Identification of a Peroxisomal Targeting Signal at the Carboxy Terminus of Firefly Luciferase

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Abstract. Translocation of proteins across membranes of the endoplasmic reticulum, mitochondrion, and chloroplast has been shown to be mediated by targeting signals present in the transported proteins. To test whether the transport of proteins into peroxisomes is also mediated by a peptide targeting signal, we have studied the firefly luciferase gene that encodes a protein transported to peroxisomes in both insect and mammalian cells. We have identified two regions of luciferase which are necessary for transport of this protein into peroxisomes. We demonstrate that one of these, region II, represents a peroxisomal targeting signal because it is both necessary and sufficient for directing cytosolic proteins to peroxisomes. The signal is no more than twelve amino acids long and is located at the extreme carboxy-terminus of luciferase. The location of the targeting signal for translocation across the peroxisomal membrane therefore differs from the predominantly amino-terminal location of signals responsible for transport across the membranes of the endoplasmic reticulum, chloroplast, or mitochondrion.

THE compartmentalization of biochemical functions in eukaryotic cells requires mechanisms capable of directing the flow of macromolecules to their proper destinations either within or outside the cell. Because these mechanisms play such critical roles, much attention has been focused on the targeting of proteins to intracellular organelles and the secretory pathway. The machinery dedicated to protein targeting must be able to recognize proteins destined for a particular organelle and also aid their transport into the organelle. Whereas part of the selectivity of this process must reside in the translocating apparatus itself, numerous studies have shown that the proteins themselves have targeting signals that act in concert with the sorting machinery (for reviews see Blobel, 1980; Schmidt and Mishkind, 1986; Douglas et al., 1986; von Figura and Hasilik, 1986). Signals responsible for directing proteins to the endoplasmic reticulum/secretion pathway (Kreil, 1981), mitochondria (Schatz and Butow, 1983), chloroplasts (Schmidt and Mishkind, 1986), the nucleus (Kalderon et al., 1984), and for retention in the endoplasmic reticulum (Munro and Pelham, 1987) reside in the polypeptide sequence of the protein, whereas transport to lysosomes is mediated by phosphorylated mannose residues that are added during posttranslational modification of the polypeptide chain (von Figura and Hasilik, 1986). Of the various transport processes, only those which target proteins to the endoplasmic reticulum/secretion pathway, mitochondria, chloroplasts, or peroxisomes require the translocation of proteins across a lipid bilayer. Though much is known about the sequences that guide proteins to the first three of these organelle systems, little information is available regarding signals that target proteins to peroxisomes.

Fortunately, a substantial amount of information does exist regarding the biogenesis of peroxisomal proteins, much of which has important implications for the mechanism of protein translocation into peroxisomes (for review see Lazarow and Fujiki, 1985). Several studies have demonstrated that peroxisomal proteins are synthesized on free polysomes and that their import into the organelle occurs posttranslationally (Goldman and Blobel, 1978; Fujiki et al., 1984; Miura et al., 1984) and without any detectable modification of the protein (Robbi and Lazarow, 1982; Fujiki et al., 1984).

In a previous paper, we demonstrated that firefly (*Photinus pyralis*) luciferase (*Photinus*-luciferin:oxygen 4-oxidoreductase [decarboxylating, ATP-hydrolyzing], EC 1.13.12.7), which catalyzes a light-producing bioluminescent reaction, is localized to peroxisomes in cells of the firefly lantern organ (Keller et al., 1987). When the cDNA encoding luciferase (de Wet et al., 1987) is expressed in mammalian cells, the enzyme is found in the peroxisomes of these cells as well, indicating a high degree of evolutionary conservation in the mechanism that sorts peroxisomal proteins (Keller et al., 1987). This in vivo model system is ideally suited for the identification of sequences involved in peroxisomal targeting because altered versions of the firefly luciferase gene, for which there is no mammalian homologue, can be introduced into mammalian cells and the resultant polypeptides easily localized by immunofluorescence. In the experiments presented in this paper we have used deletions, linker insertions, and gene fusions to identify regions of the luciferase protein involved in its transport to peroxisomes. We show that alterations in either of two regions of luciferase abolish its transport into peroxisomes. One of these regions, com-

prising a short sequence of amino acids at the extreme carboxy-terminus of luciferase, acts as a peroxisomal targeting signal because it is both necessary and sufficient for the targeting of heterologous cytosolic proteins to peroxisomes.

Materials and Methods

Reagents

The guinea pig antibody against firefly luciferase has been previously described (Keller et al., 1987). The rabbit antibodies against bovine catalase and mouse dihydrofolate reductase (DHFR)[1] were gifts from A. Schram and G. Schatz, respectively. The chloramphenicol acetyltransferase (CAT)-2 mAb-producing cell line was obtained from C. Gorman. The 12- and 10-bp oligodeoxynucleotide linkers and the Hind III termination-codon linker were gifts from D. Donoghue.

Plasmids

The plasmids pRSVL and pSV2L, which contain the wild-type luciferase gene under the transcriptional control of the Rous Sarcoma Virus long-terminal repeat and SV-40 early region, respectively, are described elsewhere (de Wet et al., 1987). pSV2LN2, pSV2LN6, pSV2LN7, and pSV2LN8 are derivatives of pSV2L in which the second, sixth, seventh, and eighth Taq I sites within the luciferase gene, respectively, were converted to Nru I sites. This was accomplished by filling in the ends of the DNAs linearized with Taq I with the Klenow fragment of polymerase I and religating the blunt ends. pSV2LNX7 and pSV2LNX8 were created by the insertion of 10-bp Xho I linkers (GCCTCGAGGC) into the Nru I sites of pSV2LN7 and pSV2LN8, respectively, so as to restore the proper translational reading frame.

