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Molecular Biology of Bacterial Bioluminescence

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INTRODUCTION

Bioluminescent organisms are widely distributed in nature and comprise a remarkably diverse set of species (20, 59, 60, 62, 81). Among the light-emitting species are bacteria, dinoflagellates, fungi, fish, insects, shrimp, and squid. This set of organisms includes terrestrial, freshwater, and marine species from almost 50% of the different phyla in the animal and plant kingdoms (59). The enzymes that catalyze the bioluminescence reactions in these organisms are called luciferases, and in most cases the substrates are designated as luciferins (34). However, consistent with the apparent absence of a strong evolutionary relationship between many of the light-emitting systems, significant differences exist between the bioluminescence reactions as well as the structures of the luciferases (enzymes) and luciferins (substrates) from different organisms. Aside from light emission, only the requirement for O_2 for the bioluminescence reactions has been clearly recognized as a common feature of the luminescence systems. This property was initially recognized more than three centuries ago by Robert Boyle, who demonstrated that air (O_2) was required for light emission by rotting wood containing luminescent fungi or bacteria (16). A little more than two centuries later, Dubois demonstrated, by performing classic in vitro experiments with hot- and cold-water extracts from the luminous fire beetle and the boring clam, the requirements for luciferase, luciferin(s), and oxygen in these luminescence systems (34).

Luminous bacteria are the most abundant and widely distributed of the light-emitting organisms and are found in

marine, freshwater, and terrestrial environments. The most common habitats are as free-living species in the ocean, as saprophytes growing on dead fish or meat, as gut symbionts in the digestive tracts of marine fish, as parasites in crustacea and insects, and as light organ symbionts in the teleost fishes and squid (60). These bacteria are all gram-negative motile rods and can function as facultative anaerobes (8, 103).

Almost all luminous bacteria have been classified into the three genera *Vibrio*, *Photobacterium*, and *Xenorhabdus*, with most of the species being marine in nature (8, 20). Only *Xenorhabdus* species infect terrestrial organisms (52). The light-emitting bacteria that have been investigated in most detail are *Vibrio harveyi*, *V. fischeri*, *Photobacterium phosphoreum*, *P. leiognathi*, and *Xenorhabdus luminescens*. Because there are many different isolates and sources of each species, significant differences may exist between the *lux* systems from luminescent strains of the same species. Other species of luminescent bacteria are *V. logei*, *V. splendidus*, *V. cholerae* (58), a freshwater species, and one species (*Altermonas haneda*) classified in the genus *Alteromonas* (68).

Because most strains classified in the genus *Photobacterium* have been isolated as specific light organ symbionts of fishes, it appears that this may be their natural habitat (61). Luminescent bacteria found as symbionts in deep-water fish are primarily *P. phosphoreum*, whereas symbiotic luminescent bacteria in fish in shallow and temperate waters are *P. leiognathi* or *V. fischeri*. In contrast, *V. harveyi* strains have not yet been found as specific light organ symbionts, although they are readily isolated in other marine habitats. *X. luminescens* has only been found infecting terrestrial organisms primarily acting in symbiosis with nematodes in a parasitic infection of caterpillars (144). Interestingly enough, this luminescent bacterium has recently been isolated from human wounds (52).

Cloning and expression of the DNA coding for luciferases from different luminescent organisms, including marine and terrestrial bacteria (4, 9, 26, 29, 43, 51, 54, 84, 143), fireflies and click beetles (33, 154), and the jellyfish (*Aequorea* spp.) (78, 112) and the crustacean ostracod (*Vargula* spp.) (145) have provided the basis for the rapid expansion in our knowledge of the molecular biology of luminescence. The recognition by researchers in other fields of the ease and sensitivity of detection of light emission has led, in turn, to the widespread and expanding application of the cDNA or genes coding for luciferases as reporters of gene expression and regulation as well as sensors of metabolic function in the cells of diverse prokaryotic and eukaryotic organisms. Consequently, significant impetus has been given to further increasing our understanding of the molecular biology of the luminescence systems and characterization of the structures and properties of the luciferases and luciferins.

This review focuses on recent developments in the molecular biology of the *lux* system from luminescent bacteria. Particular attention is given to the organization of the *lux* genes from the different luminescent bacteria, the regulation of expression of luminescence, and the application of the *lux* genes as reporters of gene expression.

Several reviews have been published since 1985 on bacterial bioluminescence covering various aspects of the biochemistry, physiology, and molecular biology of bacterial bioluminescence (20, 60, 62, 80, 81, 86, 134). There have also been some short reviews describing applications of the *lux* genes (77, 87, 124, 138). Techniques for cloning the *lux* genes and screening for the luminescent phenotype have been described in detail (45, 95, 130); additional methodology is detailed in specific papers. A list of references on the molecular biology of the luminescent systems from different organisms including bacteria has recently been published (137).

FUNCTIONS ENCODED BY THE *lux* GENES

Luciferase

The light-emitting reaction in bacteria involves the oxidation of reduced riboflavin phosphate ($FMNH_2$) and a long-chain fatty aldehyde with the emission of blue-green light. Because the structure of the substrates are relatively simple ($FMNH_2$ and RCHO) and are part of and/or closely related to the normal metabolites in the cell, the term luciferin has generally not been used to refer to the substrates for the reaction catalyzed by bacterial luciferases. This reaction is as follows:

$$FMNH_2 + RCHO + O_2 \longrightarrow FMN + H_2O + RCOOH + h\nu \text{ (490 nm)}$$

The reaction is highly specific for $FMNH_2$. Modification of the flavin ring or removal of the phosphate group decreases the activity significantly (90).

The natural aldehyde for the bioluminescence reaction is believed to be tetradecanal on the basis of identification of this compound in lipid extracts, the preference for tetradecanal by luciferases at low (nonsaturating) substrate concentrations, and the specificity of the *lux*-specific fatty acid reductase system, which catalyzes the synthesis of the fatty aldehyde substrate (89, 92, 115, 132, 147). However, differences in aldehyde specificity do exist among different bacterial luciferases. Particularly noteworthy are the high luminescent responses of *V. harveyi* and *X. luminescens* luciferases to nonanal and decanal at saturating substrate concentrations, whereas higher light intensity can be obtained with dodecanal for *P. phosphoreum*, *P. leiognathi*, and *V. fischeri* luciferases (27, 88, 126, 143). This property may be very important in terms of expression of light emission in vivo in prokaryotic and eukaryotic cells missing the aldehyde substrate, because decanal appears to cross the cell membrane much more readily than longer-chain aldehydes do.

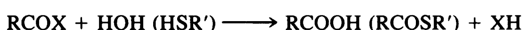
The mechanism of the bioluminescence reaction catalyzed by luciferase and the intermediates in the reaction have been studied extensively (62, 80) primarily because of the very slow turnover of the enzyme. The reduced flavin, $FMNH_2$, bound to the enzyme, reacts with O_2 to form a 4a-peroxyflavin. This complex interacts with aldehyde to form a highly stable intermediate, which decays slowly, resulting in the emission of light along with the oxidation of the substrates. Luciferase undergoes only a single catalytic cycle in most assays, because the rate of chemical oxidation of $FMNH_2$ is higher than the turnover rate of luciferase in the bioluminescence reaction. Consequently, the decay of luminescence with time is a first-order process and reflects the turnover number of the enzyme under the assay conditions. Half times for turnover are dependent on experimental conditions, including the particular luciferase and aldehyde. For example, *X. luminescens* luciferase has the lowest turnover rates, with half times of 3 and 20 s with decanal and dodecanal (143), respectively.

Bacterial luciferase is a heterodimeric enzyme of 77 kDa, composed of α and β subunits with molecular masses of 40 and 37 kDa, respectively. The two polypeptides, encoded on

closely linked adjacent genes, *luxA* and *luxB* in the *lux* operon, appear to have arisen by gene duplication (7) since there is about 30% identity in the amino acid sequence between the α and β subunits of all bacterial luciferases (5, 63, 143). Luciferase is produced in very large amounts at least in the marine bacteria, and the *luxA* and *luxB* genes can readily be expressed in *Escherichia coli*, providing abundant sources of protein for purification and/or application.

Aldehyde Biosynthesis

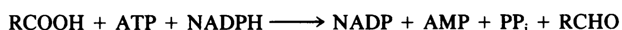
The synthesis of aldehydes for the bioluminescence reaction is catalyzed by a multienzyme fatty acid reductase complex containing three proteins, a reductase, a transferase, and a synthetase (114, 116, 118). These three polypeptides are encoded by *luxC*, *luxD*, and *luxE*, respectively, found in the *lux* operons of all luminescent bacteria (12, 13, 44, 86). The transferase subunit catalyzes the transfer of activated fatty acyl groups to water as well as other oxygen and thiol acceptors, with the enzyme being acylated during the course of the reaction.



$$\mathrm{RCOX + HOH\ (HSR') \longrightarrow RCOOH\ (RCOSR') + XH}$$

Maximum rates of cleavage occur with the substrates tetradecanoyl-acyl carrier protein (ACP), tetradecanoyl coenzyme A, and *p*-nitrophenyl tetradecanoate, demonstrating the high specificity for 14-carbon acyl groups (86). The ability to cleave acyl-ACP in extracts of luminescent bacteria and in *E. coli* transformed with the *luxD* gene appears to be exclusively catalyzed by the transferase subunit, providing a highly specific assay for detection of the expression of *luxD* (19).

The primary reaction catalyzed by the fatty acid reductase complex is the reduction of fatty acids to aldehydes:



$$\mathrm{RCOOH + ATP + NADPH \longrightarrow NADP + AMP + PP_i + RCHO}$$

This reaction is catalyzed by the reductase and synthetase components. The synthetase activates the fatty acid, resulting in the formation of a fatty acyl-AMP intermediate that is tightly bound to the enzyme. In the presence of the reductase, the acyl group is transferred first to the synthetase and then to the reductase before being reduced by NADPH to aldehyde (151). The reductase component can also directly reduce acyl coenzyme A and has often been referred to as acyl coenzyme A reductase (114, 116).

Because all the components of the fatty acid reductase complex are specifically acylated during the enzyme reactions, it has been possible to identify the transferase, reductase, and synthetase polypeptides in luminescent bacteria in vivo or in extracts by labeling with radioactive fatty acids (152). These polypeptides have molecular masses of 54 kDa (reductase), 42 kDa (synthetase), and 33 kDa (transferase) in the different luminescent strains and form a multienzyme complex of 5×10^5 Da in *P. phosphoreum* (153). This complex consists of a central tetramer of reductase subunits, each of which interacts with a synthetase subunit that in turn binds weakly to the transferase subunits.

Flavoproteins

A flavoprotein with subunits of molecular mass of about 24 kDa (107) appears to be part of the *lux* systems of most *Photobacterium* species. Recently, the flavin prosthetic group was suggested to be flavin mononucleotide covalently linked at the 6-position to the β or δ carbon of tetradecanoic acid (73a). The function of this protein, encoded by *luxF*, is unknown; however, it is homologous in amino acid sequence to the luciferase subunits and thus appears to have arisen by gene duplication (136). Its function may be related to the physiological and/or environmental niche of certain *Photobacterium* species, because it has not been found in *Vibrio* or *Xenorhabdus* strains and is not necessary for expression of light by the *Photobacterium lux* system in *E. coli*.

