as controls. Digested DNA was run on a 1.5% agarose/0.5× TBE gel overnight at 25 mA. Before transfer, the DNA in the gel was denatured with 0.5 M NaOH/1.5 M NaCl for 1 hour and neutralized with two changes of 3 M NaCl/0.5 M Tris, pH 7.5 for 45 minutes. DNA was transferred to nitrocellulose membrane by upward capillary electrophoresis in 10× SSC buffer overnight. After overnight

![FIG. 1. Schematic diagram of the Tg-FLIP plasmid. The Tg-FLIP transgene in pCDNA3.1(-)/Myc-HisA is depicted showing the relative positions of the rat thyroglobulin (Tg) promoter, the FLIP sequence including the tag sequences for a c-myc peptide and a 6 histidine peptide. Restriction enzyme sites (XhoI and DntIII) used for constructing the transgene as well as those used for Southern blot are shown. The relative positions for annealing of polymerase chain reaction (PCR) primers (TGP1, TGP4 and E8 forward) are also shown.](image-url)
transfer, the membrane was rinsed with 2× SSC. DNA was cross-linked to the membrane using a Stratalinker (Stratagene, La Jolla, CA) at 120,000 μJ/cm².

Southern blotting was conducted by first incubating the membrane with 1× prehybridization solution (6× SSC, 10× Denhardt’s, 0.1% sodium dodecyl sulfate [SDS], 0.5 μg/mL salmon sperm DNA) in a roller bottle for 30 minutes at 65°C. Tg-FLIP plasmid digested with XbaI and DraIII was gel purified for use as a probe. Twenty-five nanograms of probe was labeled with [−γ32P]dATP using a Random Primers DNA Labeling System (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. The labeled probe was purified using G-50 Sephadex Quick Spin Columns (Roche, Alameda, CA) for radiolabeled DNA purifica- tion following manufacturer’s instructions. The probe was added to 20 mL 1× prehybridization solution and boiled for 5 minutes. The prehybridization solution was replaced with the boiled probe solution and incubated overnight at 65°C. This solution was then removed and the blot washed 3 times in 6× SSC for 10 minutes at 65°C, followed by a wash with 2× SSC/0.5% SDS for 10 minutes at 65°C. Bands were visualized by exposing the blot to x-ray film.

**Thyroid recovery and morphologic evaluation**

Transgene-positive and transgene-negative mice were humanely sacrificed by an overdose of anesthetic using a protocol approved by our animal use committee. The thyroids from these animals were then dissected and the tissue used in subsequent analyses. For histology, the thyroid glands were removed with part of the trachea still attached. For cryosections tissue was snap-frozen in OCT (Sakura, Finetek, USA, Inc., Torrance, CA) on dry ice, and stored at −70°C. For paraffin-embedded sections tissue was fixed in 10% buffered formalin (Fisher, Middletown, VA). Sections were cut to 5 μm and stained with hematoxylin and eosin (H&E) to examine thyroid structure. For analysis of RNA and proteins, the thyroid glands were separated from the trachea and removed.

**RNA isolation and RT-PCR**

Mouse thyroid tissue was added directly to TriZol (Invitrogen Life Technologies), then stored at −70°C. Excised thyroid tissue equaled approximately 1–3 mg in weight, so when necessary thyroids from DNA-positive or DNA-negative littermates were combined to provide adequate amounts of RNA for analysis. Small pieces of 7–12 mg of liver tissue or submandibular gland were also collected as control tissue and RNA was isolated in a similar manner. To obtain RNA, the tissue was thawed and homogenized using a micropestle, then RNA isolation was completed according to the vendor’s protocol. One microgram of RNA was used in a reverse transcription (RT) reaction with 12.5 ng/μL oligo (dT)₁₈ primer in 1× first strand buffer, 10 mM DTT, 0.5 mM dNTPs, 0.5 U/μL RNase inhibitor (Roche), and with or without 10 U/μL Superscript II reverse transcriptase (Gibco Life Technologies, Gaithersburg, MD) for 1 hour at 42°C. A duplicate reaction without RT was also performed as a negative control. Two microliters of each RT or no RT sample was then used in a PCR reaction with a primer that annealed to the 5’ end of the FLIP transcript (E8 forward) and the TGP4 primer (5’-TGGACG AGGATG AGACCG AG-3’) using the same reaction conditions used to screen tail DNA. Amplification of the β-actin (or β-globin) sequence was also performed as described above to confirm that the RT reactions had produced good cDNA for PCR. RT-PCR reaction products were visualized on a 1.5% agarose gel containing ethidium bromide.