The luciferase deletion mutants were made as follows. ΔN11 was created by digesting pRSVL with Hind III and Nar I, making the ends flush using the Klenow polymerase and inserting a Hind III initiation-codon linker (CCATCGTAAGCTTACGATGG) onto the ends before transformation of *Escherichia coli* with the DNA. ΔN16 was created in a similar manner except that digestion of pRSVL was with Hind III and Xba I and the Hind III initiation-codon linker was 4 bp longer (CCCCATCGTAAGCTTACGATGGGG). ΔN58 was created by digesting pSV2LN2 with Hind III and Nru I, converting the Hind III site to a blunt end and religating the ends together before transformation of *E. coli* with the DNA. This clone utilizes an internal ATG, which is normally amino acid 59 of luciferase, for initiation of protein synthesis. ΔC12 was created by the insertion of a Hind III termination-codon linker (TCAATCAGTCAAGCTTGACTGATTGA) at the fourth Xho II site of luciferase (nt 1913) after filling in the ends with Klenow polymerase, resulting in termination of the protein at a site 36 bp upstream from the stop codon of luciferase. The mutant ΔC20 was made by the insertion of the same termination-codon linker at the eighth Taq I site of luciferase (nt 1892), 60 bp upstream from the stop codon, after filling in the site using Klenow polymerase.

Linker-insertion mutations were created in the following manner. A plasmid containing the luciferase gene was partially digested with one of the following restriction enzymes: Alu I, Dpn I, FnuD II, Hae III, Rsa I, or Taq I. After digestion, linear DNA cleaved only at one site was purified. The DNA resulting from Taq I digestion was further treated with Klenow polymerase to make the ends blunt. Oligodeoxynucleotide linkers encoding either Bam HI (CGCGGATCCGCG), Eco RI (CCGGAATTCCGG), Hind III (CCCAAGCTTGGG), or Xho I (GCCTCGAGGC or GCACTCGAGTGC) recognition sites were then inserted between the ends of the DNAs, which were then used to transform *E. coli*. The length of linker was chosen so that the proper reading frame was maintained in each case. The various linker-insertion mutants obtained were analyzed by restriction mapping and named for the number of the amino acid of luciferase at the amino-terminal side of the insertion followed by the one-letter designations of the four amino acids that were inserted.

The gene fusion LL1 was created by inserting the 1,433-bp Hind III-to-Cla I fragment of pSV2L between the Hind III and Nar I sites of pRSVL. LL2 was constructed by inserting the 1,072-bp Xho I-to-Bam HI fragment of pSV2LNX7, encoding the carboxy-terminal 85 amino acids of luciferase, between the Xho I and Bam HI sites of pSV2LNX8, which lacks the region of luciferase coding for the last 20 amino acids of luciferase.

1. *Abbreviations used in this paper*: CAT, chloramphenicol acetyltransferase; DHFR, dihydrofolate reductase; LI, linker insertion.

CAT-luciferase gene fusions were made as follows. A 664-bp Hind III-to-Sca I fragment contains the entire coding region of CAT except for the carboxy-terminal nine amino acids. CAT-LC104 was constructed by inserting the above CAT fragment between the Hind III and Eco RV sites of pSV2L. The CAT fragment was also inserted between the Hind III and Nru I sites of pSV2LN6 to create CAT-LC95 and between the Hind III and the seventh Dpn I sites of the luciferase gene in pSV2L to make CAT-LC12. DHFR-LC104 and DHFR-LC95 were constructed as follows. pSV2DHFR (Subramani et al., 1981) was partially digested with Acc I, the ends blunted using Klenow polymerase, and the DNA was then digested with Hind III. The resultant 618-bp fragment contains the entire DHFR coding sequence except for the carboxy-terminal five amino acids. This DNA was inserted between the Hind III and Eco RV sites of pSV2L to create DHFR-LC104 and between the Hind III and Nru I sites of pSV2LN6 to create DHFR-LC95.

Cell Lines and Transfections

Conditions for growth of CV-1 monkey cells and the procedure for DNA transfection were as described (Keller et al., 1987).

Immunofluorescence

Cells transfected with the deletion and linker-insertion mutants of luciferase, as well as the luciferase-luciferase gene fusions, were processed for double indirect immunofluorescence as previously described (Keller et al., 1987). In this procedure, fixed cells were first labeled with a guinea pig antibody that recognizes the firefly luciferase and a rabbit antibody against bovine catalase followed by a fluorescein-conjugated goat anti-guinea pig IgG antibody and a rhodamine-conjugated goat anti-rabbit IgG antibody. The CAT-luciferase gene fusions were analyzed by treating the cells transfected with these fusion genes in the same manner as just described except that a monoclonal anti-CAT antibody was used in place of the guinea pig anti-luciferase antibody and the secondary labelings were done with a fluorescein-conjugated goat anti-mouse IgG antibody. The culture medium in which the CAT-2 cell line was grown was used as the source of the anti-CAT antibody. The wild-type DHFR and the DHFR-luciferase fusions were localized with a rabbit anti-DHFR antibody followed by a rhodamine-conjugated goat anti-rabbit IgG antibody. Because the anticatalase and the anti-DHFR antibodies were both raised in rabbits, the co-localization of the DHFR-luciferase fusions and catalase was accomplished using the guinea pig anti-luciferase antibody which recognizes the carboxyterminal portion of the protein.

