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Peroxisome Assembly Factor 1: Nonsense Mutation in a Peroxisome-Deficient Chinese Hamster Ovary Cell Mutant and Deletion Analysis

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A cDNA encoding 35-kDa peroxisome assembly factor 1 (PAF-1), a peroxisomal integral membrane protein, was cloned from Chinese hamster ovary (CHO) cells and sequenced. The CHO PAF-1 comprised 304 amino acids, one residue shorter than rat or human PAF-1, and showed high homology to rat and human PAF-1: 90 and 86% at the nucleotide sequence level and 92 and 90% in amino acid sequence, respectively. PAF-1 from these three species contains a conserved cysteine-rich sequence at the C-terminal region which is exactly the same as that of a novel cysteine-rich RING finger motif family. PAF-1 cDNA from a peroxisome-deficient CHO cell mutant, Z65 (T. Tsukamoto, S. Yokota, and Y. Fujiki, J. Cell Biol. 110:651–660, 1990), contained a nonsense mutation at the codon for Trp-114, resulting in premature termination. Truncation in PAF-1 of either 19 amino acids from the N terminus or 92 residues from the C terminus maintained the peroxisome assembly-restoring activity when tested in both the Z65 mutant and the fibroblasts from a Zellweger patient. In contrast, deletion of 27 or 102 residues from the N or C terminus eliminated the activity. PAF-1 is encoded by free polysomal RNA, consistent with a general rule for biogenesis of peroxisomal proteins, including membrane polypeptides, implying the posttranslational transport and integration of PAF-1 into peroxisomal membrane.

Two different aspects of peroxisomes have been extensively studied in recent years. First, the peroxisome may be used as a model organelle to investigate the mechanism of protein translocation and organelle assembly. Topogenic signals specific to peroxisomal transport have been identified, both in vivo and in vitro, by several groups of investigators, including our own (for a review, see reference 24). One type of signal is the -Ser-Lys-Leu-COOH (SKL) motif located at the C termini of a dozen peroxisomal enzymes (9, 10, 22, 23), and another consists of the N-terminal extension peptides of 3-ketoacyl-coenzyme A thiolase (25, 31). Second, human peroxisomal disorders are of clinical consequence and are considered to be excellent model systems for the study of biogenesis and the physiological significance of peroxisomes. Among such autosomal recessive disorders, peroxisome deficiency cerebrohepatorenal Zellweger syndrome, a prototype, is the most severe disease, with patients having an average life span of 6 months (18). Several lines of evidence are consistent with the notion that all peroxisomal proteins are synthesized normally but that the assembly of peroxisomes is impaired in patients with such disorders (37). Four to nine complementation groups have been reported in generalized peroxisome deficiency disorders, implying that several genes are essential for peroxisome biogenesis (2, 27, 30, 38). Somatic animal cell mutants defective in the biogenesis of peroxisomes have been isolated and shown to be useful for the search for genes responsible for human peroxisomal disorders such as Zellweger syndrome (29, 34, 40). By transfection of a cDNA library to cell mutants and then genetic functional complementation analysis, we isolated a cDNA encoding 35-kDa peroxisome assembly factor 1 (PAF-1), comprising 305 amino acids, which restores assembly of peroxisomes and complements the anomalies in one of the peroxisome-deficient Chinese hamster ovary (CHO) cell mutants, Z65 (33). Furthermore, recently delineated was the primary defect in a peroxisome-deficient patient with Zellweger syndrome who belonged to the same complementation group as Z65 (30). In order to search for genes involved in formation of peroxisomes, yeast mutants defective in transport of peroxisomal polypeptides have also been isolated (3, 4, 11, 19, 36).

In this report, as a step toward understanding the structure and function relationship of PAF-1, we have isolated a cDNA for CHO PAF-1 and compared its sequence with those of rat and human PAF-1. PAF-1 is revealed to be highly conserved among these three species and a member of a novel cysteine-rich sequence, RING finger motif family (20). PAF-1 sequence of the mutant Z65 contained a nonsense mutation at the codon for Trp-114 resulting in the premature termination of PAF-1 that affected the assembly of peroxisomes in Z65. Moreover, deletion analysis of PAF-1 suggested that the N-terminal part of PAF-1 is essential to the function and that the ~90 residues at the C-terminal region can be truncated.

MATERIALS AND METHODS

Materials. Oligonucleotides used are AAAGACAGCAT CAGAGAAGATATG (primer H1, nucleotides [nt] −21 to 3 of rat PAF-1), ACAGGATCCGAGAAGATATG (primer H2, nt −17 to 3) (rat), CGAGGATCCATGGCTG GCAGA (primer H3, nt −9 to 12) (rat/CHO), GTATGCT GTGTGCACCATTG (primer H4, nt 312 to 331 of CHO PAF-1), CAACTTGGTTTCTAAAGAGCATT (primer T1, nt 929 to 907) (rat), CTTGGATCCTAAAGAGCATT (primer T2, nt 926 to 907) (rat), GACAGAATTCATCCTCCCTCAG AGGAAACA (primer T3, nt 954 to 925) (CHO), AAGAT

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GCTAAATGACGGTTTC (primer T4, nt 391 to 371) (CHO), TTCCTCTAACCATCTACC (probe WD, nt 351 to 334 of CHO PAF-1), and TTCCTCTAATCATCTACC (probe MT, nt 351 to 334 of Z65 PAF-1), where the initiation codon ATG (primers H1, H2, and H3) or a termination codon TAG (primers T1 and T2, in complementary orientation) is underlined. Oligonucleotides H2, H3, T2, and T3 contain site mutations (asterisks above mutations) to create restriction sites. The oligonucleotides were synthesized on a DNA synthesizer (Applied Biosystems model 380A) by cyanoethyl phosphoramidite chemistry. A cDNA library of CHO cells in a pcD vector was a generous gift from M. Nishijima and O. Kuge.