**Protein isolation and Western blot**

Mouse tissue protein was isolated from TriZol homogenized tissue after extraction of RNA, using the manufacturer’s protocol (Invitrogen, Carlsbad, CA). Protein was suspended in 1% SDS and insoluble material pelleted at 13,000g for 10 minutes. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL) and at least 10 μg of each sample was separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrophoretic transfer to nitrocellulose membrane. The membrane was subsequently blocked in 5% milk in phosphate-buffered saline (PBS) with 0.05% Tween-20 (PBS-T). Protein bands corresponding to FLIP-myc/His were identified by Western blot using a mouse monoclonal antibody (mAb) to the c-myc peptide, clone 9E10 (Roche or BACo) at 2 μg/mL diluted in 5% milk/PBS-T followed by a peroxidase-conjugated anti-mouse immunoglobulin G (IgG), Fc-specific Ab (Jackson Laboratory) and detected using a chemiluminescent substrate, ECL-Plus (Amersham, Piscataway, NJ). Lysates from COS-1 cells stably transfected with the CMV-FLIP plasmid were used as a positive control for FLIP expression.

![Fig. 2](image-url)

**FIG. 2.** Expression of Tg-FLIP plasmid in the TNT T7 Quick Coupled transcription/translation system (Promega, Madison, WI). One microgram of Tg-FLIP, Tg-Fas, or CMV-FLIP were added to the TNT 17 quick reaction. After the transcription/translation was complete, a 3-μL aliquot of each reaction was separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The dried gel was exposed on an X-film at −70°C for 16 hours. The film then was developed.
Results

In vitro transcription and translation of Tg-FLIP

The Tg-FLIP plasmid was confirmed to have the correct sequence of the Tg promoter and insertion site in the vector with sequence analysis. In order to verify the Tg-FLIP construct further, the TNT-coupled transcription/translation system was applied according to manufacturer’s protocol (Promega, Madison, WI). Under in vitro transcription/translation conditions, the Tg-FLIP construct expressed the FLIP protein of the predicted size (22.8 kd), which was the same size produced by the CMV-FLIP (Fig. 2). It is obvious that CMV promoter is much more potent than the Tg promoter. As a negative control, the Tg-Fas construct did not express the same size band.

Screening of Tg-FLIP/H11003 CBA/J mice for transgene

A total of 81 mice were produced from the Tg-FLIP transgene microinjection. Of these, 12 Tg-FLIP–positive founder mice were identified (designated numbers 811, 813, 814, 826, 827, 828, 833, 837, 840, 842, 857, and 984). The founder mice were bred and crossed with the CBA/J strain to produce Tg-FLIP transgenic mice. Eleven of the 12 founder mice produced positive progeny, as demonstrated by PCR of tail DNA from litters of founders 826 and 857 (Fig. 3).

Confirmation of transgene insertion by Southern blot

Southern blots confirmed the presence of the Tg-FLIP transgene in 8 of the founder lines. The number of transgene copies detected in each founder mouse varied (Fig. 4). Four

![Screening of Tg-FLIP × CBA/J mice for transgene](image)

**FIG. 3.** Polymerase chain reaction (PCR) of mouse tail DNA for Tg-FLIP (Tg-E8) in litters using TGP1 and TGP4 primers. PCR amplification of two different founder lines is shown. An expected band of 1278 bp identified positive and negative littermates. Negative controls included a sample without DNA and a sample with DNA from a negative mouse. Diluted Tg-FLIP plasmid was used as a positive control.
founder lines contained a Southern fragment of 727 bp, which indicated the insertion of multiple gene copies into the genome in tandem (numbers 826, 833, 840, and 857). Of these, two lines appeared to contain significantly more copies of the Tg-FLIP transgene as demonstrated by the intensity of both the 1291bp and the 727-bp fragments (numbers 826 and 857).