Regulatory and Other Functions

New *lux* genes, *luxG* and *luxH*, coding for 25-kDa polypeptides of unknown function have just been identified (141, 142). *luxG* has been found in all strains of luminescent bacteria except *Xenorhabdus* strains and consequently may code for a function associated with the marine environment. *luxH* is part of the *V. harveyi* but not the *V. fischeri lux* operon and therefore may code for a polypeptide involved in a specific role related to the luminescence system of *V. harveyi*. New *V. harveyi lux* gene loci have recently been identified that code for regulatory functions necessary for luminescence (85, 133; unpublished data). Regulatory functions, encoded by *luxR* and *luxI* of *V. fischeri* and involving the synthesis of a receptor protein and autoinducer, respectively, have also been characterized (43, 44) and are described in a later section.

Functions Related to Luminescence

Lumazine and yellow fluorescence protein. Proteins that affect the wavelength of the emitted light, lumazine and yellow fluorescence protein have been isolated from *Photobacterium* and *Vibrio* species, respectively. The lumazine proteins shift the color of the light to shorter wavelengths than 490 nm, and as a result it has been proposed that the initial excited state in the luminescence reactions cannot be a flavin derivative, which would be expected to emit light at higher wavelengths (80). A strain of *V. fischeri* that emits yellow light at 540 nm has also been investigated, and a yellow fluorescence protein has been identified that is responsible for the change in the color of the light (42, 122). At present, the genes coding for these proteins have not been identified.

Flavin reductases. NAD(P)H flavin reductases that can catalyze the reduction of free FMN in solution have been purified to homogeneity from luminescent bacteria (66, 93). Because such enzymes would probably be responsible for catalyzing the formation of $FMNH_2$ for the luminescence reaction, the possibility that *lux* genes code for flavin reductases has always been considered. However, at present the genes responsible for synthesis of the flavin reductases have not been identified. Further studies on these enzymes from luminescent bacteria are clearly warranted in view of their ability to couple the formation of NAD(P)H and light emission. Addition of flavin reductases and NAD(P)H to assays for bacterial luciferases will result in continuous light emission due to the regeneration of $FMNH_2$ and thus will eliminate the necessity for rapid mixing devices for injection of $FMNH_2$ combined with the light detection equipment. Consequently, scintillation counters and/or photographic film can be used to record the luciferase activity.

Aldehyde degradation. An aldehyde dehydrogenase with a high specificity for NADP has been purified from *V. harveyi* (18). Although this enzyme can catalyze the synthesis as well as the oxidation of aldehydes and has specificity and properties similar to those of the reductase component involved

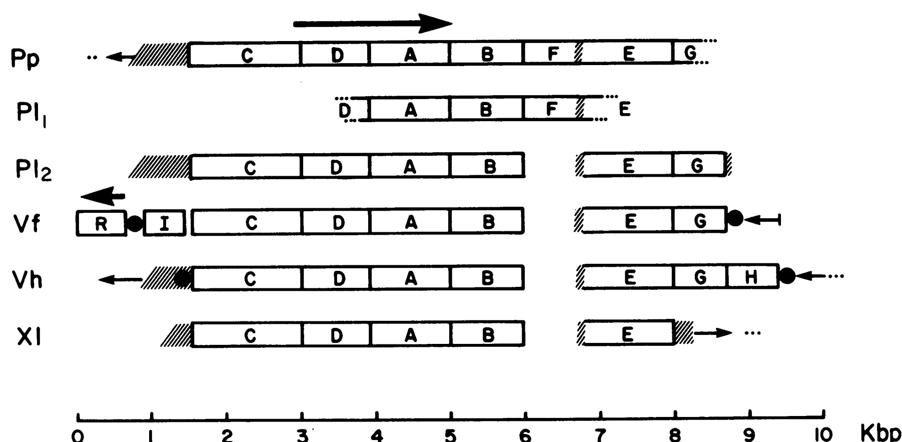


FIG. 1. *lux* gene organization for *P. phosphoreum* (Pp), *P. leiognathi* (Pl), *V. fischeri* (Vf), *V. harveyi* (Vh), and *X. luminescens* (Xl). The nucleotide sequences have been determined for all regions represented. Only the genes that have been demonstrated to be part of the *lux* systems are labeled with letters. Transcription for most genes is from left to right unless indicated otherwise (by arrows). Extended regions of DNA with only short coding regions (<40 codons) are indicated by the diagonal lines. Open reading frames not identified as part of the *lux* system and unrelated to the other genes are shown by arrows with the dots representing incomplete coding regions. The 5′ and 3′ ends of the *lux* mRNA that have been located by S1 nuclease and/or primer extension are shown by the solid circles. Currently, nucleotide sequences are published for *P. phosphoreum* (*luxF* [136]), *P. leiognathi* (Pl_1) (*luxABF* [6, 65]), *V. fischeri* (*luxIR* [30, 47], *luxCDABE* [6, 53], and *luxG* [141]), *V. harveyi* (upstream and *luxC* [94, 100], *luxD* [97], *luxAB* [25, 70], *luxE* [69], and *luxGH* and downstream [142]), and *X. luminescens* (*luxAB* [143]). In addition, locations of genes based on nucleotide sequence data completed on *P. leiognathi* (Pl_2), *P. phosphoreum*, and *X. luminescens* strains in our laboratory and not yet published are included. The strains are *P. phosphoreum* NCMB 844, *P. leiognathi* (Pl_1) 741 and 554 (two closely related strains), *P. leiognathi* (Pl_2) ATCC 25521 (neotype strain), the related *V. fischeri* MJ-1 and ATCC 7744 (neotype strain), *V. harveyi* B392, and *X. luminescens* ATCC 29999. Other *X. luminescens* strains have been cloned that are closely related (54, 69a) as well as *lux* genes from a symbiont of the flashlight fish, *K. alfredi* (63).

in aldehyde synthesis, its expression is under different control from that of the fatty acid reductase polypeptides encoded by *luxC*, *luxD*, and *luxE* (17). Further work is required to clearly establish its specific relationship to the luminescence system.

lux GENE ORGANIZATION

lux Structural Genes (*luxCDABFE*)

lux genes have been cloned from a number of luminescent bacterial strains including *V. fischeri*, *V. harveyi*, *P. phosphoreum*, *P. leiognathi*, and *X. luminescens*. In all cases, *luxA* and *luxB* genes coding for the luciferase subunits and *luxCDE* genes coding for the fatty acid reductase complex have been identified. In addition, other *lux* genes (*luxF*, *luxG*, *luxH*, *luxI*, and *luxR*) as well as unlinked *lux* gene loci have been identified in specific luminescent strains. Outlined in Fig. 1 are the *lux* gene organizations for different luminescent bacterial strains.

The order of the genes coding for the luciferase (*luxAB*) and fatty acid reductase (*luxCDE*) enzymes is the same [*luxCDAB(F)E*] in all operons, with *luxC* and *luxD*, which code for the reductase and transferase polypeptides of the fatty acid reductase, flanking the luciferase genes upstream and *luxE*, which codes for the synthetase, being downstream. An additional gene (*luxF*) is located between *luxB* and *luxE* in most but not all *Photobacterium* species and is missing in the *Vibrio* and *Xenorhabdus lux* systems. As the sequences of this gene, denoted as *luxG* (65) and *luxN* (6) in *P. leiognathi* 554 and *P. leiognathi* 721, respectively, are virtually identical and homologous to the *luxF* gene of *P. phosphoreum* (136), the latter designation has been adopted for this gene in all species. The recent determination of the nucleotide sequence of the *lux* genes of a different *P. leiognathi* strain (ATCC 25521) (79a), isolated directly from the light organ of a pony fish, has shown that the *luxF* gene is absent in some *Photobacterium* species (Fig. 1). As the exact origin of the other *P. leiognathi* strains is unknown, it is possible that the presence of the *luxF* gene coincides with a specific environmental niche of *P. phosphoreum* and some strains (Pl_1) of *P. leiognathi*.

Upstream and Regulatory Genes

Upstream of the *luxC* gene in the *V. fischeri lux* system are two regulatory genes, *luxI* and *luxR*, which are missing or not located at these positions in other luminescent species. The *luxI* gene is located immediately in front of *luxC* and is part of the same operon (right operon). The *luxI* gene product is believed to be involved in production of the autoinducer required for induction of the luminescence system (43, 44). Mutations in this gene can be complemented by the addition of the autoinducer for the *V. fischeri lux* system.

Transcribed in the opposite direction on the left operon is the *luxR* gene. Mutations in this region block expression of the *V. fischeri lux* system (43). The *luxR* gene product has been proposed to function as a receptor for the autoinducer (46). This complex then stimulates transcription of the right operon.

In contrast, the DNA upstream of *luxC* in the other *lux* operons from marine bacteria is very A+T rich and an open reading frame greater than 40 codons is not present for at least 600 nucleotides in the *V. harveyi*, *P. phosphoreum*, or *P. leiognathi lux* systems and at least 200 nucleotides in the *X. luminescens lux* system (100; unpublished data). Upstream of this noncoding region in *V. harveyi* and *P. phosphoreum*, divergent genes are transcribed in the same direction as *luxR*; however, these genes are not homologous to

luxR or to each other, indicating that they are probably not part of the *lux* system (94). Moreover, transposon insertions have not been isolated in this region that affect expression of the *V. harveyi* luminescence system (85). Regulatory *lux* gene loci have, however, been detected elsewhere on the *V. harveyi* genome (85, 133; unpublished data).

mRNA Initiation Sites

The start sites of the mRNA for the right and left *lux* operons of *V. fischeri* have been identified by S1 nuclease mapping (47, 128) between *luxI* and *luxR*. In contrast, the start site of the *V. harveyi lux* mRNA identified by S1 nuclease and primer extension was located only 24 nucleotides from the initiation codon of *luxC* (142). Recent experiments (139a) have raised the possibility that promoter sites are located 50 to 100 bp farther upstream under some conditions. However, whether this difference in mRNA initiation sites arises by modulation by regulatory proteins affecting transcription or processing of the mRNA is as yet unknown.

Downstream *lux* Genes

Downstream of *luxE* is the *luxG* gene in the *lux* operons of all marine bacteria so far investigated. This gene in the *Vibrio* systems has been shown to be encoded on mRNA that is induced during the development of luminescence (141, 142). The function of the *luxG* gene product is unknown. Transposon insertions in this region of *V. fischeri* or *V. harveyi* have not been found that disrupt the expression or regulation of the luminescence system (43, 85). However, the presence of this gene could be related to the physiological habitat of the marine luminescent bacteria as *luxG* is not present at this location in *Xenorhabdus* species.

An additional *lux* gene (*luxH*) is found downstream of *luxG* in the *V. harveyi lux* system but not in the *V. fischeri* or *P. leiognathi lux* systems. As transposon mutagenesis has failed to produce any Lux$^-$ phenotypes with insertions in this gene (85), it seems likely that the gene product is not involved in the general regulation or expression of the *lux* system in *V. harveyi*. It is possible that the gene product modulates the expression of the system under specific physiological conditions that may be related to the natural habitat of the bacteria. Alternately, these downstream genes may be required for an essential function relating to the survival and/or symbiosis of the bacteria in the marine environment.

