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Localization, trafficking, and temperature-dependence of the *Aequorea* green fluorescent protein in cultured vertebrate cells

(intracellular reporter/fusion protein/dexamethasone/transcription)

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ABSTRACT **The localization, trafficking, and fluorescence of *Aequorea* green fluorescent protein (GFP) in cultured vertebrate cells transiently transfected with GFP cDNA were studied. Fluorescence of GFP in UV light was found to be strongest when cells were incubated at 30°C but was barely visible at an incubation temperature of 37°C. COS-1 cells, primary chicken embryonic retina cells, and carp epithelial cells were fluorescently labeled under these conditions. GFP was distributed uniformly throughout the cytoplasm and nucleus independent of cell type examined. When GFP was fused to PML protooncogene product, fluorescence was detected in a unique nuclear organelle pattern indistinguishable from that of PML protein, showing the potential use of GFP as a fluorescent tag. To analyze both function and intracellular trafficking of proteins fused to GFP, a GFP–human glucocorticoid receptor fusion construct was prepared. The GFP–human glucocorticoid receptor efficiently transactivated the mouse mammary tumor virus promoter in response to dexamethasone at 30°C but not at 37°C, indicating that temperature is important, even for function of the GFP fusion protein. The dexamethasone-induced translocation of GFP–human glucocorticoid receptor from cytoplasm to nucleus was complete within 15 min; the translocation could be monitored in a single living cell in real time.**

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* possesses the property of accepting energy by a radiation-free energy-transfer process from an excited-state blue fluorescent protein during the aequorin bioluminescence reaction to emit light in the green wavelength (1–3). The protein has an absorbance maximum at 400 nm, with a minor peak at ≈480 nm, and its fluorescence-emission spectrum shows a sharp peak at 508 nm, with a shoulder at 545 nm (3). Chemical studies have shown that native GFP is a 27-kDa protein (4, 5) containing a cyclized tripeptide chromophore (5, 6). Molecular cloning and expression of GFP cDNA in *Escherichia coli* have led to amino acid-sequence determination of the protein and to the location of the chromophore in the primary structure (7, 8). GFP expressed in *E. coli* has also been shown to possess spectroscopic properties identical to the native protein (8–10). Recent studies have indicated that formation of the chromophore may depend on molecular oxygen or temperature (10–12). In addition, various mutant forms of GFP with different fluorescence excitation and emission spectra have been produced through mutagenesis (10, 12, 13).

The characteristic properties of GFP make this protein a good candidate for use as a molecular reporter to monitor patterns of protein localization, gene expression, and intracellular protein trafficking in living cells. Several such experiments have been recently carried out in *Caenorhabditis elegans* (8, 14), *Drosophila melanogaster* (15), and a mammalian cell line (16). In this study, expression constructs encoding GFP alone and GFP fused to other regulatory proteins were prepared, and expression of the proteins was analyzed in vertebrate cell lines and primary embryonic cells. Results show that the incubation temperature of GFP-expressing cells critically affects the intensity of green fluorescence and that under optimal conditions intracellular localization and trafficking of proteins can be visualized in a single living cell by fluorescence microscopy.

MATERIALS AND METHODS

Plasmids. The cloned GFP cDNA (pHis-AGP) (9) was used as a template for PCR to include a Kozak's translation start site and a 3′ linker sequence. The amplified DNA fragment was ligated to pCMX (17), generating pCMX-GFP. To prepare pCMX-PML–GFP, a *Mlu* I site was added to the 5′ end of the GFP coding sequence through PCR and inserted at the *Mlu* I site in pCMX-epi-PML (18). The human glucocorticoid receptor (hGR) cDNA, with a truncated 5′ coding region, was isolated from pGR107 (19) by *Sal* I–*Bam*HI digestion and ligated at the cognate sites of pCMX-GFP. The expression vectors used have already been described: pCMX-βGAL (17) and for wild-type hGR (19), amino-terminal truncated hGR mutant (ΔAD) (20), and mouse mammary tumor virus promoter (MMTV)-LUC (firefly) (20).