Detection of mRNA for transgenic FLIP in the thyroids of Tg-FLIP mice

RT-PCR of RNA extracted from the mouse thyroids demonstrated message for FLIP-myc/His in Tg-FLIP–positive mice, but not in DNA-negative mice, nor in other tissues in the transgene positive mice. A PCR product of 538 bp was

FIG. 4. A Southern blot with DNA from the 12 Tg-FLIP founder mice probed with radiolabeled transgene sequence. The Tg-FLIP transgene is cut twice by SalI producing a major fragment of 1291 bp and two end fragments of 579 bp and 148 bp. A 1291-bp band was detected in 8 mice (*). A 727-bp band was also present in 4 mice indicating tandem insertion. The Tg-FLIP plasmid was included as a positive control and yielded expected bands of 2287, 1291, and 1031 bp.

FIG. 5. Reverse transcriptase-polymerase chain reaction (RT-PCR) products from mouse thyroid and liver tissues obtained from mouse lines containing relatively high, medium (A) and very low (B) transgene copy number. A negative control of no cDNA and a positive control of Tg-FLIP (Tg-E8) plasmid were included in each set. RNA treated with RT (+) and without RT (−) are shown for each sample. RNA from a transgene negative littermate is also shown (B).
amplified from RT reactions from thyroid RNA (Fig. 5). The same RNA from a RT reaction without enzyme did not produce a band, indicating that genomic DNA was not present or did not produce the band seen in these samples. FLIP message was clearly produced in thyroids from 8 out of the 12 lines, which were originally identified as positive for the transgene by PCR of tail DNA (numbers 811, 813, 826, 828, 833, 840, 842, and 857). Message was detectable in all mice confirmed as positive for DNA by Southern blot, even in the lines demonstrating very low copy number.

Detection of FLIP-Myc/His protein in thyroids of Tg-FLIP mice

Western blot analyses detected Myc-tagged FLIP protein in the thyroids of some of the Tg-FLIP mRNA-positive mice (Fig. 6). In contrast, FLIP-myc/his was uniformly not expressed in the liver tissues of these mice, nor in transgene-negative animals (Fig. 5). FLIP-Myc/His protein was detected in thyroid samples from four FLIP-positive mouse lines (numbers 811, 826, 842, and 857). The level of FLIP-Myc/His expression differed between the mouse lines. Furthermore, the level of protein expression for each line was correlated to the relative copy number of the transgene identified by Southern blot. Tg-FLIP line 857 demonstrated the greatest amount of FLIP protein by Western blot and also the heaviest Tg-FLIP DNA bands by Southern blot. However, some mouse lines that had fewer copies of Tg-FLIP DNA while still producing detectable amounts of mRNA failed to produce detectable amounts of FLIP-Myc/His protein in the thyroid (numbers 828 and 833), suggesting that a certain number of Tg-FLIP DNA copies is necessary to generate sufficient amounts of FLIP protein detectable by Western blot.

FLIP protein expression in the thyroid is not detrimental to the development of mice or their thyroid glands

We observed Tg-FLIP transgenic mice for more than a year, and during this period these animals remained apparently healthy. Adult mice were able to breed and

FIG. 6. Western blot of mouse tissue proteins detected FLIP-myc/his (E8-myc/his) using an anti-c-myc monoclonal antibody (mAb). Thyroid and liver tissues from several positive and one negative mouse from different founder lines are shown. The expected size of the FLIP-myc/his protein is 22.8 kd, shown clearly in the lysate from an FLIP-expressing COS-1 transfected (pFLIP) (pE8).
showed no obvious differences in appearance and activity compared with their control littermates. Histologic examination on H&E-stained sections of thyroids from transgenic animals obtained at various time points did not reveal any gross differences between transgenic mice and their gender- and age-matched control littermates (data not shown). In addition, no difference in thyroid morphology was observed.

