Identification of Peroxisomal Targeting Signals Located at the Carboxy Terminus of Four Peroxisomal Proteins

Stephen John Gould, Gilbert-Andre Keller, and Suresh Subramani

Department of Biology and Center for Molecular Genetics, B-022, University of California at San Diego, La Jolla, California 92093

Abstract. As part of an effort to understand how proteins are imported into the peroxisome, we have sought to identify the peroxisomal targeting signals in four unrelated peroxisomal proteins: human catalase, rat hydratase:dehydrogenase, pig D-amino acid oxidase, and rat acyl-CoA oxidase. Using gene fusion experiments, we have identified a region of each protein that can direct heterologous proteins to peroxisomes. In each case, the peroxisomal targeting signal is contained at or near the carboxy terminus of the protein. For catalase, the peroxisomal targeting signal is located within the COOH-terminal 27 amino acids of the protein. For hydratase:dehydrogenase, D-amino acid oxidase, and acyl-CoA oxidase, the targeting signals are located within the carboxy-terminal 15, 14, and 15 amino acids, respectively. A tripeptide of the sequence Ser-Lys/His-Leu is present in each of these targeting signals as well as in the peroxisomal targeting signal identified in firefly luciferase (Gould, S. J., G.-A. Keller, and S. Subramani. 1987. *J. Cell Biol.* 105:2923–2931). When the peroxisomal targeting signal of the hydratase:dehydrogenase is mutated so that the Ser-Lys-Leu tripeptide is converted to Ser-Asn-Leu, it can no longer direct proteins to peroxisomes. We suggest that this tripeptide is an essential element of at least one class of peroxisomal targeting signals.

A variety of organelles exist within eukaryotic cells, each responsible for particular cellular functions. To maintain this compartmentalization, the cell must be able to direct proteins to their proper subcellular locations. The transport of proteins into peroxisomes, the endoplasmic reticulum, mitochondria, and chloroplasts all involve translocation across one or more lipid bilayers. In each of these systems, the delivery of a protein into the organelle is mediated, in part, by targeting sequences present within the polypeptide (Blobel and Dobberstein, 1975; Dobberstein et al., 1977; Hase et al., 1984; Gould et al., 1987). Many signal sequences have been identified that can direct proteins into either the endoplasmic reticulum, mitochondria, or chloroplast. These signals are located at or near the amino terminus of the protein and are usually removed during the transport process, though there are exceptions to these generalizations (Palmiter et al., 1978; Zimmerman et al., 1979; Zwizinski and Neupert, 1983; Wickner and Lodish, 1985; Stuart et al., 1987; and Chua, N.-H., A. Grossman, S. Bartlett, and G. Schmidt; unpublished observations). There are only two reports of targeting signals that sort proteins to peroxisomes. We identified a peroxisomal targeting signal (PTS)[1] in luciferase at the carboxy terminus of the protein (Gould et al., 1987); this PTS is contained within the peptide: leu-ile-lys-ala-lys-lys-gly-gly-lys-ser-lys-leu-COOH. More recently, Small et al. (1988) reported that two nonoverlapping PTSs exist within 118- and 119-amino acid-long segments of *Candida tropicalis* acyl-CoA oxidase (PXP4).

Comparison of a number of PTSs should reveal important features of the sequences that direct proteins to peroxisomes. We have sought to expand the list of known PTSs. Because the PTS of luciferase is located at the COOH terminus of the protein, we asked whether COOH-terminal segments of other peroxisomal proteins also encoded PTSs. Chimeric genes were constructed in which the carboxy termini of several peroxisomal proteins were fused to the COOH-terminal end of genes encoding cytosolic proteins. The peroxisomal proteins chosen for these studies were human catalase (Bell et al., 1986), rat hydratase:dehydrogenase (H:D) (Osumi et al., 1985), pig D-amino acid oxidase (DAAOX) (Ronchi et al., 1982), and rat acyl CoA oxidase (AOX) (Miyazawa et al., 1987). We find that a short carboxy-terminal region of each of these peroxisomal proteins is capable of acting as a PTS in gene fusion experiments. Comparison of the amino acid sequences of these PTSs reveals that they contain a common tripeptide of the sequence Ser-Lys/His-Leu. We also demonstrate that a point mutation which changes the lysine within this tripeptide to an asparagine inactivates the PTS from the rat H:D.

Materials and Methods

Reagents

The rabbit antibody against bovine catalase was a gift from A. Schram,

1. *Abbreviations used in this paper*: AOX, acyl-CoA oxidase; CAT, chloramphenicol acetyltransferase; DAAOX, D-amino acid oxidase; DHFR, dihydrofolate reductase; H:D, hydratase:dehydrogenase; PTS, peroxisomal targeting signal.

University of Amsterdam (Amsterdam, The Netherlands). The CAT-2 cell line producing the anti-chloramphenicol acetyltransferase (CAT) mAb was a gift from C. Gorman, National Cancer Institute, Bethesda, MD. All oligodeoxynucleotides were purchased from Operon Technologies, Inc. (San Pablo, CA). The human catalase cDNA was a gift of R. Hallewell, Chiron Corp., Emeryville, CA.