Isolation of rat and CHO PAF-1 cDNAs. A rat liver cDNA library in pcD2 vector (33) and a cDNA library of CHO cells constructed in the pcD vector were screened by colony hybridization with a [^{32}P]*Pvu*II-*Eco*RI fragment of rat PAF-1 cDNA (33) as a probe. Positive clones were excised with *Bam*HI; cDNA was subcloned into pUC18 or Bluescript SK(−) (Stratagene). The nucleotide sequence was determined by the method of Sanger et al. (28). From a rat liver cDNA library, at least four independent PAF-1 cDNA clones, J, K, T, and U, were isolated. Three full-length clones, K, T, and U, restored assembly of peroxisomes in a peroxisome-deficient CHO mutant, Z65, as previously noted, with a PAF-1 cDNA clone D (33). Nucleotide sequencing revealed that clone K was exactly the same as clone D (33) and that clones T and U contained a 110-bp-long additional sequence with an in-frame stop codon and a *Bam*HI site between nucleotide residues −18 and −17 of clone K (33). It is noteworthy that human genomic clones for PAF-1 contain an intron at the same position (28a). In this report, a *Bam*HI-*Eco*RI fragment of clone T was used for deletion analysis.

RNA analysis. Total RNA and free and membrane-bound polysomal RNAs were isolated from rat liver, as previously described (26). Isolation of RNA from CHO cells and Northern (RNA) blot analysis were performed as described elsewhere (30).

PCR amplification (reverse transcription-PCR). In experiment 1, cDNA was synthesized from poly(A)$^+$ RNA of CHO cells with an oligo(dT) primer, by PCR with primers H1 and T1 and *Taq* polymerase (Boehringer-Mannheim) in a buffer recommended by the manufacturer. A faint band of ~900 bp was further amplified by the second PCR with H2 and T2 primers and then subcloned into Bluescript SK(−).

In experiment 2, the first strand of cDNA was synthesized at 37°C for 60 min with 4 μg of total RNA from CHO cells, 0.1 pmol of antisense primer T3, 0.67 mM deoxynucleoside triphosphates (dNTPs), and 600 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) in 30 μl of a buffered solution supplied by the manufacturer. A 1/15 aliquot of the cDNA synthesized was used for PCR amplification with 1 U of Vent DNA polymerase (New England Biolabs), using 20 μM dNTPs and 10 pmol (each) of primers H3 and T3 in the supplied buffer. The amplified fragment was digested with *Bam*HI-*Eco*RI and subcloned into a vector, pcD2MCS, that had been constructed as follows. *Bam*HI, *Cla*I, and *Sal*I sites of vector pcDV1 and two *Bam*HI sites of vector pL2 were filled in with Klenow enzyme. Those ligated were the *Kpn*I-*Hin*dIII fragment of pcDV1 containing the *amp* gene, the *Hin*dIII-*Cla*I (filled in with Klenow) fragment containing the *neo* gene, and the *Kpn*I-*Sac*I (blunted with T4 DNA polymerase) fragment of a multicloning site derived from Bluescript SK(−).

Southern blot analysis. Genomic DNA was prepared from normal rat liver as previously described (21) and digested with several restriction enzymes. The digests were separated on a 1% agarose gel and transferred onto a Zeta-Probe membrane (Bio-Rad) which was then prehybridized for 4 h at 65°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate (SDS)–5× Denhardt's solution containing 0.5 μg of salmon sperm DNA per ml. Hybridization was done at 65°C for 16 h with a rat PAF-1 cDNA *Pvu*II-*Eco*RI fragment labelled with [^{32}P]dCTP by using a random primer DNA labelling kit (Takara Shuzo, Kyoto, Japan); washing was done twice at room temperature and once at 65°C with 2× SSC–0.1% SDS and twice at 65°C, sequentially with 1× SSC–0.1% SDS and then 0.1× SSC–0.1% SDS.

PCR with allele-specific oligonucleotide analysis. Genomic DNA was prepared from the wild-type and Z65 CHO cells as previously described (30). PCR was performed for 40 cycles of 94°C for 0.5 min, 52°C for 1 min, and 72°C for 1.5 min, in 50 μl of a solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 1 μg of genomic DNA, 10 pmol (each) of H4 and T4 primers, 200 μM dNTPs, and 1.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus). The reaction mixture was subjected to extraction with phenol-chloroform and subsequent precipitation with ethanol. The resulting DNA precipitate was dissolved in 10 mM Tris-HCl–1 mM EDTA, pH 8.0. The PCR products or cDNA clones were diluted in 0.4 M NaOH and spotted onto a Hybond-N$^+$ membrane (Amersham). Hybridization was performed at 30°C with 2.5 pmol of ^{32}P-labelled oligonucleotide probe, WD or MT, in 6× SSC, 5× Denhardt's solution, and 0.05% sodium PP_i. The membrane was washed twice in 5× SSC–0.1% SDS at 54°C for 15 min.

