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Replacement of the *p16/CDKN2* Gene Suppresses Human Glioma Cell Growth[1]

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ABSTRACT

The *p16/CDKN2* gene has many features of a growth suppressor gene: it maps to 9p21, a frequent region of loss of heterozygozity in a variety of tumor types; it encodes an inhibitor of cyclin-dependent kinase 4; and its homozygous deletion is common in tumor-derived cell lines. However, the lower frequency of alteration of the gene in primary tumor tissue as compared to the cognate tumor cell lines has brought this interpretation into question. We have assessed the growth suppressive function of *p16/CDKN2* by gene transfer. The introduction of full-length p16/CDKN2 cDNA caused marked growth suppression in p16/CDKN2-null human glioma cells, but was without significant effect in those cells with endogenous wild-type *p16/CDKN2* alleles. These results provide functional evidence in support of the hypothesis that the *p16/CDKN2* gene is a functional growth suppressor gene, at least in gliomas.

INTRODUCTION

The clonal evolution of human tumors appears to derive from an accumulation of genetic alterations that are advantageous to growth (1). This hypothesis has been tested most rigorously in human colon cancer (2) and gliomas (3). In the latter tumor type, a series of events occurring in a relatively defined order have been identified and include loss of heterozygozity for chromosome 17p, mutation of the *p53* gene, deletion of chromosome 9p, amplification and alteration of the epidermal growth factor receptor gene, and monosomy of chromosome 10 (3). Among these lesions, deletions involving chromosome region 9p21 appear to be the most common structural abnormality observed in grade III and grade IV malignant gliomas, where they are detected in up to one half of primary tumor samples (3, 4) and up to two thirds of glioma cell lines (4, 5). Recently, a candidate tumor suppressor gene, *p16/CDKN2*, located between the *IFN-α* and *methylthioadenosine phosphorylase* loci in the 9p21 region has been reported (6–8).

$p16^{INK4}$ belongs to a newly described class of cyclin-dependent kinase inhibitory proteins that also includes $p15^{INK4B}$, $p21^{WAF1}$, and $p27^{KIP1}$ (6) and which may provide a connection between tumor suppression and cell cycle regulation. Four distinct lines of evidence promote the candidacy of the *p16/CDKN2* gene as a growth suppressor: (*a*) It maps to 9p21, a chromosome region frequently deleted in many tumor types including glioma, malignant melanoma, leukemia, osteosarcoma, and carcinomas of lung, kidney, ovary, pancreas, breast, bladder, head and neck, and esophagus (7–12). (*b*) Homozygous deletions and intragenic mutations of the *p16/CDKN2* gene itself are frequent in cell lines derived from these human tumors (7–14). (*c*) Mutations in the *p16/CDKN2* gene appear to underlie some cases of familial malignant melanoma (10, 14, 15). (*d*) The p16/CDKN2 product specifically inhibits cyclin-dependent kinase 4-cyclin D activity *in vitro* (16), suggesting that the $p16^{INK4}$ protein may be a negative regulator of proliferation (6, 16).

On the other hand, two observations have challenged this interpretation: (*a*) Although *p16/CDKN2* gene alterations are common in cultured cell lines, the frequency of homozygous deletions and mutations in primary uncultured tumors is much lower (12–14, 17, 18). (*b*) Transfer of p16/CDKN2 cDNA of the originally published sequence into human esophagus, lung, liver, ovary, and mesothelioma tumor cell lines had only a modest and variable growth inhibitory effect (19).

To specifically address the hypothesis that the *p16/CDKN2* gene is a growth suppressor in human glioma cells, we have transfected full-length p16/CDKN2 cDNA into cultured glioma cells having either deleted or wild-type endogenous *p16/CDKN2* alleles. The introduction of the *p16/CDKN2* gene led to a pronounced growth suppression in cells lacking the endogenous gene but had minimal effects on cells with wild-type alleles in support of the contention that the *p16/CDKN2* gene can function as a growth suppressor in human glioma.

MATERIALS AND METHODS

Cell Lines. The glioma cell lines used in this study were described previously (20, 21) and were derived from glioblastomas except LN-319 and U-373MG, which originated from astrocytomas, and D-247MG, which was derived from a gliosarcoma. CCD-43SK, a normal skin fibroblast cell line used, was obtained from the American Type Culture Collection (Bethesda, MD).