Polycistronic *lux* mRNA

Polycistronic mRNA that extends from the start of *luxC* to the end of *luxH* in *V. harveyi* has been identified by Northern (RNA) blotting (97, 99). However, the mRNAs present in largest amounts appear to start in front of *luxA* or *luxD* and terminate after *luxB*, consistent with the high levels of expression of luciferase encoded by *luxA* and *luxB* encompassed within this region. The set of polycistronic *lux* mRNAs could arise from internal initiation and termination within the *lux* operon or, could arise via processing of the polycistronic mRNA extending from *luxC* to *luxH*.

mRNA Termination Sites

Termination sites have been identified by S1 nuclease mapping and shown to occur in a poly(T) region immediately after a G+C-rich hairpin loop of high stability, typical of rho-independent termination sites. This termination site occurs just after *luxH* in *V. harveyi* (142) and *luxG* in *V. fischeri* (141). For *V. harveyi*, a convergent gene terminates at another stable hairpin loop just after the termination site for the *lux* operon. Interestingly, for the *V. fischeri lux* operon, the termination site for the *lux* operon is bidirectional and thus serves as a termination signal for a convergent gene. Transfer of these hairpin loops by conjugation back into the luminescent bacteria in front of a reporter gene has also demonstrated that they function as efficient termination sites in vivo whereas DNA upstream of these sites inside the *lux* operon does not cause termination.

In this regard, RNA sequences with the capability of forming hairpin loops with high stability are located before *luxE* in all the *lux* operons either just after *luxB* in *Vibrio* (6) and *Xenorhabdus* (143) strains or after *luxB* or *luxF* in *Photobacterium* strains (65). These hairpin loops appear to serve to stabilize the upstream mRNA and not to terminate transcription at least in the *Vibrio* systems, consistent with the high levels of mRNA coding for *luxA* and *luxB*.

Amino Acid Sequences Encoded by the Luciferase Genes

The primary structures of the proteins encoded by the corresponding *lux* genes from different luminescent bacteria have diverged significantly, as shown by a comparison of the amino acid sequences of the α and β subunits of luciferase, encoded by *luxA* and *luxB*, respectively, for seven distinct strains from three genera (Fig. 2 and 3). The sequence of the *luxA* gene product has been conserved to a much greater degree (54 to 88% identity) than that of the *luxB* gene product (45 to 77% identity between different strains) (Table 1), consistent with the proposal that the α subunit primarily controls the kinetic properties of luciferase (24, 88, 91). On the basis of these identities, the luciferases, and perhaps the luminescent strains as well, can be classified into two distinct groups, with the *V. fischeri*, *P. phosphoreum*, and two *P. leiognathi* luciferases in one group and the *V. harveyi*, *X. luminescens*, and *Kryptophanaron alfredi* luciferases in the other. The *luxA* gene from the *K. alfredi* luciferase was isolated from a symbiont of the flashlight fish, *Kryptophanaron alfredi*. Although the symbiont has not been cultured in the laboratory, the similarities in sequence and properties of the *K. alfredi* luciferase and the *V. harveyi* luciferase suggest that *K. alfredi* is related to *V. harveyi* (63). Specific deletions (or additions) of codons in the genes are also consistent with two classes of bacterial luciferases (e.g., addition of two codons near the 5′ end of *luxB* and deletion of a codon in the central region of *luxA* occur specifically in the *V. fischeri*, *P. phosphoreum*, and *P. leiognathi lux* genes). Moreover, the specificities of the luciferases for aldehydes are also consistent with this classification (27, 63, 88, 126, 143). A more detailed classification of the luminescence systems and strains should also take into account the divergence in sequence of the other *lux* genes as well as differences in *lux* gene organization (Fig. 1).

Because *luxA* and *luxB* arose by gene duplication with approximately 30% identity in amino acid sequence between any α and β subunit independent of bacterial source, it appears that the luciferase genes must have arisen prior to divergence of the bacterial strains. Similarly, the *luxF* gene product is homologous to both the α and β subunits (136). Because the amino acid sequence encoded by *luxF* is closely related to the sequence of the β subunits encoded by the *luxB* genes from all strains (approximately 30% identity) and yet the *luxF* gene is present only in *P. phosphoreum* and

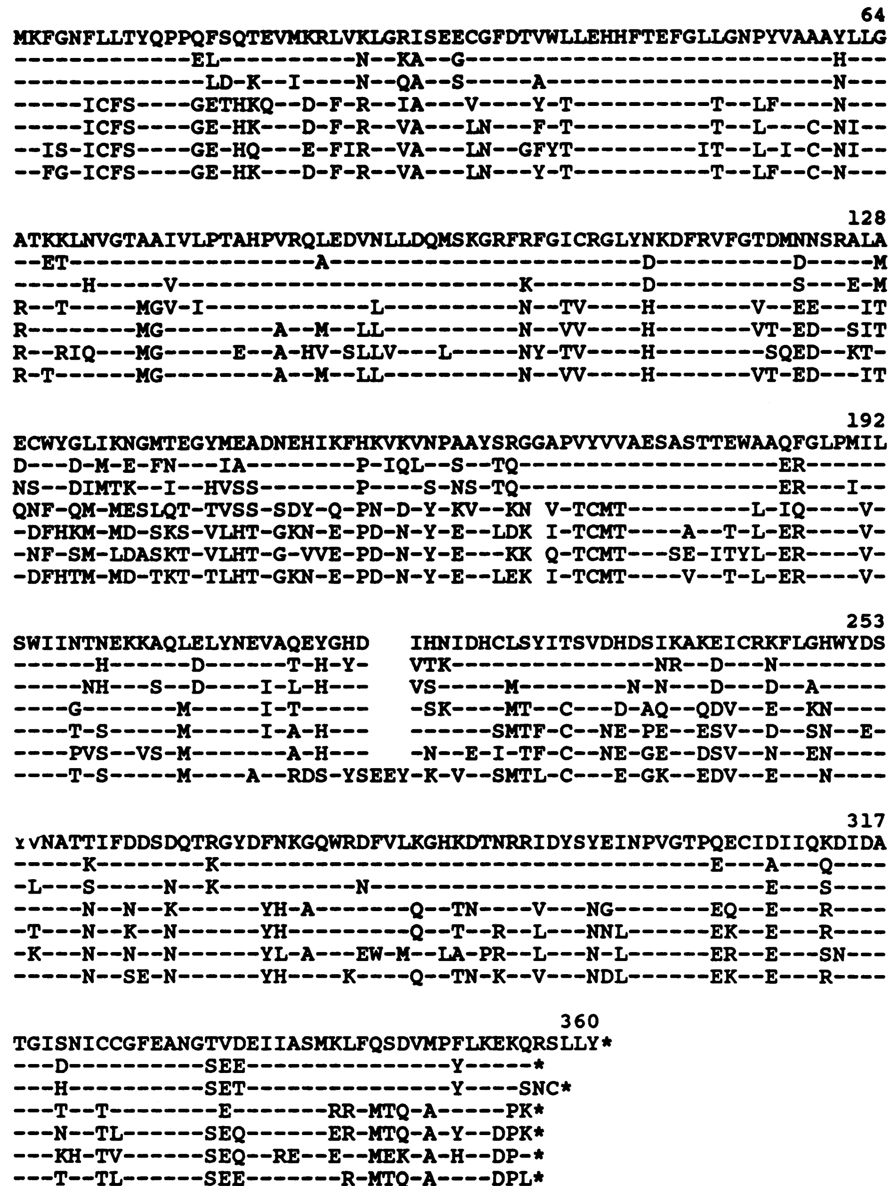


FIG. 2. Comparison of the amino acid sequences encoded by the *luxA* genes of bacterial luciferases. The sequences in order from the top are *X. luminescens* (143), *V. harveyi* (25), *K. alfredi* (63), *V. fischeri* 7744 (6), *P. leiognathi* (Pl_1) 554 (65), *P. leiognathi* (Pl_2) (79a), and *P. phosphoreum* (unpublished data). A dash indicates identity with the *X. luminescens* sequence, and gaps have been introduced at a few places to give maximum alignment. Sequences for *P. leiognathi* (Pl_1) 741 and *V. fischeri* MJ-1 are not given because these strains have greater than 97% identity with the respective strains given above.

some *P. leiognathi* strains (Fig. 1), it is likely that the *luxF* and *luxB* genes arose by gene duplication prior to divergence of the bacterial strains and that then *luxF* was lost in some species during the course of evolution.

Differences in the sequences and properties of luciferases and other *lux* gene products can be readily generated by mutation of the cloned genes (5, 155). Deletion of 10 to 15 codons at the 3′ end of the coding region of the *luxB* gene has generated truncated luciferase variants that could not fold at higher temperatures but were capable of folding at lower temperature to form stable and functional luciferase (139). Interestingly, bacterial luciferases in which the carboxyl terminal of the subunit and the amino terminal of the β subunit have been linked by a short polypeptide by fusing the *luxA* and *luxB* genes have very similar properties with respect to folding at different temperatures (49). Identification of the sites of fatty acyl transfer in the reductase, transferase, and synthetase subunits (117) encoded by *luxC*, *luxD*, and *luxE* are also being investigated by site-specific mutagenesis of the respective gene (52a). The variation in amino acid sequences, resulting in turn in proteins with different properties, will be important for understanding the function and structure of the proteins involved in luminescence as well as for the application of luciferase and the *lux* genes in light-emitting detection systems.

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                                                               62
MKFGLFFLNFINSTTVQEQSIVRMQEITEYVDKLN  FEQILVYENHFSDNGVVGAPLTVSGFL
----------M--KRSSD-V-EE-LDTAH---Q-K  -DTLA-------N----------A---
----------QKDGITS-ETLDN-VKTVTLI-STKYH-NTAF-N-H---K--I----I AA---
-N--------QLKGMTS-AVLDN-IDTIAL---DEYH-KTAF-N-H---K--I----M-AAS--
-N--------QPEGMTS-MVLDN-VDTVAL---DDYH-KRAV-S-H---K--II-E---AIS--
-N--------QPEN-SS-TVLDN-INTVSL---DYKN-TTA--N-H---K--I----M-AAS--

                                                              126
LGLTEKIKIGSLNHIITTHHPVAIAEEACLLDQLSEGRFILGFSDCEKKDEMHFFNRPVEYQQQ
--M-KNA-VA----V-------RV---------M-----AF-------SAD-R-----TDS-F-
----N-LH-----QV-------RV----S----M-------------SDF--E--K-HIPSR--
-----RLH-----QV-------R-----S----M-D------L---VSDF--D--K-QRDS---
----KR-E-----QV-------R-G-QTG----M-Y---V--L---VNDF--D--K-KRSS---
-----RLH-----QV-------R-----S----M-DS-----L---VNDF--D--K-QRDS--L

                                                              190
LFEECYEIINDALTTGYCNPDNDFYSFPKISVNPHAYTPGGPRKYVTATSHHIVEWAAKKGIPL
--S--HK-----F-----H-N---------------F-E---AQF-N---KEV------L-L--
Q--A--------------H-Q----D---V-I---C-SDN--KQ--S---KEV-M-----AL--
---A----L--GI--N--YAN----N-----I---CISKENLKQ-IL---MGV--------L--
Q--A----L-E----N--QA-D--FN--R------CIS  EVKQ-IL-S-MGV-----R--L--
Q--A--D---E-I--N--QAN----N--R--I---CLSKENMKQ-IL-S-VSV-------AL--

