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In situ hybridization of nucleus basalis neurons shows increased β-amyloid mRNA in Alzheimer disease

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ABSTRACT **To determine which cells within the brain produce β-amyloid mRNA and to assess expression of the β-amyloid gene in Alzheimer disease, we analyzed brain tissue from Alzheimer and control patients by *in situ* hybridization. Our results demonstrate that β-amyloid mRNA is produced by neurons in the nucleus basalis of Meynert and cerebral cortex and that nucleus basalis perikarya from Alzheimer patients consistently hybridize more β-amyloid probe than those from controls. These observations support the hypothesis that increased expression of the β-amyloid gene plays an important role in the deposition of amyloid in the brains of patients with Alzheimer disease.**

Alzheimer disease (AD) is a progressive neurodegenerative disease that is the most common cause of intellectual failure in the elderly (1). In AD, clusters of degenerating neurites called senile or neuritic plaques are found throughout the cerebral cortex, and the severity of dementia correlates well with the number of plaques present (2). Senile plaques frequently contain an amyloid core composed of 5- to 10-nm filaments that stain metachromatically with Congo red. In most cases, amyloid filaments are also found in the walls of cerebral vessels (3, 4). Individuals with Down syndrome (trisomy 21) who are over the age of 40 invariably develop central nervous system pathology essentially identical to that seen in AD (5, 6).

A 4.2-kDa polypeptide, referred to as the amyloid β protein or A4, has been isolated from the amyloid in plaque cores and in vessels of AD and Down syndrome brains. Sequencing of the β proteins isolated from plaque cores and vessels has shown that they have an essentially identical 28-amino acid sequence (7, 8) not found in other amyloid proteins (9). Using oligonucleotides based on this sequence, several groups (10–13) have isolated cDNA clones that encode A4 as part of a 695-residue β-amyloid precursor protein (11). With these clones, the gene encoding β-amyloid has been mapped to the 21q11.2–21q21 region of chromosome 21 (10–13). RNA gel blot analyses indicate that the β-amyloid gene is expressed in a wide variety of tissues. Goldgaber *et al.* (10) detected a 3.5-kilobase (kb) mRNA in mouse, rat, and bovine brain, and in human thymus. Kang *et al.* (11) detected 3.4-kb and 3.2-kb mRNAs in human embryonic cerebral cortex. Tanzi *et al.* (12) identified a 3.7-kb mRNA in each of the many human fetal tissues and control adult human brain regions that they examined. This group also examined AD cerebellum and frontal cortex and found the same 3.7-kb mRNA. Bahmanyar *et al.* (14) analyzed β-amyloid mRNA in monkey and human hippocampus and prefrontal cortex by *in situ* hybridization. In both monkey and human tissue, β-amyloid mRNA was present in a subset of prefrontal neurons and in neurons from all hippocampal fields. The distribution of β-amyloid mRNA observed in this study bore some resemblance to the distribution of neurofibrillary tangles in AD, but β-amyloid mRNA was also expressed in neurons that don't normally form tangles.

St. George-Hyslop *et al.* (15) have performed a genetic linkage analysis on four pedigrees showing the familial form of AD, a variant that displays an autosomal-dominant pattern of inheritance. Their analysis indicates that two markers close to the β-amyloid locus, D21S16 and D21S1/D21S11, are linked to the genetic defect in familial AD. Delabar *et al.* (16) have analyzed leukocyte DNA from patients with "sporadic" AD. They report that a segment of chromosome 21 bearing the β-amyloid and *ETS2* genes is duplicated (three copies per diploid genome, rather than two) in each of three AD patients and none of seven controls. Duplication of a segment of chromosome 21 in AD patients but not in controls has also been reported by Schweber and Tuscon (17). To determine which cells within the brain produce β-amyloid mRNA and to evaluate the question of increased expression of the β-amyloid gene in AD, we analyzed β-amyloid mRNA by *in situ* hybridization (18–22).

MATERIALS AND METHODS

Tissue blocks containing the nucleus basalis of Meynert or Brodmann area 21 cerebral cortex were dissected from a series of fresh AD and control brains. These blocks were rapidly frozen by immersion in isopentane cooled with liquid nitrogen and stored at −70°C.