Reverse Transcriptase-PCR, Cloning, and Sequencing of *p16/CDKN2*. First-strand p16/CDKN2 cDNA synthesis was accomplished using a Stratascript RT-PCR kit (Stratagene, La Jolla, CA). Amplification of p16/CDKN2 full-length cDNA was performed as a two-step procedure. Two overlapping fragments were amplified using primers 5′-CGGAGAGGGGGAGAACAGACAACG-3′ (sense) and 5′-CCAGGAAGCCCTCCCGG-3′ (antisense) for the 5′ fragment, and primers 5′-CCCGCCACTCTCACCCG-3′ (sense) and 5′-ACCTTCGGTGACTGATGATCTAAG-3′ (antisense) for the 3′ fragment of the gene. Then, the isolated 5′ and 3′ fragments were mixed in equimolar amounts and a recombinant PCR reaction (22) was performed using primers 5′-CGGAGAGGGGGAGAACAGACAACG-3′ and 5′-ACCTTCGGTGACTGATGATCTAAG-3′ modified to contain *Bam*HI and *Xho*I sites, respectively. PCR conditions were 65°C for annealing and 72°C for extension, in the presence of 2% DMSO (American Type Culture Collection), for 35 cycles. p16/CDKN2 cDNA was subcloned into *Bam*HI/*Xho*I sites of the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA), and at least 10 individual clones per cell line were isolated, pooled, and sequenced using a Sequenase kit (USB, Cleveland, OH). A clone containing the wild-type p16/CDKN2 cDNA, derived from CCD-43SK fibroblasts, was designated as pCDKN2WT. This clone contained eight additional amino acids at its NH_2 terminus as compared to the originally reported sequence (16) but identical to recent revisions (19, 23). Moreover, codon 35 was GGG (glycine), consistent with many other reported sequences (11, 19, 24), but different from the original published clone (16) in which codon 35 was GTG (valine).

Nucleic Acid Isolation and Blot Analysis. Genomic DNA was extracted using standard procedures (25). Ten μg genomic DNA from each cell line were digested with *Eco*RI, separated by electrophoresis, blotted onto nylon membranes, and hybridized with a ^{32}P- labeled p16/CDKN2 cDNA probe covering nucleotides 25–960 (16). Genomic DNA integrity was determined by reprobing the Southern blots with the *ABL* gene, located on the chromosome 9q arm.

RNA was isolated using Trizol reagent (GIBCO-BRL, Gaithersburg, MD). A 5′ region p16/CDKN2-specific ^{32}P-labeled probe containing 172 base pairs (nucleotides −40 to 132) was generated by PCR using primers 5′-CG-

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GAGAGGGGGAGAACAGACAACG-3′ (sense) and 5′-GCCTCCGACCG-TAACTATTCGG-3′ (antisense). Twenty μg total cellular RNA were electrophoresed through a 1% denaturing agarose gel and blotted onto nylon membranes. Northern blot hybridization and washes were performed using Quickhyb solution (Stratagene, La Jolla, CA). RNA quality and blotting efficiency were confirmed by reversible methylene blue staining of the blot (26).

Western Blot Analysis. Cells (4×10^4 cells/well) in a 12-well plate were cultured overnight in medium containing 10% fetal bovine serum, washed with 1X PBS, lysed with 200 μl lysis buffer (0.064 M Tris-HCl pH 6.8, 1.28% SDS, 12.8% glycerol, 1.28% 2-mercaptoethanol, 0.25% bromophenol blue), and then boiled. Cell lysates were size fractionated through SDS-PAGE and transferred onto nitrocellulose membranes as described (27). Membranes were probed with anti-p16/CDKN2 rabbit polyclonal antibody (PharMingen, San Diego, CA) and proteins were detected by chemiluminescence (Amersham Corp., Arlington Heights, IL).

Transfection Assays. Transfections were performed using the calcium phosphate method (28) in duplicate dishes and in at least four independent experiments. Briefly, 20 μg of either pCDKN2WT or pcDNA3 were used per 10-cm dish. Transfections were terminated after 12 h. Forty-eight-h posttransfection, cells were split at a 1:5 dilution and maintained for 7 days in G418-containing (Geneticin; GIBCO-BRL) media. Trypan blue excluding cells were counted using a hemocytometer.