Results

Deletions at Either End of Luciferase Abolish Peroxisomal Localization

The firefly luciferase is transported to peroxisomes in both insects and mammalian cells (Keller et al., 1987). With the aim of identifying regions of this protein involved in its import into peroxisomes, we constructed mutant forms of the luciferase gene. The plasmids pRSVL and pSV2L (de Wet et al., 1987) were used for the construction of deletions in the luciferase gene. Both wild-type and mutant genes (Fig. 1 *A*), under the transcriptional control of the Rous Sarcoma Virus long-terminal repeat were transfected into CV-1 cells which were subsequently processed for double indirect immunofluorescence using anticatalase and antiluciferase antibodies. In a previous paper (Keller et al., 1987), colocalization of catalase, a peroxisomal matrix protein, and wild-type luciferase was used to demonstrate the peroxisomal location of luciferase, and this same assay was used to differentiate peroxisomal from nonperoxisomal localization in this report. For comparison, we again show the peroxisomal localization of luciferase (Fig. 1 *B*) which is superimposable on the distribution of catalase (Fig. 1 *C*). The removal of either 12 (ΔC12) or 20 (ΔC20) amino acids from the carboxy-terminus of the protein caused these mutant proteins to re-

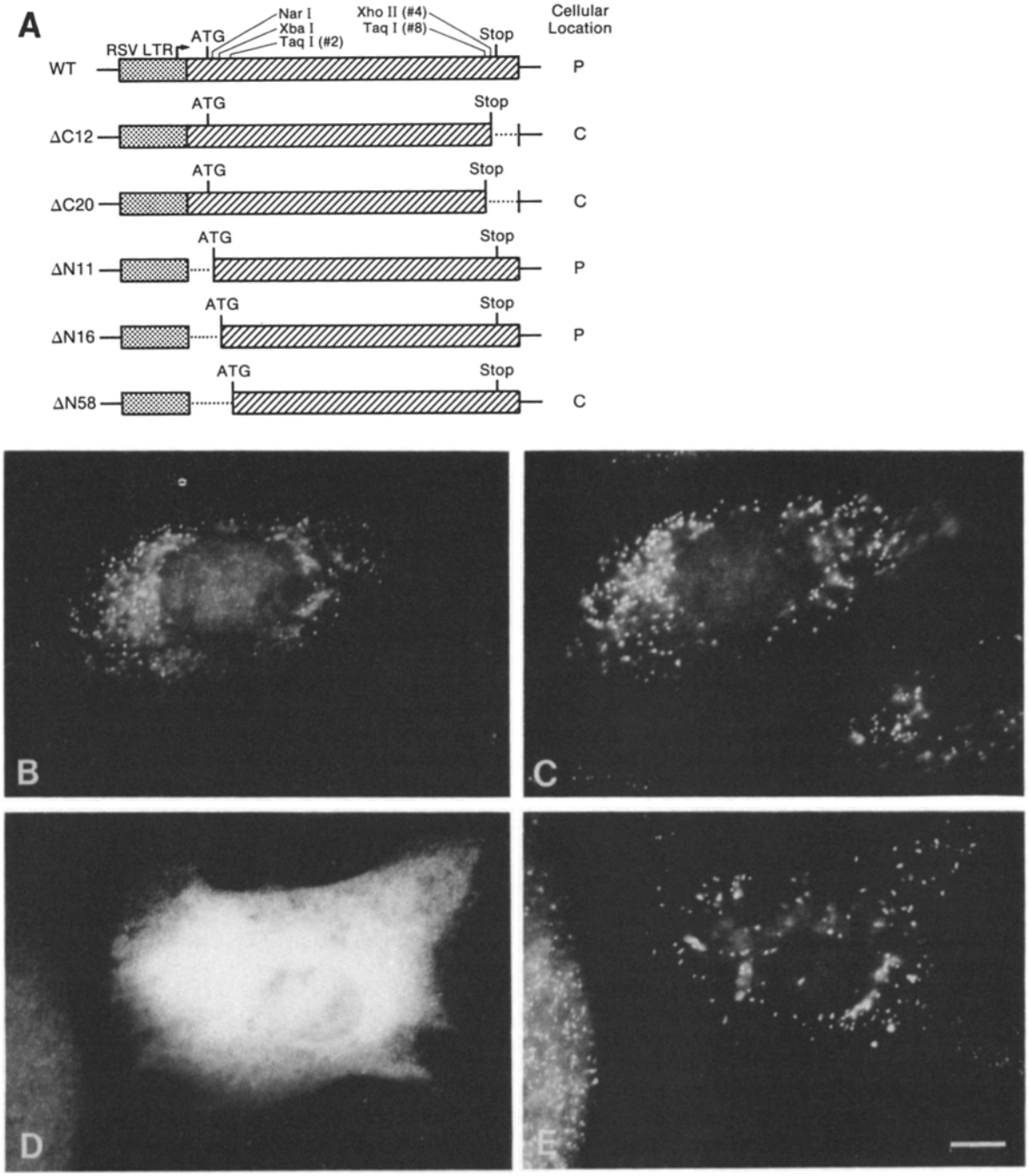

Figure 1. Deletions at both ends of the luciferase gene abolish peroxisomal targeting of luciferase. (*A*) Wild-type and mutant luciferases. Hatched portion is the luciferase gene; dotted lines represent regions deleted from pRSVL; stippled box is the RSV-LTR; and thin line denotes the plasmid sequences. Relevant restriction enzyme sites used for the construction of the mutants are shown. ΔN and ΔC represent deletions from the amino- and carboxy-terminus of the protein, respectively. Numerals refer to number of amino acids removed from that end of the protein. Subcellular localization of each mutant is indicated on the right. (*B* and *C*) Distribution of fluorescein and rhodamine labels showing localization of luciferase and catalase, respectively, in cells transfected with wild-type luciferase. (*D* and *E*) Distribution of fluorescein and rhodamine labeling depicting the cytoplasmic distribution of the mutant ΔC12 relative to the peroxisomal marker catalase. Bar, 10 μm.

main in the cytoplasm rather than being sorted to peroxisomes. The immunolocalization of the mutant ΔC12 is shown in Fig. 1 *d* and the catalase labeling of the same cell is represented in Fig. 1 *e*. The same type of localization was observed for mutant ΔC20 (data not shown). Other mutants (ΔC85 and ΔC95) that lacked the last 85 or 95 amino acids of luciferase were also not peroxisomal (data not shown). In contrast, the removal of either 11 (ΔN11) or 16 (ΔN16) amino acids from the amino-terminus of luciferase had no apparent effect on its transport to peroxisomes because the mutant proteins had the same immunofluorescence staining pattern as the wild-type protein (data not shown). However, when 58

amino acids were deleted from the amino-terminus of luciferase (ΔN58), the protein was not transported to peroxisomes and was found instead in the cytoplasm of the cell (data not shown).

