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Firefly luciferase is targeted to peroxisomes in mammalian cells

(catalase/peroxisomal targeting/protein sorting)

GILBERT-ANDRE KELLER*, STEPHEN GOULD*, MARLENE DELUCA†, AND SURESH SUBRAMANI*‡

Departments of *Biology and †Chemistry, University of California, San Diego, La Jolla, CA 92093

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ABSTRACT **Although several enzymes known to reside in peroxisomes have been studied extensively, no cis-acting amino acid sequences involved in the transport of these proteins to peroxisomes have been described. As a first step towards the determination of a putative peroxisomal targeting sequence, we have expressed the cDNA encoding the firefly luciferase [*Photinus*-luciferin:oxygen 4-oxidoreductase (decarboxylating, ATP-hydrolyzing), EC 1.13.12.7] in monkey kidney cells and found that the product of the gene is transported to peroxisomes. Luciferase is derived from the firefly (*Photinus pyralis*) and is synthesized and stored in the cells of the firefly's lantern organ, where it is also found in peroxisomes. The fact that this protein is similarly targeted in cells from such different organisms suggests that the process of protein transport to peroxisomes has been highly conserved through evolution.**

The eukaryotic cell contains distinct organelles, each highly specialized for its particular functions. To maintain this organization, the cell must efficiently direct proteins to their proper subcellular locations. Previous studies dealing with the sorting of proteins to subcellular compartments have demonstrated the necessity of specific sequences or protein modifications for the transport of proteins to the endoplasmic reticulum (1, 2), lysosomes (3), chloroplasts (4), mitochondria (5), and the nucleus (6–8). Therefore, it is reasonable to assume that analogous cis-acting sequences are involved in the transport of peroxisomal proteins to peroxisomes. At present, virtually nothing is known about the signals that sort peroxisomal proteins.

We recently expressed the cloned firefly luciferase gene in CV-1 cells, a monkey kidney cell line. In transfected cells, the gene product was shown by indirect immunofluorescence to be present in small vesicular structures (9). In this paper, we show by double-immunofluorescence experiments that firefly luciferase [*Photinus*-luciferin:oxygen 4-oxidoreductase (decarboxylating, ATP-hydrolyzing), EC 1.13.12.7] is transported into the peroxisomes of transfected mammalian cells. We also demonstrate by immunocryoelectron microscopy that luciferase is localized within peroxisomes in the cells of the firefly lantern. Since luciferase is not endogenous to mammalian cells, expression of firefly luciferase and altered derivatives of the gene in mammalian cells should provide a valuable model system for future studies on the transport and uptake of proteins into peroxisomes.

MATERIALS AND METHODS

Vectors. The plasmid pRSVL, whose construction is described elsewhere (9), contains the full-length luciferase cDNA under the transcriptional control of the promoter from the Rous sarcoma virus (RSV) long terminal repeat (Fig. 1). At the 3′ end of the luciferase gene are the splicing and polyadenylylation sequences from the simian virus 40 (SV40) early region.

Transfections. CV-1 monkey kidney cells were plated onto coverslips placed in 10-cm dishes; 24 hr later, the cells were transfected by the calcium phosphate procedure of Parker and Stark (10). The coverslips were removed 36–48 hr after transfection, and the cells were used for immunofluorescence microscopy.

Production of Antibodies. Rabbit antibodies to bovine catalase and goat antibodies to rat catalase were gifts from A. Schram and P. Lazarow, respectively. Firefly luciferase was purified as described by Green and McElroy (11). Antibodies against the firefly luciferase were raised in rabbits and guinea pigs. All of the antibodies were purified by affinity chromatography with columns containing bovine catalase or firefly luciferase linked to AcA 22 (Pharmacia).

Affinity-purified, cross-adsorbed goat antibodies against rabbit or guinea pig IgG were prepared as in other studies (12). For immunofluorescence microscopy, secondary antibodies were conjugated to rhodamine or fluorescein by standard procedures. For immunoelectron microscopy, guinea pig antibodies against rabbit IgG were adsorbed on 5-nm and 12-nm gold adducts. Guinea pig antibodies against goat IgG were adsorbed on 12-nm gold adducts as described (13).