Cell Culture and Transfection. COS-1 cells, chicken embryonic retina cells, and EPC (fish cell line derived from carp epithelial tumor) cells were maintained in Dulbecco's modified Eagle's medium, without phenol red, supplemented with 10% fetal bovine serum. For EPC cells, 25 mM Hepes was added to the culture medium. For dexamethasone treatment, resin/charcoal-stripped fetal bovine serum was used. Retina cells were dissected from 8-day-old chicken embryos and cultured as described (21). Transfection was done in 6-cm tissue culture dishes by the calcium phosphate-DNA precipitation method for 6–9 hr at 37°C using 6 μg of DNA (17). After washing the DNA precipitate, the following was carried out: (*i*) COS-1 cells were kept for 24 hr at 37°C and then incubated for 36 hr at a specified temperature, (*ii*) retina cells were incubated at 30°C for 48 hr, and (*iii*) EPC cells were maintained at 30°C, including the transfection period (22). For luciferase assays, indicated expression vectors (0.2 μg) were cotransfected with MMTV-LUC (0.5 μg) and pCMX-βGAL (0.2 μg), in 24-well tissue culture dishes. Luminescence was measured in triplicate samples by using a Berthold (Nashua, NH) luminometer, according to the manufacturer's directions.

Microscopy and Image Analysis. Before microscopic observation, the medium was removed and replaced with phosphate-buffered saline buffer. The cells were examined by using a

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Abbreviations: GFP, green fluorescent protein; hGR, human glucocorticoid receptor; DAPI, 4′,6-diamidino-2-phenylindole; MMTV, mouse mammary tumor virus promoter.

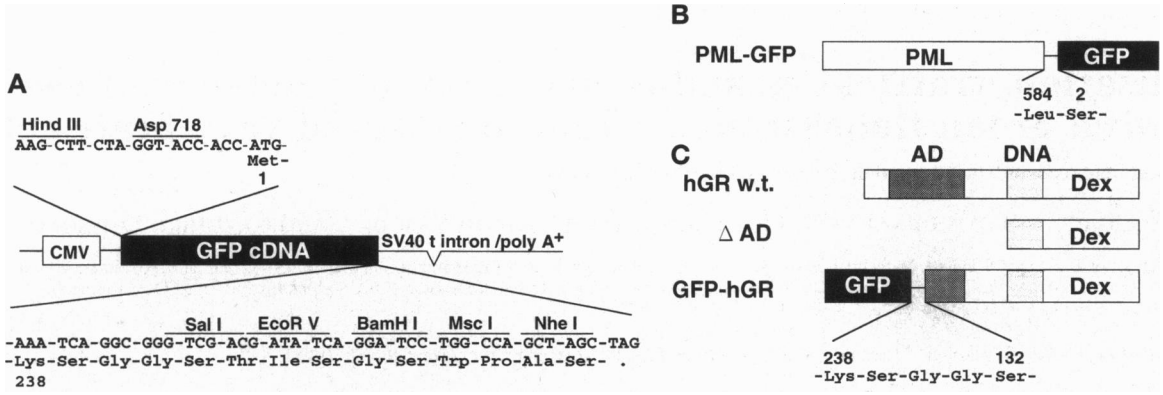

FIG. 1. Construction of GFP expression plasmids and structure of GFP fusion proteins. (*A*) Schematic structure of pCMX-GFP, encoding full-length GFP for expression in vertebrate cells. GFP cDNA is transcribed from human cytomegalovirus (CMV) immediate early promoter followed by a simian virus 40 small t intron and poly(A)$^+$ signal. The 5′ end of the cDNA was modified to encode a Kozak's consensus sequence (ACCACC-ATG). To the carboxyl-terminal lysine codon of the GFP cDNA, 13 amino acid residues were added to accommodate several restriction enzyme sites. (*B*) Structure of PML–GFP fusion protein. Full-length GFP was fused at the carboxyl terminus of PML protein through *Mlu* I sites in the cDNAs. In this construct, PML protein lacks the last tryptophan residue. (*C*) Structure of full-length hGR (hGR w.t.), amino-terminal truncated hGR (ΔAD), and GFP–hGR fusion proteins. A synthetic initiation codon was placed in front of the DNA-binding domain (DNA) of hGR to generate ΔAD protein, which lacks the amino-terminal transactivation domain (AD) (20). In GFP–hGR, GFP was fused amino-terminal to an hGR that lacks the first 131 amino acid residues. Dex, dexamethasone.

