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Engineering a bioluminescent indicator for cyclic AMP-dependent protein kinase

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cDNA coding for the luciferase in the firefly *Photinus pyralis* was amplified *in vitro* to generate cyclic AMP-dependent protein kinase phosphorylation sites. The DNA was transcribed and translated to generate light-emitting protein. A valine at position 217 was mutated to arginine to generate a site RRFS and the heptapeptide kemptide, the phosphorylation site of the porcine pyruvate kinase, was added at the *N*- or *C*-terminus of the luciferase. The proteins carrying phosphorylation sites were characterized for their specific activity, pI, effect of pH on the colour of the light emitted and effect of the catalytic subunit of protein kinase A in the presence of ATP. Only one of the recombinant proteins (RRFS) was significantly different from wild-type luciferase. The RRFS mutant had a lower specific activity, lower pH optimum, emitted greener light at low pH and when phosphorylated it decreased its activity by up to 80%. This latter effect was reversed by phosphatase. This recombinant protein is a good candidate to measure for the first time cyclic AMP-dependent phosphorylation in live cells.

INTRODUCTION

A universal feature of eukaryotic cells is the ability of physiological agonists, such as hormones, growth factors and neurotransmitters, components of the body's defence system, non-host antigens and other pathogens and drugs, to interact with the plasma membrane and trigger molecular events within the cell. These agents initiate a molecular sequence that starts with the generation of an intracellular signal, such as Ca^{2+}, cyclic AMP, inositol trisphosphate or diacylglycerol, and ends with a physiological or pathological event in the cell (Campbell, 1983; Berridge & Irvine, 1989). These events include movement, secretion, transformation, division, defence and death. The timing and magnitude of the end response in each cell depends on the timing and location of both the intracellular signals and the covalent modifications they induce. A particular cell will only undergo an end response if the right sequence of molecular thresholds has occurred (Campbell, 1983, 1988, 1990).

Measurement and imaging of intracellular Ca^{2+} using fluorescent and bioluminescent indicators (Campbell, 1983; Cobbold & Rink, 1987) has established that one explanation for gross heterogeneity in individual cell responses is variation in the timing and the location of the intracellular Ca^{2+} signal. In neutrophils, for example, four subpopulations have been defined, including one group showing no response at all (Hallett *et al.*, 1990; Davies *et al.*, 1991). A major problem in elucidating the molecular basis of heterogeneity within a cell population is the lack of a method for measuring and manipulating covalent modification of proteins in live cells. The purpose of the work reported here was to engineer cyclic AMP-dependent protein kinase phosphorylation sites into firefly luciferase, such that a change in colour and/or light intensity occurred after phosphorylation and dephosphorylation (Campbell, 1989).

Benzothiazole luciferases occur only in luminous beetles. They contain approx. 550 amino acids, and require ATP, Mg^{2+} and O_2, as well as a common luciferin, to generate light (Campbell, 1988). Just a few amino acid changes can cause the colour to shift from green to green–yellow, yellow or red (Wood *et al.*, 1989*a*,*b*). Recognition sites for protein kinase A (Cohen, 1988) have been added to α-interferon to allow high-specific-activity labelling for binding studies (Li *et al.*, 1989). In a previous study the heptapeptide kemptide (LRRASLG) (Zetterqvist *et al.*, 1976; Kemp *et al.*, 1977) was chemically coupled to extracted luciferase from the firefly *Photinus pyralis*. *Photinus* luciferase (*Photinus*-luciferin:oxygen 4-oxidoreductase; EC 1.13.12.7) emits yellow light with a peak intensity at 565 nm. The coupled kemptide shifted the colour of the light emitted to the red and phosphorylation shifted it even further (Jenkins *et al.*, 1990). Here PCR was used followed by transcription–translation *in vitro* (Sala-Newby *et al.*, 1990*a*,*b*) to change an amino acid sequence VRFS (217–220) (de Wet *et al.*, 1987) to RRFS, or to add kemptide to the *N*- or *C*-terminus of the protein.