                                                              254
IFKWDDSNDVRYEYAERYKAVADKYDVDLSEIDHQLMILVNYNEDSNKAKQETRAFISDYVLEM
V-R-----AQ-K---GL-HE--QAHG--V-QVR-K-TL---Q-V-GEA-RA-A-VYLEEF-R-S
T---E-NLETKER--IL-NKT-QQ-G--I-DV----TVIA-L-S-RST-QE-V-EYLK--IT-T
TYR-S-TLAEKEN-YQ--LT--AENN--ITHV---FPL---I-P-RDI----M-DY-RG-IA-A
TYR-S--LAEKEK-YQ--L---KENNI-V-N----FPL---I--NRRI-RD-V-EY-QS--S-A
TYR-S-TLEDKEILYK--LE--A-HNI-V-NVE--FPL---L-H-RDV-H--AT-YLVS-IA-V

                                                              317
HP NENFENKLEEIIAENAVGNYTECITAAKLAIEKCGAKSVLLSFEPMNDLMSQKNVINIVDD
YS -TD--Q-MG-LLS---I-T-E-STQ--RV---C---ADL-M---S-E-KAQ-RA--DV-NA
Y- QMDRDE-INC--E-----SHDDYYEST---V--T-S-NI-----S-A-FKGV-EI-DMLNQ
Y- -TDQ-E-I--L-KQH---TED-YYESS-Y-L--T-S-N------S-KNKAAVIDL--M-NE
Y-TDP-I-LRV--L-EQH---KVD-YYDSTMH-VKVT-S-NL-----S-KNKDDVTKL--MFNQ
Y- HL-QQQ-IA-L-SQH-I-TDNDYYESTLN-L-RT-S-N------S-KNHDDVVK---M-NE

