

## NOTES

### Red Fluorescent Protein (DsRed) as a Reporter in *Saccharomyces cerevisiae*

FERNANDO RODRIGUES,<sup>1,2</sup> MARTIJN VAN HEMERT,<sup>2</sup> H. YDE STEENSMA,<sup>2,3</sup>  
MANUELA CÔRTE-REAL,<sup>1\*</sup> AND CECÍLA LEÃO<sup>1</sup>

*Centro de Ciências do Ambiente, Departamento de Biologia, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal,<sup>1</sup> and Institute of Molecular Plant Sciences, Clusius Laboratory, Leiden University, 2333 AL Leiden,<sup>2</sup> and Kluyver Institute for Biotechnology, Delft University of Technology, 2628 BC Delft,<sup>3</sup> The Netherlands*

Received 9 January 2001/Accepted 16 March 2001

**We describe the utilization of a red fluorescent protein (DsRed) as an in vivo marker for *Saccharomyces cerevisiae*. Clones expressing red and/or green fluorescent proteins with both cytoplasmic and nuclear localization were obtained. A series of vectors are now available which can be used to create amino-terminal (N-terminal) and carboxyl-terminal (C-terminal) fusions with the DsRed protein.**

Green fluorescent protein (GFP) is a powerful tool for identifying the subcellular localization of proteins and to monitor gene expression. The protein is capable of producing a strong green fluorescence when excited by blue light, without any exogenously added substrate or cofactor (3). Events inside living cell can thus be visualized in a noninvasive way (3, 12, 16). For *Saccharomyces cerevisiae*, a series of plasmids has been developed for the expression of N-terminal and C-terminal in-frame fusions with the protein of interest (4, 5, 16). These so-called pUG vectors have proved useful for localization and expression studies in this yeast.

Recently, fluorescent proteins have been described that emit light with a wavelength different from that of GFP. These comprise blue-, cyan-, and yellow-shifted mutants of GFP and the newly isolated DsRed (1, 6, 20). The latter is a red-emitting fluorescent protein. The longer wavelength of the emitted light minimizes problems associated with light scattering and autofluorescence of the cells (21, 23). Fluorescent proteins with different emission colors are valuable for in vivo multilabeling experiments, allowing comonitoring of several events (8, 22).

In this study, we show the use of the red fluorescent protein DsRed as a reporter in yeast cells. Based on the pUG plasmids, a new set of vectors expressing *DsRed* was constructed, allowing the production of amino-terminal (N-terminal) and carboxyl-terminal (C-terminal) fusion proteins. Our results indicate that *DsRed* can be expressed in *S. cerevisiae*, and the protein can be targeted specifically to the nucleus. Finally, we show that cells with nuclei labeled with either red or green fluorescent proteins can be used to follow mating in vivo.

The isogenic strains W303-1A (*Mata*) and W303-1B (*Mata*) of *S. cerevisiae* (*ade2-1 his3-11,15 ura3-1 leu2-3,112 trp1*) were used. *Escherichia coli* XL1-Blue was used as the bacterial host for plasmids (2). *E. coli* strains were grown in Luria-Bertani

medium (LB) at 37°C (19). Yeast strains were grown in YPD (17) or, for selective purposes, in a synthetic medium (24).

The plasmid pR1 was constructed by digesting the vector pUG36 with *Xba*I. The product thus obtained was ligated to the 681-bp *Xba*I fragment of the pDsRed vector (Clontech). U. Güldener and J. H. Hegemann, Düsseldorf, Germany, kindly provided all pUG vectors used in this study. The sequences of all these vectors are available in the MIPS website (<http://www.mips.biochem.mpg.de/proj/yeast/info/tools/hegemann/gf-p.html>).