McGeer *et al.* (23) have shown, and we have confirmed (unpublished observations), that nucleus basalis neurons with diameters >20 μm are cholinergic as assessed immunocytochemically for choline acetyltransferase. Whitehouse *et al.* (24) have shown, and many laboratories including our own (25) have confirmed, that in AD there is a marked loss of nucleus basalis neurons with diameters >20 μm. In the present study, we analyzed nucleus basalis neurons with diameters >20 μm that were clustered into a readily identifiable nucleus located between the lateral anterior commissure and medial optic tract. All seven of the AD cases evaluated in this study showed the expected reduction in cholinergic neurons (25), and all had neuritic plaques throughout the cerebral cortex in numbers well above those required for a diagnosis of AD.

Oligonucleotide probes were synthesized as described by Caruthers *et al.* (26). We prepared a probe for β-tubulin mRNA [bases 1190–1213 of the human DB1 sequence from Hall *et al.* (27)], a probe for β-amyloid mRNA [bases 1885–1908 in the A4 region of the sequence from Kang *et al.* (11)], and an arbitrary sequence noncomplementary probe. These probes, which were 24 bases long and had G+C contents of 62–67%, were labeled with ^{125}I-labeled dCTP to specific activities of $>10^8$ cpm/μg using terminal deoxynucleotidyltransferase. RNA gel blot analysis of poly(A)$^+$ RNA from rat brain, liver, kidney, and human basal ganglia

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Abbreviation: AD, Alzheimer disease.

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with our probe for β-amyloid mRNA showed single bands of ≈3.7 kb similar to those illustrated by Tanzi *et al.* (12).

Thaw-mounted 12-μm cryostat sections cut from flash frozen blocks were fixed 5 min in freshly prepared 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate (pH 7), treated 10 min with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8), delipidated by passage through an ethanol series of increasing concentrations [70% (vol/vol), 1 min; 80% (vol/vol), 1 min; 95% (vol/vol), 2 min; 100% (vol/vol), 1 min] and through chloroform (5 min), rehydrated [100% (vol/vol) ethanol, 1 min; 95% (vol/vol), 2 min] to ensure optimal probe exposure, and hybridized overnight at 50°C with 20 μl of hybridization solution [1× SSC (1× SSC is 0.15 M NaCl/0.015 M trisodium citrate, pH 7.0), salmon sperm DNA at 1 mg/ml, tRNA at 1 mg/ml, 10% (wt/vol) polyethylene glycol, and 1–3 nM probe] under baked glass coverslips. Coverslips were removed in 0.2× SSC, and unbound probe was removed by washing four times for 15 min in 0.2× SSC at 37°C and twice for 60 min in 0.2× SSC at room temperature. After two brief rinses in water, sections were air-dried, dipped in Kodak NTB-2 emulsion, exposed for 4–6 days, developed for 2 min with Kodak D-19, rinsed in water, and fixed in Kodak Rapid Fix for 1.5 min. After copious rinsing in distilled water, sections were stained with 0.4% cresyl violet in water for ≈30 sec, dehydrated, and mounted.

To quantitate the hybridization of the β-amyloid or β-tubulin probes to mRNA within nucleus basalis perikarya, we identified each perikaryon at ×1000 magnification, superimposed a 243-μm^2 grid within the perikaryon, and counted the grains within the grid. In each tissue block, the average grain count per perikaryon following hybridization with the three probes was obtained by analyzing 50–60 neurons. Evaluating this number of cells reduced the SEM of the average grain count per perikaryon to 5–11% of the mean for the β-amyloid and β-tubulin probes and to 9–16% of the mean for the noncomplementary probe. Grain counts are expressed per 600 μm^2 [the average area of a cholinergic perikaryon in our Nissl-stained material (25)].

RESULTS

We hybridized sections from each tissue block with an oligonucleotide probe for β-amyloid mRNA, an oligonucleotide probe for β-tubulin mRNA that served as a positive control, and an arbitrary sequence noncomplementary oligonucleotide that served as a control for nonspecific binding. In each tissue block analyzed, the three probes were applied to three adjacent serial sections.