In vitro mutagenesis of PAF-1 cDNA. In order to construct PAF-1 mutants with N-terminal deletions, a *Nco*I site was introduced at the initiator methionine of rat PAF-1 cDNA by site-directed mutagenesis. Fragments from the *Nco*I site to the *Eco*O109I, *Eco*RII, *Bst*BI, or *Sph*I site were cleaved out. Other mutants were obtained by site-directed mutagenesis with synthetic oligonucleotides (16). All mutations were assessed by nucleotide sequencing. The resulting mutant PAF-1 cDNAs were subcloned into a *Bam*HI-*Eco*RI site of the pcD2MCS vector and separately transfected into CHO cells and human fibroblasts and then selected with G418, as previously described (30, 33). Peroxisomes in the CHO cells and the fibroblasts were detected by staining with rabbit antibodies to rat and human catalases, respectively, and fluorescein isothiocyanate-labelled anti-rabbit immunoglobulin G second antibody (30).

Nucleotide sequence accession number. The nucleotide sequence of a DNA encoding PAF-1 of Chinese hamster was submitted to the GenBank database, together with those of rat PAF-1 cDNA clones T and U, under accession numbers D30618, D30616, and D30617, respectively.

RESULTS

Southern and Northern analysis. In Southern blot analysis of genomic DNA from rat liver, a single band was noted in all of the digests with *Eco*RI, *Bam*HI, *Hin*dIII, and *Pst*I, suggesting that PAF-1 is likely to be a single-copy gene (Fig. 1).

To examine whether PAF-1 can be induced by peroxisome proliferators such as clofibrate, a hypolipidemic drug that induces peroxisomal proteins, including β-oxidation enzymes (26), total liver RNA from normal and clofibrate-treated rats was analyzed by Northern blot. By treatment with clofibrate, the level of acyl-coenzyme A oxidase mRNA increased ~50-fold, while β-actin mRNA changed 1.1-fold, as assessed by RNA dot blot analysis with the respective cDNAs (not shown). Nearly the same amounts of RNA from normal and clofibrate-

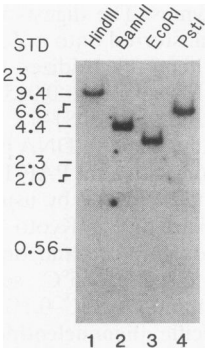

FIG. 1. Southern analysis of rat genomic DNA. Rat genomic DNA (10 μg) was digested with the restriction enzymes indicated above, separated, transferred onto a membrane, and probed with a ^{32}P-labelled *Pvu*II-*Eco*RI fragment of rat PAF-1 cDNA. DNA size markers (in kilobases) are on the left. Exposure, 2 days.

treated rats bound to the PAF-1 cDNA, suggesting that PAF-1 is not noticeably induced by clofibrate (Fig. 2, lanes 3 and 4). The intracellular site of synthesis of PAF-1 was also studied by Northern blot analysis of free and membrane-bound polysomal RNAs from the liver of a clofibrate-treated rat. In vitro translation of RNA followed by immunoprecipitation of catalase and preproalbumin, marker proteins for synthesis on free and membrane-bound polysomes, respectively (6, 7, 26), revealed that free and membrane-bound RNAs were 90 and 98% pure, respectively, confirming adequate separation of two types of polysomes (not shown). An RNA band hybridized with PAF-1 cDNA was predominant in free polysomal RNA (Fig. 2, lanes 5 and 6), strongly suggesting the synthesis of PAF-1 on free polysomes, in accordance with a general rule for biogenesis of peroxisomal soluble and membrane polypeptides (1, 17, 24).

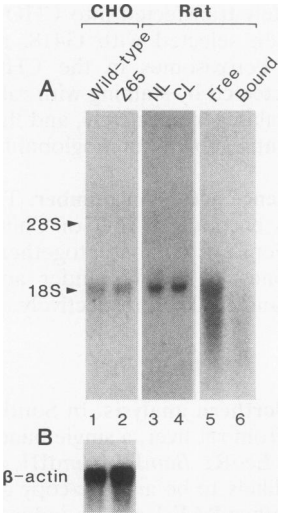

FIG. 2. RNA blot analysis of PAF-1. RNA was separated, transferred to a nylon membrane, and hybridized with ^{32}P-labelled cDNA probes for rat PAF-1 (A) and human β-actin (B). Lanes: 1 and 2, poly(A)$^+$ RNA (8 μg) from wild-type and Z65 CHO cells, respectively; 3 and 4, total RNA (20 μg) from the livers of a control (NL) and a clofibrate-treated (CL) rat, respectively; 5 and 6, total RNA (25 μg) of free and membrane-bound polysomes, respectively, from a clofibrate-treated rat. RNA standards are on the left. Exposure: panel A, lanes 1 to 4, 19 days; lanes 5 and 6, 24 days; panel B, 5 h.