Plasmids

Manipulations of DNA were performed as described (Maniatis et al., 1982). DNA sequence analysis was performed by the method of Sanger et al. (1977) as modified by Chen and Seeburg (1985). Construction of the luciferase mutant RSVLΔC104 was accomplished by cleaving the plasmid pRSVL (de Wet et al., 1987) with Eco RV (at nucleotide position 1642 in the luciferase gene) and ligating a Hind III linker onto the ends of the DNA. This linker had the sequence 5'-TCAATCAGTCAAGCTTGACTGATTGA-3' and contained stop codons in all three reading frames. The plasmid pRSVHCAT contained the Nco I-Sal I fragment of the human catalase cDNA (Bell et al., 1986) inserted in the Xho I site of pRSV-AnX between the RSV promoter and the SV-40 early region polyadenylation signal (An). The plasmid pRSVL-HCAT404 was constructed by ligating the 3,169-bp Eco RV-Bgl I fragment of pRSVL between the Pvu II and Bgl I sites of pRSVHCAT. pRSVL-HCAT145 was constructed by digesting pLI-446-CTRV (Gould et al., 1987) with Xho I and Bam HI, isolating the 4,800-bp piece of DNA, and ligating it with the 703-bp Xho I-Bam HI fragment of pRSVHCAT. pRSVLHCAT27 was created by cleaving pRSVL with Eco RV and Bam HI followed by isolation of the 4,800-bp fragment. The plasmid pRSVHCAT was cleaved with Rsa I and Bam HI and the 350-bp fragment was isolated. These two fragments were ligated together to make the plasmid pRSVLHCAT27.

The reconstruction of the 3' end of the CAT gene was accomplished as follows. Two oligodeoxynucleotides were hybridized to one another and then inserted into pUC19 between the Hind III and Bam HI sites to generate pUC-C-CAT. These oligodeoxynucleotides had the following sequences: 5'-AGCTTAGTACTGCGATGAGTGGGAGGGCGGGGCCGGCG-3' and 5'-GATCCGCCGGCCCCGCCCTCCCACTCATCGCAGTACTA-3'. When hybridized together they created a 34-bp fragment of DNA with a Hind III overhang on one end and a Bam HI overhang on the other. They were also designed so that they encoded the carboxy-terminal nine amino acids of CAT, introduced a site for a blunt-end-generating restriction enzyme (Nae I) that cuts immediately after the last codon of the gene, and allowed insertion of the 664-bp Hind III to Sca I fragment of the CAT gene between the Hind III and Sca I sites of pUC-C-CAT to generate pCATC. The construction of fusions between the CATC gene and the DNAs containing COOH-terminal sequences of the peroxisomal protein is outlined in Fig. 1.

Cell Culture, Transfections, and Immunofluorescence

Conditions for growth and transfection of CV-1 monkey kidney cells and the procedure for immunofluorescence were as described (Keller et al., 1987), except where noted in the figure legends.