The results of trial hybridizations to sections from the nucleus basalis of Meynert and Brodmann area 21 cerebral cortex of controls are illustrated in Fig. 1. After hybridization with the β-tubulin probe, many grains were clustered over neuronal perikarya in both the cerebral cortex (Fig. 1*A*) and nucleus basalis (Fig. 1*B*) as expected given the substantial amount of β-tubulin produced by these cells. Hybridization with the noncomplementary probe produced very few grains above the emulsion background in either the cerebral cortex (Fig. 1*C*) or nucleus basalis (Fig. 1*D*). Hybridization with the β-amyloid probe labeled large neuronal perikarya in both the cerebral cortex (Fig. 1*E*) and nucleus basalis (Fig. 1*F*). Grain counts over glia and endothelial cells in nucleus basalis sections showed that these cells were specifically labeled by the β-tubulin probe, but the β-amyloid probe produced no specific grains (number of grains with β-amyloid probe − number of grains with noncomplementary probe) over either of these cell types.

To evaluate the effect of AD on β-tubulin and β-amyloid mRNA, we performed experiments in which nucleus basalis sections from AD and control patients were processed side-by-side. In our initial experiment, we compared two AD patients with two controls. In this experiment, hybridization with the β-tubulin and noncomplementary probes gave results similar to those observed in our trial experimentation. In both control and AD patients, the β-tubulin probe (Fig. 2 *A* and *B*) reliably labeled the large cholinergic perikarya known to degenerate in AD (24), and hybridization with the noncomplementary probe produced very few grains above the emulsion background (Fig. 2 *C* and *D*). Hybridization with the β-amyloid probe labeled nucleus basalis perikarya in both the AD and control patients, but in the two AD