Immunofluorescence Microscopy. Forty-eight hours after transfection with pRSVL DNA, CV-1 cells plated on coverslips were fixed with 3% formaldehyde in phosphate-buffered saline (pH 7.2) for 15 min. They were then permeabilized with 1% Triton X-100 for 5 min, after which they were washed extensively with phosphate-buffered saline. The rabbit and guinea pig primary antibodies against catalase and luciferase were applied together at a concentration of 10 μg/ml each, and the mixture was incubated at room temperature for 10 min. The cells were washed again extensively in phosphate-buffered saline, and the rhodamine- and fluorescein-conjugated antibodies were added together at 10 μg/ml for 10 min. The cells were washed with phosphate-buffered saline and mounted in 90% glycerol/100 mM Tris, pH 8.5, containing 0.1% *p*-phenylenediamine as an antibleaching reagent. Immunofluorescence microscopy was performed on a Zeiss photoscope III microscope.

Immunocryoelectron Microscopy. Live fireflies were supplied by W. Biggley. The ventral surface of firefly lantern organs was dissected and fixed with 3% formaldehyde and 0.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 hr. Ultrathin frozen sections were prepared for cryoultramicrotomy as described by Tokuyasu (14). Immunolabeling and embedding of the frozen sections in acrylic resin LR-white (London Resin) have been described elsewhere (13). The sections were observed without poststaining in a Philips model 300 transmission electron microscope equipped with an 11-μm diameter aperture at a tension of 80 kV.

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Abbreviations: RSV, Rous sarcoma virus; SV40, simian virus 40.

‡To whom reprint requests should be addressed.

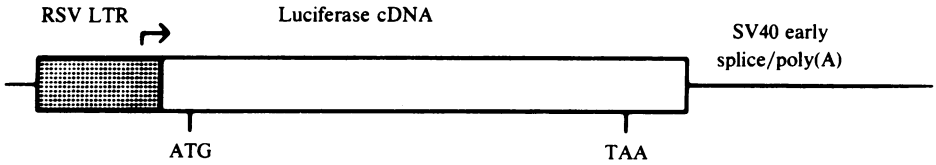

FIG. 1. The luciferase transcription unit in pRSVL. The luciferase cDNA is expressed from the RSV long terminal repeat (LTR). Other details of this plasmid are in ref. 9.

RESULTS

Previously we have expressed firefly luciferase from SV40 and RSV promoters in mammalian cells. This work established that luciferase could be used as a reporter gene to monitor promoter activity (9). Using immunofluorescence microscopy with anti-luciferase antibodies, we found that luciferase was localized to vesicular structures (Fig. 2*A*) when expressed in mammalian cells. The punctate immunofluorescence pattern was evident in all cells expressing luciferase. However, in some cells overproducing the enzyme, diffuse cytoplasmic fluorescence was detected in addition to the vesicular localization. Because the punctate pattern closely resembled the labeling pattern obtained with antibodies against catalase (G.-A.K., unpublished data), the major peroxisomal protein (15), it was of interest to determine whether the luciferase was targeted to and taken up by peroxisomes in the transfected cells.

Localization of Firefly Luciferase in CV-1 Cells by Double Immunofluorescence. CV-1 cells transfected with pRSVL were processed for double-immunofluorescence microscopy 48 hr after transfection. The results are shown in Fig. 3. Peroxisomes in the cells were visualized by immunolabeling with antibodies against bovine catalase (Fig. 3*B*). Immunolabeling for luciferase showed that few cells (in this picture only one) were expressing the gene product (Fig. 3*A*), a result expected in transient transfections. Close examination of the immunofluorescence patterns in transfected cells revealed that the vesicular structures labeled by anti-catalase and anti-luciferase staining were superimposable (Fig. 3 *C* and *D*). Since catalase is a peroxisomal enzyme, these results indicate that luciferase is transported to the peroxisomes of the transfected cells. In more than 100 transfected cells, the localization of luciferase was always peroxisomal. Furthermore, human hepatoma cells transfected with pRSVL also showed luciferase in peroxisomes (data not shown).