Zeiss Axiophot microscope, equipped with a fluorescein isothiocyanate filter set for fluorescence detection and a ×40 or ×63 water-immersion objective. Where indicated, cells were fixed with 4% (vol/vol) paraformaldehyde on the culture dish. To visualize nuclei, cells were treated with 0.1% Triton X-100 and soaked in 4′,6-diamidino-2-phenylindole (DAPI) at 50 ng/ml in phosphate-buffered saline. Photographs were taken by using Kodak Echtachrome 400, and films were subsequently processed to enhance sensitivity.

RESULTS

Detection of GFP Fluorescence in Cells. Attempts were first made to visualize fluorescence in a single cell by overexpress-

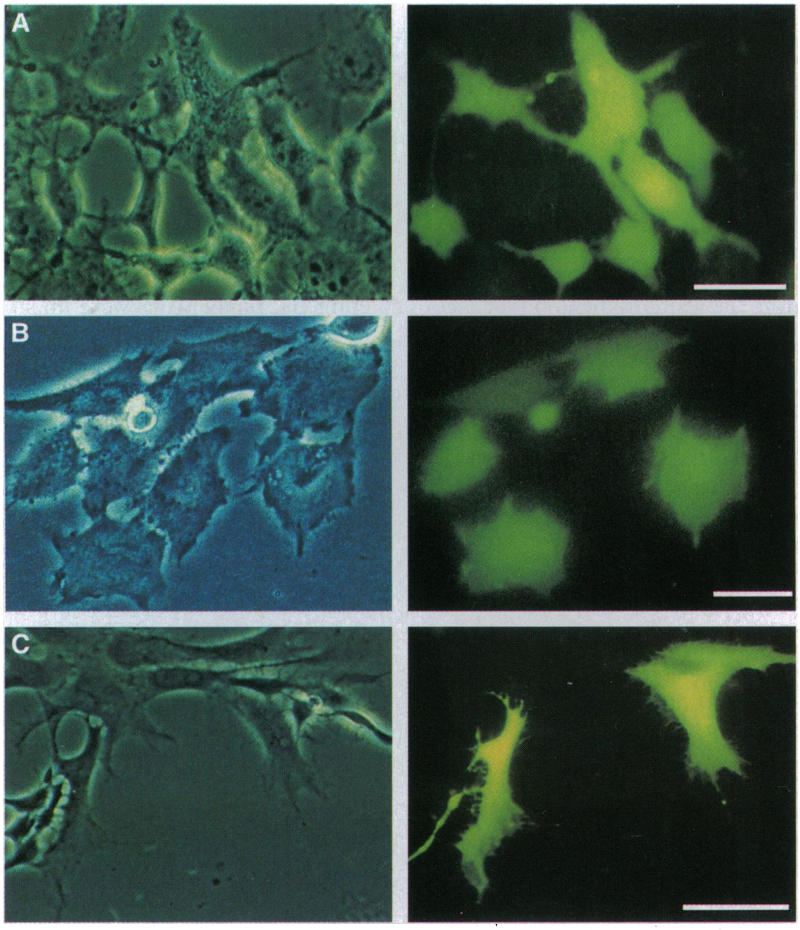

FIG. 2. Detection of fluorescence in cells expressing GFP. (*A*) COS-1 cells. (*B*) Embryonic chicken retina cells. (*C*) Carp epithelial EPC cells. (*Left*) Phase-contrast microscopy of cells. (*Right*) Fluorescence microscopy of same cells. (Bar = 25 μm.)

ing GFP in *E. coli* carrying pHis-AGP at 37°C (9). Strong green fluorescence in single cells was readily detectable under the microscope (data not shown). Subsequently, a eukaryotic expression construct of GFP, pCMX-GFP, was prepared and used for transient transfection of COS-1 cells. Fig. 1 shows a schematic diagram of GFP expression plasmids and structures of GFP fusion proteins. To monitor transfection efficiency, an expression plasmid for *E. coli* β-galactosidase (pCMX-βGAL) was cotransfected. The results showed that at 10% transfection efficiency, as judged by *in situ* staining of β-galactosidase activity, fluorescent cells were barely detectable. Similar results were obtained with CV-1 cells.