Deletion analysis of PAF-1. Next, in order to study the relation of the structure and function of PAF-1, various in vitro-mutagenized PAF-1 cDNAs were constructed and assayed for restoring activity in peroxisome assembly by transfection to Z65 cells (Fig. 3). Numerous catalase-positive particles (peroxisomes) were apparent in the wild-type CHO cells, whereas no peroxisome was seen in the mutant Z65 and mock-transfected Z65 (Fig. 3A to C). Wild-type rat PAF-1 cDNA evidently failed to restore peroxisome assembly in other mutants, Z24 (34) and ZP92 (29), consistent with the notion that these two mutants are in complementation groups different from that of Z65 (Fig. 3D and E). Peroxisomes in Z65 transfected with cDNA encoding a PAF-1 mutant with deletion of amino acid residues 2 through 19 at the N-terminal part were as numerous as in the transfectants with full-length PAF-1 cDNA, whereas no peroxisomes were seen with several PAF-1 mutants deleted in residues 2 to 27 or further (Fig. 3F to H and 4). Partial deletion mutants in the C-terminal region including that up to amino acid residue 214 from the C terminus, just after the second putative membrane-spanning region, did not influence the apparent activity of PAF-1, whereas truncation of residues from the C terminus, such as that of residues 204 to 305 and those deleted further, resulted in a loss of the activity (Fig. 3I and J and 4).

As a clue to search for a peroxisomal targeting signal of PAF-1, we examined PAF-1 mutants with a site mutation of lysine 219 to glutamic acid in the sequence Ala-Lys-Leu, residues 218 to 220, similar to a C-terminal SKL motif that has been shown to lose the topogenic activity by mutation to SEL (9, 22). Normal appearance of peroxisomes was evident, suggesting that this internal AKL is not important for PAF-1 (Fig. 3K). This is compatible with the finding that a PAF-1 mutant deleted in residues 214 to 305 was functional and presumably localized to the peroxisomal membrane. As a step to understanding the significance in PAF-1 of the cysteine-rich RING finger motif (20), cysteine 264 was replaced with serine. Peroxisomes were formed by this PAF-1 mutant, indicating that one site mutation of the motif exerts no apparent effect (Fig. 3L). Moreover, peroxisomes were evident with PAF-1 mutants with deletion of the entire sequence of the RING finger (Fig. 3I and 4).

Transfection of deletion mutants of rat PAF-1 cDNA was also done by using skin fibroblasts from a patient with Zellweger syndrome who belonged to the same complementation group as Z65 cells (30) (Fig. 5). The PAF-1 mutants with the deletion in amino acid residues 2 to 19 or 214 to 305 apparently restored peroxisome assembly (Fig. 5A and C), as noted with the wild-type PAF-1 cDNA (Fig. 4) (29). No peroxisomes were detectable in the fibroblasts transfected with the PAF-1 mutants with a deletion in residues 2 to 27, 204 to 305, or 126 to 305 (Fig. 4 and 5B and D). These results were fully compatible with those obtained with Z65 (Fig. 3 and 4).

Primary defect in CHO mutant Z65. To investigate the dysfunction of PAF-1 in mutant Z65, Northern blot analysis was performed (Fig. 2, lanes 1 and 2). An RNA band of ~1.9 kb, similar in size and amount to that from the wild-type cells, was detected, suggesting that the dysfunction of PAF-1 is unlikely to be at the transcriptional level. Next, to examine whether the PAF-1 gene of Z65 is mutated, cloning of PAF-1 cDNA from the wild-type and Z65 cells was carried out by means of PCR. In experiment 1, amplification of PAF-1 cDNA was evident with only one preparation of RNA, among several examined, from the wild-type CHO cells (not shown). Nucleotide sequence analysis revealed that all four subclones con-