MATERIALS AND METHODS

Materials

Oligonucleotide primers were prepared using an Applied Biosystems 381A DNA synthesizer and purified as 'trityl-on' oligonucleotides (100, 101) or 'trityl off' (105, 107, 108, 113, 114, T7-K). Their sequences were as follows:

100: TCATCGCTGAATACAGTTAC (3′ end antisense)
101: GGTAAAATGGAAGACGCCAAAAAC (5′ end sense)
105: CACCTAATACGACTCACTATAGGGAGAATGGAAGACGCCAAAAAC (5′ end antisense including the T7 promoter)
107: AGAACTGCCTGCCGCAGATACTCGCA (5′ end sense, underlined bases generate R codon)
108: TGCGAGAATCTGCGGCAGGCAGTTCT (3′ end antisense, underlined bases generate R codon)
113: CCTTGTCGACTTAGCCCAGGGAGGCCCGCCGCAGCAATTTGGACTTTCC (3′ end antisense with 21 bases coding for kemptide, a stop codon and a *Sal*I restriction site)
114: GGCCTCCCTGGGCGAAGACGCCAAAAAC (5′ end sense, part of kemptide)
T7-K: CACCTAATACGACTCACTATAGGGAGAATGCTGCGGCGGGCCTCCCTGGGC (5′ end sense, clamp, T7 promoter and part of the coding sequence for kemptide)

Abbreviations used: CL count, chemiluminescence count; KNt, luciferase with kemptide at *N*-terminus; KCt, luciferase with kemptide at *C*-terminus.

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The coding sequence for firefly luciferase was isolated from a cDNA library (Sala-Newby *et al.*, 1990*a*,*b*). A 2400 bp *Sal*I fragment was used as the target for amplification. Amplitaq DNA polymerase was from Perkin–Elmer Ltd., U.K., T7 RNA polymerase was from Promega, nucleotides and Sephacryl S100 were from Pharmacia and Centricon 100 cartridges were from Amicon, U.K. [γ-^{32}P]ATP (10–50 Ci/mmol), [α-^{32}P]UTP (3000 Ci/mmol), stabilized [^{35}S]methionine ($\geqslant$ 1000 Ci/mmol), RNAase inhibitor and rabbit reticulocyte lysate (N90) were purchased from Amersham International plc. Restriction enzymes, alkaline phosphatase (24 units/μl) and luciferin were from Boehringer Corp. Low-protein-binding ultrafiltration units, Ultrafree MC, were from Millipore Corp. Protein kinase A inhibitor (P-3294) and kemptide were from Sigma Chemical Co. All other AnalaR-grade reagents were from Sigma Chemical Co. and BDH Chemicals. The catalytic subunit of cyclic AMP-dependent protein kinase was generously given by Dr. K. J. Murray of Smith Kline Beecham, Welwyn, Herts., U.K.

Preparation of DNA fragments

Addition of the T7 RNA polymerase promoter (TAATACGACTCACTATAGGGAGA) (Stoflet *et al.*, 1988) and the DNA sequence coding for kemptide (CTGCGGCG-GGGGTCCCTGGGC), as well as mutation of two bases within the luciferase cDNA, were carried out using PCR (Saiki *et al.*, 1988), as previously described (Sala-Newby *et al.*, 1990*b*). Firefly (*Photinus pyralis*) cDNA (4 ng/ml) was amplified in a solution containing 10 mM-Tris/HCl (pH 8.3), 50 mM-KCl, 2 mM-$MgCl_2$, 0.01 % (w/v) gelatin, 0.2 mM each of the four deoxynucleoside triphosphates, 0.5 μM of each oligonucleotide primer and 40 units of Amplitaq DNA polymerase/ml. The cycling reactions were carried out in a Perkin–Elmer thermal cycler. Each of the 25 cycles consisted of 1 min at 94 °C, 1 min at 55 °C, 2 min at 72 °C plus a 5 s extension on each cycle. Klenow fragment of *Escherichia coli* DNA polymerase (40 units/ml) was added after the completion of the 25 cycles, and incubated for 30 min at 37 °C.

The final product was extracted once with phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.) and precipitated with 2 vol. of 7.5 M-ammonium acetate plus 2.5 vol. of ethanol. The DNA concentration was assessed visually from ethidium bromide-stained agarose gels by comparison with the bands of a standard DNA (Sambrook *et al.*, 1989).

Wild-type firefly luciferase DNA preceded by the T7 RNA polymerase promoter was prepared using oligonucleotide primers 100 and 105 (see under 'Materials'). The kemptide coding sequence was added to the 3′ end of the firefly cDNA using primers 105 and 113. The incorporation of the kemptide coding sequence at the 5′ end and the 2 bp change coding for the mutation V-217 → R were carried out in two stages (Higuchi, 1990). After the first amplification the primers were removed by filtration through Centricon 100 cartridges (Higuchi, 1990). The first stage of the introduction of kemptide at the *N*-terminus was carried out using oligonucleotide primers 114 and 100. The resulting DNA (4 ng/ml) was amplified for 25 cycles in the presence of oligonucleotide primers T7-K and 100 to produce the final product. The first stage of the preparation of the RRFS variant generated two fragments: 5′ end fragment (643 bp) was generated by amplification with oligonucleotide primers 101 and 108 and the 3′ end (1018 bp) with oligonucleotide primers 107 and 100. In the second stage the two fragments were mixed in equimolar amounts (2 μg of total DNA/ml), denatured (1 min at 94 °C) and allowed to reanneal by decreasing the temperature at 5.7 °C/min to 37 °C in amplification mixture containing primers 105 and 100 followed by 1 min extension at 72 °C. Eight to twelve cycles of amplification under normal conditions followed.