The low fluorescence intensity suggested the possibility that temperature may be a factor because *Aequorea victoria* lives in cold water (Friday Harbor, WA) and successful expression of GFP has been reported in nematodes and flies, both of which are usually maintained at 20–25°C (8, 14, 15). Experiments with temperature shifts showed that to detect fluorescence it was important to shift the incubation temperature after transfection from 37°C to 30°C for at least 4 hr before observing fluorescence (Fig. 2*A*). The strongest intensity was obtained when cells were kept at 37°C for 24 hr after removing the DNA precipitate and then incubating cells at 30°C for another 36–48 hr.

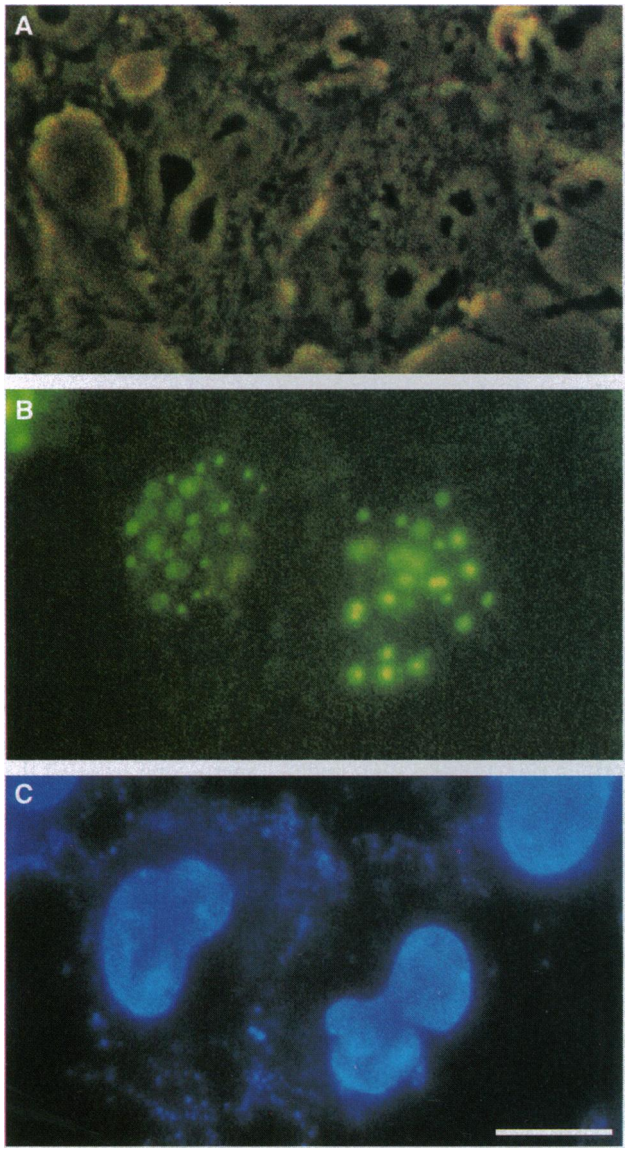

FIG. 3. Localization of fluorescently tagged PML protein in COS-1 cells. Same cells are viewed by phase contrast microscopy (*A*), fluorescence microscopy (*B*), and DAPI staining (*C*). (Bar = 10 μm.)

Similar temperature-shift experiments were done with embryonic cells in primary culture labeled with GFP. Retina cells, dissected from 8-day-old chicken embryos and maintained in culture, were transfected with pCMX-GFP at 37°C, followed by incubation at 30°C. Very good fluorescence was observed under these conditions (Fig. 2*B*), indicating that GFP can be used to label vertebrate embryonic cells, as well as immortalized cells.