RESULTS

Selection of Recipients. In order to identify appropriate recipients for *p16/CDKN2* gene transfer, we analyzed the endogenous genes of a panel of 16 glioma lines; the data are summarized in Table 1. Southern blot analysis showed that 4 (25%) of 16 cell lines (LN-215, LN-235, LN-319, and LN-Z308) carried detectable *p16/CDKN2* genes, while the other 12 (75%) of 16 cases had homozygously deleted *p16/CDKN2* genes. To rule out intragenic alterations (such as small deletions/insertions or point mutations) that would be undetectable by Southern blot analysis, we sequenced the cDNAs derived from the four p16/CDKN2-positive glioma cell lines, and found that they were all identical to the wild-type sequence obtained for pCDKN2WT (see "Materials and Methods").

Western blot analysis of the p16/CDKN2 product was consistent with the Southern blot and sequencing results, with $p16^{INK4}$ protein being detected in the glioma lines LN-215, LN-235, LN-319, and LN-Z308. These observations are in concordance with other reports showing frequent (71–87%) *p16/CDKN2* homozygous deletions in glioma-derived cell lines (7, 9).

For transfection studies, we selected five glioma lines. Three (T98G, U-87MG, and U-251MG) had endogenously deleted *p16/CDKN2* genes, while two (LN-319 and LN-Z308) contained wild-type

Table 1 *p16/CDKN2 gene and $p16^{INK4}$ protein in a panel of human glioma cell lines*

Cell line	Southern blot	Western blot	cDNA
LN-215	+[a]	+	Wild-type
LN-235	+	+	Wild-type
LN-319	+	+	Wild-type
LN-Z308	+	+	Wild-type
A-172	–	–	N/A
D-247MG	–	–	N/A
LN-18	–	–	N/A
LN-229	–	–	N/A
LN-340	–	–	N/A
LN-382T	–	–	N/A
LN-428	–	–	N/A
LN-443	–	–	N/A
T98G	–	–	N/A
U-87MG	–	–	N/A
U-251MG	–	–	N/A
U-373MG	–	–	N/A

[a] +, present; –, altered or missing; N/A, not applicable.