Two Regions of Luciferase Are Necessary for Its Transport to Peroxisomes

The necessity of other sequences for the transport of luciferase to peroxisomes was investigated using a set of linker-insertion mutations. These were created by inserting oligodeoxynucleotide linkers into restriction enzyme sites within the luciferase gene such that they would add four amino acids to the 550-amino acid luciferase protein while maintaining the proper translational reading frame. After transfection of the mutants into CV-1 cells, each was assayed for the subcellular location of the encoded protein by double indirect immunofluorescence as described for the analysis of the deletion mutants. Peroxisomal localization was typified by the staining pattern exhibited by the wild-type protein (Fig. 1 *B*) while cytoplasmic distributions were identical to that of the deletion mutant ΔC12 (Fig. 1 *D*). The names of the linker insertion (LI) mutants represent the amino acid position upstream of the insertion site, followed by the one-letter designations of the added amino acids. The data generated by analysis of the LI mutants are presented in Table I. Every mutation that resulted in a four-amino acid insertion between amino acids 47 and 262 prevented transport to peroxisomes with the exception of the mutant LI-105-QAWA. It should be noted that another LI mutant introducing a different set of four amino acids at the same site (LI-105-GIPA) did abolish peroxisomal import. These mutants define a region that represents almost the entire amino-terminal one-half of the protein (amino acids 47-261). All LI mutations located in the sequence COOH-terminal to this region, including one with an insertion 12 amino acids from the carboxy-terminus (LI-538-STRV), had no effect on the peroxisomal localization of luciferase. These data, together with those from the deletion analysis demonstrating the importance of carboxy-terminal and amino-terminal sequences, reveal that there are two regions of luciferase necessary for the localization of the protein to peroxisomes, a large one spanning much of the NH_2-terminal half of the protein (amino acids 47-261) which we refer to as region I, and a smaller one consisting of 12 or fewer amino acids at the extreme COOH-terminus, designated region II. Because the targeting signals involved in the transit of proteins into other organelles are relatively short polypeptides 10–30 amino acids in length, we felt it unlikely that region I, which is more than 200 amino acids long, would be the peroxisomal targeting signal. For this reason we focused our attention on the role that region II plays in the targeting of luciferase to peroxisomes.

Table I. Linker Insertion Mutants of Luciferase and Their Subcellular Location

Plasmid	Subcellular location
LI-12-ASRP	P
LI-47-GLEA	C
LI-52-GIPD	C
LI-53-ARIR	C
LI-57-GLEA	C
LI-105-QAWA	P
LI-105-GIPA	C
LI-164-GIPD	C
LI-187-PSLA	C
LI-261-PRGR	C
LI-320-ASRP	P
LI-423-QAWA	P
LI-446-CTRV	P
LI-456-ASRP	P
LI-465-GLEA	P
LI-508-QAWA	P
LI-508-GIPA	P
LI-513-RNSG	P
LI-522-RNSG	P
LI-538-STRV	P

The linker-insertion mutants of luciferase have been designated by the amino acid at their site of insertion and the one-letter code of the amino acids that are inserted. For example, LI-105-GIPA is a mutant containing the residues glycine, isoleucine, proline, and alanine inserted between amino acids 105 and 106 luciferase.

Carboxy-Terminal Region of Luciferase Redirects a Cytoplasmic Luciferase Mutant to Peroxisomes

If region II contained a peroxisomal signal sequence, deletions of this region should have resulted in nonperoxisomal proteins, as we have shown. In addition, we reasoned that it should be possible to redirect these mutant proteins to peroxisomes by the addition of sequences encoding region II onto their COOH-termini. Two gene fusions were constructed (Fig. 2 *A*) in which pieces of the wild-type luciferase gene that contained region II were fused in frame to certain deletion mutants of luciferase that lacked region II. The first of these, LL1, has almost the entire luciferase coding sequence (amino acids 13–550), including region II, fused at the COOH-terminus of a luciferase mutant lacking sequences that code for the last 95 amino acids of the protein. We have classified the protein produced by this fusion gene as cytoplasmic (Fig. 2, *B* and *C*), though in a small percentage of expressing cells a weak peroxisomal staining pattern was detected, suggesting inefficient transport of this fusion protein into peroxisomes. The fusion LL2 provided more convincing results. LL2 consists of the last 85 amino acids of luciferase tagged to the end of a luciferase gene lacking sequences encoding the carboxy-terminal 20 amino acids of the protein. This fusion was efficiently transported to peroxisomes (Fig. 2 *D*), as evidenced by its colocalization with catalase in the same cell (Fig. 2 *E*). This result demonstrates that a segment of luciferase contained within its last 85 amino acids was capable of redirecting a cytoplasmic luciferase mutant to peroxisomes. In these experiments we tried to redirect cytoplasmic luciferase mutants lacking region II back to peroxisomes by the addition of segments containing region II of luciferase. This was possible in one instance, with LL2, and indicated that the initial defect in targeting was due to the loss of a necessary, replaceable function rather than a nonspecific effect of the deletion.