The *yEGFP3* gene was removed from the plasmids pUG34 and pUG36 by digestion with *Xba*I followed by self-ligation. Two plasmids were obtained pSL34 and pSL36, respectively. The *DsRed* gene was obtained by PCR on the vector pDsRed with the primers P1 and P2 (Table 1). After digestion with *Bam*HI and *Xba*I, the fragment was ligated into pSL34 or pSL36 digested with the same enzymes. The resulting plasmids were named pUR34 and pUR36, respectively. pUR23 and pUR35 were obtained similarly by cloning a *Clal*- and *Xho*I-digested *DsRed* gene PCR product obtained by using primers P3 and P4 (Table 1) into the *Clal*- and *Xho*I-digested plasmids pUG34 and pUG36, respectively. The vectors pUR34NLS, pUR36NLS, and pUG36NLS were obtained by ligating an NLS1 fragment into the *Bam*HI- and *Xho*I-digested vectors pUR34, pUR36, and pUG36, respectively. The NLS1 fragment was obtained as follows. The oligonucleotide O1 and O2 (Table 1) were mixed, boiled for 5 min, and then cooled overnight in a water bath. The vectors pUR23NLS, and pUR35NLS were obtained mainly as described above. In these constructs we used the vectors pUR23 and pUR35, the enzymes *Bam*HI and *Sal*I, and the oligonucleotides O3 and O4 (Table 1). DNA manipulations and transformation procedures were performed as described elsewhere (7, 10, 18, 19).

Cells collected from middle of the exponential growth phase were used for fluorescence microscopy analysis. For colocalization of DsRed, yEGFP3, and DNA, the cells were fixed as follows. Approximately 10<sup>9</sup> cells were incubated in 100 mM

\* Corresponding author. Mailing address: Departamento de Biologia, Universidade do Minho, 4710-057 Braga, Portugal. Phone: 351-253-604314. Fax: 351-253-678980. E-mail: mcortereal@bio.uminho.pt.

TABLE 1. Sequences of the oligonucleotides used in the constructions of the plasmids

Name	Sequence (5'-3')
P1	GCTCTAGAATGAGGTCTTCCAAGAATGTT
P2	CGGGATCCAAGGAACAGATGGTGGCGTCC
P3	CCGCTCGAGCATAAGGAACAGATGGTGGCGTCC
P4	CCATCGATACCGTCGACATGAGGTCTTCCAAGAATGTT
O1	GATCGCCAAAAAAGAAGAGAAAAGGTCGTTGTTAAATAG
O2	TCGACTATTTAACAACGACCTTTCTCTCTTTTTGGC
O3	GATCCATGCCAAAAAAGAAGAGAAAAGGTCGTTGTTAAAT
O4	TCGAATTTAACAACGACCTTTCTCTCTTTTTGGCATG

phosphate buffer (pH 6.5) containing 3.7% formaldehyde at room temperature during 2 h. Fixation was also performed with 70% ethanol at 4°C during 30 min. To stain DNA, the fixed cells were resuspended in 100 mM phosphate buffer containing 4', 6'-diamidino-2-phenylindole (DAPI; 0.5 µg/ml) at room temperature for 10 min. The stained cells were washed twice and subjected to fluorescence microscopy. For fluorescence microscopy a Zeiss Axioplan microscope equipped with a mercury lamp and a 510–560 FT 580 (excitation), LP 590 (emission) filter set was used (17). The same microscope coupled to a Bio-Rad 1024 system was used for confocal laser-scanning microscopy with excitation by the 568-nm line of a krypton-argon laser and using an emission filter LP 585.