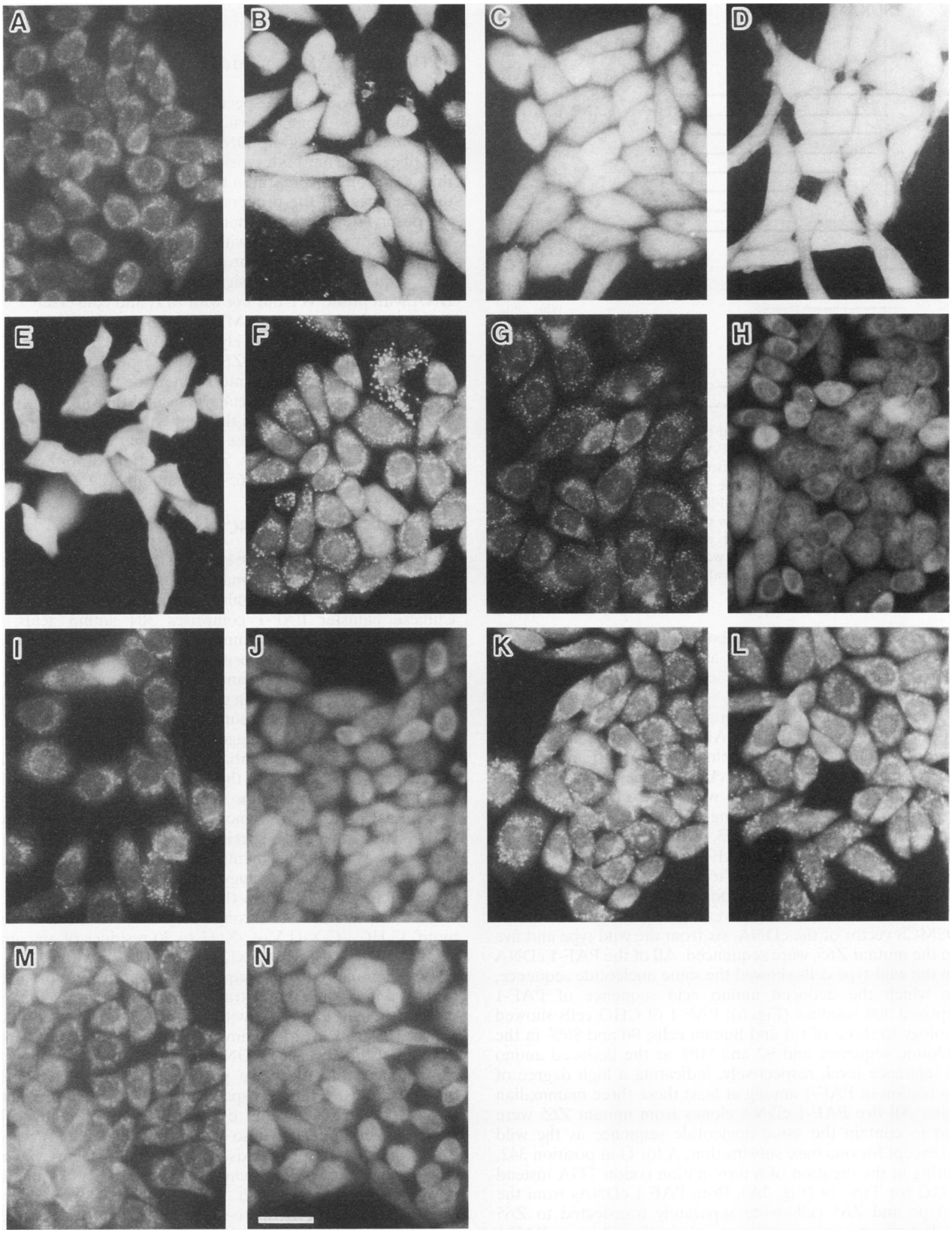

FIG. 3. Transfection of truncated and site-mutated variants of PAF-1. Peroxisome-deficient Z65 cells were transfected with deleted or site-mutated variants of rat PAF-1 (A to L) and with Chinese hamster PAF-1 (M and N); the transfectants were stained for catalase. (A) Wild-type CHO cells; (B) mutant Z65; (C) Z65 with pcD2 vector alone; (D to F) mutants Z24, ZP92, and Z65, respectively, transfected with full-length PAF-1 cDNA; (G and H) Z65 with PAF-1 truncated in the N-terminal region, at residues 2 to 19 and 2 to 27, respectively; (I and J) Z65 with PAF-1 deleted in the C-terminal part, at residues 214 to 305 and 204 to 305, respectively; (K and L) Z65 with PAF-1 site-mutated at residues K-219 and C-264 to E and S, respectively; (M and N) Z65 transfected with PAF-1 cDNAs isolated from the wild-type CHO cells and Z65, respectively. Bar, 40 μm.

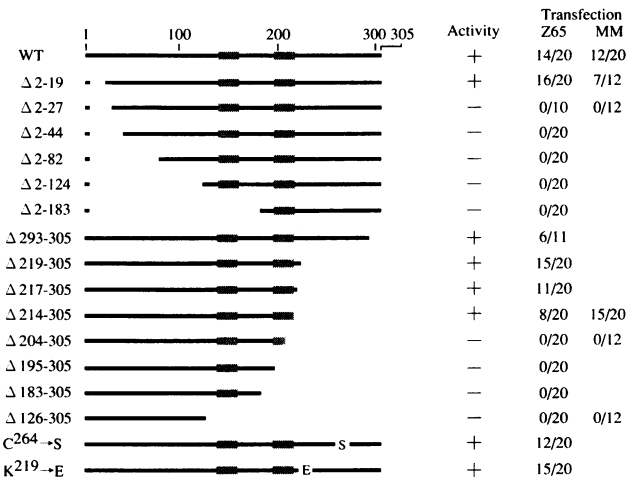

FIG. 4. Summary of deletion and mutation analysis of PAF-1. All truncated and mutated variants of PAF-1, shown by solid bars, were tested for restoring activity in peroxisome assembly (see Fig. 3 and 5). Two putative hydrophobic membrane-spanning segments are indicated by stippled bars. WT, wild-type, full-length PAF-1; deletion mutants are indicated as Δ*m*-*n*, where *m* and *n* represent amino acid residues of starting and terminal positions of deletion, respectively. Activity, where peroxisome-positive clones were predominant in 20 G418-resistant colonies counted, was indicated as positive (+).

tained several misincorporated bases. Accordingly, they were not tested for complementation assay by transfection to Z65. Nevertheless, all of the clones contained the 5′-coding sequence ATGGCTGGCAGA.