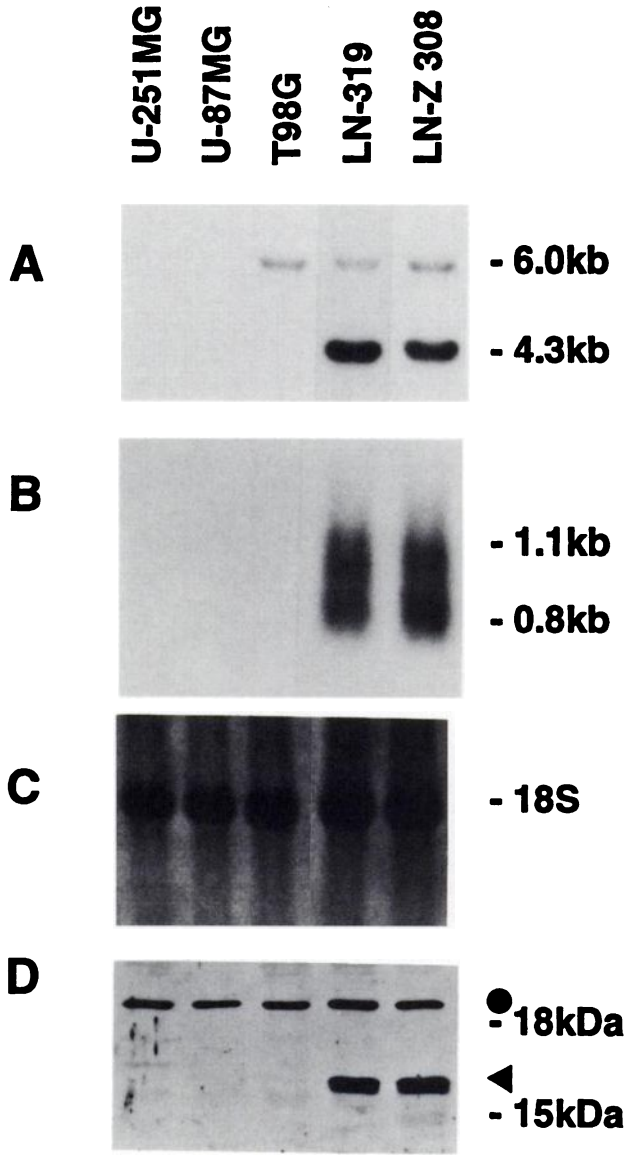

Fig. 1. Analysis of *p16/CDKN2* in human glioma cell lines. *A*, Southern blot analysis. DNA fragments were hybridized with a p16/CDKN2 probe (see "Materials and Methods"). The 4.3-kilobase band represents the *p16/CDKN2* gene, *right*. The 6.0-kilobase band may represent cross-hybridization with the $p15^{INK4B}$ (*MTS2*) gene. *B*, Northern blot analysis. Two p16/CDKN2 RNA transcripts of 0.8 and 1.1 kilobases, which hybridize with a p16/CDKN2-specific probe, *right*. *C*, methylene blue staining (26) of the nylon membrane blot in *B* to demonstrate transfer efficiency. Only 18S rRNA is shown. *D*, Western blot analysis. Size markers, *right*. ◀, $p16^{INK4}$ product; ●, nonspecific protein cross-reaction with the anti-$p16^{INK4}$ polyclonal antibody serves as an internal control.

p16/CDKN2 alleles. Southern blot analysis (Fig. 1*A*) showed a 4.3-kilobase band that represents the *p16/CDKN2* gene. A larger band of 6.0 kilobases was noted in the positive cell lines as well as in the T98G line and may represent cross-hybridization to the recently cloned $p15^{INK4B}$ gene (23). Southern blots reprobed with p*abl*K2, an *ABL* gene probe located on chromosome arm 9q, revealed bands of similar intensity in all five cell lines, confirming the integrity of the genomic DNA (data not shown). Two transcripts of 1.1 and 0.8 kilobases were apparent on the Northern blot of LN-319 and in LN-Z308, while no message could be detected in the *p16/CDKN2* gene null lines (Fig. 1*B*) although nucleic acid loading was equivalent (Fig. 1*C*). A 16–17-kDa protein was detected in LN-319 and LN-Z308, but not in the other three lines by Western blotting (Fig. 1*D*).

***p16/CDKN2* Gene Transfer.** To test the growth suppressive abilities of *p16/CDKN2*, we introduced a full-length cDNA into T98G,

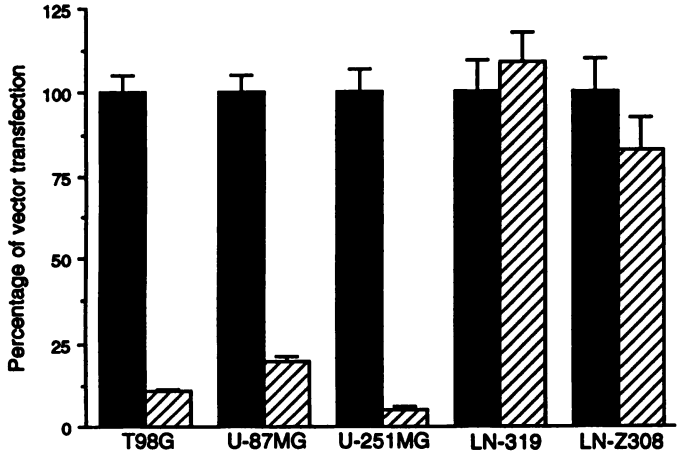

Fig. 2. Effects of *p16/CDKN2* gene introduction in human glioma cell growth. pCDKN2WT (▨) or vector alone (■) were transfected by the calcium phosphate method in duplicate 100-mm dishes. Viable cell counts were determined after G-418 selection. A typical experiment is shown. Results were reproduced in at least four independent experiments. The results are normalized in terms of percentage of vector transfection, setting the vector control to 100% in each case.