Formation of luciferase *in vitro*

PCR products (0.5–1.5 μg/25 μl of incubation mixture) were transcribed as previously described (Sala-Newby *et al.*, 1990*b*). The RNA capped using 0.5 mM-m^{7}G(5′)ppp(5′)G and 0.1 mM-GTP was precipitated twice with 0.2 vol. of 7.5 M-ammonium acetate and 2.5 vol. of ethanol. The RNA (1–100 ng) in 2 μl of 10 mM-Tris/HCl, 1 mM-EDTA (pH 7.4), 3 μl of potassium acetate and magnesium acetate to optimize their concentration (90–110 and 1.6–2.0 mM final concns. respectively) and 5 μl of rabbit reticulocyte lysate N90 were incubated for 1 h at 30 °C, and luciferase activity as chemiluminescent (CL) count was measured in a home-built luminometer (Campbell, 1988) for 10 s at room temperature in 229 μl of 20 mM-Tris/acetate/0.3 mM-dithiothreitol/0.2 mM-EDTA/1 mg of BSA/ml/12 mM-magnesium acetate/1.5 mM-ATP, pH 7.75. The reaction was started by addition of luciferin to 0.2 mM final concentration (1 ng of extracted luciferase yields 2.1×10^5 and 2.9×10^5 CL counts/10 s in the presence of rabbit reticulocyte lysate and buffer respectively). The amount of protein synthesized was measured by including 15 μCi of [^{35}S]methionine/10 μl of translation cocktail. Proteins were separated on SDS/9 % (w/v) polyacrylamide gels under reducing conditions (Laemmli, 1970) followed by fluorography and exposure to preflashed X-ray film. The luciferase bands were excised from the gel, radioactivity was measured in a liquid-scintillation counter and the amount of protein was estimated taking into account the concentration of methionine (28 μM) in the lysate.

Phosphorylation of proteins

The proteins were synthesized in 100–250 μl of rabbit reticulocyte mixture, precipitated in 64 % saturated ammonium sulphate, resuspended in 100 μl of 50 mM-Tris/Mes/1 mM-EDTA/0.3 mM-dithiothreitol (pH 7.8) for normal, and for protein with kemptide *N*-terminus (KNt) and kemptide at *C*-terminus (KCt) and pH 7.2 for RRFS, and subjected to gel filtration on a column (0.7 cm × 20 cm) packed with Sephacryl S100 and equilibrated in the corresponding buffer. Active fractions were pooled and concentrated by ultrafiltration. Protein was measured by the method of Lowry *et al.* (1951). BSA (fraction V) was used as standard.

The phosphorylation was carried out in a mixture containing 20 mM-Mes, 60 mM-sodium glycerol 2-phosphate, 30 mM-NaF, 10 mM-magnesium acetate, 1 mM-EDTA, 1 mg of BSA/ml, 1 μg each of leupeptin and pepstatin/ml and 125 μM-ATP, pH 6.8. Active fractions from the gel filtration (0.8–1.2 mg of protein/ml, of which approximately 0.1 % was luciferase) were added together with 0.5 μl of purified catalytic subunit of protein kinase A or catalytic subunit diluent (0.5 M-potassium phosphate/0.1 % Tween-20, pH 6.8) per 40 μl of mixture [the catalytic subunit can transfer 7 mmol of ^{32}P/min per μl using 20 μM-malantide as a substrate, as in Murray *et al.* (1990)]. The incubations were carried out at 30 °C for 10–20 min. Kemptide was also phosphorylated in the presence of rabbit reticulocyte that was gel-filtered under the same conditions as the variants in the presence of [γ-^{32}P]ATP (Livesey & Martin, 1988). The phosphorylated proteins were stored on ice until ready to assay.

Dephosphorylation of the luciferase

When the phosphorylated proteins were to be treated with alkaline phosphatase, the phosphorylation buffer contained no sodium glycerol 2-phosphate nor NaF. For the dephosphorylation reaction 0.7 unit of alkaline phosphatase/μl and 0.01 mM-protein kinase inhibitor (Cheng *et al.*, 1985) were added to the phosphorylation mixture.