Subsequently, one positive colony was cloned by screening a CHO cDNA library with rat PAF-1 cDNA, and its plasmid apparently contained a cDNA clone with a partial, ~500-bp sequence of PAF-1. To obtain cDNA clones including open reading frames, PCR was done with Vent DNA polymerase and with primers H3, containing the sequence ATGGCTG GCAGA at the 3′ end, and T3, with a complementary sequence of the partial CHO PAF-1 cDNA cloned above but with one substituted nucleotide to create an *Eco*RI site. Two ~950-bp fragments from RNA, one from the wild type and the other from the mutant cells, were noted. Subclones in the pcD2MCS vector of the cDNA, six from the wild type and five from the mutant Z65, were sequenced. All of the PAF-1 cDNA from the wild-type cells showed the same nucleotide sequence, from which the deduced amino acid sequence of PAF-1 comprised 304 residues (Fig. 6). PAF-1 of CHO cells showed homology to those of rat and human cells, 90 and 86% in the nucleotide sequence and 92 and 90% at the deduced amino acid sequence level, respectively, indicating a high degree of conservation of PAF-1 among at least these three mammalian species. All five PAF-1 cDNA clones from mutant Z65 were found to contain the same nucleotide sequence as the wild type, except for one base substitution, A for G at position 342, resulting in the creation of a termination codon TGA instead of TGG for Trp-114 (Fig. 7A). Both PAF-1 cDNAs from the wild-type and Z65 cells were separately transfected to Z65 cells. Peroxisomes were apparent only in the wild-type PAF-1 cDNA-transfected cells but not in the cells with the PAF-1 cDNA from the Z65 cells, indicating the dysfunction of Z65 PAF-1, presumably due to a premature termination (Fig. 3M and N). This is compatible with the findings with truncated PAF-1 mutants such as that deleted in residues 126 to 305 (Fig. 3 to 5). With these data taken together, the nonsense mutation at Trp-114 of PAF-1 appeared to be the cause of peroxisome deficiency in Z65 cells.

In PCR with allele-specific oligonucleotide analysis of genomic DNA from the wild-type and Z65 cells, nearly the same amount of ~80-bp DNA fragments was synthesized by amplification from both types of cells (not shown), suggesting that no intron was present in the CHO PAF-1 gene(s) between the sequences of the primers H4 and T4, in accordance with the observation of the human genome (30). In the hybridization of these fragments with oligonucleotide probes, each specific for the wild-type (probe WT) and mutant-type (probe MT) sequences, a strong signal was noted for the wild-type DNA with probe WT but not with MT, and conversely for the fragment of Z65 only with MT (Fig. 7B). Probes WT and MT likewise hybridized exclusively with PAF-1 cDNA clones from the wild-type and mutant Z65 cells, respectively. This result strongly suggested that the mutation in the PAF-1 gene of Z65 cells was homozygous or hemizygous and that no wild-type or no other mutant allele with the wild-type sequence at position 342 was present in Z65. The hemizygosity of PAF-1 appears more likely in CHO cells, with a notion of rather highly frequent isolation of the Z65-type mutants (29, 39, 41).

DISCUSSION

Homology higher than 90% among the primary sequences of PAF-1 from rats (33), humans (30), and Chinese hamsters implies that PAF-1 is highly conserved through evolution. Chinese hamster PAF-1 comprises 304 amino acids, one residue shorter than rat or human PAF-1. The one-amino-acid deletion is at alignment position 5, where glutamic acid and lysine are located in the rat and human sequences, respectively. These three PAF-1s, with an acidic amino acid at this position in rats, a basic residue in humans, and one deletion in CHO cells, are all functional, suggesting that the residue at alignment position 5 is not essential to the function of PAF-1. This inference is consistent with the finding in the deletion analysis of rat PAF-1, in which amino acid residues 2 through 19 at the N-terminal part can be removed without loss of the activity. PAF-1s from the three species all also contain two putative membrane-spanning segments, suggesting the importance of these regions for the topology and function of PAF-1.

A cysteine-rich region in the C-terminal part of PAF-1, at alignment positions 244 through 305, contains a RING finger motif, C_3HC_4, C-X-(I,V)-C-X(11 to 30 residues of any amino acids)-C-X-H-X-(F,I,L)-C-X(2)-C-(I,V,L,M)-X(10 to 18)-C-P-X(1)-C, common to the sequences of a number of diverse proteins, including many transcription regulators, and was thereby thought to interact with DNA (5, 20). However, PAF-1 appears less likely to be involved in DNA binding simply because of the absence of DNA in peroxisomes (15), which is compatible with alternative possible function of the RING finger, i.e., involvement in specific protein-protein interaction through binding with zinc or divalent metal ions (5). The RING finger motif was also noted in complementing genes PAS-4 and PAS-5 for peroxisome assembly-defective *pas* mutants of the yeast *Saccharomyces cerevisiae* (12). To our surprise, however, this RING finger does not appear to be essential to the peroxisome-restoring activity of PAF-1, as deduced from the truncation studies (Fig. 3I and 4). The functional significance of this motif is to be investigated. Very recently, Thieringer and Raetz (32) reported that two types of point mutations of PAF-1, Arg-124 to a stop codon and Cys-246 to Tyr, were noted in peroxisome-deficient CHO mutants apparently belonging to the same complementation group as Z65. The former mutation is similar to the incident