Carboxy-Terminal 12 Amino Acids of Luciferase Target Bacterial Chloramphenicol Acetyltransferase to Peroxisomes

The definitive test of a targeting signal is whether it directs

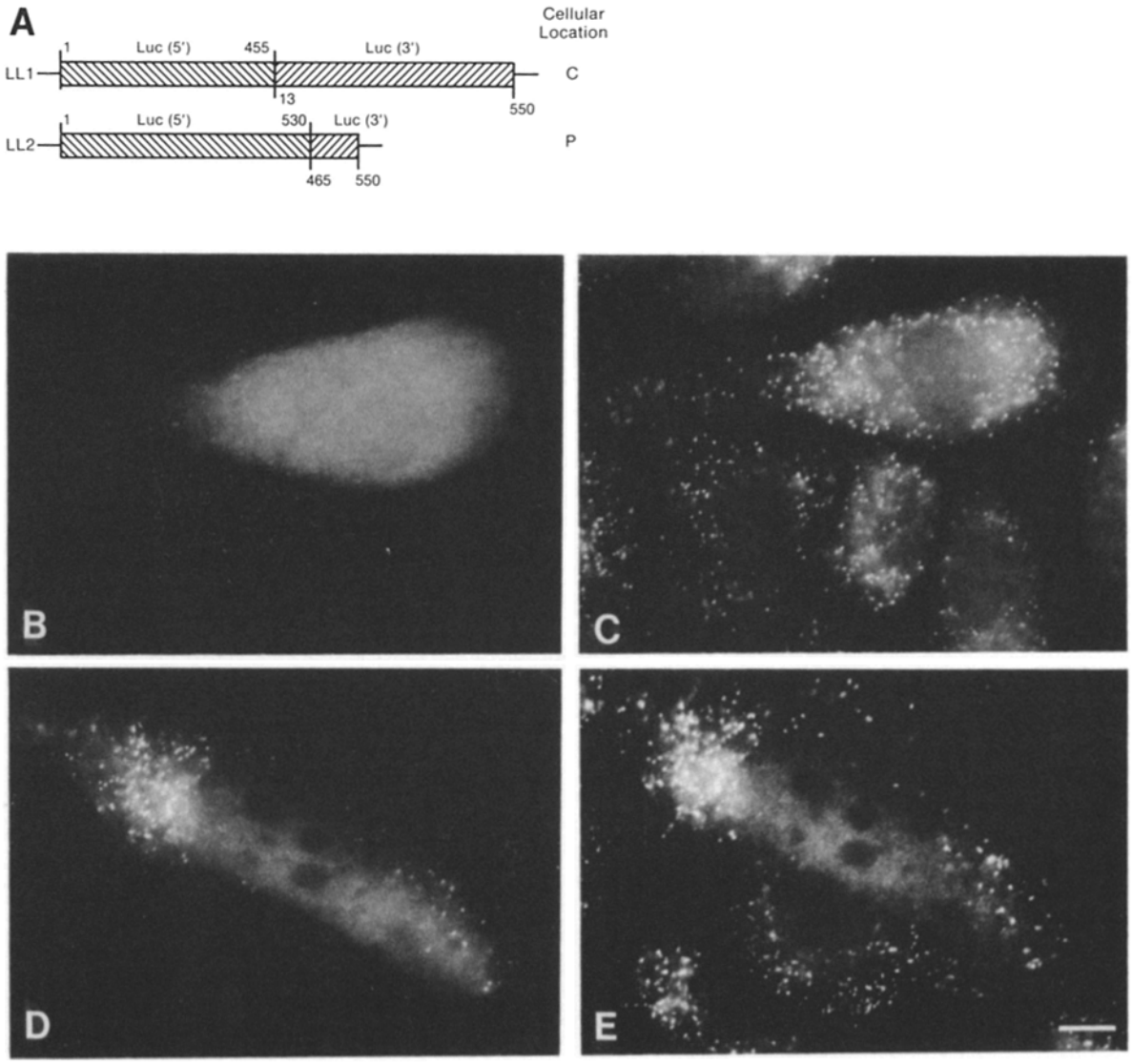

Figure 2. Luciferase–luciferase fusions and their subcellular localization. (*A*) The structure of the two luciferase–luciferase fusions. Only the relevant coding regions are presented. Portions shaded by hatching patterns represent the amino- and carboxy-terminal segments of the luciferase gene used in the fusions. The amino acids corresponding to these segments are shown above and below the diagrams. To the right, the subcellular location of each protein is noted as *C* for cytoplasmic or *P* for peroxisomal. (*B* and *C*) Subcellular localization of the LL1 fusion protein and catalase, respectively, in cells transfected with the LL1 fusion. (*D* and *E*) Peroxisomal distribution of the LL2 fusion and catalase, respectively, in cells transfected with LL2. Bar, 10 μm.

the transport of a heterologous protein to the organelle which it specifies. To apply this test to region II of luciferase, we constructed several hybrid genes in which the carboxy-terminus of luciferase was fused in-frame to the end of the bacterial CAT gene. The CAT gene has been commonly used in eukaryotic cells as a reporter gene for studying transcriptional regulatory regions (Gorman et al., 1982*b*), and it has been shown that the gene codes for a protein that is cytoplasmic when expressed in mammalian cells (Gorman et al., 1982*a*).

The structures of these CAT-luciferase fusions are outlined in Fig. 3. After transfection of the plasmids into CV-1 cells, these fusions were assayed for their subcellular location by double indirect immunofluorescence using the same procedure as before except that a monoclonal anti-CAT antibody was used instead of an antiluciferase antibody. The peroxisomes were visualized using an anticatalase antibody, and peroxisomal transport was shown by the colocalization of the CAT and catalase markers (see Methods).