       327
NIKKYHMEYT*
--V---S*
K-E-NLP*
K---NL*
K--DNLIK*
K-Q-NLPSS*
```

FIG. 3. Comparison of the amino acid sequences encoded by the *luxB* genes of bacterial luciferases. The sequences in order from the top are *X. luminescens*, *V. harveyi*, *V. fischeri*, *P. leiognathi* (Pl_1, *P. leiognathi* (Pl_2), and *P. phosphoreum* sequences. Additional details are given in the legend to Fig. 2.

REGULATION OF EXPRESSION OF THE *lux* GENES

Autoinduction

The induction of luminescence in cultures of many strains of light-emitting bacteria is a spectacular phenomenon. In dilute cultures the cells are very dim; however, at the later stages of cellular growth, luminescence may increase several thousand-fold (Fig. 4). Consequently, the development of light emission lags behind growth. During induction of luminescence, both subunits of luciferase, as well as the fatty acid reductase polypeptides, have been shown to be specifically synthesized. This growth-dependent development of luminescence appears to have some similarity with simple developmental phenomena (104) found in higher organisms and has been the most intriguing aspect of regulation of luminescence expression.

The lag in luminescence relative to cellular growth is due not only to the removal of inhibitors from the media but also to the apparent necessity in some or perhaps most strains for a small molecule produced and excreted by the cells to accumulate in the media (39, 121). These molecules have therefore been referred to as autoinducers. Consequently, the lag in development of luminescence still persists even in minimal media even though most if not all inhibitors have been removed. Only by adding the autoinducer or conditioning the media by prior growth of bacteria excreting the autoinducer can the lag be eliminated. Under conditions where the autoinducer cannot accumulate, luminescence will remain very low. Consequently, luminous bacteria living free in the ocean or growing in a chemostat are very dim compared with bacteria living as light organ symbionts or growing in confined environments (121, 148).

V. fischeri. Induction of luminescence has been best characterized for the *V. fischeri lux* system. An autoinducer, β-ketocaproyl homoserine lactone (40), excreted into the media and capable of diffusing freely across the cell membrane (71), causes induction of luminescence at an early stage of cellular growth. The *luxI* gene product is believed to be responsible for autoinducer synthesis since luminescence can be restored to *E. coli* cells transformed with *V. fischeri lux* DNA with a defective *luxI* gene by addition of the autoinducer (44). The specificity for β-ketocaproyl homo-

TABLE 1. Amino acid identities between the luciferase subunits of different strains

Strain	% Identity[a] with:						
	X. luminescens	*V. harveyi*	*K. alfredi*	*V. fischeri*	*P. leiognathi*		*P. phosphoreum*
					Pl_1	Pl_2	
X. luminescens		85	81	66	63	56	62
V. harveyi	59		84	64	61	54	62
K. alfredi	–[b]	–		65	62	54	60
V. fischeri	52	51	–		77	65	78
P. leiognathi (Pl_1)	51	49	–	65		77	88
P. leiognathi (Pl_2)	47	45	–	57	74		72
P. phosphoreum	47	48	–	61	77	68	

[a] Percent identity between the *luxA* gene products (upper right) and between the *luxB* gene products (lower left). Strains and references are given in Fig. 2 and 3 and their legends.

[b] –, The *luxB* sequence from *K. alfredi* has not yet been determined.

serine lactone is very high, because only closely related analogs can inhibit luminescence induction and only analogs with minor changes in the fatty acyl moiety can act as agonists (41). This autoinducer is species specific, causing the induction of luminescence in *V. fischeri* and *V. logei* but not in other strains (43).

A mechanism involving both positive and negative feedback loops (Fig. 5) has been proposed to explain the induction of luminescence in *V. fischeri* (46). The autoinducer is produced at a low constitutive rate during the early stages of growth. When a sufficient level of autoinducer has accumulated, interaction with the *luxR* gene product would stimulate transcription from the right operon, containing *luxI* and the *lux* structural genes. This stimulation should lead to additional autoinducer production, thus creating a positive feedback loop. Although the level of autoinducer appears to be proportional to the extent of cellular growth (101), synthesis of mRNA for the operon containing *luxI* has been shown to be induced (47). The dependence of expression of genes placed after the promoter of the right *lux* operon on the presence of both autoinducer and the *luxR* gene has provided direct in vivo evidence for this mechanism (43). A palindromic structure just upstream of *luxI* is essential for this response, with single point mutations in this region causing loss of transcriptional stimulation by the autoinducer and/or *luxR* gene product (31). In contrast, DNA located between the palindrome and the *luxR* gene appears to be inhibitory, because removal of this region stimulates the basal expression of both the right (*luxICDABEG*) and left *luxR*) operons.

High levels of the autoinducer-receptor (*luxR* gene product) complex are also proposed to turn off or limit expression of the left operon containing *luxR* (46). However, the results from different laboratories supporting a role for this complex in a negative feedback loop are not in agreement about whether the regulation is at a transcriptional or translational level (38, 46). Moreover, there is some evidence that the autoinducer may stimulate rather than repress expression of the left operon (6).

The specific role of the *luxR* gene product in the regulatory mechanism is also not yet clear. On the basis of the clustering of mutations causing inactivation, two distinct domains in the carboxyl- and amino-terminal regions of the *luxR* protein have been proposed (135). The carboxyl-terminal

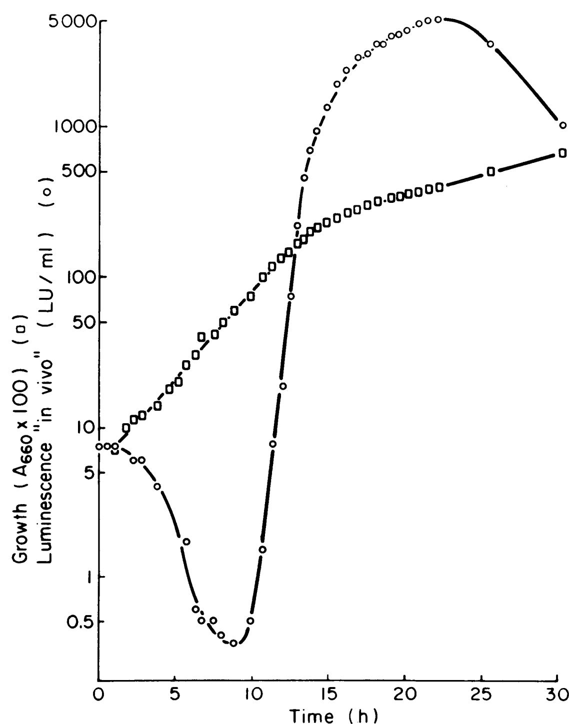

FIG. 4. Dependence of luminescence and cellular growth on time for *P. phosphoreum*. The light intensity is given in light units (LU), where 1 LU = 5×10^9 quanta per s, and growth is given by the optical density of the culture at 660 nm (A_{660}). Courtesy of L. Wall.

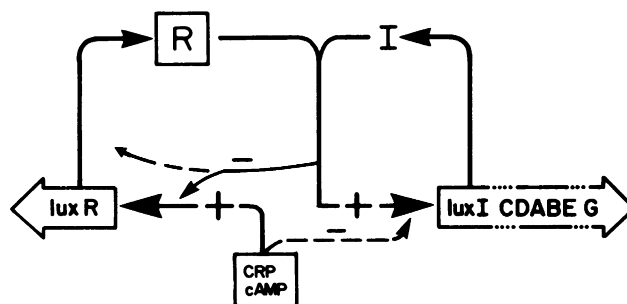

FIG. 5. Regulation of the *V. fischeri lux* system. The scheme summarizes data and models presented in the literature (37, 46, 47, 128), with the *luxR* gene product (R) and autoinducer (I) stimulating expression of the *lux* genes (*luxICDABEG*) and decreasing expression from the *luxR* gene whereas cAMP and CRP cause the reverse effects.

domain may interact with *lux* DNA because it has some sequence similarity with DNA-binding sites of transcriptional regulators involved in environmental sensing. In the amino-terminal domain, the activity of several of the mutants could be restored by high concentrations of autoinducer, implicating this region in autoinducer binding. However, direct biochemical evidence for its interaction with the autoinducer or the binding of this complex to the regulatory region of the *lux* DNA has not been obtained. The *luxR* gene product has been purified to homogeneity as a polypeptide of 24 kDa (72). Although the purified protein could bind to DNA, evidence that binding was specific or depended on the autoinducer was not obtained. However, the protein was exposed to 6 M guanidine HCl for extended periods during its purification to maintain solubility. Although this strong denaturant was removed in the final stage of purification, the protein may not have regained its native configuration, leaving open the question of its biochemical function in vitro.

V. harveyi. The unique *lux* gene organization of *V. fischeri* compared with the other *lux* strains has indicated that the regulation of luminescence induction is different and/or that the regulatory genes must be located elsewhere. Moreover, regulated expression of the *V. harveyi lux* system in *E. coli* has not been observed (98), making it necessary to study the regulation of cloned *lux* DNA after transfer back into *V. harveyi* (96).

A new *lux* gene locus (*lux* region II) has been discovered in *V. harveyi* by transposon mutagenesis (85). This locus, which codes for a protein of 205 amino acids, is flanked by two hairpin structures followed by an unusual 20-fold repeat of a heptanucleotide (133). This heptameric repeat may not be essential for expression, because all transposon insertions causing inactivation were located upstream of this region. The luminescence of *V. harveyi* containing mutations in this DNA was restored by conjugation with *lux* region II, and complementation of *E. coli* transformed with the *V. harveyi lux* structural genes (*luxCDABE*) stimulated expression. However, the regulatory function of this region was not responsive to conditioned media containing autoinducer, nor did this DNA direct the synthesis of autoinducer. Moreover, significant homology of this gene (designated as *luxR*) was not found with *luxR* of *V. fischeri*, indicating that this may be a new genetic element required for luminescence and that additional components must still be found. Recent experiments (95a) have resulted in the identification of a third *lux* gene locus in *V. harveyi* by complementation of regulatory mutants missing the ability to synthesize autoinducer (21). After conjugation of the third *lux* gene locus into the *V. harveyi* regulatory mutants, synthesis of autoinducer was restored, indicating that this locus contains regulatory or structural elements required for autoinducer synthesis. Both new *lux* gene loci appear to be unlinked to the *lux* structural genes or to each other but appear to control the expression of the *lux* structural genes.

The discovery of an autoinducer excreted by the *V. harveyi* cells whose chemical structure is closely related to that of *V. fischeri* (22), has, however, indicated that there may be close similarities in the mechanism of regulation of these *lux* systems despite the differences in *lux* gene organization. The *V. harveyi* inducer differs in chemical structure from the *V. fischeri* autoinducer in having a β-hydroxybutyryl rather than a β-ketocaproyl moiety linked to the amino group of the homoserine lactone. However, despite the close similarity in chemical structures, molecular-modeling studies have demonstrated a dramatic difference in actual conformation (Fig. 6).

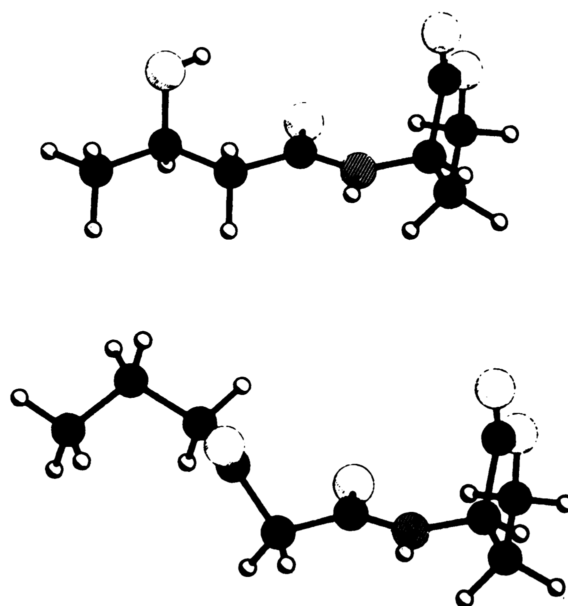

FIG. 6. Structures of the autoinducers of the *lux* systems of *V. fischeri* (bottom) and *V. harveyi* (top). The structures of highest stability have been drawn by using a program for three-dimensional molecular modeling. The *V. fischeri* autoinducer is β-ketocaproyl homoserine lactone; the *V. harveyi* autoinducer is β-hydroxybutyryl homoserine lactone. Symbols: ●, Carbon; ◍, nitrogen; ○, oxygen; ○, hydrogen. Courtesy of J. Cao.

The possibility that the *lux* autoinducers are part of a larger class of signaling molecules (allomones, pheromones, or hormones) used to sense the local nutritional or chemical environment has been suggested (39, 40, 134). This proposal is supported by the close similarity in chemical structure of the autoinducers to a regulatory molecule (A factor), isocapryl-δ-butyryl lactone which causes autoinduction of sporulation and antibiotic synthesis in certain *Streptomyces* species.

Although β-hydroxybutyryl homoserine lactone appears to be specific for induction of the *V. harveyi lux* system, evidence exists that cells of other bacterial species, including nonluminescent strains, excrete compounds into the medium that induce the luminescence system of *V. harveyi* (56, 58). If so, the autoinducers may be involved in the control of cellular functions other than luminescence. This proposal would be consistent with a role for the autoinducer as a signal of the environmental status because chemical sensors found in other systems appear to affect more than one metabolic pathway.

The mechanism for stimulation of luminescence by the *V. harveyi* autoinducer is unknown. Addition of the autoinducer causes induction of luminescence of wild-type cells at an earlier stage of cell growth and stimulates light emission of regulatory dark mutants. The luminescence of a *V. harveyi* dark mutant can be stimulated more than 1,000-fold by β-hydroxybutyryl homoserine lactone, (Fig. 7). Expression of genes placed after the *V. harveyi lux* promoter and transferred back into regulatory mutants of *V. harveyi* is also stimulated by the autoinducer, indicating that induction is occurring at the level of gene transcription. However, it is not known whether the autoinducer stimulates the expres-