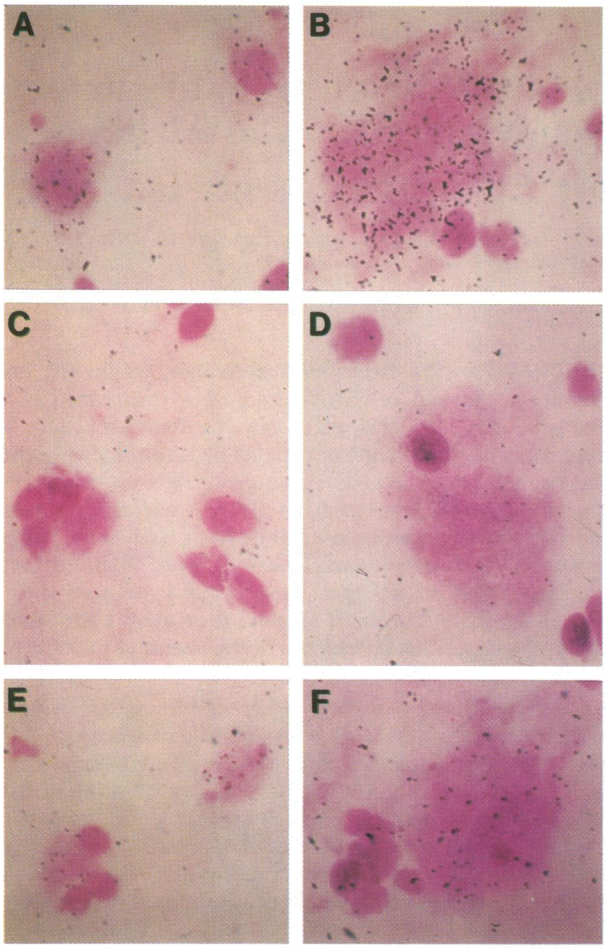

FIG. 1. *In situ* hybridization of human cerebral cortex and nucleus basalis of Meynert with ^{125}I-labeled synthetic oligonucleotides. (*A*) Cerebral cortex hybridized with the β-tubulin probe at 3.1 × 10^8 cpm/μg. (*B*) Nucleus basalis hybridized with the β-tubulin probe. (*C*) Cerebral cortex hybridized with the arbitrary sequence noncomplementary probe at 1.2 × 10^8 cpm/μg. (*D*) Nucleus basalis hybridized with the noncomplementary probe. (*E*) Cerebral cortex hybridized with the β-amyloid probe at 2.6 × 10^8 cpm/μg. (*F*) Nucleus basalis hybridized with the β-amyloid probe. The nucleus basalis neurons in *B*, *D*, and *F* are considerably larger than the cerebral cortical neurons in *A*, *C*, and *E*. In *A* and *E*, two cortical perikarya are labeled; the perikaryon in the lower left of *E* is surrounded by three satellite oligodendroglia. In *F* the cytoplasm, nucleus, and nucleolus of the large nucleus basalis perikaryon that is labeled are clearly visible. The large cluster of grains in the center of *B* is located over a single large perikaryon that stained less intensely than those in *D* and *E*. The concentration of the three probes was 1 nM for the hybridizations to cerebral cortex and 3 nM for the hybridizations to nucleus basalis. Autoradiographic exposure time was 4 days for cerebral cortex and 6 days for nucleus basalis. The cerebral cortex was taken from a 78-year-old male free of neurological disease in whom the postmortem interval was 5 hr. The nucleus basalis was taken from a 51-year-old male free of neurological disease in whom the postmortem interval was 9 hr. (Stain, 0.4% cresyl violet in water; ×600.)

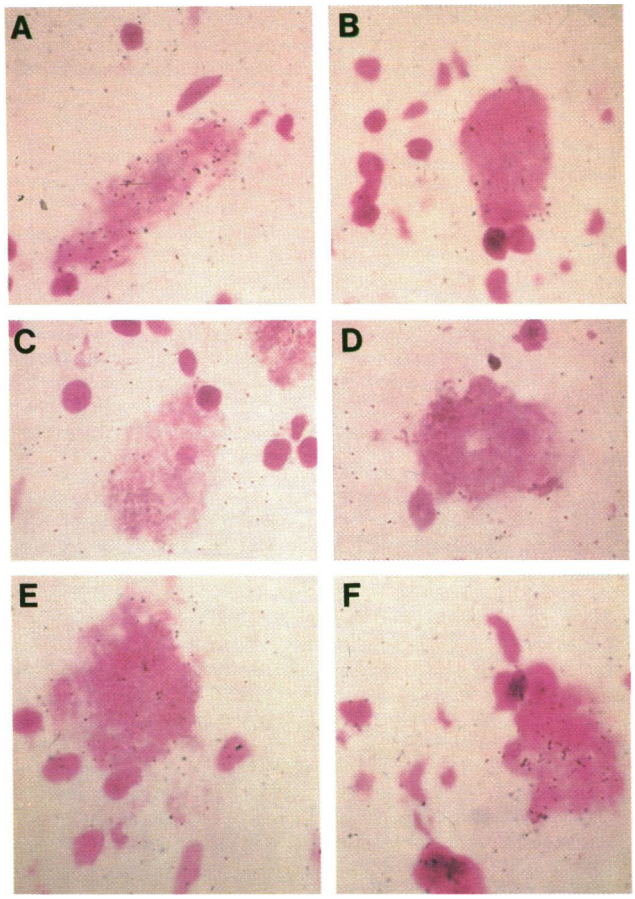

FIG. 2. *In situ* hybridization of nucleus basalis perikarya in human nucleus basalis of Meynert with ^{125}I-labeled synthetic oligonucleotides. (*A*) Control, hybridized with β-tubulin probe. (*B*) AD, hybridized with β-tubulin probe. (*C*) Control, hybridized with noncomplementary probe. (*D*) AD, hybridized with noncomplementary probe. (*E*) Control, hybridized with β-amyloid probe. (*F*) AD, hybridized with β-amyloid probe. Lipofuscin granules are present at the left edge of the soma shown in *D* and in the upper part of the soma illustrated in *E*. Hybridizations were performed with 1 nM probe, and the autoradiographic exposure time was 4 days. Counts of 100 soma on two sections exposed to noncomplementary probe showed an average of 14.1 grains per 600 μm² of soma. A control section processed in parallel that was not exposed to radiolabeled probe showed 9.6 grains per 600 μm². Thus relatively few of the grains observed were due to nonspecific binding of the radiolabeled probes employed. The mean age of the AD and control patients was 80 years, and the mean postmortem intervals were 4 and 8 hr, respectively. (Stain, 0.4% cresyl violet in water; ×600.)

patients the β-amyloid probe produced more grains over nucleus basalis perikarya (Fig. 2*F*) than it did in either of the controls (Fig. 2*E*).