An unavoidable problem encountered with mammalian and avian cells when the incubation temperature was lowered from 37°C to 30°C was a slowing in the metabolic rate. For comparison, the fish cell line EPC, derived from carp epithelial tumor and which proliferates well at 30°C (22), was transfected with pCMX-GFP and incubated at 30°C. The number of intensely fluorescent cells greatly increased over the number found with homoiothermic cells (Fig. 2*C*). The conclusions to be drawn from these observations are that temperature is an important parameter in detection of *in vivo* GFP fluorescence and that the promoter is highly active in driving transcription of cDNA for GFP.

Intracellular Localization of GFP. Fig. 2 shows that GFP appears uniformly distributed throughout the cytoplasm and nucleus. This pattern was not due to the additional 13-amino acid residues attached to the carboxyl terminus of the pCMX-GFP-encoded protein (Fig. 1*A*) because an identical pattern was observed with intact unmodified GFP (data not shown). To visualize other proteins, a host protein with an easily recognizable pattern of localization was selected. The *PML* protooncogene has been identified as a partner that undergoes reciprocal chromosomal recombination with the retinoic acid receptor α gene in acute promyelocytic leukemia (23–26). The PML protein localizes in punctate nuclear organelles called PML oncogenic domains or nuclear bodies (18, 27, 28). The GFP was fused to the carboxyl terminus of the PML protein (Fig. 1*B*), and expression plasmid pCMX-PML–GFP was used to transfect COS-1 cells. Upon incubation as described, uniform distribution of GFP fluorescence was no longer seen; instead a punctate nuclear fluorescence pattern, reminiscent of the PML oncogenic domains in fixed cells, was visualized

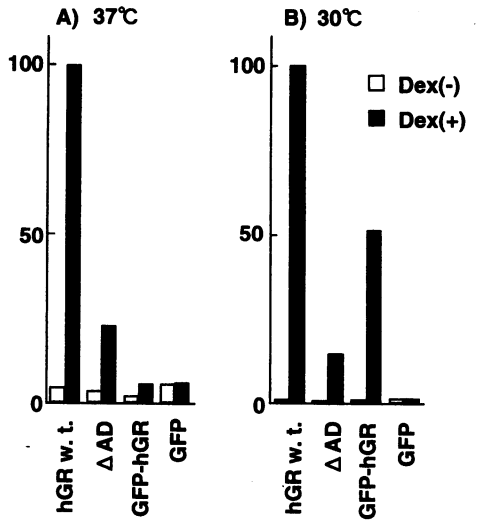

FIG. 4. Transcriptional enhancement by hGR mutants in COS-1 cells. Dexamethasone (Dex)-inducible reporter MMTV-LUC was cotransfected with expression plasmids encoding full-length hGR (hGR w.t.), amino-terminal truncated mutant ΔAD, GFP–hGR fusion, or GFP alone. Two different temperatures were tested: *A*, 37°C; *B*, 30°C. Maximum induction obtained with hGR w.t. by challenge with 1 μM Dex was taken as 100, and the relative reporter luciferase activities were plotted. All values are averages of triplicate experiments.

(Fig. 3*B*). A single fluorescent spot is called a "nuclear body," and the two clusters represent two cell nuclei. Most fluorescent spots were nuclear, but occasionally a few spots could be detected in the cytoplasm. This result showed that GFP can be used as an efficient tag to trace distribution of the host protein to small nuclear organelles such as PML oncogenic domains in living cells.

Properties of GFP–hGR Fusion Proteins in COS-1 Cells. To carry out real-time imaging of protein trafficking in a single living cell, pCMX-GFP–hGR was prepared by fusing GFP to a mutant hGR containing intact DNA- and ligand-binding domains (Fig. 1*C*). The advantages of this fusion protein are as follows: (*i*) it contains the functional properties of a transcription factor that can be addressed through hormone-dependent transactivation of reporter genes such as MMTV-LUC (20) and (*ii*) hGR, which is cytoplasmic in the absence of hormone, translocates to the nucleus upon binding to hormone (for review, see ref. 29).