Discussion

Our studies demonstrated that transgenic mice overexpressing FLIP in their thyrocytes had no thyroid or developmental abnormalities. The FLIP (E8) transgene has been previously transfected into multiple types of cells and documented to inhibit apoptosis induced by death receptors, such as CD95 and FLICE (14–6). In our study, FLIP expression on thyrocytes of transgenic mice was driven by the Tg promoter, which has been previously shown to specifically express target molecules in thyrocytes (18,19). Using Southern blotting techniques we have clearly shown that the FLIP transgene was present in the thyrocytes of 8 transgenic lines (of a total of 12 generated), and it was not found in any thyroids of control mice. The expression of FLIP in thyrocytes from these transgenic mice was further confirmed at RNA and protein levels, as demonstrated by RT-PCR and Western blot analysis, respectively. To confirm that the FLIP molecule under the control of Tg was specifically expressed in the thyroid, we screened for FLIP mRNA and protein in non-thyroid tissues. The result demonstrated that the FLIP transgene was only expressed in thyroid tissue, documenting the specificity of the Tg promoter. Therefore, we created transgenic animal in which FLIP protein was specifically produced in thyrocytes.

Although some of the mouse lines with low copies of the FLIP transgene failed to produce detectable FLIP protein by Western blot, the transcription of the FLIP transgene was still demonstrable with RT-PCR. The reason for this is not entirely clear, however, there are several possible explanations. One of them is that the detection of mRNA with RT-PCR is more sensitive than detecting protein with Western blots. Another possible explanation is that low levels of protein may be related to the insertion sites in the genome or trans elements that interfere with transcription and/or translation (20). This could also be the reason for the negative mRNA results in the 3 founder lines that produced transgene positive progeny as detected by PCR. Furthermore, it also is possible that post transcriptional modifications and/or mRNA degradation may also contribute to the low levels of FLIP protein below the threshold of Western blot in some of these animals.

Several studies have shown that FLIP can act not only as a tumor-progression factor (21,22), but also as a contributing factor to the development of autoimmune disease (23). For example, FLIP expression has been shown to correlate with resistance against death receptor-induced apoptosis in B-cell lymphomas, and FLIP-transfected tumor cell lines develop more aggressive tumors in vivo (21,22). Conversely, administering chemotherapeutic drugs to sensitized cells that are resistant to death receptor-induced apoptosis often correlates with decreased expression of FLIP (24). Several reports have also tied FLIP expression to thyroid diseases. Thyrocytes of Graves disease have been reported to be associated with an increased FLIP level, which makes thyrocytes resistant to Fas-mediated apoptosis. It has been suggested that Fas-FLIP signaling in thyrocytes may stimulate the proliferation of thyrocytes in Graves disease (23). However, there also have been reports showing that FLIP fails to function as an anti-apoptotic molecule in some cell death pathways (25–8). There is also a study indicating that binding of FLIP to FADD and caspase-8 is insufficient to block apoptosis induced by the death receptors, suggesting that other mechanisms may be required (26). In our transgenic mice, FLIP apparently exerts neither antiapoptotic nor proapoptotic effects sufficient to change the phenotype of these animals. Significant lymphocyte infiltration or other evidence of inflammation was also not observed.

This report is the first to describe the thyroid-specific expression of FLIP in a transgenic mouse model. Our results demonstrate that the presence of FLIP in thyrocytes does not appear to interfere with the development of thyroid and induce thyroiditis in an unchallenging environment. It would be interesting to know whether expression of FLIP in thyrocytes would contribute to the pathogenesis of thyroiditis following insults, such as infection, radiation, or chemical administration.

In conclusion, transgenic mice with thyroid-specific expression of FLIP do not show apparent alterations in the development and dysfunction of the thyroid. Although these results show that FLIP-producing thyrocytes lack the pathologic features such as malignancy or autoimmunity, additional factors may be necessary for them to appear. Future studies where interventions such as the administration of autoantigen (TSHR, Tg, thyroid peroxidase) through various immunization protocols will make this transgenic model useful for better understanding how death pathways influence thyroid diseases.

Acknowledgments

We gratefully acknowledge Dr. C. Vincenz for the viral FLIP in pCDNA3.1 (-)/Myc-HisA plasmid; Dr. T.J. Giordano for help with histologic identification; and Mary E. Van Antwerp for help with manuscript preparation.

This work was supported by the National Institutes of Health Grants R01 A137141, P60DK20572, and DK58771.

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