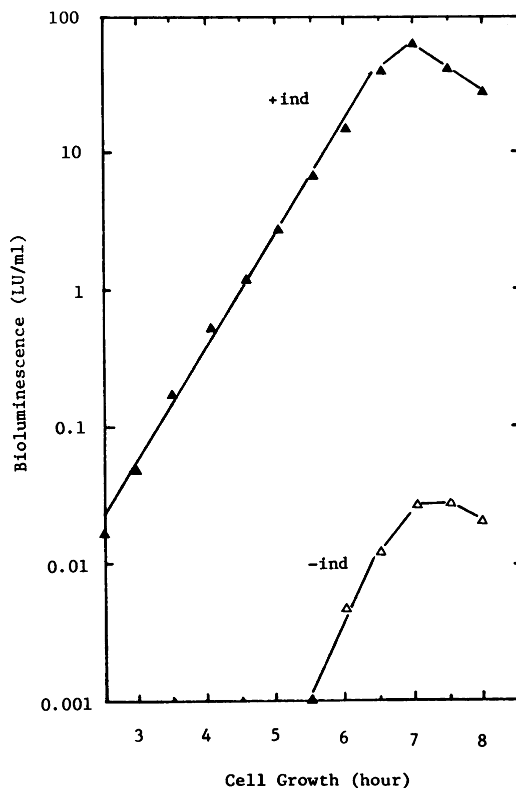

FIG. 7. Stimulation of luminescence of a *V. harveyi* dark mutant with β-hydroxybutryl homoserine lactone. The cells were grown in the absence (△) and presence (▲) of 10 μg of autoinducer per ml and reached the same cell density after 8 h. Reproduced from reference 22 with permission.

sion of the *lux* system directly by interacting with a receptor protein that binds to the *lux* promoter or whether it affects the expression of other genes whose gene product stimulates luminescence expression. A mechanism for control of the *V. harveyi lux* system similar to that proposed for regulation of luminescence of the *V. fischeri lux* system can be considered. Such a mechanism must take into account the different locations of the promoter and regulatory regions for the *lux* structural genes of *V. harveyi* and *V. fischeri*, with the promoter being in front of *luxC* and not *luxI* in the former case (140). A positive feedback loop controlling synthesis of the autoinducer as well as the *lux* proteins in *V. harveyi* could occur if an autoinducer-receptor complex interacted at two different sites. In this instance, one of the recently discovered *lux* gene loci could produce a protein analogous to the *luxR* gene product of *V. fischeri*.

Catabolite Repression

Luminescence of both *V. fischeri* and *V. harveyi* is inhibited by growth in the presence of glucose (37, 55, 102). The temporary repression of luminescence in *V. fischeri* can be relieved by cyclic AMP (cAMP). Repression by glucose can also be observed in *E. coli* (36) transformed with the *V. fischeri lux* operons. Luminescence is very low in mutants of *V. fischeri* and transformed *E. coli* (35) missing the cAMP receptor protein (CRP) or cAMP. These components were found to stimulate expression of the left operon containing *luxR* but to inhibit expression of the right operon containing the *lux* structural genes. However, if both operons were present, luminescence was stimulated by CRP and cAMP presumably as a result of the higher levels of the *luxR* gene product. A consensus sequence for the binding of CRP has been located in the center of the intergenomic region between *luxR* and *luxI*. Consequently, the autoinducer-receptor complex and the CRP-cAMP complex would bind to sites in the operator area of the *V. fischeri lux* operons, with binding to the former site stimulating expression of the right operon and binding to the latter stimulating expression of the left operon (Fig. 5). Moreover, such interactions could also lead to inhibition of expression of the divergent operon by the respective complexes.

In *V. harveyi*, both permanent and transient catabolite repression occur (83, 102). The *lux* operon transferred into *E. coli* is not repressed by glucose; however, transfer of the *lux* promoter with a reporter gene back into *V. harveyi* clearly demonstrated that transcription from this promoter was inhibited by glucose (96). A consensus sequence for CRP-cAMP binding has been located 400 nucleotides from the start site for mRNA. However, the absence of repression by glucose in *E. coli* indicates that potential binding at this site by CRP-cAMP may not affect the expression of the *lux* operon directly and is more consistent with the control of expression of other genes (100).

Induction of luminescence in *V. harveyi* is stimulated by the addition of arginine (104). Recent experiments have demonstrated that expression from the *lux* promoter transferred back into *V. harveyi* is also stimulated, indicating that the control of expression by arginine is at the level of transcription (95a).

Other Regulatory Controls

The involvement of *lexA* protein in control of the luminescence system has been proposed (146). Much higher levels of expression of the *V. fischeri lux* system were found in *lexA* mutants of transformed *E. coli*, and lower levels were found in *recA* mutants. A *lexA* consensus sequence has been found as part of a palindrome in the operator region of the *V. fischeri* region (6, 146). Deletion of this palindrome or mutation to prevent base pairing drops expression from the right operon, implicating this palindrome and the *lexA* sequence in an essential role in controlling luminescence. Detailed biochemical studies showing specific binding to the putative binding sites on the DNA and effects on transcription in vitro will have to be performed to establish the role of any of these proteins.

The sigma 32 (σ^{32}) protein (htpr), which is required for response to stress such as heat shock in bacteria and is involved in transcription initiation, has also been implicated in a positive regulatory role in expression of the *V. fischeri lux system* (146, 149). However, even though the response to autoinducer occurs preferentially in htpr$^+$ and not in htpr$^-$ strains of *E. coli* transformed with the promoter and operator region of the *V. fischeri lux* system, expression can still be observed in the absence of σ^{32} protein in hptr strains at high levels of autoinducer.

EXPRESSION OF THE *lux* GENES IN PROKARYOTES AND EUKARYOTES

Gene Transfer into Prokaryotes

The *lux* genes can be routinely transferred into *E. coli* by transformation by using a variety of different plasmids. High

TABLE 2. *lux* gene transfer into bacteria

Species	Gene transfer system	Integration into host	*lux* genes transferred[a]	Reference(s)
Citrobacter koseri	Transformation	−	Vf *luxAB*	10, 111
Escherichia coli	Conjugation, transformation, transduction	±	Vf, Vh, Pp, Pl, Xl, *lux*	
Klebsiella aerogenes	Transformation	−	Vf *luxAB*	10
Pseudomonas fluorescens	Conjugation	−	Vf *luxABCDE*	129
Salmonella typhimurium	Transformation, transduction	−	Vf *luxAB*	67, 111, 138
Shigella flexneri	Transformation	−	Vf *luxAB*	111
Vibrio parahaemolyticus	Transduction	+	Vf *luxABCDE*	48
Xenorhabdus luminescens	Conjugation	−	Vf *luxABCDE* Vh *luxABCD*	129, unpublished data
Plant pathogens				
Agrobacterium radiobacter	Conjugation	+	Vh *luxAB*	11
Agrobacterium rhizogenes	Conjugation	±	Vh *luxAB*, Vf *luxABCDE*	11, 129
Agrobacterium tumefaciens	Conjugation	±	Vh *luxAB*, Vf *luxABCDE*	11, 129
Bradyrhizobium japonicum	Conjugation	+	Vh *luxAB*	82, 106
Erwinia amylovora	Conjugation	−	Vf *luxABCDE*	129
Erwinia caratovora	Conjugation	−	Vf *luxABCDE*	129
Pseudomonas glumae	Conjugation	−	Vf *luxABCDE*	129
Pseudomonas putida	Conjugation	+	Vh *luxAB*	11
Pseudomonas syringae	Conjugation	−	Vf *luxABCDE*	129
Rhizobium meliloti	Conjugation	±	Vh *luxAB*, Vf *luxABCDE*	11, 129
Rhizobium leguminosarum	Conjugation	+	Vh *luxAB*	11
Xanthomonas campestris	Conjugation	±	Vf *luxABCDE*	129, 131
Marine bacteria				
Vibrio harveyi	Conjugation	−	Vh *luxABCDEFGH*, Pp *luxABCDE*	57, 96, unpublished data
Vibrio fischeri	Conjugation	−	Vh *luxABCD*	Unpublished data
Photobacterium phosphoreum	Conjugation	−	Vh *luxABCD*	Unpublished data
Cyanobacteria				
Anabaena spp.	Conjugation	−	Vf *luxAB*, Vh *luxAB*	125
Gram-positive bacteria				
Bacillus megaterium	Transformation	−	Vf *luxAB*	23
Bacillus subtilis	Transformation	−	Vf *luxAB*, Vh *luxDAB*	23, 73
Lactobacillus casei	Transformation	−	Vf *luxAB*	1
Lactococcus lactis	Transformation	−	Vf *luxAB*	1
Listeria monocytogenes	Transformation	−	Vf *luxAB*	67
Staphylococcus aureus	Transformation	−	Vf *luxAB*	10
Streptococcus lactis	Transformation	−	Vf *luxAB*	138
Streptomyces coelicolor	Transformation	−	Vh *luxAB*	123

[a] Vf, *V. fischeri*; Vh, *V. harveyi*; Pp, *P. phosphoreum*; Pl, *P. leiognathi*; Xl, *X. luminescens*.

expression of most *lux* genes requires a promoter on the plasmid. Only for the *V. fischeri lux* system has it been clearly established that the *lux* promoters can function in *E. coli*. This result could arise from the inability of *E. coli* RNA polymerase to recognize the transcription initiation sites in the *lux* DNA from other luminescent bacteria as a result of the absence of the appropriate regulatory *lux* genes and/or regulatory molecules in *E. coli*.

lux DNA has been transferred into a multitude of different prokaryotic species (Table 2), including many plant and mammalian pathogens. Transfer of DNA into the enteric bacteria, including *E. coli*, as well as into the gram-positive bacteria, has generally been accomplished directly by transformation. However, for phytopathogenic and marine bacteria, conjugation with *E. coli* containing recombinant *lux* DNA has been necessary. Both biparental and triparental mating have been used depending on whether the genes required for conjugation and the *lux* DNA are on the same or different plasmids.

Transduction of the *lux* DNA by using bacteriophages has been accomplished in a few cases (48, 138, 150). By including genetic elements that allow recombination with the host, the *lux* DNA can also be integrated into the bacterial genome. Insertion of *lux* genes into the transposon mini-Mu and transduction with helper phages resulted in their integration into the genomes of *E. coli* and *V. parahaemolyticus*. Similarly, *lux* DNA was integrated into the genomes of phytopathogenic bacteria by insertion into the Tn*5* (11) or Tn*4431* (131) transposons or into other DNA (106) on the plasmid that was capable of effective recombination. Integration into the genome may be advantageous because it avoids the effects of variable gene dosage that may occur when the *lux* genes are expressed on a plasmid vector.

Applications of the *lux* Genes Transferred into Prokaryotes

Growth, distribution, and viability. The ability to introduce the *lux* phenotype into different bacterial species provides a convenient method for rapidly screening for the presence of specific bacteria. Because very low levels of light can be

measured, only a short period is required for transcription and translation of the *lux* genes before there will be sufficient synthesis of luciferase (138, 150) for detection of light. The primary difficulty in this approach appears to be in defining the host range of the viral or plasmid vector for different bacterial strains. A vector with broad specificity could readily introduce the *lux* phenotype into species other than those being screened, leading to a false-positive response, whereas a vector with too narrow a specificity may not transfer the *lux* genes into all strains of a given species and may therefore give a negative response. However, despite these limitations, this approach still has considerable potential as an early warning detection system for contaminating bacteria and, coupled with other tests, may prove to be a significant value. Transduction of *Escherichia* (150) and *Salmonella* (138) species by viral vectors containing recombinant *luxAB* DNA has demonstrated that within 1 h after infection as few as 10 to 100 cells could be detected. Transformation or conjugation of *lux* DNA was about 1,000-fold less sensitive for detection of bacteria than was transduction of *lux* genes (150).

The expression of the *lux* genes in different bacterial species also provides a simple and sensitive system for monitoring the growth and distribution of the bacteria in the environment. In plants, the bacterial luminescent phenotype has been used to observe the movement of *Xanthomonas* species causing black rot in cauliflower (129), the infection of potato slices by *Erwinia* species (129), and the distribution of *Bradyrhizobium* species in soybean root nodules (106).

Tests of susceptibility to different antibiotics or toxic materials can also be readily performed (150) because agents that disrupt or kill the bacteria will almost always destroy metabolism, thus eliminating light emission. Therefore, the presence in milk of antibiotics, bacteriophages, and/or other toxic components that could kill the *Lactobacillus* starter culture used for fermentation can be monitored by the loss of light emission by using *Lactobacillus* transformed with the *lux* genes (1). Similarly, the effect of biocides acting as preservatives can be rapidly analyzed by their toxicity to specific bacterial species (e.g., *Escherichia*, *Salmonella*, and *Bacillus* species) as well as by the recovery of the bacteria from sublethal injury by the increase in luminescence (138).