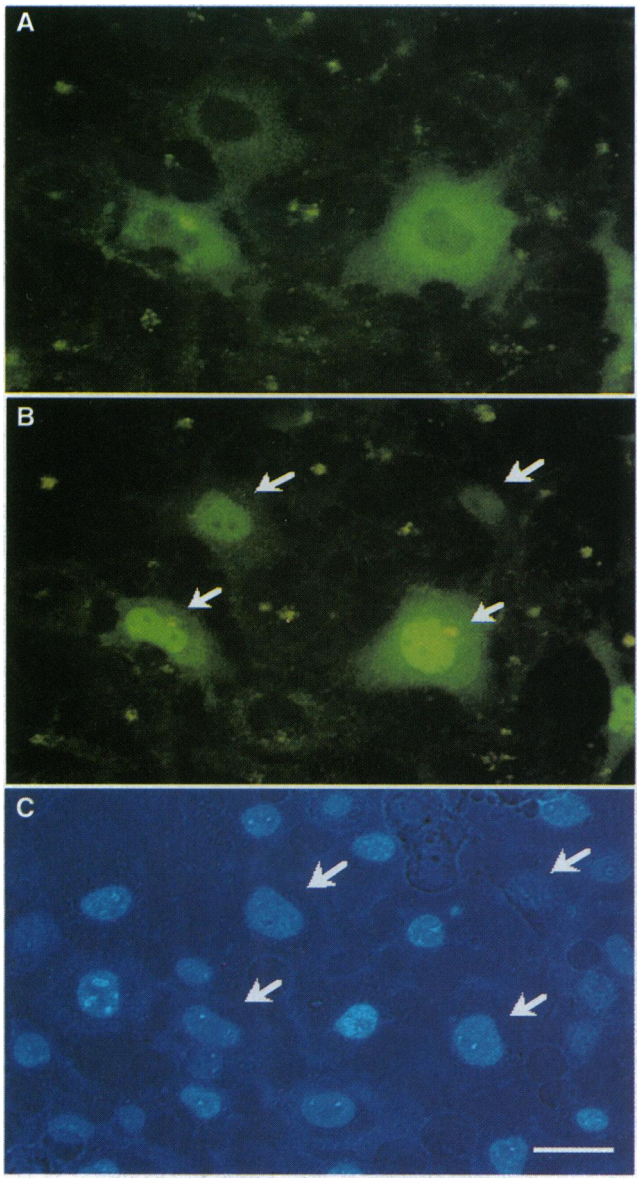

FIG. 5. Nuclear transfer of GFP–hGR fusion protein in COS-1 cells. (*A*) COS-1 cells were transfected with pCMX-GFP–hGR, and fluorescence was observed mainly in the cytoplasm. (*B*) Fluorescence seen 30 min after addition of 1 μM dexamethasone to COS-1 cells in *A*. Arrows indicate nuclear fluorescence from translocation of fusion protein in response to hormone. (*C*) Same field after DAPI staining. (Bar = 25 μM.)

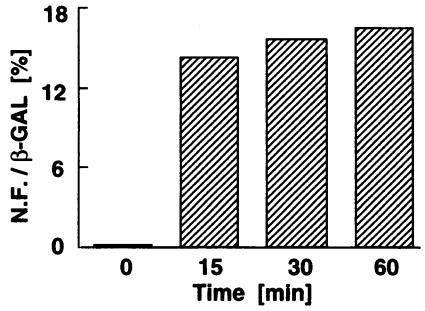

FIG. 6. Time course of increase in number of COS-1 cells with positive nuclear fluorescence after dexamethasone addition. Cells were transfected with pCMX-GFP–hGR and pCMX-βGAL, after which they were incubated for 0, 15, 30, and 60 min with 1 μM dexamethasone and then fixed. Number of cells showing nuclear fluorescence (N.F.) was determined, and cells were then analyzed for β-galactosidase (β-GAL) activity *in situ*. Percentages of fluorescent cells against β-galactosidase-positive cells at each time point were calculated and plotted.