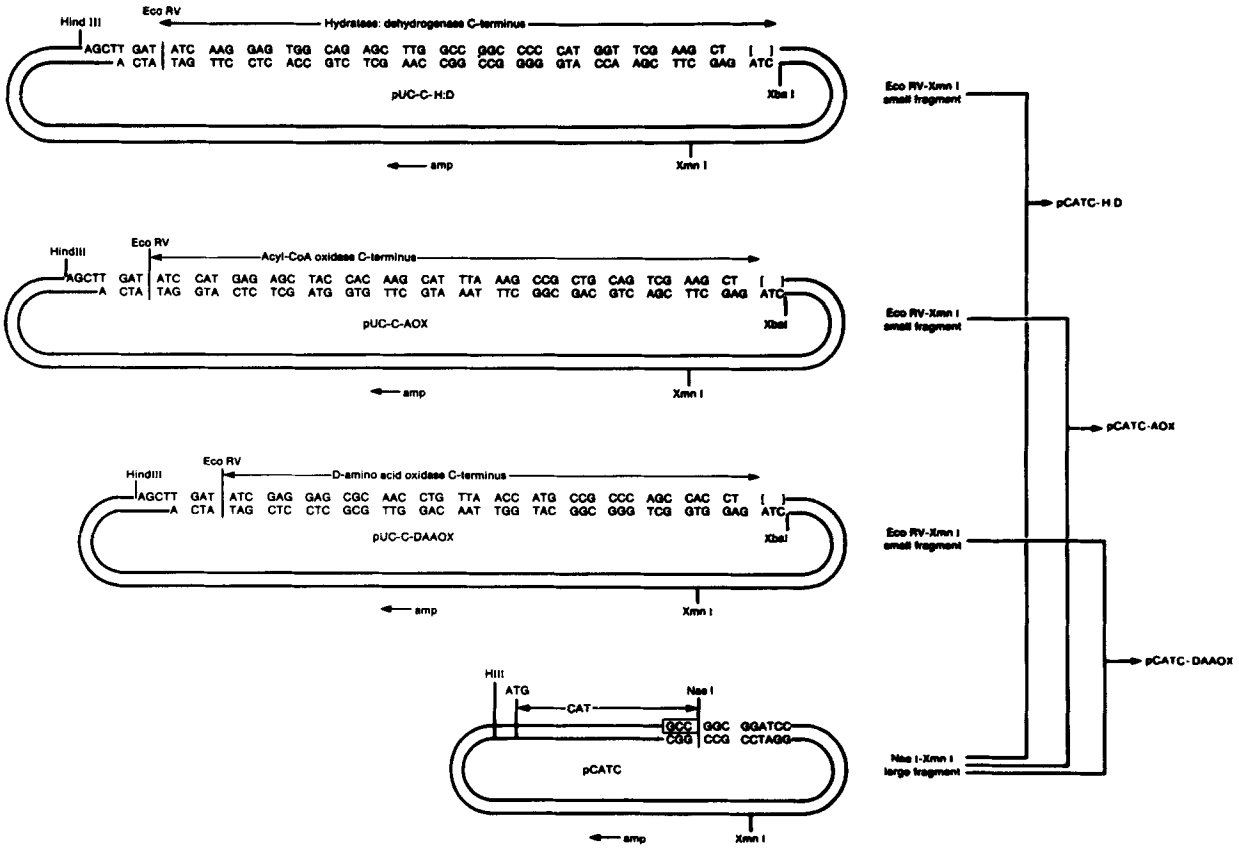

Figure 1. Outline of the construction of CATC-PTS gene fusions. Oligodeoxynucleotide pairs encoding either the COOH-terminal 15 amino acids of rat H:D, COOH-terminal 14 amino acids of pig DAAOX, or COOH-terminal 15 amino acids of rat AOX (sequences presented in the figure) were hybridized together and ligated between the Hind III and Xba I sites of pUC19 to create the plasmids pUC-C-H:D, pUC-C-DAAOX, and pUC-C-AOX, respectively. Each of these plasmids was cleaved with Xmn I and Eco RV and the smaller fragment was isolated. Each was then cloned between the Nae I and Xmn I sites of the ~2,600-bp fragment derived from pCATC to create the plasmids pCATC-H:D, pCATC-DAAOX, and pCATC-AOX. The entire open reading frame of these plasmids was then inserted into the eukaryotic expression vector pSV2. This was accomplished by cleaving the plasmids pCATC-H:D, pCATC-DAAOX, and pCATC-AOX with Hind III and Bam HI, isolating the small fragments, and ligating each between the Hind III and Bgl II sites of pSV2DHFR (Subramani et al., 1981). The resultant plasmids pSV2CATC-H:D, pSV2CATC-DAAOX, and pSV2CATC-AOX were subsequently used in transfection experiments.

Results

Identification of a PTS in Human Catalase

We showed earlier that wild-type firefly luciferase is transported to peroxisomes when expressed in mammalian cells (Keller et al., 1987). Further, we demonstrated that deletion mutants lacking 12 or more amino acids from the COOH terminus of firefly luciferase were cytoplasmic (Gould et al., 1987). To identify additional PTSs, we tested whether segments of another peroxisomal protein can redirect a cytosolic form of luciferase into peroxisomes when fused onto the COOH terminus of such a mutant. Gene fusions were constructed that attached the regions encoding the COOH-terminal 404, 145, or 27 amino acids of the human catalase gene to the 3′ end of a mutant luciferase (LΔC104) lacking the coding region for the COOH-terminal 104 amino acids of luciferase (Fig. 2). After transfection of each gene into CV-1 monkey kidney cells, the intracellular distribution of the expressed proteins was determined by double indirect immunofluorescence. The colocalization of a protein with catalase (a peroxisomal marker) identifies it as a peroxisomal protein. The micrographs show the subcellular localization of the mutant luciferase (Fig. 3 *A*) and the luciferase–catalase fusion proteins L-HCAT404 (Fig. 3 *C*), L-HCAT145 (Fig. 3 *E*), and L-HCAT27 (Fig. 3 *G*). The localization of endogenous catalase in the same cells is shown in the images in the right column (Fig. 3, *B*, *D*, *F*, and *H*). The deletion mutant of luciferase was cytosolic as were the two largest of the luciferase–catalase fusion proteins. However, the luciferase–catalase fusion protein L-HCAT27 was transported into peroxisomes, demonstrating that the COOH-terminal 27 amino acids of catalase contains sufficient information to target a heterologous protein to peroxisomes. The two larger fusions containing either 404 or 145 amino acids of catalase were not imported into peroxisomes, even though they contain the COOH-terminal 27 amino acids of catalase.

Identification of PTSs in RAT H:D, Pig DAAOX, and Rat AOX

To investigate whether other peroxisomal enzymes also contained a PTS at their carboxy terminus, we constructed gene fusions in which DNAs encoding the carboxy-terminal amino acids of either rat H:D, pig DAAOX, or rat AOX were fused to the 3′ end of the CAT gene. To make the CAT gene more amenable to gene fusions at the 3′ end of its coding region, we altered the DNA sequence of this gene without changing the predicted amino acid sequence. The modified CAT gene (CATC) contained a unique restriction enzyme site allowing blunt-end cleavage on the 3′ side of the last codon of the gene. Sequences encoding the carboxy terminus of the peroxisomal proteins mentioned above (H:D, DAAOX, and AOX) were fused to the carboxy terminus of the CATC gene. The strategy used in the construction of these gene fusions is outlined in Fig. 1 of Materials and Methods.

The plasmids pSV2CAT (Gorman et al., 1982), pSV2CATC-H:D, pSV2CATC-DAAOX, and pSV2CATC-AOX were transfected into CV-1 cells. 48 h later, transfected cell populations were examined by double indirect immunofluorescence. In these experiments, the gene fusions were detected with an anti-CAT antibody. The anti-catalase antibody was used to visualize the peroxisomes in the cells. Micrographs showing the immunofluorescent staining of transfected cell populations are presented in Fig. 4. The cytosolic distribution of wild-type CAT is demonstrated at the top left (Fig. 4 *A*). Below it are micrographs showing the localization of the fusion proteins CATC-H:D (Fig. 4 *C*), CATC-DAAOX (Fig. 4 *E*), and CATC-AOX (Fig. 4 *G*). The peroxisomes of the cells (visualized by immunofluorescent labeling for endogenous catalase) are shown to the right of each frame (Fig. 4, *B*, *D*, *F*, and *H*). While the wild-type CAT protein was distributed throughout the cytosol, the fusion proteins CATC-H:D, CATC-DAAOX, and CATC-AOX each colocalized with catalase. This experiment demonstrated that the carboxy-terminal 15 amino acids of rat H:D, 14 amino acids of pig DAAOX, and 15 amino acids of rat AOX were each capable of acting as PTSs. Similar results have been obtained when these sequences are appended to the COOH terminus of mouse dihydrofolate reductase (DHFR). Our results with the DHFR fusions will be presented elsewhere.