Fig. 4, *A* and *B* confirms the cytoplasmic distribution of the wild-type CAT protein. All CAT-luc fusion proteins in which region II was added to the COOH-terminus of CAT were transported to peroxisomes. Fusion proteins consisting of either the COOH-terminal 104 (CAT-LC104), 95 (CAT-LC95), or 12 (CAT-LC12) amino acids of luciferase appended to the carboxy-terminus of CAT all colocalized with catalase in peroxisomes (Fig. 4, *C–H*). We conclude that a segment of luciferase contained in its last 12 amino acids is both necessary and sufficient to direct a protein to peroxisomes.

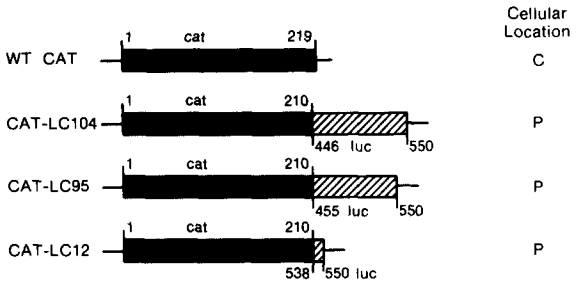

Figure 3. Structure of the CAT-luciferase fusion genes. The CAT and luciferase sequences are designated by the solid and hatched bars, respectively. Numerals above each bar refer to the amino acid positions of CAT at the ends of the CAT sequences. The wild-type CAT gene encodes 219 amino acids and the fusions encode the first 210 before the luciferase sequences begin. Numerals below the bars represent the amino acid positions at the boundaries of the luciferase segment present in the fusion gene. Wild-type luciferase is 550 amino acids long and each fusion contains carboxy-terminal fragments of luciferase. Only the relevant coding regions of the constructs are shown. The subcellular localization of each fusion protein, *P* for peroxisomal or *C* for cytoplasmic, is noted on the right side of the figure.

Carboxy-Terminal Region of Luciferase Also Targets Mouse Dihydrofolate Reductase to Peroxisomes

To test further the ability of the COOH-terminus of luciferase to act as a peroxisomal signal sequence, we asked whether it could be used to transport a second cytoplasmic protein into peroxisomes. For this we chose the mouse DHFR gene that has previously been used to demonstrate the activity of mitochondrial signal sequences (Hurt et al., 1984, 1985; Horwich et al., 1985). In the studies just cited, amino-terminal mitochondrial targeting signals were fused to the amino-terminus of DHFR, whereas in our experiments carboxy-terminal fragments of luciferase were fused to the carboxy-terminus of DHFR. Utilizing a restricton enzyme site five amino acids from the COOH-terminus of DHFR, we fused portions of the luciferase gene encoding either 104 (DHFR-LC104) or 95 (DHFR-LC95) amino acids of luciferase onto the 3′ end of the DHFR gene (Fig. 5 *A*). Fig. 5 *B* shows the cytoplasmic distribution of wild-type DHFR. The peroxisomal localization of fusion DHFR-LC95 is shown in Fig. 5, *C–E*. The immunolabeling of fusion DHFR-LC104 demonstrated that it was peroxisomal also (data not shown). The results obtained with these DHFR-luciferase and the CAT-luciferase fusions provide compelling evidence for the existence of a peroxisomal targeting signal at the carboxy-terminus of luciferase.

Discussion

We have recently shown that the firefly luciferase is a peroxisomal enzyme when expressed in either mammalian or insect cells. To study the process of protein transport to peroxisomes we created a number of deletion mutations, LI mutations, and gene fusions using the firefly luciferase gene. Each of these constructs has been tested for peroxisomal transport. These experiments have allowed us to define two distinct regions of the protein, the structural integrity of each being necessary for the transport of luciferase to peroxisomes. One of these, region II, consists of 12 amino acids located at the extreme carboxy-terminus of luciferase and has the sequence Leu-Ile-Lys-Ala-Lys-Lys-Gly-Gly-Lys-Ser-Lys-Leu-COOH. To be defined as a peroxisomal signal sequence, a polypeptide segment must be both necessary and sufficient to direct peroxisomal transport. The following observations indicate that the COOH-terminal 12-amino acid segment of luciferase meets these criteria. First, deletions that remove this sequence from luciferase cause the protein to remain in the cytoplasm, demonstrating that it is necessary for transport. Second, a segment of luciferase containing region II is able to rescue a cytoplasmic mutant of luciferase. Third, when fragments of luciferase containing this segment are fused onto the COOH-termini of the heterologous cytoplasmic proteins CAT and DHFR, the fusion proteins are efficiently transported to peroxisomes.

Our analysis of mutant luciferases also identified a second region (region I) of the protein required for the transport of luciferase into peroxisomes. It begins ~50 amino acids from the amino-terminus and spans the amino-terminal half of the protein (amino acids 47–261). Mutations in this segment cause the proteins to remain in the cytoplasm even though they contain the intact peroxisomal targeting signal (region II). This was also the situation with the deletion ΔN58 and the fusion LL1. These results demonstrate that whereas the signal defined by region II is capable of directing proteins to peroxisomes, only some polypeptides are permissive for transport to peroxisomes. One possible explanation for this result is that these proteins may be insoluble and therefore not competent for transport. Alternatively, the mutations in these proteins may interfere with the folding or accessibility of the targeting signal or hinder the transit of the protein through the peroxisomal membrane. This interpretation is not without precedent. Though the nuclear targeting signal in SV40 T-antigen is capable of transporting a heterologous cytoplasmic protein (pyruvate kinase) to the nucleus (Kalderon et al., 1984), this ability has been found to be context dependent, because the nuclear location signal only functions properly when inserted into certain locations of this protein (Roberts et al., 1987). In addition, Hurt and Schatz (1987) have shown that the mouse DHFR, a cytoplasmic protein, contains a cryptic mitochondrial targeting signal which can be exposed through alteration of the gene to yield a protein that is transported to mitochondria.