In a second experiment, we compared five AD and five control patients well-matched for age (AD, 77.6 ± 2.0 years; control, 74.8 ± 4.2 years) and postmortem interval (AD, 9.6 ± 3.2 hr; control, 9.1 ± 1.8 hr). The number of specific grains (number of grains with probe for mRNA − number of grains with noncomplementary probe) over cholinergic nucleus basalis perikarya are expressed per 600 μm² [the average area of a cholinergic perikaryon in our Nissl-stained material (25)] (Table 1). After hybridization with the β-amyloid probe, the mean number of specific grains over nucleus basalis perikarya in each of the AD patients was greater than in any of the controls. Overall, the mean number of specific "β-amyloid grains" over nucleus basalis perikarya in the five AD patients (18.1 ± 5.3 grains) was 3.8 times that observed in the five controls (4.7 ± 0.2 grains) ($P < 0.01$). After hybridization with the β-tubulin probe, the mean number of specific grains over nucleus basalis perikarya in the AD patients (22.5 ± 1.5 grains) was 33% less than that in the controls (33.5 ± 2.3 grains) ($P < 0.05$).

Table 1. Grains localized over nucleus basalis perikarya after *in situ* hybridization with ^{125}I-labeled synthetic oligonucleotides

	Grains, no. per 600 μm² of soma			
	β-Amyloid probe		β-Tubulin probe	
Patient	AD	Control	AD	Control
1	11.9 ± 2.0	5.3 ± 1.4	27.0 ± 2.6	23.5 ± 2.5
2	6.4 ± 1.6	4.6 ± 1.4	23.3 ± 2.6	32.7 ± 2.5
3	24.5 ± 1.9	5.0 ± 1.1	26.0 ± 2.1	38.3 ± 2.5
4	9.3 ± 1.2	5.0 ± 1.8	17.2 ± 1.9	31.5 ± 2.6
5	38.5 ± 6.1	3.5 ± 1.1	18.8 ± 1.7	41.3 ± 2.9
Mean	18.1 ± 5.3*	4.7 ± 0.2	22.5 ± 1.5**	33.5 ± 2.3

Hybridizations were performed with 1 nM probe, and the autoradiographic exposure time was 5 days. In each case, the average grain count per perikaryon following hybridization with the β-amyloid, β-tubulin, and noncomplementary probes was obtained by analyzing 50–60 neurons. The values shown are the mean ± SEM for the specific grains over nucleus basalis perikarya (number of grains with probe for mRNA − number of grains with noncomplementary probe). AD and control patients were well-matched for age (AD, 77.6 ± 2.0 years; control, 74.8 ± 4.2 years) and postmortem interval (AD, 9.6 ± 3.2 hr; control, 9.1 ± 1.8 hr).
*$P < 0.01$, **$P < 0.05$ by Mann–Whitney test for AD versus control.

Fig. 3 shows frequency histograms of the nucleus basalis somata over which specified numbers of grains were produced by hybridization with the β-tubulin, noncomplementary, and β-amyloid probes (composite data from the five AD and five control patients in our second experiment are depicted). In AD, nucleus basalis neurons hybridized reduced amounts of β-tubulin probe as illustrated by the shift in the β-tubulin histogram to the left and increased amounts of β-amyloid probe as illustrated by the shift in the β-amyloid histogram to the right. Following hybridization with the β-amyloid probe, >50% of the nucleus basalis perikarya in the AD patients had 16 grains or more, whereas <10% of the perikarya in control patients showed this number of grains.

DISCUSSION

RNA gel blot analysis of RNA from normal and Down syndrome (trisomy 21) fetal brain by Tanzi *et al.* (12) showed an increase in the β-amyloid transcript in Down syndrome consistent with a 50% increase in gene dosage. Similar analysis of RNA from an AD and a control brain showed equal expression in AD and control cerebellum and less β-amyloid transcript in AD as compared to control frontal cortex (12). RNA gel blot analyses are important in evaluating AD, but they provide no information on the cells producing particular mRNAs and are, therefore, difficult to interpret in AD brain where neurons are lost and where there is an accompanying proliferation of astrocytes. We, therefore, examined β-amyloid mRNA in AD by *in situ* hybridization.