In certain cells that expressed the fusion proteins, we observed a substantial decrease in the amount of catalase detectable by the anti-catalase antibody. This made colocalization difficult to represent photographically (see Fig. 4, *G* and *H*) due to the loss in resolution through several steps of reproduction. The lower levels of catalase in the transfected cells may be due to competition between the fusion protein and catalase for entry into the peroxisome.

A Mutation in the Conserved Peptide of the PTS Abolishes Peroxisomal Import

In the process of sequencing clones of pUC-C-H:D (see Fig. 1), a mutant was isolated that encoded an altered amino acid sequence. This mutation resulted in replacement of the lysine at the penultimate position in the CATC-H:D fusion protein with an asparagine. This mutation falls within a conserved tripeptide present in each of the PTSs we have identified (see Discussion). The plasmid containing this mutant was designated as p-UC-C-H:D-N. The DNA encoding the H:D-N sequence was fused to the 3′ end of the CAT gene in the same manner as the wild-type H:D sequence (Fig. 1). The fusion

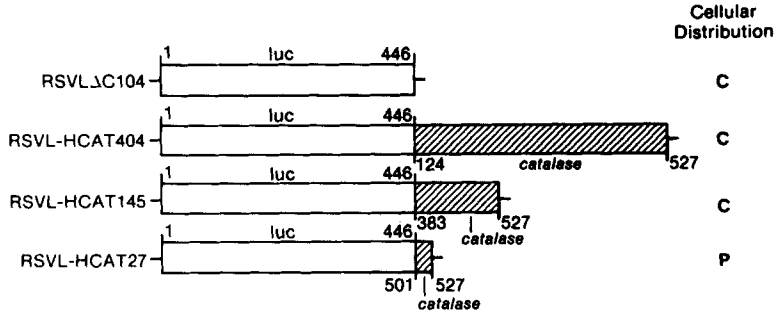

Figure 2. Structure of the luciferase deletion and the luciferase–catalase fusion genes. Luciferase mutant RSVLΔC104 lacks the coding region for the COOH-terminal 104 amino acids of luciferase (*luc*). RSVL-HCAT404, RSVL-HCAT145, and RSVL-HCAT27 encode proteins containing amino acids 1–446 of luciferase followed by the COOH-terminal 404, 145, or 27 amino acids, respectively, of human catalase. The intracellular distribution of each mutant has been noted. *C*, cytosolic. *P*, peroxisomal.