Localization of Luciferase in the Firefly Lantern Using Immunoelectron Microscopy. In view of the peroxisomal localization of luciferase in mammalian cells, we wished to determine whether luciferase was also peroxisomal in the firefly. The firefly lantern is comprised of specialized cells called photocytes, which contain large amounts of luciferase (11). Within the photocytes are large numbers of diaminobenzidine-positive granules known as photocyte granules (16). Immunoelectron microscopy experiments performed previously on thin plastic sections of photocytes from *Photuris sp.*, a closely related species, had indicated that luciferase was localized to the photocyte granules (17). However, these earlier studies could not assign luciferase unambiguously to peroxisomes because the diaminobenzidine reaction product was seen over the mitochondria and tracheoles as well as the photocyte granules. To resolve this issue and to show that the antibodies we used for the immunofluorescence studies of transfected cells were cross-reacting with the authentic firefly luciferase, electron microscopic localization of both catalase and luciferase was performed on frozen sections of *Photinus pyralis* lanterns.

The results of this experiment (Fig. 4*A*) showed the presence of catalase in single-membrane-bound structures within the photocytes. Two antibodies, one raised against rat catalase and the other against rabbit catalase, were able to recognize the insect catalase and showed that it was present within the photocyte granules. Since these granules contain catalase they are, by definition, peroxisomes (15). The double-immunolabeling experiment (Fig. 4*B*) showed that luciferase and catalase colocalized to these structures. This result provides unambiguous evidence that luciferase is localized to peroxisomes within the photocytes.

DISCUSSION

Though a variety of important metabolic pathways and as many as 40 different enzymes (18) have been identified in peroxisomes or in glyoxysomes from various sources, the mechanism by which proteins are targeted to these organelles remains an enigma. An examination of the amino acid

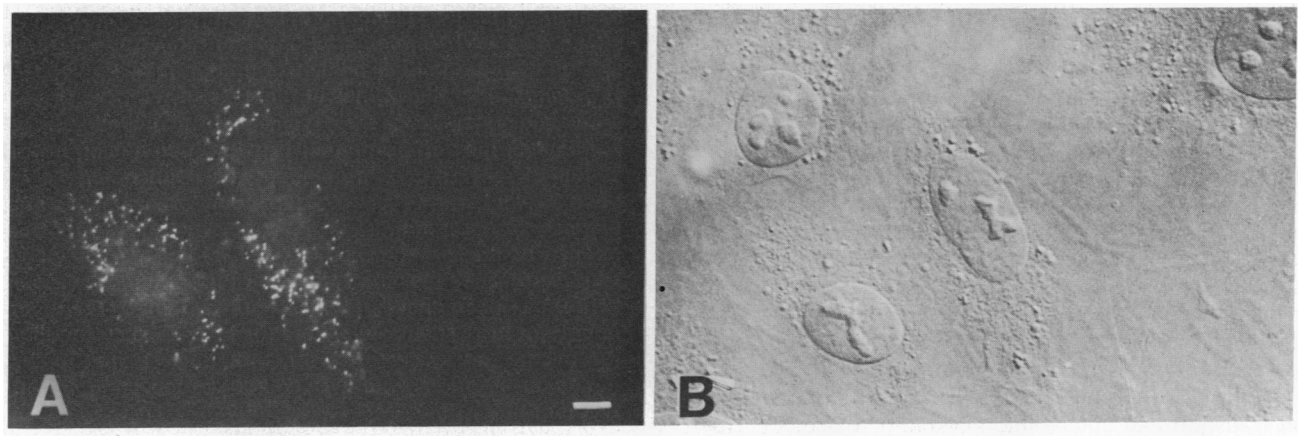

FIG. 2. Localization of luciferase in CV-1 cells transfected with pRSVL. (*A*) Single indirect immunolabeling with rabbit antibody against the firefly luciferase, followed by visualization with rhodamine-conjugated goat anti-rabbit IgG. Two cells in the field are positively labeled for luciferase, mainly in particulate structures scattered throughout the cytoplasm. (*B*) Micrograph of the same field using Nomarski optics. (Bar = 10 μm.)