Effect of pH on activity and colour of the light emitted by the variants

Chemiluminescence from the enzymes was measured by diluting them 40-fold into an assay mix with pH ranging from 6 to 9 containing mixtures of 50 mM-Mes and 50 mM-Tris to give the desired pH, 0.3 mM-dithiothreitol, 0.2 mM-EDTA, 1 mg of BSA/ml, 12 mM-magnesium acetate, 0.2 mM-luciferin and 1.5 mM-ATP. Colour was assessed using a dual-wavelength luminometer fitted with narrow-band pass-interference filters, with a maximal transmission at 603 nm (red) and 545 nm (green) of 30.2 and 35.3% respectively (Campbell *et al.*, 1985). The light produced by the luciferase reactions was measured simultaneously at the two wavelengths and the ratio of activity at 603 nm to activity at 545 nm was calculated. The ratio was corrected for the transmission of the filters, but not for the spectral sensitivity of the photomultiplier tubes, which at 603 nm was approximately 10% of its value at 545 nm.

RESULTS

Characterization of PCR products

The PCR was used to amplify cDNA coding for wild-type firefly luciferase and for variants containing putative protein kinase A-recognition sites at position 217–220 (referred to as RRFS), kemptide at *N*-terminus (referred to as KNt) or kemptide at *C*-terminus (referred to as KCt).

The PCR products were characterized using three criteria: size on agarose-gel electrophoresis, formation of ^{32}P-labelled mRNA of the correct size on glyoxal/agarose-gel electrophoresis and translation *in vitro* of the mRNA to form light-emitting protein. This protein was compared with wild-type synthetic luciferase for molecular mass, specific activity, pH profile and colour and with firefly tails luciferase when appropriate. The PCR generated a single DNA band apparently of the correct predicted size for all the recombinant DNA, i.e. for wild-type and RRFS the predicted size is 1682 bp, for KCt the predicted size is 1703 bp, and for KNt a major band is present at the predicted size 1703 bp with a minor band at 380 bp (Fig. 1). The yields were 1–3 μg of DNA/0.1 ml of reaction mixture. No bands were seen without addition of primers or when template DNA was omitted.

Transcription of the PCR products with T7 RNA polymerase generated a major band of ^{32}P-labelled capped mRNA of the correct length, i.e. 1650 bp. Small quantities of longer and shorter mRNA products were observed (Fig. 2). The latter could not generate light-emitting protein because removal of 12 amino acids at the *C*-terminus reduces the activity by 99% (Sala-Newby *et al.*, 1990*b*). The yields of capped mRNA were 4–8 molecules of RNA per DNA molecule, the lower yields corresponding to DNA coding for KNt. No mRNA was detected in gels when the DNA transcribed lacked T7 promoter in spite of the detection of [^{32}P]UTP incorporation equivalent to 0.05 molecule of RNA per DNA molecule. mRNA generated light-emitting protein (Table 1) and a major ^{35}S-labelled protein band of the expected molecular mass (60 kDa) on SDS/PAGE (Fig. 3).

Characterization of the recombinant variants

The new proteins were characterized using three criteria: specific activity (i.e. CL counts/μg of RNA and CL counts/ng of protein), effect of pH 6–9 on their activity and colour of the light emitted as assessed by the ratio of chemiluminescence at 603 nm to 543 nm.

The CL counts/10 s per ng of protein indicated the effect the modifications had on the catalytic activity of the protein. Luciferase with kemptide at the *N*- or *C*-terminus had a specific activity similar to that of the wild-type and extracted luciferase. However, the specific activity of the RRFS variant was only 10–15% of that of wild-type luciferase (Table 1). The specific chemiluminescent activity estimated per μg of RNA differed between the variants by a greater factor than the activity per μg