Reporters of gene expression. By using the *lux* genes as reporters of gene expression, the strength and regulation of transcription from various promoters can be readily monitored. Light emission has been used to detect and measure the strength of promoters under temporal and morphological or spatial regulation during sporulation, mycelium development, and/or germination of *Bacillus* (23, 138) and *Streptomyces* (123) species; to identify promoters involved in lateral motion by flagella in *V. parahaemolyticus* (48) and in osmotic regulation in *E. coli* (111); to measure the regulation of nitrogen-fixing genes in *Bradhyrhizobium* species (82); and to analyze the response of promoters of genes involved in opine catabolism to acetosyringone and to growth of the transconjugated *Agrobacterium* species on wounded plant tissue (64, 120).

Transfer of *lux* DNA into dark mutants of bioluminescent bacteria has allowed analysis of the control of the *lux* promoters in their natural habitat. Analysis of *lux* promoters introduced into light-emitting species is perhaps the only instance when it is necessary to use a gene other than the *lux* genes as a reporter of gene expression (96). This obvious exception, however, points out the general applicability of the system, since background activity for the *lux* system will be encountered only in naturally luminescent species.

The *lux* genes can also be used as reporters in assays that are not directly involved in measurement of the control of gene expression at the promoter level. By use of two *luxA* genes inactivated by mutation at different sites, as well as *luxB*, light emission was used to measure recombination in *E. coli* (105). Similarly, transformed *E. coli* containing *luxA* and *luxB* with a nonsense mutation has been applied to the study of tRNA-mediated nonsense suppression. Light emission was found to be at least 80 times more sensitive than expression of β-galactosidase as a sensor of nonsense suppression (127).

Other applications. Other reporter enzymes or genes can be detected by light emission by coupling their assays to the bacterial luminescence reaction (77). For example, a highly sensitive luminescence assay has been developed for DNA probes conjugated with glucose-6-phosphate dehydrogenase (3). The NADH reaction product was used to reduce FMN (catalyzed by FMN reductase) to $FMNH_2$, which in turn gave a luminescent signal in the reaction catalyzed by luciferase. Because a relatively large number of enzymes can produce NAD(P)H, luminescence assays can be used for the detection of a variety of reporter enzymes or genes with different properties.

Selection of Luciferase Genes

Both *V. harveyi* and *V. fischeri luxAB* genes have been used for expression of luciferase in different bacterial strains. The selection of the most suitable luciferase genes is dependent on a number of factors such as stability, substrate specificity, and activity of luciferase, to name only a few. Because most bacteria have sufficient levels of $FMNH_2$ and O_2, bacteria containing the recombinant *luxAB* genes need only a supply of fatty aldehyde to emit light. Tetradecanal generally gives the highest luminescent response in assays with bacterial luciferases in vitro and is believed to be the naturally occurring aldehyde (89, 92, 147). However, tetradecanal is much less soluble and gives a lower response in vivo than shorter-chain aldehydes on addition to bacteria containing recombinant luciferase genes, presumably owing to its greater difficulty in entering the cell. Because the response of *V. harveyi* luciferase is higher with decanal than dodecanal in vitro and the reverse specificity is true for *V. fischeri* luciferase, decanal has generally been used for in vivo expression of cells containing the *V. harveyi luxAB* genes and dodecanal for in vivo expression of cells containing the *V. fischeri luxAB* genes.

Figure 8 compares the aldehyde specificity of the luciferase reaction in vitro and the luminescent response in vivo of *E. coli* containing the recombinant *luxAB* genes from *X. luminescens*, *V. harveyi*, and *V. fischeri*. Higher responses are obtained in vivo with decanal than with dodecanal in all cases despite the differences in aldehyde specificity in vitro, presumably since decanal can more readily enter the cell. However, the preferred aldehyde for use in vivo is clearly nonanal for all three *lux* systems. Because both *V. harveyi* and *X. luminescens* luciferases can give higher responses with nonanal, the use of these bacterial *lux* systems may be preferred. Moreover, selection of the *V. harveyi* or *X. luminescens luxAB* genes for expression at higher temperatures (37°C) would also be recommended because these *lux* systems appear to have a higher thermal stability than the *V. fischeri lux* system. In this regard, the *X. luminescens lux* system may prove to be the best system for expression in different bacteria grown under various conditions because the aldehyde specificities of the *V. harveyi* and

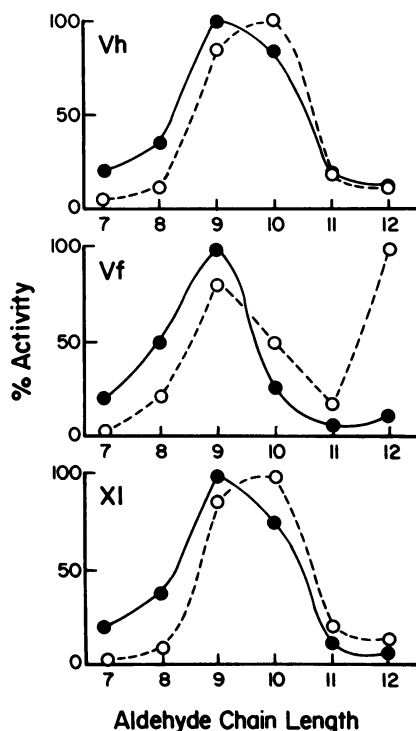

FIG. 8. Aldehyde specificities of the luminescence system in *E. coli* containing recombinant *luxAB* genes from *V. harveyi* (Vh) *V. fischeri* (Vf), and *X. luminescens* (Xl). The *luxAB* genes were ligated into the pT7 plasmid under the control of a T7 RNA polymerase promoter and transformed into *E. coli*. The maximum response in vivo was obtained by vortexing 1 μl of the aldehyde into 1.0 ml of culture (●). Luminescence reached a maximum within 30 s and then decreased slowly. In vitro reactions were conducted by injection of 50 μM $FMNH_2$ into 0.05 M phosphate–0.2% bovine serum albumin (pH 7.0) containing 10 μl of 0.1% aldehyde for *V. harveyi* and *X. luminescens* luciferases and 75 μl of 0.2% aldehyde for *V. fischeri* luciferase (○). The data are plotted as a percentage of the highest response obtained for aldehydes from heptanal to dodecanal for in vivo and in vitro reactions for *V. harveyi*, *V. fischeri*, and *X. luminescens* luciferases.

X. luminescens luciferases are almost identical and the thermal stability of the *X. luminescens* luciferase is even higher ($t_{1/2}$ > 3 h at 45°C) than that of the *V. harveyi* luciferase ($t_{1/2}$ = 5 min at 45°C) (143).

Aldehyde-Independent Expression

Addition of aldehyde to bacteria containing the recombinant *luxAB* genes is not required for light emission if the *luxCDE* genes responsible for aldehyde synthesis are also transferred. Because the aldehyde precursors are probably intermediates or end products in fatty acid biosynthesis (e.g., tetradecanoyl-ACP), they should be available in most if not all bacteria. Consequently, the expression of *V. fischeri* luciferase in phytopathogenic bacteria containing the *luxCDABE* genes did not require the addition of aldehyde (120, 129, 131). These genes are readily transferred as one unit (*luxCDABE*) from the cloned *V. fischeri lux* DNA. Although the *V. harveyi luxCDABE* genes have been constructed as one unit in the pT7 plasmid by combining a number of DNA fragments extending from the *Sac* I site at 1.0 kbp to the *Hin*dIII site at 7.4 kbp (Fig. 9) (97), this DNA cannot be readily excised and transferred into other vectors because of the presence of many common restriction sites within the *luxCDABE* genes and the limitation in unique restriction sites flanking these genes (Fig. 9). Such sites need to be generated by site-specific mutagenesis or less common sites should be used. Alternatively, the *X. luminescens luxCDABE* genes, which are flanked by unique *Eco*RI sites, could be used (Fig. 9). The expression, properties, and stability of the *luxCDE* products as well as the *luxAB* gene products are important in selection of the best system in this regard. Both the *V. harveyi luxCDABE* system and the *X. luminescens luxCDABE* system give very high light intensity at 37°C in *E. coli* in analogous pT7 plasmid constructions (143).

Expression in Eukaryotic Systems

Fusion of *luxA* and *luxB*. In contrast to expression in bacterial systems, expression in most eukaryotic systems requires that a separate promoter precede each gene. Because two promoters would be necessary to effectively express the *luxA* and *luxB* genes coding for the native heterodimeric luciferase, the generation of a monocistronic luciferase by fusion of the *luxA* and *luxB* genes has provided added impetus to the application and expression of the bacterial luciferase genes in eukaryotic systems. Shown in Fig. 10 is the restriction map of *V. harveyi luxAB* and a fused *luxAB* gene generated by addition of one nucleotide and the replacement of a second nucleotide in the 29-bp intergenomic region between *luxA* and *luxB* (14, 15). As a result, the stop codon of *luxA* was eliminated and the α and β subunits encoded by *luxA* and *luxB* were placed in frame linked by a decapeptide. A variety of monocistronic *V. harveyi* luciferases with between 1 and 22 amino acids linking the amino acid sequences of the α and β subunits of luciferase have been generated by similar procedures (Table 3).

The activities of the fused luciferases in Table 3 are those relative to the native enzyme expressed in the analogous construction in *E. coli*. Because the free β subunit is also expressed owing to internal translation initiation even if the *luxB* Shine-Dalgarno sequence is eliminated or altered, luciferase activities cannot be attributed to the fused luciferase alone in many cases but reflect to a large degree the complementation of the fused luciferase with the free β subunit. Only for the fused genes in which the methionine codon of *luxB* has been changed may the activity be attributed to the fused luciferase alone, and even in these cases, data are needed to support this conclusion. Indeed, alteration of only a single nucleotide in the methionine initiation codon is not sufficient to block internal initiation of translation from *luxB* (108). At least two nucleotides of the ATG codon have been altered in the three cases given in Table 3. Fusion of the *luxB* and *luxA* genes in the reverse orientation (*luxB-luxA*) has also been accomplished (108); however, the activity was very low and could easily reflect complementation of the fused β polypeptide with free α subunit arising from internal initiation of translation at *luxA*.

Although relatively high activities are obtained if the linker contains 10 amino acids and the activity is low in most cases when only a single amino acid links the α and β polypeptides, the level of activity appears to be dependent on not only the number but also the sequence of amino acids. Moreover, the activity of the fused luciferase in extracts of *E. coli* is highly dependent on the temperature of cellular growth, whereas the activity of the native enzyme remains relatively constant (49, 50). This effect has been proposed to

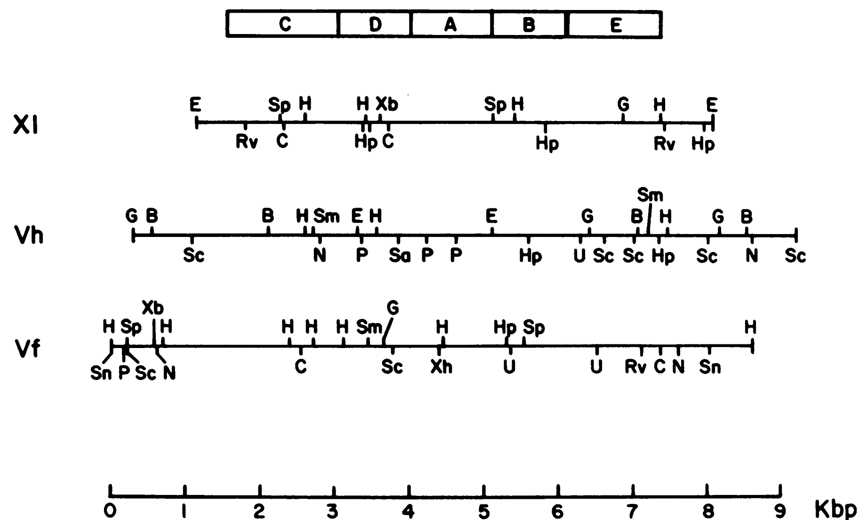

FIG. 9. Restriction maps of *X. luminescens* ATCC 29999 (Xl), *V. harveyi* B392 (Vh), and *V. fischeri* ATCC 7744 (Vf) *lux* DNA. All restriction sites for *Bam*HI (B), *Bgl*II (G), *Cla*I (C), *Eco*RI (E), *Eco*RV (Rv), *Hin*dIII (H), *Hpa*I (Hp), *Nco*I (N), *Pst*I (P), *Pvu*II (U), *Sac*I (Sc), *Sal*I (Sa), *Sma*I (Sm), *Sna*BI (Sn), *Sph*I (Sp), *Xba*I (Xb), and *Xho*I (Xh) are included except *Eco*RV, *Cla*I and *Sna*BI sites in the *V. harveyi lux* system. Complete nucleotide sequences for the above DNA extending across the entire region and including the exact location of all *lux* genes are available on a floppy disk upon request.

be due to incorrect folding of the fused luciferase at higher temperatures, because the fused enzyme isolated at lower temperatures has the same thermal stability as the native enzyme (49). However, this property appears to be somewhat dependent on the amino acids connecting the two subunits, because the fused luciferases with decapeptide linkers of YLIFSQKERD and VINIFEKERD have similar activities (40 to 50%) relative to the native enzyme (Table 3) and yet are expressed at different temperatures (23 and 30°C, respectively). These differences may also reflect in part the replacement of the methionine initiation codon in *luxB* by codons for different amino acids (Q and K) in the fused luciferases. Development of new, improved luciferases that are expressed at high levels at higher temperatures would clearly be beneficial.