With COS-1 cells, pCMX-GFP–hGR was cotransfected with MMTV-LUC, and transfection efficiency was determined by using cotransfected pCMX-βGAL. When cells were kept at 37°C, transfection with pCMX-GFP–hGR gave no transactivation, whereas wild-type hGR and truncated hGR lacking the amino-terminal transactivation domain (ΔAD) induced reporter luciferase activity in response to dexamethasone, a synthetic glucocorticoid (Fig. 4*A*). However, efficient transactivation by GFP–hGR was seen when the incubation temperature was shifted to 30°C. This result showed that GFP can be fused to a transcription factor without affecting its function of either binding DNA or interacting with the transcription machinery (Fig. 4*B*).

Further evidence of GFP localization and nuclear translocation was obtained as follows. Without dexamethasone, COS-1 cells transfected with pCMX-GFP–hGR showed localization of the fusion protein, as evidenced by green fluorescence, primarily in the cytoplasm (Fig. 5*A*). When dexamethasone (1 μM) was added, followed by incubation at 30°C for 30 min, fluorescence shifted into the nucleus (Fig. 5*B*).

To estimate the rate of GFP–hGR nuclear transfer induced by dexamethasone, COS-1 cells were cotransfected with pCMX-GFP–hGR (5 μg) and pCMX-βGAL (1 μg), and after visualization of cytoplasmic fluorescence, cells were challenged with hormone for 0, 15, 30, and 60 min at 37°C, followed by paraformaldehyde fixation to stop further protein transfer. The number of cells with nuclear fluorescence was determined, and percentages were calculated from the number of β-galactosidase-positive cells on the same plate. Fig. 6 shows that nuclear transfer was almost complete within 15 min. Further, direct observation under a microscope revealed that some cells completed their nuclear transfer of GFP–hGR within 5 min.

DISCUSSION

Since the report of Chalfie *et al.* (8), there has been wide interest in the application of GFP to solving biological problems. In this study, we have concentrated on GFP labeling in vertebrate cell systems. Our results show that incubation temperature is important in the detection of GFP fluorescence in mammalian, avian, and fish cells. Further, functional activity of fusion protein GFP–hGR also appears to depend on temperature. The underlying cause of this temperature dependence is still unknown, but it may be associated with the folding and/or redox state of GFP (11) within the cell. A number of factors appear to be involved because (*i*) fluorescence of highly purified recombinant GFP (11) dissolved in 100 mM ammo-

nium bicarbonate, pH 8.0, did not show the same temperature dependence as the protein *in vivo*, (*ii*) incubation time required at 30°C was relatively short (4 hr), (*iii*) fluorescence was greater when a 24-hr incubation period at 37°C preceded the temperature shift, presumably due to GFP accumulation, and (*iv*) once the cells began to fluoresce at 30°C, they remained fluorescent when the temperature was shifted back to 37°C (at least for an additional 48 hr).

These results suggest various possibilities for using GFP as an intracellular reporter—for example, in labeling chicken embryos by retroviral gene transfer and shifting the temperature down during incubation. Marshall *et al.* (16) have recently reported the detection of GFP targeted to membrane in a human cell line after transient transfection with an expression construct. Even though the gfp10 GFP (7) used by Marshall *et al.* (16) differs at seven amino acid positions from our GFP (9), their protein has also been found to exhibit temperature sensitivity when expressed in yeast cells (K. Kohno, personal communication). Both the work by Marshall *et al.* (16) and our work clearly indicate that GFP may be used to visualize intracellular components within a vertebrate cell. Visualization of intracellular protein trafficking as seen with GFP–hGR should also allow experiments to be designed to address important biological questions. For example, immediate applications would be the study of nuclear translocation of other transcription factors (e.g., NF-κB, aryl hydrocarbon receptors, sterol regulatory element-binding protein 1, and STAT proteins), synaptic vesicle transfer, exocytosis, stress-induced cytoskeletal reorganization, and protein secretion.