U-87MG, U-251MG, LN-319, and LN-Z308 cells and compared the resultant growth response to that when the cells were transfected with empty vector sequence. The results in Fig. 2 show that the cell number was reduced in the presence of the *p16/CDKN2* gene to (mean ± SE) 11.0% ± 0.4% of vector control for T98G, 19.6% ± 1.4% for U-87MG, and 5.2% ± 1.0% for U-251MG. On the other hand, for the wild-type p16/CDKN2 glioma lines, the observed percentages of vector transfection were 109.0% ± 8.6% for LN-319 and 82.4% ± 10.0% for LN-Z308. Therefore, in each of the p16/CDKN2-negative cell lines, replacement of this gene resulted in marked growth suppression, while in the p16/CDKN2 wild-type cell lines there was no significant growth inhibition by the introduction of the exogenous *p16/CDKN2* gene. Moreover, since these results were obtained with the bulk population of transfected cells, the possibility of clonal variation is minimized.

To confirm that the growth inhibitory effect was the consequence of the expression of the introduced *p16/CDKN2* gene, we analyzed transfectants of a null background glioma line (U-251MG) and a wild-type background glioma line (LN-319). Cells were harvested and these studies were performed 7–10 days posttransfection. Fig. 3*A* demonstrates expression of the transfected *p16/CDKN2* gene as the 0.95-kilobase transcript of the exogenous *p16/CDKN2* gene seen in U-251MG, which can be readily distinguished from the 0.8- and 1.1-kilobase endogenous transcripts present in LN-319. The level of RNA expression obtained from pCDKN2WT was roughly comparable to the level of endogenous RNA expression in the wild-type cells. Moreover, $p16^{INK4}$ protein expression was demonstrated by Western blot analysis of the transfected cells after selection. As expected, no $p16^{INK4}$ protein was detected in vector-transfected U-251MG cells. However, the levels of the $p16^{INK4}$ protein were indistinguishable among pCDKN2WT-transfected U-251MG, vector-transfected LN-319, or pCDKN2WT-transfected LN-319. The other glioma lines used for transfections revealed similar Northern and Western blot results (data not shown).

These data indicate that the growth suppression of p16/CDKN2-negative glioma cells transfected with pCDKN2WT was achieved at levels of protein expression very similar to that of the p16/CDKN2 present endogenously in wild-type glioma cells. Since the introduction of additional copies of *p16/CDKN2* into a wild-type background had negligible effects in $p16^{INK4}$ protein expression (despite a moderate increase in p16/CDKN2 RNA expression), it is possible that the endogenous *p16/CDKN2* gene may be subject to homeostatic autoregulation.

DISCUSSION

Genomic deletion mapping studies implicate a gene on chromosome 9p in the progression of astrocytic tumors (3). Thus far, it has been unclear whether the *p16/CDKN2* gene is the target of the 9p21 deletions seen in gliomas, or whether the actual target is another recessive tumor suppressor gene located nearby (17, 29). Here, we have shown that the expression of $p16^{INK4}$ (at levels comparable to those in glioma cells expressing endogenous *p16/CDKN2*) specifically suppresses the growth of p16/CDKN2-negative glioma cells. In contrast, p16/CDKN2 wild-type glioma cell growth was not affected by introduction of additional copies of *p16/CDKN2*. These results support the hypothesis that the *p16/CDKN2* gene is a functional suppressor of growth in glioma cells.