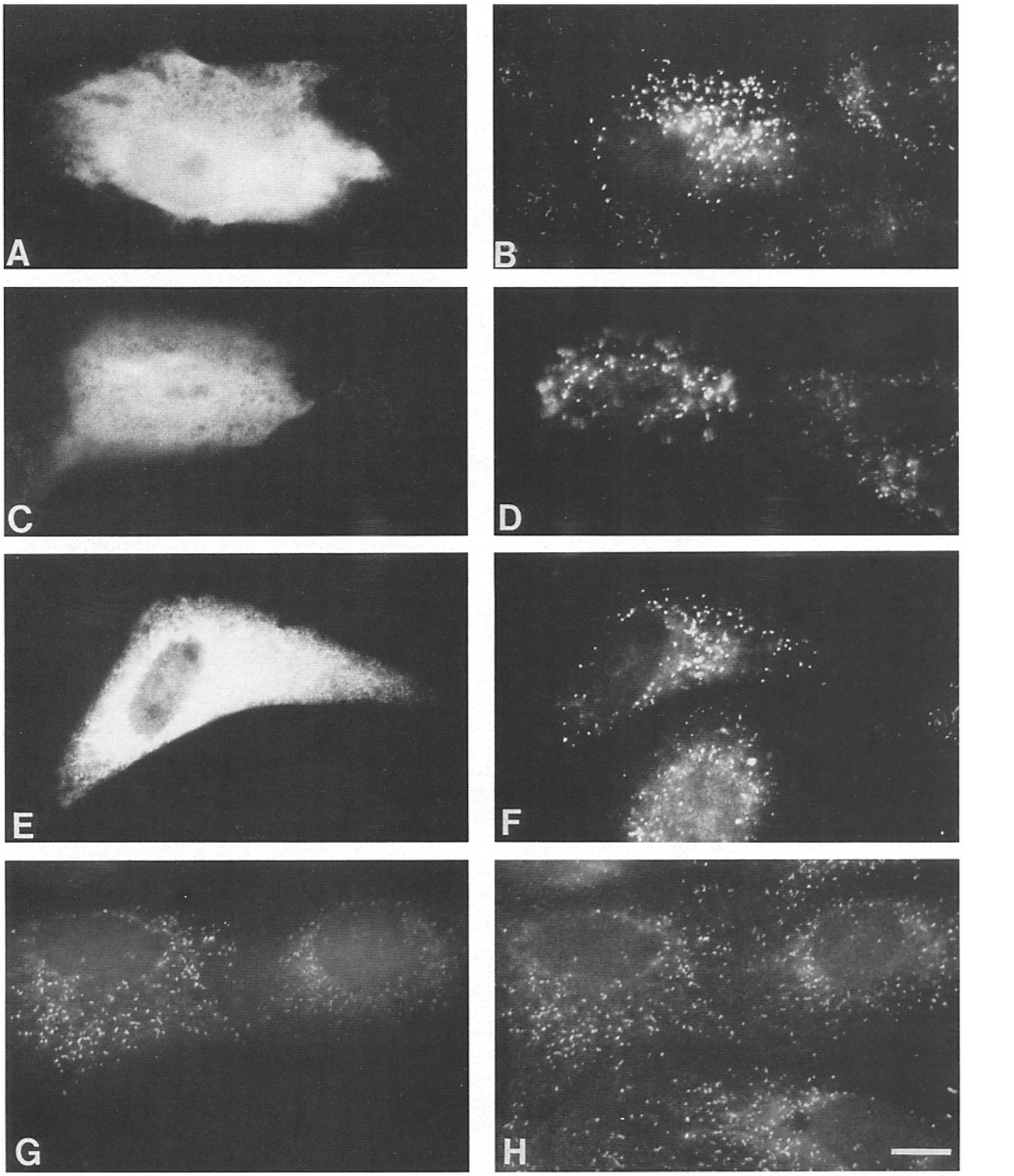

Figure 3. Distribution of the mutant luciferase and the luciferase–catalase fusion proteins. Monkey kidney CV-1 cells were transfected with each of the DNAs described in Fig. 2. Cells were processed for double indirect immunofluorescence using a guinea pig antibody that recognizes firefly luciferase and a rabbit antibody specific for catalase. The primary labeling was followed by staining with a fluorescein conjugate of a goat anti-guinea pig Ig antibody and a rhodamine conjugate of a goat anti-rabbit Ig antibody. Micrographs on the left depict the immunofluorescent localization of (*A*) the luciferase mutant RSVLΔC104 and the luciferase–catalase fusions (*C*) RSVL-HCAT404, (*E*) RSVL-HCAT145, or (*G*) RSVL-HCAT27. To the right (*B*, *D*, *F*, and *H*) are corresponding images of the cells stained for catalase, a marker of peroxisomes. Bar, 10 μm.

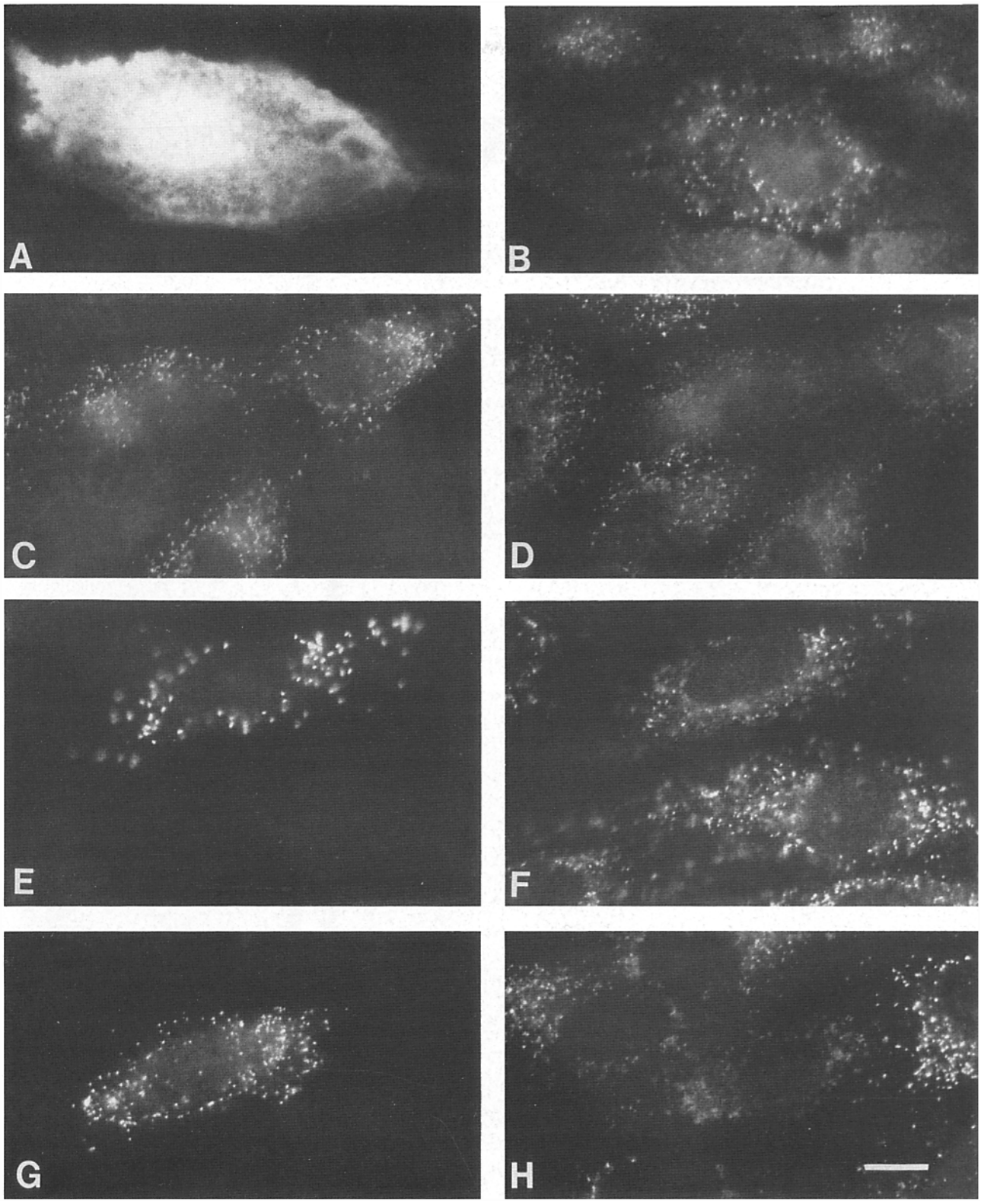

Figure 4. Immunofluorescent localization of CAT and the CAT–PTS fusion proteins. CV-1 cells were transfected either with pSV2CAT, pSV2CATC-H:D, pSV2CATC-DAAOX, or pSV2CATC-AOX. The cells were processed for double indirect immunofluorescence using a mAb that recognizes the bacterial CAT protein and a rabbit anti-catalase antibody. The secondary antibodies used were a fluorescein conjugate of a goat anti-mouse Ig antibody and a rhodamine conjugate of a goat anti-rabbit Ig antibody. Micrographs on the left are of the distribution of either (*A*) wild-type CAT or the fusions (*C*) CATC-H:D, (*E*) CATC-DAAOX, or (*G*) CATC-AOX. Micrographs on the right (*B*, *D*, *F*, and *H*) show the location of the peroxisomes within these cells as detected by immunofluorescent labeling for catalase. Bar, 10 μm.