The experiments presented in this paper have shown that the targeting signal of luciferase is contained within the sequence LIKAKKGGKSKL. We have searched for homologies to this sequence in several other peroxisomal proteins for which sequences are known: rat acyl-CoA oxidase (Miyazawa et al., 1987), catalase (Furuta et al., 1986), bifunctional hydratase:dehydrogenase (Osumi et al., 1985), thiolase (Arakawa et al., 1987), and the *Candida tropicalis* acyl-CoA oxidase genes (Okazaki et al., 1986). No significant homologies to this segment of luciferase could be detected. This is not surprising considering that no sequence homologies exist for secretory or mitochondrial targeting signals.

Peptide targeting signals have been shown to mediate the translocation of proteins across the membranes of the endoplasmic reticulum, mitochondria, and chloroplasts. We have presented evidence that suggests that protein trans-

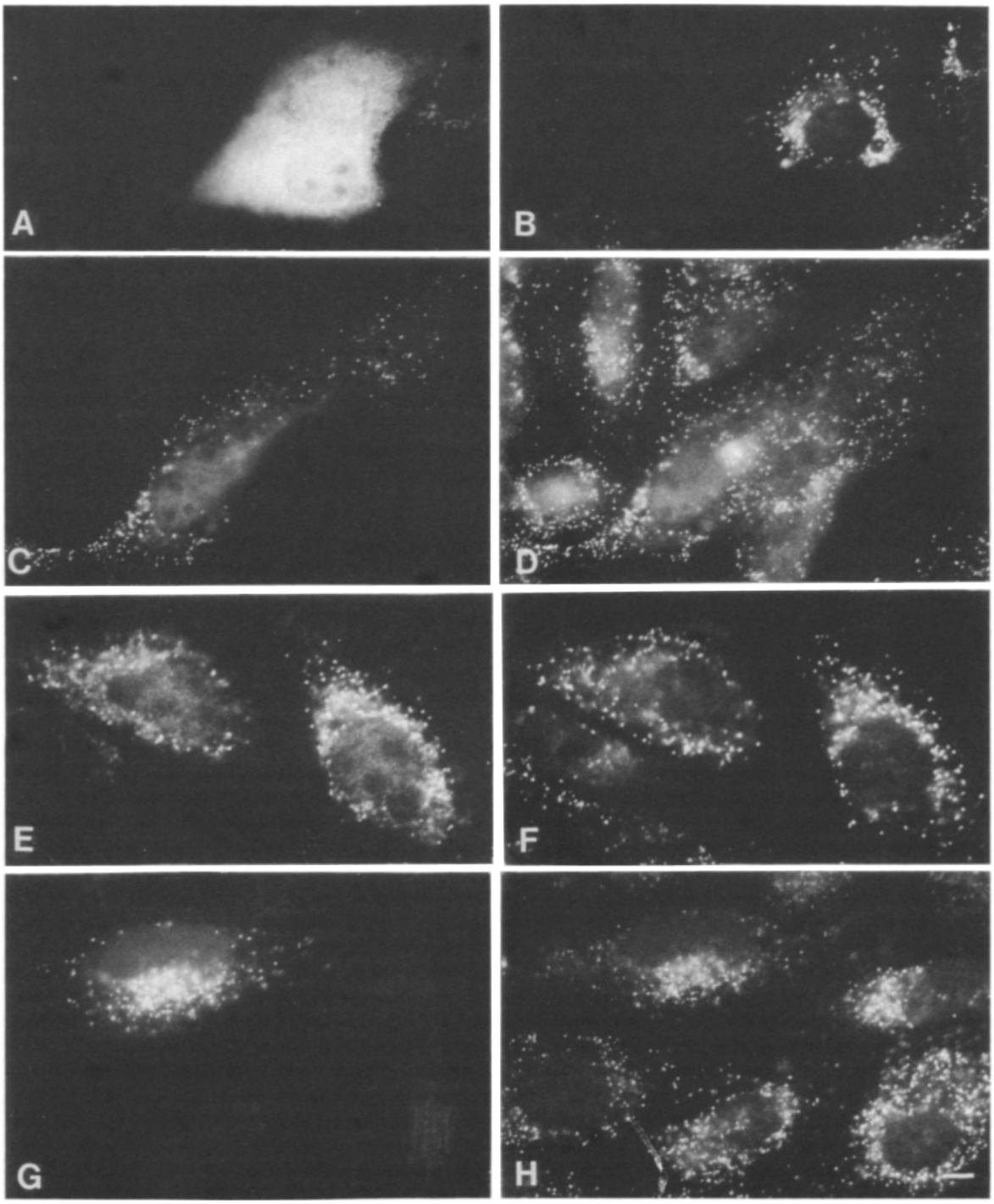

Figure 4. The CAT–luciferase fusions are transported to peroxisomes. Left column of panels shows the fluorescein staining of transfected cells. This represents the distribution of CAT or the various CAT–luciferase fusions, whereas the rhodamine stainings of the same fields presented in the right column show the distribution of catalase, and therefore peroxisomes, in the cells of the same field. (*A* and *B*) Cells transfected with wild-type CAT; (*C* and *D*) CAT-LC104; (*E* and *F*) CAT-LC95; and (*G* and *H*) CAT-LC12. Bar, 10 μm.

port into peroxisomes is also mediated by a peptide-targeting signal. The carboxy-terminal location of the peroxisomal targeting signal indicates that import of the protein into peroxisomes is obligatorily posttranslational, which is in agreement with previous results demonstrating the posttranslational import of proteins into peroxisomes (for review see Lazarow and Fujiki, 1986). Also, the location of the targeting signal at the COOH-terminus of the protein is unusual when compared with the typically amino-terminal location of targeting signals for other types of transmembrane translocation.