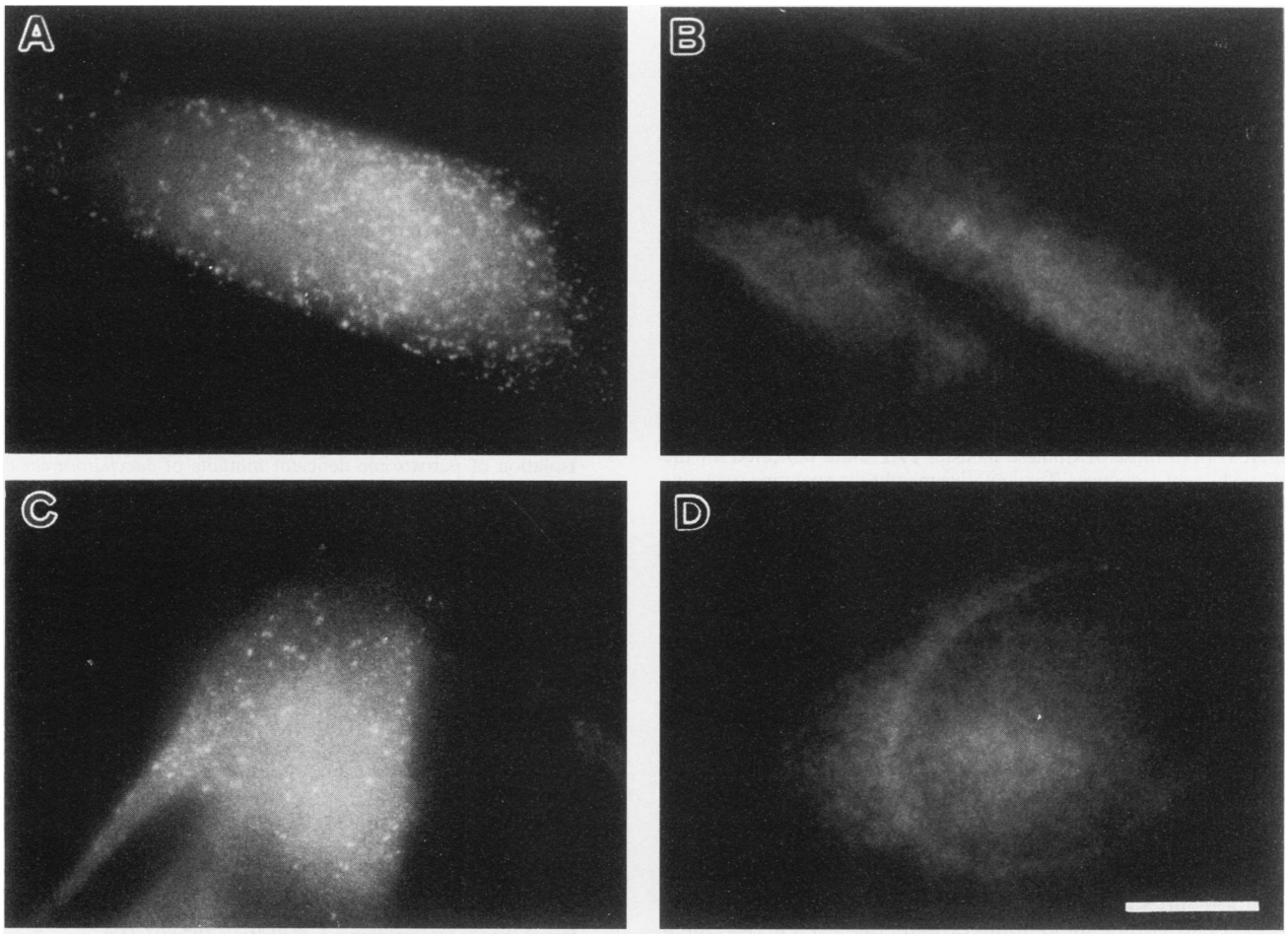

FIG. 5. Transfection of rat PAF-1 truncated at N- and C-terminal parts to fibroblasts from a peroxisome-deficient Zellweger patient. Fibroblasts, from a patient with Zellweger syndrome, that belong to the same complementation group as Z65 (30) were transfected with truncated forms of rat PAF-1. (A and B) PAF-1 deleted in the N-terminal region, at residues 2 to 19 and 2 to 27, respectively; (C and D) PAF-1 with truncation at the C-terminal part, residues 214 to 305 and 204 to 305, respectively (see Fig. 3G-J). Bar, 25 μm.

which occurred in Z65 and is consistent with the results of the deletion study described in this report. The latter disagrees with our results, which suggest that the cysteine-rich motif appears to be dispensable, although the possibility of instability of PAF-1 caused by this mutation cannot be excluded. It would be worthwhile to examine whether this mutant PAF-1 could restore the peroxisome assembly.

The primary sequence of PAF-1 with deletion of amino acid residues 2 through 19 at the N terminus and residues 214 to 305 in the C-terminal part appears to be essential for peroxisome-restoring activity, although the possibility of intracellular degradation, improper targeting, or failure in expression of several PAF-1 mutants, thereby showing no peroxisome-restoring activity, cannot be excluded. However, the results in transfection to Z65 cells of deletion mutants of PAF-1, particularly those truncated in residues 2 to 19, 2 to 27, 204 to 305, and 214 to 305, are unambiguously consistent with the findings made by using peroxisome-deficient fibroblasts from a Zellweger patient, implying that such a possibility is less likely (Fig. 3 to 5).