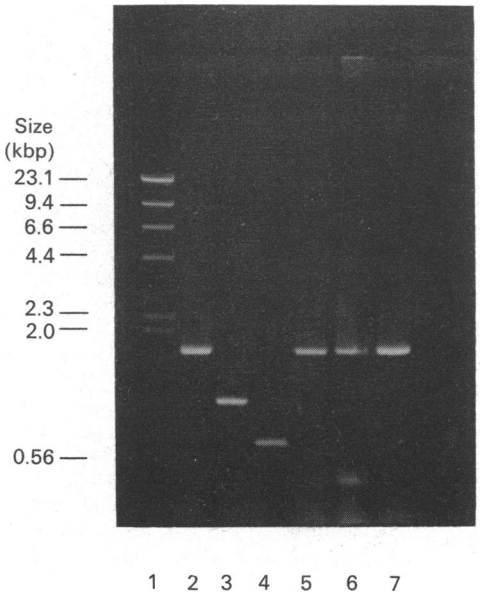

Fig. 1. Agarose-gel electrophoresis of cDNAs prepared by PCR

Wild-type firefly cDNA was amplified with oligonucleotides 105–100 (lane 2). The fragments of DNA that were used to prepare RRFS variant are shown in lanes 3 and 4. They correspond to PCR products obtained using oligonucleotide primers 107–100 (3′ end) and 101–108 (5′ end). The firefly RRFS cDNA is shown in lane 5; it was prepared by the amplification of DNA from lanes 3 and 4 in the presence of primers 105–100. cDNAs coding for variants with kemptide at *N*- and *C*-terminus are shown in lanes 6 and 7 respectively. Size markers were *Hin*dIII-digested λ DNA (lane 1).

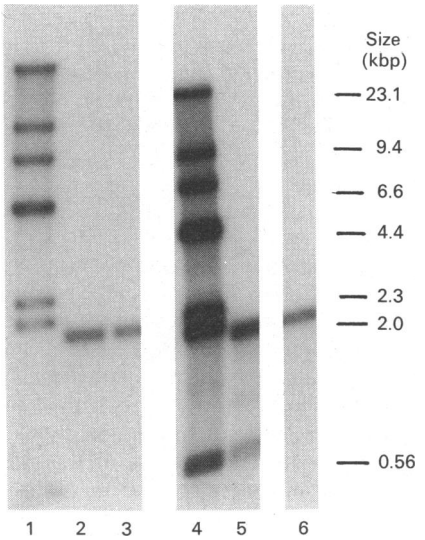

Fig. 2. Transcription products of the cDNAs

cDNAs produced by PCR were transcribed using T7 RNA polymerase and the ^{32}P-labelled mRNAs were separated by glyoxal/agarose-gel electrophoresis, dried and autoradiographed as described in the Materials and methods section. The size markers were ^{32}P-labelled *Hin*dIII-digested λ DNA (lanes 1 and 4) (Sambrook *et al.*, 1989). RNAs for the recombinant proteins are shown as follows: RRFS (lane 2), wild-type (lane 3), KNt (lane 5), KCt (lane 6).

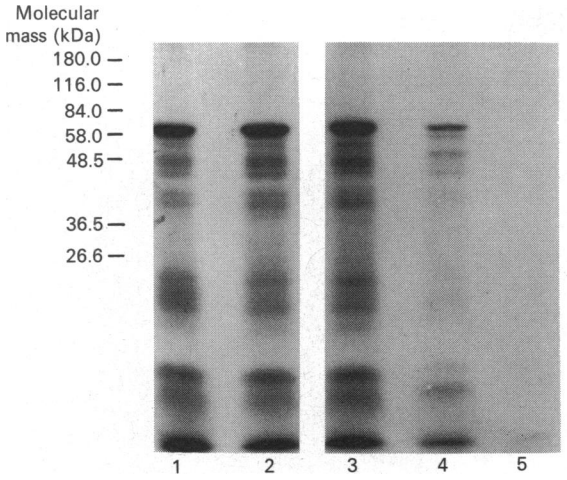

Fig. 3. Synthesis *in vitro* of recombinant proteins

mRNAs were translated using rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The proteins were separated by SDS/PAGE under reducing conditions. RRFS (lane 1), wild-type (lane 2), KCt (lane 3), KNt (lane 4) and the products in the absence of mRNA (lane 5) are shown. Prestained molecular-mass (Da) markers were: α_2-macroglobulin (180000), β-galactosidase (116000), fructose 6-phosphate kinase (84000), pyruvate kinase (58000), fumarase (48500), lactate dehydrogenase (36500) and triose phosphate isomerase (26600).

Table 1. Specific activity of the luciferases

The values in parentheses indicate the number of independent DNA amplifications. Results are expressed as means ± S.E.M. and when only two determinations were made the range is given.

Variant	CL counts/10 s per ng of protein	CL counts/10 s per μg of RNA	Protein/RNA (mol/mol)
Wild-type synthetic	$(2.6\pm0.5)\times10^5$ (3)	$(3.0\pm0.6)\times10^7$ (5)	1.04
RRFS	$(3.0\pm1.0)\times10^4$ (6)	$(3.9\pm1.3)\times10^5$ (6)	0.11
KNt	$(2.1$–$3.7)\times10^5$	$(4.2$–$4.7)\times10^6$	0.14
KCt	$(2.1$–$2.0)\times10^5$	$(3.7$–$8.9)\times10^6$	0.27
Extracted	2.1×10^5	–	–

of protein. All the variants with phosphorylation sites showed less activity per μg of RNA than the wild-type variant (Table 1). The number of molecules of protein produced per molecule of RNA in the translation assay, estimated from the two specific activities, confirmed that the normal synthetic wild-type enzyme yielded up to nine more copies of RNA than the other three (Table 1). The lower levels of translation shown could reflect differences in the secondary structure of the mRNA with the translation assay being optimized for the wild-type synthetic RNA. An additional effect due to a change in the codon usage cannot be ruled out. The RNA coding for KNt also contained an RNA band at approx. 500 bp (Fig. 2, lane 6) which would not translate into active protein as this would reduce the specific activity estimated per total RNA.