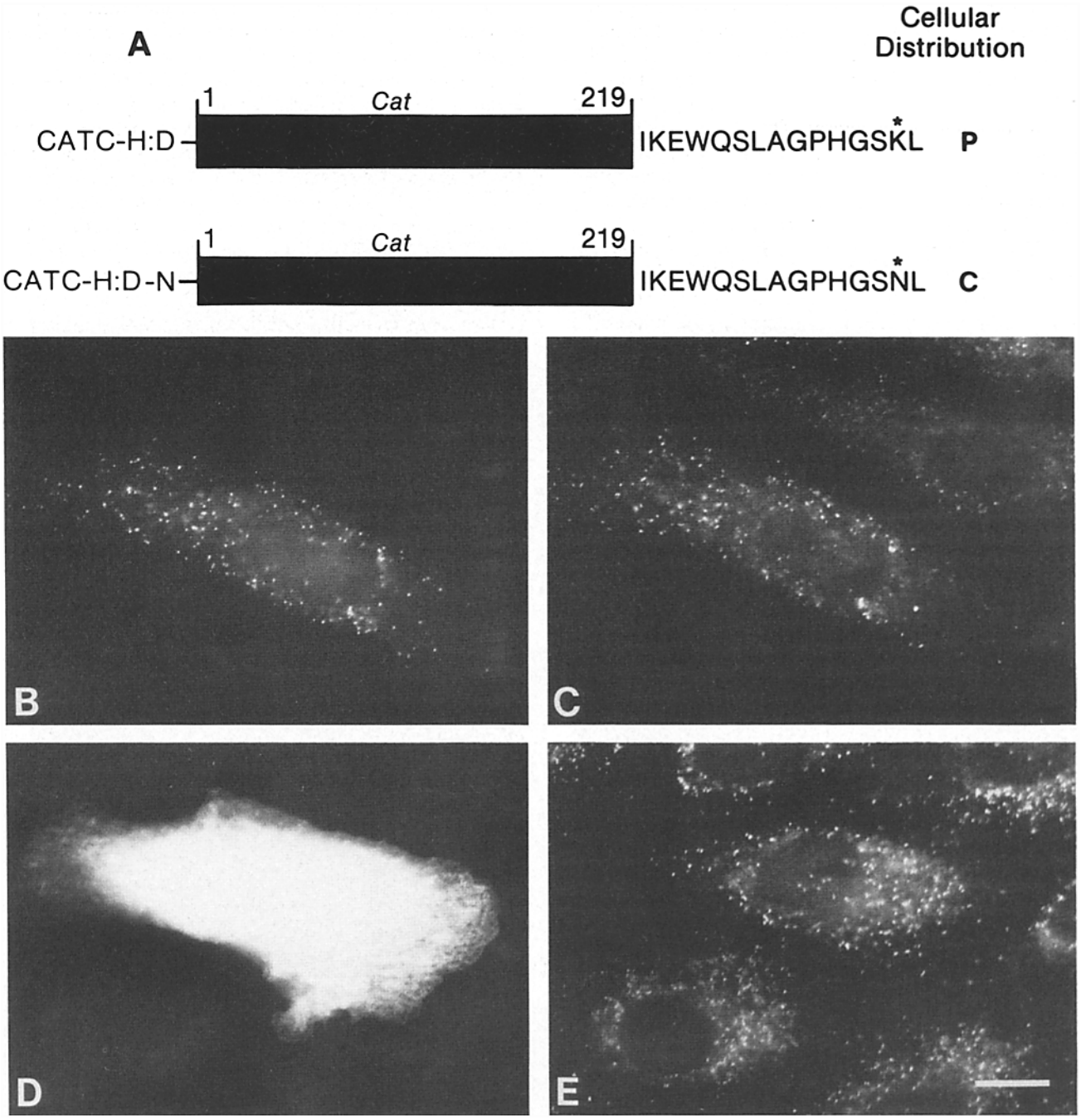

Figure 5. Effect of the Lys to Asp mutation on the distribution of the CATC-H:D fusion protein. (*A*) The predicted structures of the fusions between CAT and the COOH-terminal 15 amino acids of the H:D protein. The wild-type (CATC-H:D) and mutant (CATC-H:D-N) fusions are shown. CV-1 cells were transfected either with pSV2CATC-H:D or pSV2CATC-H:D-N). After transfection, the cells were processed for double indirect immunofluorescence as described in the legend to Fig. 4. Distribution of (*B*) the CATC-H:D fusion protein and (*C*) catalase in the same cell. Localization of (*D*) the CATC-H:D-N fusion protein and (*E*) catalase in the same cell. Bar, 10 μm.

gene was transfected into mammalian cells and the distribution of the fusion protein determined by double indirect immunofluorescence. As shown in Fig. 5 *B*, the fusion protein containing the wild-type H:D sequence was transported to peroxisomes. In contrast, the fusion protein containing the Lys to Asn mutation was found in the cytoplasm (Fig. 5 *D*). Thus the Lys to Asn point mutation inactivated the PTS derived from rat H:D.