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1. Johnson, F. H., Shimomura, O., Saiga, Y., Gershman, L. C., Reynolds, G. T. & Waters, J. R. (1962) *J. Cell. Comp. Physiol.* **60,** 85–103.
2. Morin, J. G. & Hastings, J. W. (1971) *J. Cell. Physiol.* **77,** 313–318.
3. Morise, H., Shimomura, O., Johnson, F. H. & Winant, J. (1974) *Biochemistry* **13,** 2656–2662.
4. Prendergast, F. G. & Mann, K. G. (1978) *Biochemistry* **17,** 3448–3453.
5. Shimomura, O. (1979) *FEBS Lett.* **104,** 220–222.
6. Cody, C. W., Prasher, D. C., Westler, W. M., Prendergast, F. G. & Ward, W. W. (1993) *Biochemistry* **32,** 1212–1218.
7. Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G. & Cormier, M. J. (1992) *Gene* **111,** 229–233.
8. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. & Prasher, D. C. (1994) *Science* **263,** 802–805.
9. Inouye, S. & Tsuji, F. I. (1994) *FEBS Lett.* **341,** 277–280.
10. Heim, R., Prasher, D. C. & Tsien, R. Y. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 12501–12504.
11. Inouye, S. & Tsuji, F. I. (1994) *FEBS Lett.* **351,** 211–214.
12. Heim, R., Cubitt, A. B. & Tsien, R. Y. (1995) *Nature (London)* **373,** 663–664.
13. Delagrave, S., Hawtin, R. E., Silva, C. M., Yang, M. M. & Youvan, D. C. (1995) *Bio/Technology* **13,** 151–154.
14. Sengupta, P., Colbert, H. A. & Bargmann, C. I. (1994) *Cell* **79,** 971–980.
15. Wang, S. & Hazelrigg, T. (1994) *Nature (London)* **369,** 400–403.
16. Marshall, J., Molloy, R., Moss, G. W. J., Howe, J. R. & Hughes, T. E. (1995) *Neuron* **14,** 211–215.
17. Umesono, K., Murakami, K. K., Thompson, C. C. & Evans, R. M. (1991) *Cell* **65,** 1255–1266.
18. Dyck, J. A., Maul, G. G., Miller, W. H., Jr., Chen, J. D., Kakizuka, A. & Evans, R. M. (1994) *Cell* **76,** 333–343.
19. Hollenberg, S. M., Weinberger, C., Ong, E. S., Cerelli, G., Oro, A., Lebo, R., Thompson, E. B., Rosenfeld, M. G. & Evans, R. M. (1985) *Nature (London)* **318,** 635–641.
20. Hollenberg, S. M. & Evans, R. M. (1988) *Cell* **55,** 899–906.
21. Okada, T. S., Yasuda, K., Araki, M. & Eguchi, G. (1979) *Dev. Biol.* **68,** 600–617.
22. Kamei, Y., Yoshimizu, M. & Kimura, T. (1987) *Fish Pathol.* **22,** 147–152.
23. Kakizuka, A., Miller, W. H., Jr., Umesono, K., Warrell, R. P., Jr., Frankel, S. R., Murty, V. V. V. S., Dmitrovsky, E. & Evans, R. M. (1991) *Cell* **66,** 663–674.
24. de Thé, H., Lavau, C., Marchio, A., Chomienne, C., Degos, L. & Dejean, A. (1991) *Cell* **66,** 675–684.
25. Goddard, A. D., Borrow, J., Freemont, P. S. & Solomon, E. (1991) *Science* **542,** 1371–1374.
26. Kastner, P., Perez, A., Lutz, Y., Rochette-Egly, C., Gaub, M.-P., Durand, B., Lanotte, M., Berger, R. & Chambon, P. (1992) *EMBO J.* **11,** 629–642.
27. Weis, K., Rambaud, S., Lavau, C., Jansen, J., Carvalho, T., Carmo-Fonseca, M., Lamond, A. & Dejean, A. (1994) *Cell* **76,** 345–356.
28. Koken, M. H. M., Puvion-Dutilleul, F., Guillemin, M. C., Viron, A., Linares-Cruz, G., Stuurman, N., de Jong, L., Szostecki, C., Calvo, F., Chomienne, C., Degos, L., Puvion, E. & de Thé, H. (1994) *EMBO J.* **13,** 1073–1083.
29. Akner, G., Wikström, A.-C. & Gustafsson, J.-Å. (1995) *J. Steroid Biochem. Mol. Biol.* **52,** 1–16.