Effect of phosphorylation and dephosphorylation

Initial experiments using kemptide as a substrate for protein kinase A indicated that rabbit reticulocyte lysate inhibited phosphorylation of kemptide (results not shown). Gel filtration

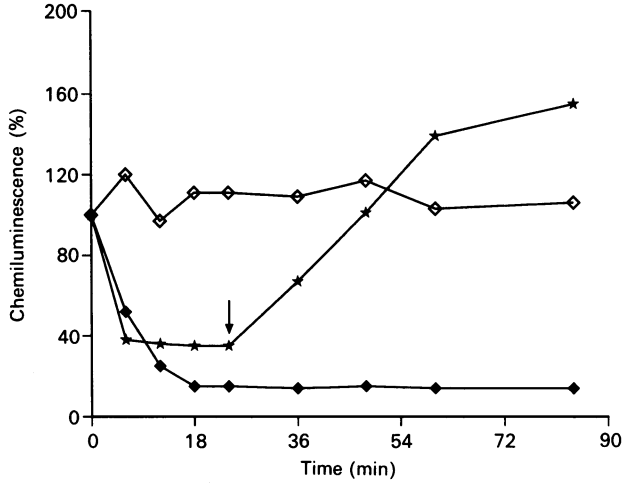

Fig. 4. Effect of phosphorylation–dephosphorylation on the activity of RRFS luciferase (V-217 → R)

Partially purified variant RRFS was incubated at 30 °C as described in the Materials and methods section in the presence of kinase diluent only (◇), protein kinase A catalytic subunit with (◆) and without (★) phosphate inhibitors. At 24 min alkaline phosphatase and protein kinase A inhibitor were added (↓). Samples were taken from the tubes at various times up to 90 min, diluted immediately 40-fold into luciferase assay mixture pH 7.2 and the chemiluminescence was measured for 10 s. Activity at time 0 was measured before addition of kinase. Results are presented as percentage of activity at time 0. Each point is a mean of two and a representative experiment is shown. Experiments were carried out with protein produced from two separate PCRs.

removed the inhibitory activity and 1.7 ± 0.2 ($n = 3$) nmol of phosphate (1.3–1.4 mol when phosphatase inhibitors were omitted) was incorporated into kemptide after 20 min incubation per 40 μl of reaction mixture.

Incubation of the RRFS luciferase variant with protein kinase A catalytic subunit in the presence of ATP resulted in a decrease in its catalytic activity to $19\pm4\%$ ($n = 5$) within 20 min, and remained at this level for the duration of the experiment, i.e. 90 min (Fig. 4). When alkaline phosphatase was added to the phosphorylated RRFS luciferase, the chemiluminescent activity increased to control levels within 30 min. No effect of protein kinase A was observed on the activity of wild-type luciferase, nor on recombinant luciferases with kemptide at the *N*- or *C*-terminus, at any pH (Figs. 5*a* and 5*b*).

Attempts to demonstrate a change in pI between the various recombinant luciferases, using isoelectric focusing, were unsuccessful, because of artifactual bands generated from the focusing procedure. However, the major band for recombinant and extracted luciferase had the same pI (6.6).

Recombinant wild-type luciferase had a pH optimum of around 7.8, identical with that of the extracted luciferase (Fig. 6*a*). Addition of kemptide at the *N*- or *C*-terminus appeared to have no effect on the pH profile (Figs. 5*b* and 6*a*). Similarly these three recombinant proteins had similar colour shifts to the red at acidic pH (Fig. 6*b*). In contrast the RRFS mutant luciferase showed both an altered pH profile with optimum activity at pH 7.2 (Figs. 5*a* and 6*a*) and a shift in colour to the green at acid pH (Fig. 6*b*). The inhibitory effect of phosphorylation on RRFS activity was most marked at its optimum pH (Fig. 5*a*). The ratio of light emission at 603 nm/543 nm measured at pH 7.5 changed from 0.16 to 0.32 after phosphorylation, indicating that the light became redder. Since the detection system used for activity measurements was less sensitive to red light, this red shift may