Discussion

Five Peroxisomal Proteins Contain Carboxy-Terminal PTSs

We demonstrated earlier that the PTS in luciferase was located at the carboxy terminus of the protein (Gould et al., 1987). To assess the generality of the COOH-terminal loca-

tion of PTSs in peroxisomal proteins, we created gene fusions encoding proteins containing the carboxy-terminal 27 amino acids of human catalase, 15 amino acids of rat H:D, 14 amino acids of pig DAAOX, or 15 amino acids of rat AOX added on to the carboxy terminus of a cytosolic protein (CAT, DHFR, or a mutant luciferase). The observation that each of the fusion proteins is targeted to peroxisomes demonstrates that the carboxy terminus of each of these peroxisomal proteins contains a PTS. We have now identified five PTSs that are active in mammalian cells. None are greater than 27 amino acids long and each is found at the extreme carboxy terminus of the protein. They represent the only carboxy-terminal targeting signals identified that are involved in the transport of proteins across a lipid bilayer. The location of the PTS implies that these proteins must be imported into the organelle posttranslationally. This is consistent with previous observations of posttranslational import of peroxisomal proteins into the organelle (Goldman and Blobel, 1978; Fujiki and Lazarow, 1985; Small and Lazarow, 1987; Small et al., 1988).

The Nature of the PTS in Peroxisomal Proteins

In Table I we have listed carboxy-terminal sequences of several proteins known to reside in peroxisomes, including those in which a PTS has been identified. The only feature shared by the five PTSs we have studied is the presence of the tripeptide Ser-Lys/His-Leu somewhere in the sequence. This homology is striking and raises the possibility that this peptide is is an important element of at least one class of PTSs. Our result with the mutant H:D-N clone, in which this tripeptide is altered by replacement of the Lys residue with an Asn, adds support to the hypothesis that this tripeptide may play an essential role in the activity of these PTSs. Recent mutagenesis results which show that the Ser-Lys-Leu peptide in the PTS of luciferase is required for import reinforce this idea (Gould, S. J., G.-A. Keller, and S. Subramani, manuscript in preparation).

The Ser-Lys/His-Leu tripeptide is located at the extreme COOH terminus in firefly luciferase, rat AOX, rat H:D, and pig DAAOX. In catalase, however, the conserved tripeptide is located 10 amino acids from the COOH terminus. If this peptide is the PTS, then its location in catalase must mean that this sequence can function at an internal location, provided it is accessible for recognition by the sorting apparatus. An alternative possibility that cannot be ruled out at present is that catalase contains a different PTS present within its COOH-terminal 27 amino acids. An examination of the sequences of ~12 peroxisomal proteins reveals that the conserved sequence, or some conservative variant of it (Ser to Ala, Lys to His or Arg, Leu to Ile), is present within all of the peroxisomal sequences we have analyzed so far.

We have searched the Protein Identification Resource data bank (Bethesda, MD) for sequences that have the tripeptide Ser-Lys/His-Leu at their COOH terminus. 15 proteins were identified that end in this tripeptide. Of these, four were of bacterial, mitochondrial, or chloroplast origin. Also, two of the proteins (pig DAAOX and rat H:D) studied in this report were identified in the search. The remaining eukaryotic members of this group consisted of six isolates of alpha-amylase (MacDonald et al., 1980; Hagenbuchle et al., 1980; Kluh, 1981; Nakamura et al., 1984; Pasero et al., 1986), a rat testis-specific basic protein (Kistler et al., 1975), carboxypeptidase I from barley (Sorensen et al., 1986), bombolitin V from bumblebee (Argiolas and Pisano, 1985), and nodulin-35 protein from soybeans (Nguyen et al., 1985). The subcellular location of the rat testis-specific protein is not known. Alpha-amylase, carboxypeptidase, and bombolitin V (or their precursors) enter the secretory pathway. If Ser-Lys/His-Leu does function as a PTS, why is it that these proteins are not found in the peroxisome? Since previous reports have shown that peroxisomal proteins are imported into the organelle posttranslationally (Goldman and Blobel, 1978; Fujiki and Lazarow, 1985; Small and Lazarow, 1987; Small et al., 1988), one possibility is that these proteins are recognized by the signal recognition particle for cotranslational translocation across the endoplasmic reticulum membrane and are destined to insert into this membrane before any putative PTS has been synthesized. This hypothesis predicts that signals for secretion of eukaryotic proteins take precedence over PTSs. The dominance of secretory signal sequences over those for nuclear transport, another sorting mechanism that is posttranslational, has already been demonstrated (Lee et al., 1987). An alternative possibility is that the sequence is cryptic and not used as a PTS in certain contexts, as has been observed for mitochondrial targeting