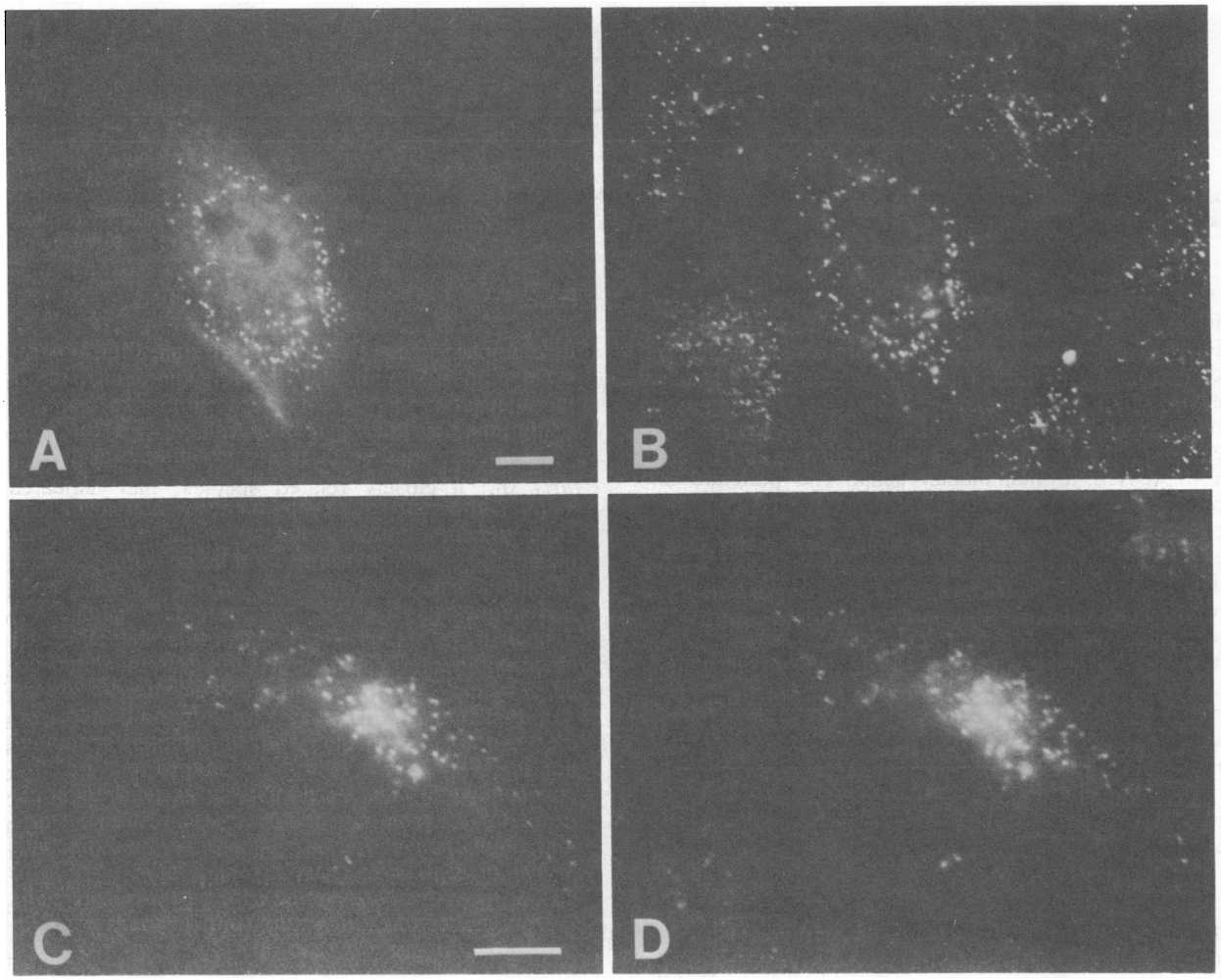

FIG. 3. Peroxisomal localization of luciferase by double immunofluorescence. CV-1 cells transiently transfected with pRSVL were processed for double indirect immunofluorescence as outlined. The cells were simultaneously labeled for luciferase (*A* and *C*) and catalase (*B* and *D*). The punctate pattern obtained with the antibody against catalase is seen in all cells and shows the location of the peroxisomes (*B*). The immunolabeling for luciferase is seen only in one transfected cell in the field (*A*). The cytoplasmic and punctate labeling pattern for luciferase is characteristic of cells expressing high levels of the enzyme. Note the superimposability of the punctate patterns for luciferase and catalase in the transfected cell. Another transfected cell immunolabeled for luciferase (*C*) and catalase (*D*) is shown at a higher magnification. In this cell, luciferase shows a more limited and localized labeling pattern that is totally superimposable on the labeling for catalase. (Bars = 10 μm.)

sequences of several known peroxisomal enzymes (19–23) has not revealed any common structural or sequence similarities that may represent a putative peroxisomal targeting signal.

Currently there appears to be no model system that is entirely satisfactory for the elucidation of the mechanism of protein targeting to peroxisomes. Although cDNA clones for some peroxisomal enzymes have been characterized (19–21), the immunochemical localization of their gene products and those of mutant genes would be difficult to study because the cells normally produce these proteins. In principle, this problem could be surmounted by using mutant cell lines lacking one of these genes, but we know of none that fit this description. An alternative approach for studying this problem would involve the