Applications. The fused luciferase has been expressed in a number of eukaryotic systems extending from mammalian to plant to insect cells as well as in *Saccharomyces cerevisiae* and in vitro in reticulocyte lysates (Table 4). Although the number of applications of fused luciferase in eukaryotes is much smaller than that of using the *lux* gene from fireflies, the levels of expression appear to be comparable (87). Table 4 gives the amount of native luciferase that would have to be synthesized to account for the light emission in eukaryotic systems containing the fused recombinant *luxA-luxB* gene. These values are dependent on many factors including the particular vector; the host; growth, assay, and other experimental conditions; and whether the genes are expressed in a transient or stable manner. However, the results do illustrate that the relative levels of luciferase synthesized vary widely depending on the system and that very high levels of bacterial luciferase can be expressed in eukaryotic systems. Indeed, the levels of luciferase (6%) in the insect, *Spodoptera frugiperda* are much higher than those in eukaryotic cells infected by a vaccinia virus containing the cDNA coding for firefly luciferase (119). However, it should be noted that the eukaryotic cells in which high-level expression of bacterial luciferase has been obtained are generally grown at temperatures below 30°C. Additional examples of expression of the fused *luxA-luxB* gene, particularly in mammalian cells, will be necessary for determining whether bacterial luciferases can be used as effective reporters of gene expression at high temperature. However, it appears clear that at lower temperatures bacterial luciferase can be expressed very effectively in eukaryotic cells.

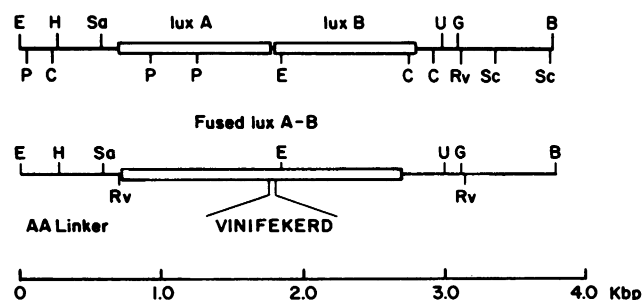

FIG. 10. Restriction map of *V. harveyi luxAB* genes and a fused *luxA-luxB* gene. Restriction sites are *Bam*HI (B), *Bgl*II (G), *Cla*I (C), *Eco*RI (E), *Hin*dIII (H), *Eco*RV (Rv), *Pst*I (P), *Pvu*II (U), *Sac*I (Sc), and *Sal*I (Sa). The fused *luxA-luxB* gene was generated by addition of a G residue before the *luxA* stop codon and replacement of a T by a G residue to eliminate a second stop codon between *luxA* and *luxB* (15). An *Eco*RV site was also introduced by site-specific mutagenesis immediately upstream of the initiation codon of *luxA-luxB* so that the fused gene can be transferred after restriction with *Eco*RV without any upstream ATG initiation codons.

Currently, insect cells infected by baculovirus containing recombinant fused *luxA-luxB* DNA linked by 10 codons (Fig. 10) express the highest level of luciferase. The amount of luciferase is so large in cells infected with *luxA-luxB*$^+$-baculovirus that the fused luciferase can be detected simply by protein staining (Fig. 11). The level of expression is as high as that for another reporter gene (β-galactosidase) and corresponds to approximately 10% of the protein. In contrast to expression in the bacterial systems, internal initiation of translation does not occur. Consequently, free luciferase β subunits were not observed even though the

TABLE 3. Fusions of *V. harveyi* bacterial luciferase

No. of codons in linker[a]	Linker amino acids[b]		Growth temp (°C)[c]	% Activity[d]		Reference
				In vivo	In vitro	
0 (wt)	..LKEKQ	MKF...		100	100	
1	....	D	30	0.04		14
1	....	L		4		108
1	....	P G.....		19	22	2
2	....	SR				74
6	...LRPA	GMQALL	28	9		109
10	....	VINIFEKERD	30	80	50	15
10	....	VINIFEKERD Q.....	30	40	15	15[e]
10	....	YLIFSQKERD	23 (37)	90 (8)		49
10	....	YLIFSQKERD K.....	23 (37)	50 (0.002)		49[e]
18	....	NSSSVPGDPRPAGMQALL	28	70		109
18	....	NSSSVPGDRRPAGMQALL	28	50		109
22	....	NSSSVPGDRSLHARPAGMQALL	28	70		109

[a] Number of amino acids between the position of the last codon of *luxA* and the first codon of *luxB*. wt, Wild type.
[b] The *luxA* sequence is on the left, and the *luxB* sequence is on the right.
[c] The activity of the fused luciferase is highly sensitive to the growth temperature (49, 109).
[d] Activities in transformed *E. coli* relative to the unlinked wild-type *luxA* and *luxB* genes expressed in the analogous construction.
[e] The purified fused luciferases had specific activities close (50 to 80%) to that reported for the native *V. harveyi* luciferase.

methionine initiation codon of *luxB* is retained in the fused *luxA-luxB* gene in the recombinant baculovirus.

Detection of Luciferase Activity

Bacterial luciferase activity can be detected in extracts of cells over an extremely wide range and at very low levels. The luminescent response is linearly dependent on the amount of luciferase over a 10^7-fold range, with less than 1 pg of luciferase capable of being detected (Fig. 12). Activity in solutions containing luciferase and aldehyde was measured either by injecting $FMNH_2$ into the assay media and recording the maximum luminescent response reached in less than 1 s or by using a continuous assay which involved regeneration of $FMNH_2$ by an NAD(P)H:FMN oxidoreductase. The latter assay gave a relatively constant emission of light (<0.5% decrease per min), allowing quantitative measurement of light in a scintillation counter even after 1 to 2 h or on photographic film exposed for this period (Fig. 12). By using these assays, the luciferase activity of the extract from less than one insect cell infected with baculovirus could be detected.

Advantages and Disadvantages of Using *lux* Genes for Gene Expression

The high sensitivity, linear dependence of light intensity on the amount of luciferase over a very wide range (Fig. 12), and assay times of only seconds or minutes are unique features of luminescence assays. Assays for other enzymes are at least 10^2 to 10^3 times less sensitive, generally require much longer assay times, and often involve separation of reaction products that are radioactive. Moreover, most assays for enzymes from nonluminescent organisms are restricted to relatively narrow limits for the amount of enzyme and time of assay so that the response is directly proportional to the enzyme, conditions that may be difficult to achieve for routine analysis of samples expressed to different levels.

The absence of endogenous luciferase activity in nonluminescent organisms and cells clearly permits the widespread use of *lux* genes as reporters of gene expression. Lower limits of detection are restricted only by the background noise (dark current) of the phototube or the sensitivity of the film. Although detectors equipped with fast-injection devices are necessary for assays requiring rapid mixing with $FMNH_2$, coupled assays in which NAD(P)H:FMN oxidoreductases are used to generate a constant level of $FMNH_2$ will give continuous emission of light, permitting the use of scintillation counters, which are available in most laboratories. Moreover, the cost of substrates (FMN, aldehyde) is extremely low for the bacterial luminescence assay.

Currently, the major disadvantage in the use of the bacterial luciferase genes as reporters of gene expression resides in the necessity to use a monocistromic fused *luxA-luxB* gene for expression under a single promoter in eukaryotic cells. Although bacterial luciferases, particularly from *V. harveyi*

TABLE 4. Expression of bacterial luciferase in eukaryotes

Eukaryotic system	Amt of luciferase (μg)[a]	Reference(s)
Mammalian cells		
Mouse fibroblasts	0.06	28
Plants		
Daucas carota (carrot cells, protoplasts)	–	76
Nicotiana tabacum (tobacco plants)	0.4	50, 75, 76, 79, 108, 109
Nicotiana plumbaginifolia (protoplasts)	–	74
Insect cells		
Drosophila melangoster	–	2
Spodoptera frugiperda	60	113
Yeasts		
Saccharomyces cerevisiae	16	2, 15, 50, 74
In vitro		
Reticulocyte lysates	0.3[b]	15

[a] Amount of luciferase with the same specific activity as the native enzyme necessary to account for the highest level of activity found in 1 mg of extracted protein.
[b] In micrograms of native luciferase synthesized per hour per milligram of RNA.

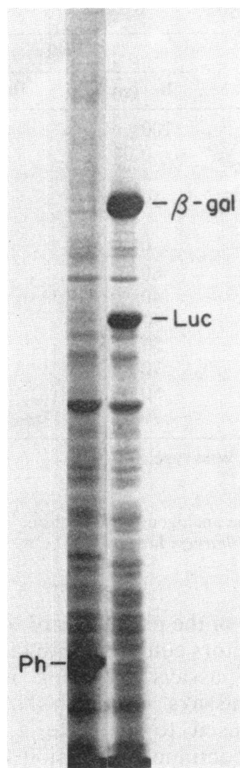

FIG. 11. Sodium dodecyl sulfate-gel electrophoresis of extracts of insect cells infected with baculovirus contains the fused *luxA-luxB* gene from *V. harveyi*. Insect cells from *S. frugiperda* were infected with the wild-type baculovirus (left lane) and baculovirus with the *lacZ* gene coding for β-galactosidase (β-gal) under the P10 promoter and the *luxA-luxB* gene coding for the fused luciferase (Luc) replacing the gene for the polyhedron capsid protein (Ph) (right lane). Cells were grown at 28°C for 72 h. Data courtesy of C. Richardson (113a).

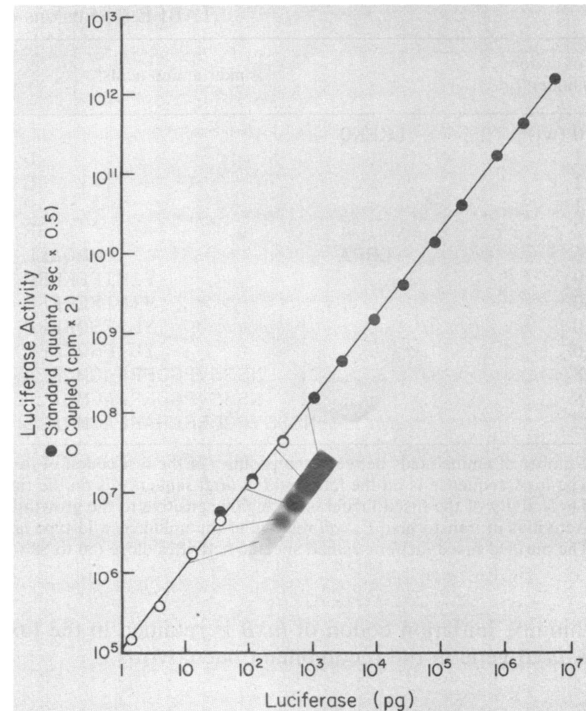

FIG. 12. Dependence of the luminescence response on the amount of *V. harveyi* luciferase. Assays were conducted by injecting $FMNH_2$ into a solution containing decanal and luciferase (injection assay) and recording the maximum response reached in 1 s (●) or by measuring the counts per minute (for 0.2 min) in a scintillation counter set on single-photon mode (continuous assay) in an assay containing *V. harveyi* NAD(P)H-FMN oxidoreductase to constantly regenerate $FMNH_2$ (○). The latter assay was also used to detect the luminescent response on photographic film after exposure for 2 h.

and *X. luminescens*, are quite stable, the fused luciferase cannot fold efficiently at 37°C (49). This property would therefore limit the use of the fused bacterial *lux* gene in eukaryotic cells to plant, insect, and yeast cells grown at temperatures at or below 30°C, which give very high levels of expression. Development of new *luxA-luxB* constructions coding for bacterial luciferases which can refold efficiently at 37°C will be necessary to obtain levels of expression in mammalian cells comparable to the expression of firefly luciferase. This goal appears reasonable in view of the number of different *luxA* and *luxB* genes available (Fig. 1 to 3).

The direct measurement of in vivo function without disruption of the cell membrane and loss of cell viability has been one of the main reasons for the widespread interest in the application of the *lux* genes. Detection of in vivo function will be of value because it would be possible to more readily analyze the spatial as well as temporal expression of genes in different organisms and cells. This goal has already been accomplished with bacteria because shorter-chain aldehydes can readily cross the cell membrane and a sufficient supply of $FMNH_2$ appears to be present in most if not all bacteria. Moreover, in contrast to other luminescence systems, the genes (*luxCDE*) for synthesis of a substrate (aldehyde) can be supplied, eliminating the need for any exogenous additions to generate light. In eukaryotes, generation of in vivo luminescence is more difficult because the availability of $FMNH_2$ appears to be much more limited, although aldehydes can apparently cross the cell membrane.

Although the simplicity of the assay and maintenance of cell viability are clear advantages in measurement of in vivo function, there are some disadvantages. In particular, interpretation of the intensity of light emission in intact cells is much more complicated than analysis of enzyme levels in cell extracts. Changes in intensity of in vivo luminescence are dependent not only on the amount of functional luciferase but also on the availability of the substrates ($FMNH_2$, aldehyde, and O_2), for the luminescence reaction, which in turn can be affected by the expression of other genes. Consequently, obtaining quantitative results would be more difficult than measurement of in vitro activity and the chances of misinterpreting changes in in vivo light intensity would be much higher.

At present, the bacterial *lux* system appears to be the system of choice for expression in prokaryotic cells, whereas firefly luciferase has been expressed most often in eukaryotic cells (32, 110, 119). However, the very high level of expression of bacterial luciferase in eukaryotic cells grown at lower temperatures indicates that the bacterial *lux* system can be successfully used in higher organisms. Because the number of bacterial *lux* systems producing luciferases with different properties (e.g., specificity and stability) is rapidly increasing, further improvements in the future should lead to an expansion in the applications of the bacterial *lux* genes for expression in both prokaryotic and eukaryotic cells.

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ADDENDUM IN PROOF

Genes for the *V. fischeri* yellow fluorescence protein, *luxY* (T. O. Baldwin, M. L. Treat, and S. C. Daubner, Biochemistry **29**:5509–5515, 1990), and the *P. phosphoreum* lumazine protein, *LumP* (D. C. Prasher, D. O'Kane, J. Lee, and B. Woodward, Nucleic Acids Res. **18**:6450, 1990), have recently been cloned. The *LumP* gene was found linked to the *P. phosphoreum lux* operon and is located about 600 bp upstream of *luxC*, corresponding to the open reading frame transcribed in the direction opposite that of *luxC* in Fig. 1.

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