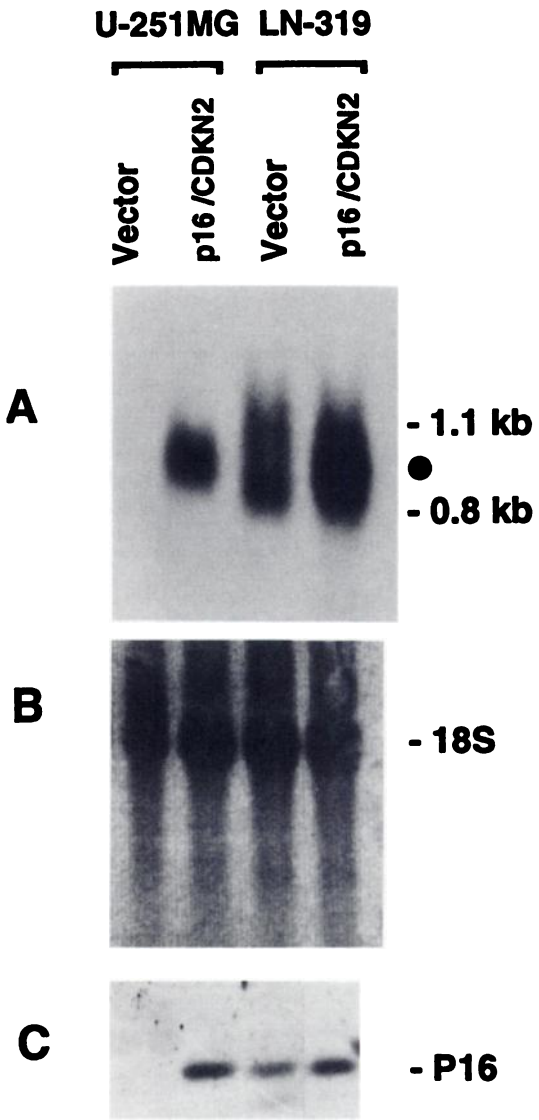

Fig. 3. Expression of endogenous and transfected *p16/CDKN2* gene in glioma cells. *A*, Northern blot analysis. The 0.8- and 1.1-kilobase p16/CDKN2 endogenous transcripts, *right*. ●, 0.95-kilobase size transcripts of the exogenous *p16/CDKN2* gene introduced by transfection. *B*, methylene blue staining (26) of the nylon membrane blot in *A* to demonstrate transfer efficiency. Only 18S rRNA is shown. *C*, Western blot analysis. $p16^{INK4}$ product, *right*. The other human glioma cell lines used in this study revealed similar Northern and Western blot patterns of p16/CDKN2 expression (data not shown).

Other investigators suggested that transfection of the *p16/CDKN2* gene inhibits growth and is selected against in the transfected cells, explaining the absence of detection of $p16^{INK4}$ in their expanded populations (19). Since we could clearly detect $p16^{INK4}$ protein expression by Western blot in our selected transfectants, this discrepancy could be attributed either to the transfection of an incomplete p16/CDKN2 cDNA (19, 23) or, perhaps, to cell-specific differences.

Four of the cell lines we analyzed expressed the wild-type *p16/CDKN2* gene. The mechanism or mechanisms by which these cells override $p16^{INK4}$ growth suppression remains unclear. One possibility is amplification or overexpression of targets such as cyclin-dependent kinase 4 (30, 31) or cyclin D. An alternate to this may be the inactivation of another growth inhibitory pathway, such as that involving p53-$p21^{WAF1}$. Since $p16^{INK4}$ is an inhibitor of the CDK4-cyclin D complex, while p53 is an activator of transcription of another cyclin-dependent kinase-inhibitory protein ($p21^{WAF1}$) (6), the expression of either wild-type *p16/CDKN2* or wild-type *p53* would predictably be growth suppressive and cells with inactivation of either one or both of these genes could be expected to have a survival advantage. We have characterized the *p53* gene in two of the four wild-type p16/CDKN2 glioma cell lines studied and found it mutated (LN-319) or rearranged (LN-Z308), consistent with this notion (21). Additionally, among the eight p16/CDKN2-negative glioma cell lines for which the *p53* gene has been characterized (21), six had inactivation of at least one allele of *p53* (LN-18, LN-229, LN-428, U-251MG, U-373MG, and T98G) and two had wild-type *p53* alleles (D-247MG and U-87MG). Thus, we could not identify a glioma line simultaneously containing intact *p53* and *p16/CDKN2* genes, supporting the hypothesis that the inactivation of at least one of these two genes is necessary for cell growth. Conversely, the inactivation of one of these two genes may not be sufficient to allow glioma cell growth. For example, a comparison of the growth of LN-319 and LN-Z308 to the growth inhibition effected in the p16/CDKN2-replaced T98G and U-251MG lines shows that although the genetic backgrounds in terms of these two genes in both groups appear to be similar (intact *p16/CDKN2* and mutated *p53*), their growth responses likely depend on additional factors. Nevertheless, the present results indicate that p16/CDKN2 growth inhibition is independent of the p53 status of the glioma cell lines used. Others have also suggested that the *p16/CDKN2* gene is often mutated or deleted in cell lines containing wild-type *p53*, and is often found intact in cell lines containing mutated *p53* genes (13, 14).

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