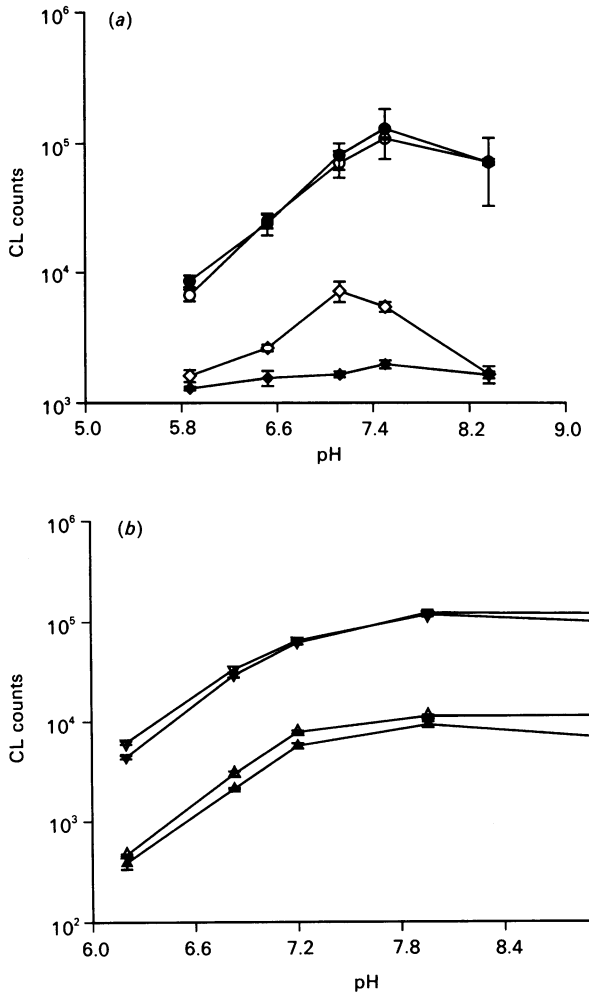

Fig. 5. Effect of pH and protein kinase A on the activity of recombinant luciferases

The variants were incubated for 15 min in the presence of protein kinase A (closed symbols) or kinase diluent only (open symbols) as described in the Materials and methods section. The resulting enzyme activity was then measured in triplicate (mean ± S.E.M.) at various pH values. (*a*) Wild-type (●, ○) and RRFS (◆, ◇). (*b*) KNt (▲, △) and KCt (▼, ▽).

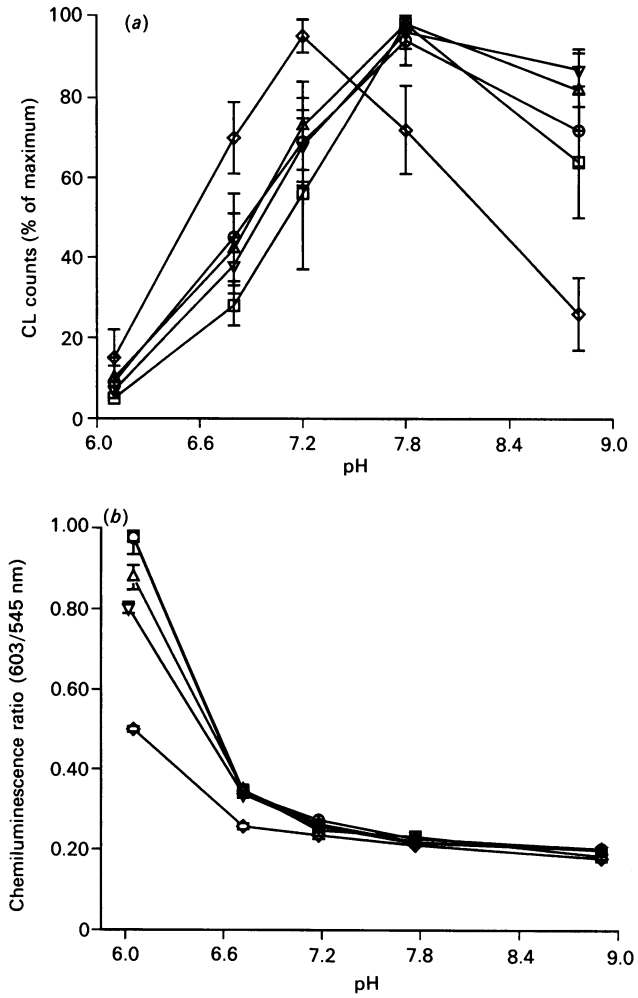

Fig. 6. Effect of pH on the activity and colour of the light emitted by luciferase variants

(*a*) pH optimum curve. The results are expressed as % of maximal activity (mean ± S.E.M.) from three to five experiments, each in triplicate. ○, Wild-type recombinant; □, extracted luciferase; ◇, RRFS; △, KNt; ▽, KCt. (*b*) pH effect on the colour of the light produced. The ratio of chemiluminescent counts at 603 nm and 545 nm was measured in triplicate at each pH (mean ± S.E.M.). ○, Wild-type recombinant; □, extracted luciferase; ◇, RRFS; △, KNt; ▽, KCt. The luciferase from firefly tails was purified as described by Sala-Newby *et al.* (1990*b*).

partly explain the decrease in activity for the phosphorylated enzyme.