Our studies on three mutually distinct, peroxisome-defective CHO mutants, Z24 (34), Z65 (34), and recently isolated ZP92 (29), suggested that assembly of peroxisomes is impaired in these mutants, although there is normal synthesis of peroxisomal polypeptides. The formation of peroxisomes was restored in a mutant, Z65, by expression of the transfected cDNA encoding PAF-1 (33). In this report, we searched for the primary defect in the impaired assembly of peroxisomes in Z65 and found a nonsense point mutation in the PAF-1 gene causing the premature termination at residue Trp-114, which presumably resulted in synthesis of immature and nonfunctional PAF-1. This finding was assessed by back-transfection of this mutated PAF-1 cDNA to Z65, in which no peroxisomes were formed. A nonsense point mutation at Trp-124 in the PAF-1 gene was noted in a CHO mutant, ZR-82 (32). Moreover, we recently observed in a Zellweger patient, patient 1, the premature termination of PAF-1 by a homozygous nonsense mutation at residue Arg-119 very similar to those in Z65 and ZR-82, resulting in the dysfunction of PAF-1, which was apparently the cause of the Zellweger syndrome (30). Taken together, the mutation in this region of the PAF-1 gene in human and CHO cells may not be coincidental but might be more frequent than those in other parts of the sequence.

Synthesis of PAF-1 on cytoplasmic free polysomes is compatible with the general idea of a posttranslational mechanism of peroxisome assembly (1, 17, 35). As a step toward the understanding of peroxisome biogenesis, it is important to study the mechanism of transport and integration of peroxiso-

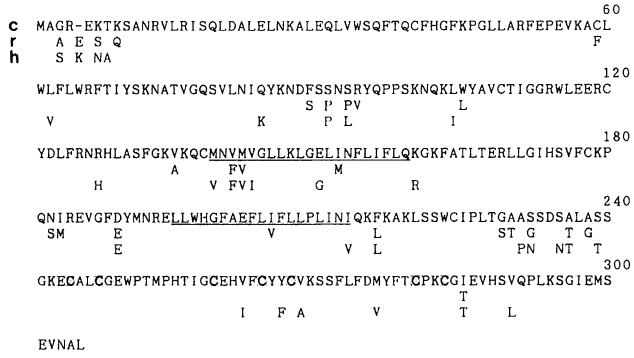

FIG. 6. Amino acid sequence of Chinese hamster PAF-1. The deduced amino acid sequence of Chinese hamster PAF-1 (c) is shown, in comparison to rat (r) and human (h) sequences; only amino acids different from those of Chinese hamster PAF-1 are indicated for the rat and human sequences. Two putative membrane-spanning segments are underlined; cysteine residues in the novel cysteine-rich RING finger sequence are highlighted by shading.

mal membrane proteins, particularly those with known function, such as PAF-1 and Pas3p of *S. cerevisiae* (13). PAF-1 does not appear to be induced by a peroxisome proliferator, clofibrate, that enhances transcription of several peroxisomal proteins such as β-oxidation enzymes (7, 26). Among other peroxisomal membrane polypeptides, the 22-kDa integral membrane protein is not inducible (6), while the 70-kDa membrane protein, a member of the ATP-binding cassette transporters (14), is inducible. Of 21 patients of one complementation group, two point mutations (a donor splice site mutation and a missense mutation) in the human gene for the 70-kDa peroxisomal membrane protein, one each in one allele of 2 patients with Zellweger syndrome, were noticed (8). Functional significance, however, of the 70-kDa membrane protein in peroxisome biogenesis has not been elucidated. Membrane proteins essential for assembly of peroxisomes, some of which (e.g., PAF-1) appear not to be induced in a chemically significant amount, may function as key proteins, in a catalytic manner, mediating peroxisomal formation.

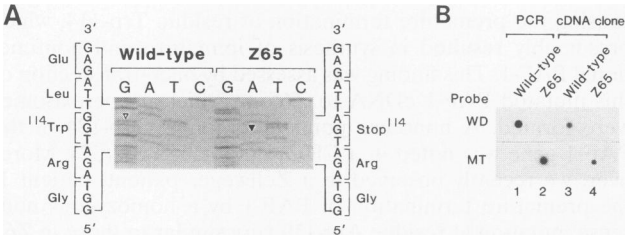

FIG. 7. Nonsense mutation of PAF-1 in CHO mutant Z65. (A) Sequence analysis of PAF-1 cDNA. cDNA for PAF-1 was isolated from the CHO mutant Z65 and sequenced. A part of the sequencing autoradiogram where a point mutation was noted is shown. A point mutation changes a codon for Trp-114, TGG, in the wild type (open arrowhead) to a stop codon, TGA, in the mutant Z65 (solid arrowhead). (B) Allele-specific oligonucleotide analysis for PAF-1 mutation. PCR products from the wild-type and Z65 genomic DNA (lanes 1 and 2) and PAF-1 cDNA clones from the wild-type and Z65 cells (lanes 3 and 4) were hybridized with ^{32}P-labelled, wild-type and mutant oligonucleotide probes WD and MT. Exposure, 1 h.

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