establishment of an *in vitro* system for peroxisomal transport, such as the one established for the import of proteins into mitochondria (5). Wild-type and mutant proteins, synthesized *in vitro*, could then be tested for their transport and uptake into isolated peroxisomes. However, this strategy necessitates the maintenance of the functional integrity of the peroxisomes through the various stages of purification and the definition of the energy and cofactor requirements of the system.

Our observation that the firefly luciferase is targeted to peroxisomes in mammalian cells demonstrates that the luciferase gene provides an excellent model system to investigate the signal involved in peroxisomal targeting. The absence of the luciferase gene in mammalian cells makes it possible to monitor the subcellular localization of the wild-type and mutant versions of the protein by using simple immunofluorescence microscopy techniques. Eventually the *in vitro* approach could also be utilized to further define the mechanism of peroxisomal targeting. In addition, the finding that luciferase is localized to peroxisomes in both fireflies and in monkey cells demonstrates that the signals and intracellular machinery involved in peroxisomal targeting are highly conserved through evolution.

Though the biological significance of the peroxisomal localization of luciferase in fireflies is not understood at present, the presence of luciferase in this organelle is intriguing. McElroy and Seliger (24) have proposed that luciferases evolved from proteins whose original purpose was to detoxify small amounts of oxygen as cells adapted to the increasingly oxidative atmosphere of the earth and that light production was incidental to this process. The evolution of peroxisomes has been attributed to similar environmental pressures (25).