Our study indicates that β-amyloid mRNA is produced by neurons in the nucleus basalis and Brodmann area 21 cerebral cortex and is in good agreement with the study of Bahmanyar *et al.* (14) who found expression of β-amyloid mRNA in hippocampal pyramidal neurons and neurons of the prefontal cortex. We have, so far, been unable to demonstrate β-amyloid mRNA in glia or capillary endothelial cells, but additional studies with higher-specific-activity probe, longer exposure times, or both are necessary before concluding that these cells do not express the β-amyloid gene.

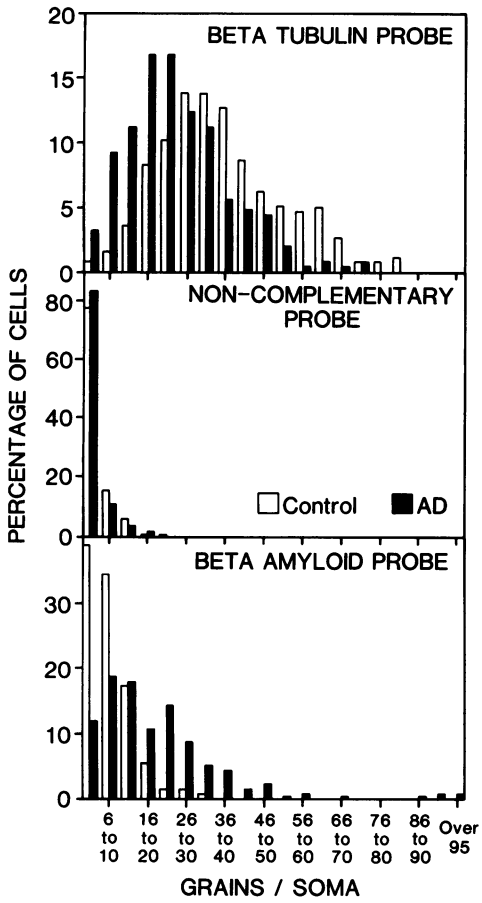

FIG. 3. Grains localized over cholinergic perikarya in human nucleus basalis after *in situ* hybridization with ^{125}I-labeled synthetic oligonucleotides. Histograms show composite data from an experiment in which sections from five AD and five control patients were hybridized with β-tubulin (*Top*), noncomplementary (*Middle*), and β-amyloid (*Bottom*) probes and were processed in parallel. Bars show the percentage of perikarya over which the indicated number of grains were present. Each AD and control histogram is based on grain counts of >250 perikarya. Counts of 500 soma on 10 sections exposed to radiolabeled noncomplementary probe showed an average of 3.5 grains per 600 μm^2 of soma. A control slide processed in parallel but not exposed to radiolabeled probe showed 2.9 grains per 600 μm^2. Thus very few of the grains observed were due to nonspecific binding of the radiolabeled probes employed. Mean values for specific binding in each case and additional information on this experiment are shown in Table 1.

Studies, such as those by Cox *et al.* (20), Lawrence and Singer (21), and Young *et al.* (22), indicate that *in situ* hybridization reflects the cellular level of the mRNA probed. In our experiments, nucleus basalis perikarya from AD patients consistently hybridized more β-amyloid probe than those from controls and, on average, hybridized less β-tubulin probe. This observation of increased β-amyloid mRNA in a neuronal population degenerating in AD suggests that increased expression of the β-amyloid gene may play an important role in the deposition of amyloid in the brains of patients with Alzheimer disease.

In preliminary analysis of cerebral cortical neurons in AD, we have not seen the marked increase in β-amyloid hybridization observed in nucleus basalis neurons. This suggests that the severity of neuronal degeneration, which is greater in nucleus basalis than in cerebral cortex, may be correlated with the increase in expression of the β-amyloid gene.

Note Added in Proof. Three reports have been published (28–30) in which large numbers of familial and sporadic AD patients were carefully analyzed. In these studies, no AD patients were found in which the β-amyloid gene was duplicated. On the basis of these findings, we conclude that the increased expression of the β-amyloid gene observed in our study cannot be attributed to increased gene dosage.

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