DISCUSSION

The results presented demonstrate that DNA amplification coupled to transcription–translation *in vitro* allowed the generation and characterization of firefly luciferase variants containing phosphorylation sites. Only one of the variants (RRFS) showed a decrease in its activity when incubated with the catalytic subunit of protein kinase A in the presence of ATP, and the effect was reversed by addition of alkaline phosphatase (Figs. 4, 5*a* and 5*b*). The enzyme activity per unit of protein of the wild-type variant and the luciferases with kemptide at the *N*- or *C*-terminus were indistinguishable from that of the extracted luciferase (Table 1). The activity expressed per unit of RNA was more variable and lower for all the variants with phosphorylation sites than for the wild-type. The pH–activity profile for KNt, KCt and wild-type were very similar, but RRFS had a lower pH optimum (Fig. 6*a*). The colour of the light emitted was assessed by measuring the ratio of activities at 603 nm and 545 nm (Fig. 6*b*). At alkaline pH no significant differences were detected but as the pH was decreased the variant RRFS had a significantly lower ratio, indicating that the light emitted was greener than for all the others. As chemiluminometers contain photomultipliers which are more sensitive to green than red light this colour change cannot explain the decrease in specific activity measured. The activity was measured under saturating concentrations of ATP and luciferin, suggesting that the $V_{max.}$ was decreased.

Several beetle luciferases have now been cloned: *Photinus pyralis*, *Pyrophorus plagiophthalamus* and *Luciola cruciata* (de Wet *et al.*, 1987; Wood *et al.*, 1989*a*,*b*; Tatsumi *et al.*, 1989). Spectral changes are known to occur in the light emitted by firefly luciferase in response to changes in pH and temperature, and in the presence of heavy metals (Seliger & McElroy, 1964). Work on four click-beetle luciferases that show 94–99 % sequence homology demonstrated that a small number of amino acid

substitutions were responsible for the different colours displayed by the luciferases. The spectral shift between luciferases yellow–green and yellow belong to the amino acid set, R-223, L-238 → E, V with the effect probably being due to R-223 → E (Wood *et al.*, 1989*a*,*b*). Since all the beetle luciferases use the same luciferin, the colour of the light emitted in the reaction must depend on the environment around the emitter (i.e. oxyluciferin). The oxyluciferin can exist as a monoanion (ketonic form) or dianion (enolic form) at acid and basic pH respectively. The presence of an arginine in position 223 of the click-beetle yellow–green luciferase seemed to be responsible for a shift to the green in the light it emitted. The change V-217 → R-217 that generated RRFS in *Photinus* luciferase introduced a basic amino acid in that area of the protein and also resulted in a shift to the green of the light emitted, suggesting that a positive charge there stabilized the oxyluciferin dianionic form, the green emitter.

The phosphorylation of the RRFS variant by the catalytic subunit of protein kinase A decreased its activity, and dephosphorylation reversed the effect. The decrease in activity was accompanied by a spectral shift to the red that can account for part of the lower activity measured. The other two variants, KNt and KCt, showed no detectable differences from the wild-type luciferase in any aspect. The luciferase with kemptide at the *C*-terminus was expected to show properties different from the normal luciferase in view of the fact that the removal of 12 amino acids at the *C*-terminus nearly abolishes activity (Sala-Newby *et al.*, 1990*b*). The removal of three amino acids (results not shown) and the addition of the seven amino acids from kemptide at the *C*-terminus did not affect the catalytic properties. This could be important when using firefly luciferase or its variant in eukaryotic cells because the last three amino acids of the *C*-terminus contain a peroxisomal targetting signal (Keller *et al.*, 1987; Gould *et al.*, 1987).

The RRFS variant provides, for the first time, an indicator potentially useful for measuring protein phosphorylation in intact cells, and has also highlighted a domain within the enzyme that results in changes in colour in response to a change in charge. Recognition peptides for other kinases could thus be engineered in this region of the protein, thereby establishing a universal strategy for measuring any protein kinase and visualizing it in living cells (Hooper *et al.*, 1990).

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