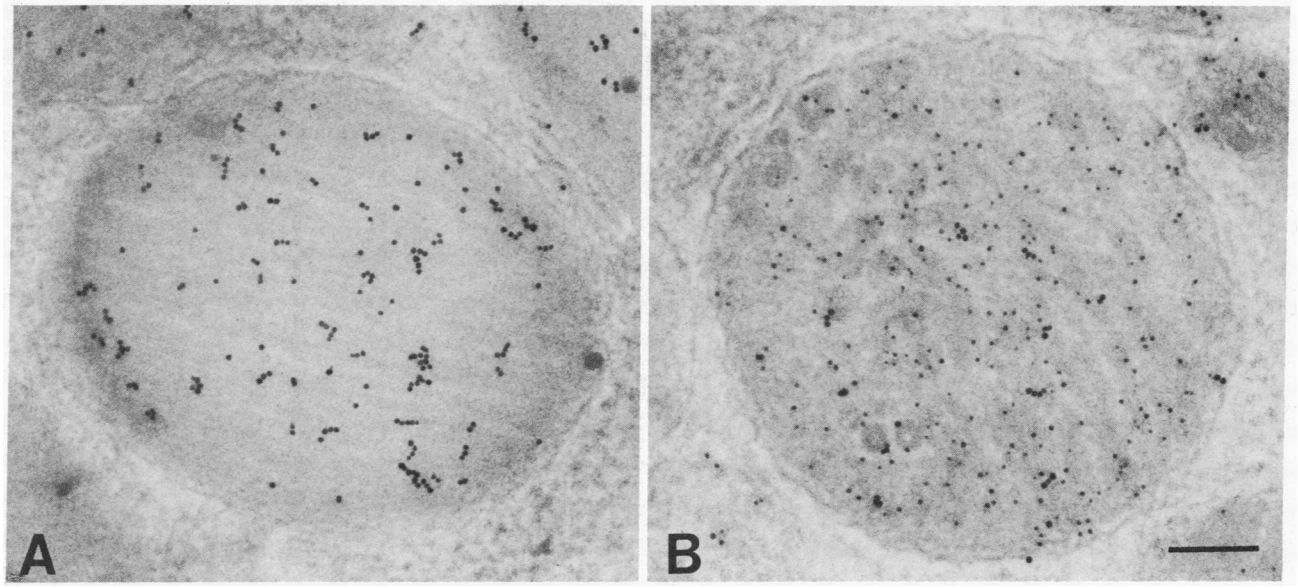

FIG. 4. Peroxisomal localization of luciferase in the firefly lantern using immunocryoelectron microscopy. (*A*) Ultrathin frozen section through the central region of the firefly photocyte immunolabeled with a rabbit antibody against bovine catalase followed by visualization with 12-nm gold adducts of guinea pig antibodies to rabbit IgG. Gold particles are mainly associated with the tubular structures visible in the matrix of the photocytes. (*B*) Another frozen section of the same specimen as in *A* immunolabeled with a goat antibody against rat catalase and a rabbit antibody against luciferase. Catalase and luciferase were visualized with 12-nm and 5-nm gold adducts of guinea pig antibodies against goat and rabbit IgG, respectively. Luciferase and catalase colocalize within the peroxisomes. (Bar = 0.1 μm.)

It will be of interest to see if other eukaryotic luciferases are also peroxisomally localized.

Several laboratories have demonstrated that synthesis of peroxisomal proteins occurs on free polyribosomes and that a detectable cleavage event does not normally occur during transport into peroxisomes (26–34). For the few peroxisomal and glyoxysomal proteins shown to undergo a posttranslational reduction in size, it is not clear whether the modification plays any role in peroxisomal targeting (34–36).

The synthesis of luciferase may very well follow the pattern established for the majority of peroxisomal proteins. Previous experiments have shown that the luciferase protein produced in mammalian cells migrates according to its predicted molecular mass on denaturing polyacrylamide gels and that it is indistinguishable in size from the *in vitro* translation product of the gene (9). These results imply that a detectable cleavage event, such as those shown to occur in some other types of protein transport (1, 2, 4, 5), does not occur for luciferase. However, it does not rule out the possibility that some posttranslational modification of luciferase, not detectable as a size difference on gels, may play a role in the transport of the protein to peroxisomes.

Recently, the firefly luciferase gene has been expressed in a variety of eukaryotes such as yeast (M.D., unpublished data), dictyostelium (P. Howard and R. Firtel, unpublished data), and plants (37). If the protein is also peroxisomal in these cells, the biology of these different systems can be exploited to unravel genetically and biochemically the pathways involved in the targeting of luciferase and other proteins to peroxisomes.

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