The results presented in this paper raise a number of questions. First, is the carboxy-terminal location of the peroxisomal targeting signal an absolute requirement for import

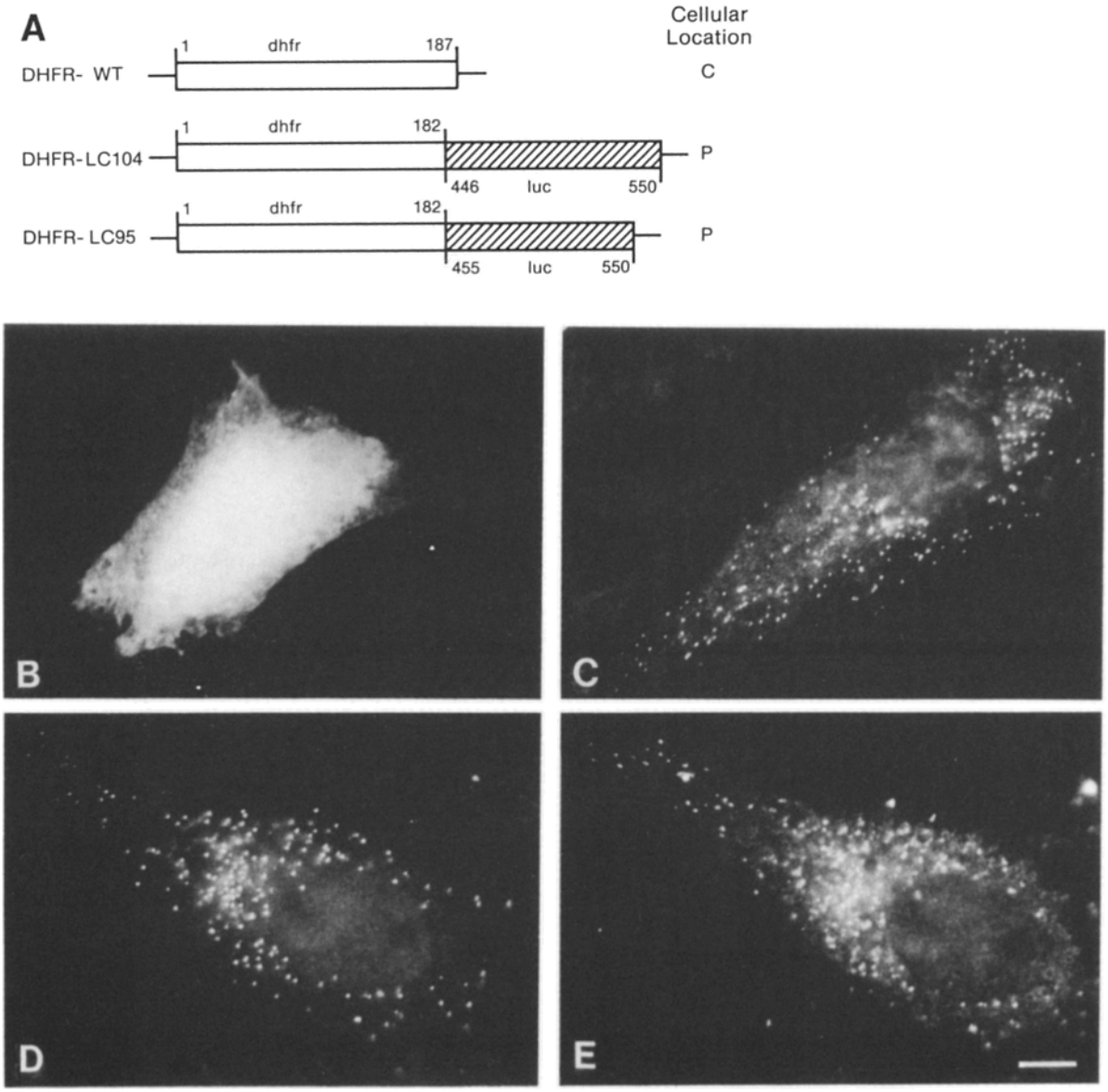

Figure 5. Fusion proteins between luciferase and DHFR are also transported to peroxisomes. (*A*) The coding regions of wild-type DHFR and the two DHFR-luciferase fusion proteins. To the right, cellular location of the proteins produced are indicated as either *P* for peroxisomal or *C* for cytoplasmic. Open and hatched boxes denote DHFR and luciferase sequences, respectively. The amino acids in the DHFR and luciferase segments are above and below each diagram. (*B*) Distribution of the rhodamine label showing the cytoplasmic distribution of DHFR in cells transfected with the wild-type gene. (*B* and *C*) Distribution of the DHFR–luciferase fusion and (*E*) catalase, respectively, in cells transfected with the fusion DHFR-LC95. The same type of staining pattern was observed when the fusion was detected using an anti-DHFR (*C*) or an antiluciferase antibody (*D*). Bar, 10 μm.

or is the targeting signal able to function when placed elsewhere within a protein? Second, though we have shown that the last twelve amino acids are sufficient to direct a heterologous protein to peroxisomes, is it possible that shorter peptides may act as a peroxisomal targeting signal? Third, which residues or features of this targeting signal are critical for its action? Further work will be directed toward addressing these questions and will hopefully shed more light on the process of peroxisomal protein translocation and the complex mechanisms that control the traffic of proteins in the eukaryotic cell.

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