Table I. Carboxy-Terminal Sequences of Several Peroxisomal Proteins

Protein	Sequence	Reference
Firefly luciferase	Arg-Glu-Ile-Leu-Ile-Lys-Ala-Lys-Lys-Gly-Gly-Lys-*Ser-Lys-Leu*	de Wet et al., 1987
Pig DAAOX	Val-Leu-Glu-Glu-Arg-Asn-Leu-Leu-Thr-Met-Pro-Pro-*Ser-His-Leu*	Ronchi et al., 1982
Rat AOX	His-Glu-Ser-Tyr-His-Lys-His-Leu-Lys-Pro-Leu-Gln-*Ser-Lys-Leu*	Miyazawa et al., 1987
Rat peroxisomal H:D	Leu-Lys-Glu-Trp-Gln-Ser-Leu-Ala-Gly-Pro-His-Gly-*Ser-Lys-Leu*	Osumi et al., 1985
Human catalase	Asn-Ala-Glu-Lys-Pro-Lys-Asn-Ala-Ile-His-Thr-Phe-Val-Gln-Ser-Gly-*Ser-His-Leu*-Ala-Ala-Arg-Glu-Lys-Ala-Asn-Leu	Bell et al., 1987
Soybean uricase	His-Gly-Ser-Ile-Gln-Ala-Ser-Leu-Ser-Arg-Leu-Trp-*Ser-Lys-Leu*	Nguyen et al., 1985
Rat peroxisomal thiolase	Gly-Thr-Gly-Met-Gly-Ala-Ala-Ala-Val-Phe-Glu-Tyr-Pro-Gly-Asn	Hijikata et al., 1987
D. melanogaster xanthine dehydrogenase	Glu-Ile-Pro-Glu-Pro-Gly-Ser-Phe-Thr-Pro-Trp-Asn-Ile-Val-Pro	Keith et al., 1987
C. tropicalis PXP4	Glu-Arg-Phe-Glu-Lys-Ser-Asp-Glu-Thr-Ala-Ala-Ile-Leu-Ser-Lys	Okazaki et al., 1986
H. polymorpha methanol oxidase	Asn-Phe-Arg-Leu-Gly-Thr-Tyr-Glu-Glu-Thr-Gly-Leu-Ala-Arg-Phe	Ledeboer et al., 1985

The underlined amino acids have been shown to function as PTSs. The italicized amino acids represent the conserved Ser-Lys/His-leu tripeptide present at or near the carboxy terminus of several peroxisomal proteins and within all five PTSs we have identified. The last four proteins do not contain this three-amino acid sequence at their COOH terminus but they do contain it (or a conservative variant) at an internal location.

signals (Hurt and Schatz, 1987). The last member of the proteins terminating in Ser-Lys/His-Leu is the soybean nodulin-35 (soybean uricase). Nguyen et al. (1985) have demonstrated that this protein is indeed located within the peroxisomes. Thus, the subcellular location of the proteins discussed above is not inconsistent with the hypothesis that the sequence Ser-Lys/His-Leu can serve as a PTS.

Recently, Small et al. (1988) identified two internal PTSs within PXP4, one of two AOXs of *C. tropicalis* (Okazaki et al., 1986). They reported that proteins containing amino acids 1-118 or 309-427 of the PXP4 protein fused to the amino terminus of DHFR are imported into yeast peroxisomes in vitro (PXP4 is 709 amino acids long). Though the POX-4 gene (encoding PXP4) and the rat AOX gene share a certain degree of homology (Miyazawa et al., 1987), neither of the putative PTSs of POX-4 contain a tripeptide similar to the one we have found to be a common constituent of higher eukaryote PTSs.

At least two hypotheses could account for the difference between the types of PTSs we have isolated and those identified by Small et al. (1988). One is that the mechanism by which proteins enter peroxisomes has, through evolution, diverged to such an extent that there is no longer structural (and therefore functional) homology between the PTSs active in yeast and those active in higher eukaryotes. Another possibility is that there are at least two (and possibly more) pathways by which proteins can enter peroxisomes, either in yeast or in mammalian systems. If this were the case, structurally different types of PTSs (such as those we have identified and those presented by Small et al. [1988]) would be expected. We favor the latter of these hypotheses for two reasons. First, the signals that direct proteins into the secretory pathway (Briggs and Gierasch, 1986), the nucleus (Kakidani and Ptashne, 1988; Webster et al., 1988; Lech et al., 1988), and the mitochondria (Cheng et al., 1987) have each been shown to function in both yeast and mammalian cells. This indicates that protein-sorting mechanisms in general have been highly conserved through evolution. A priori, one would expect the same to be true for peroxisomal import. Second, when expressed in yeast (*S. cerevisiae*), luciferase was transported to a peroxisome-like organelle, suggesting that this higher eukaryote peroxisomal protein contains sufficient information to direct it to peroxisomes in yeast (Keller, G.-A., S. J. Gould, M. Schneider, M. DeLuca, S. Howell, and S. Subramani, unpublished observations). Though our results indicate that a higher eukaryote peroxisomal protein (luciferase) can be imported into mammalian, insect, plant, and yeast peroxisomes, it is possible that the converse may not hold and yeast peroxisomal proteins may not be competent for import into higher eukaryote peroxisomes. It will be of great interest to see whether the POX-4-DHFR proteins imported into yeast peroxisomes in vitro can be imported into peroxisomes of mammalian cells in vivo.

Even though the Ser-Lys/His-Leu tripeptide may be the essential element of at least one type of PTS, it is clear that this alone is not always sufficient to direct proteins to peroxisomes. The evidence for this is twofold. First, there are many eukaryotic proteins which contain a Ser-Lys/His-Leu somewhere in their sequence but are not transported to peroxisomes (>100 such proteins were identified in the Protein Identification Resource protein sequence data bank). Second, we have observed that several fusion proteins which contain a PTS (as identified in other gene fusion experiments) are nevertheless not transported into perosixomes. Examples included in this report were the two luciferase–catalase fusions that were not transported to peroxisomes (L-HCAT404 and L-HCAT145) even though they contained the sequences encoding the PTS of catalase. Similar results have been documented previously (Gould et al., 1987). These findings are consistent with a model in which the ability of a PTS to function is dependent on the accessibility of the targeting signal within the three-dimensional configuration of the protein. Similar properties have been documented for the nuclear targeting signal of SV-40 large T antigen (Roberts et al., 1987).

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