**Expression and subcellular localization of the DsRed protein.** To assess whether the *DsRed* gene was expressed in *S. cerevisiae*, the *yEGFP3* gene in the vector pUG36 was replaced by *DsRed* (4). When the resulting plasmid, pR1, was introduced into *S. cerevisiae*, the cells emitted a red bright fluorescence when illuminated with UV light (Fig. 1I). We developed a similar set of vectors, based on the pUG plasmids, expressing a red fluorescent protein, DsRed. pUR23 and pUR35 allow the production of C-terminal fused proteins and contain as selective markers the *HIS3* and *URA3* genes, respectively. The vectors for N-terminal fusions were called pUR34 (*HIS3*) and pUR36 (*URA3*). While this study was in preparation, Baird and coworkers reported the multimeric nature of DsRed (1, 9). For some purposes, this oligomerization can be troublesome since it may lead to misinterpretation of the results. Nevertheless, the new set of DsRed vectors that we described may be very useful, since genes cloned on pUG vector can be easily transferred to pUR vectors and vice versa. This allowed us to test whether DsRed oligomerization interferes with trafficking of the host protein. To use *DsRed* as a reporter gene, the resulting protein should (i) not carry any specific target information, (ii) result in a functional protein when fused in frame, and (iii) not be toxic when expressed in *S. cerevisiae* cells. The *in silico* analysis of the coding sequence of *DsRed* and *yEGFP3* did not show significant differences with respect to the predicted localization (data not shown). Our data demonstrated that when *DsRed* was expressed in *S. cerevisiae* a bright red fluorescence was spread uniformly over the cell (Fig. 1I). This result is in agreement with the localization of the protein in the cytoplasm, indicating that the protein does not have its own functional targeting signal in this organism. The expression of *DsRed* did not appear to have any toxic effect on cell growth, as judged by specific growth rates. In addition, ca. 90% of the cell population emitted a red bright signal, and this signal was

stronger in cells grown in liquid media, with high rates of oxygenation, than in cells from solid media.

The NLS (PKKKRKV132) of the simian virus 40 large T antigen was used as a single cluster for nuclear targeting of DsRed in *S. cerevisiae* (11, 13). A dimer of oligonucleotides encoding this nuclear localization signal (NLS) was cloned in frame in all pUR, resulting in a redistribution of the fluorescent signal to a single subcellular compartment (Fig. 1IIB). As expected, counterstaining of the cells with DAPI confirmed the nuclear localization of DsRed-NLS (Fig. 1III A, -B, and -C) and *yEGFP3*-NLS (data not shown). Mozdy et al. have recently used the DsRed protein with a mitochondrial target in *S. cerevisiae* (15).

The fluorescence of the DsRed persisted after treatment of the cells with either ethanol or formaldehyde. This allows examination of preparations fixed with these chemicals and the utilization of antibodies. Clontech commercializes antibodies for DsRed protein that are specific and do not recognize *yEGFP3*.

**Double labeling and potential applications.** Clones of *S. cerevisiae* expressing *DsRed-NLS* and *yEGFP3* showed emission of both fluorescences (Fig. 1III). These cells showed green fluorescence in the cytoplasm and red, orange, or yellow fluorescence in the nuclei. The different colors of the nucleus may be attributed to colocalization of the two proteins. It has been demonstrated that some GFP can diffuse into the nuclei without any signal (22). Nevertheless, differences in the superposition of the green and red fluorescence in the cytoplasm and nuclei may occur. This fact may also explain the observed range colors of nuclei. Reinforcing this idea, cells expressing both *DsRed-NLS* and *yEGFP3-NLS* gave indeed rise to bright yellow fluorescent nuclei (data not shown).

Taking advantage of the double labeling, we followed mating *in vivo*. One of the mating types of the strain *S. cerevisiae* W303 was transformed with plasmid expressing *DsRed-NLS*, and the other was transformed with plasmid expressing *yEGFP3-NLS*, and conjugation was induced. Time-lapse digital imaging in a confocal laser-scanning microscope allowed tracking the dynamics of the mating process in *S. cerevisiae*. An example of the images obtained is shown in Fig. 1IV, where both nuclei have moved toward the shmoo tips. Our data demonstrated that DsRed-NLS can be used to stain the nuclei of live cells in a manner that is compatible with the use of *yEGFP3* and confocal laser-scanning microscopy. This has proven to be difficult with the existing chemical fluorescent dyes for the nucleus (M. van Hemert, unpublished data). In conclusion, our data show that *DsRed* is well suited as a reporter gene for *S. cerevisiae* and

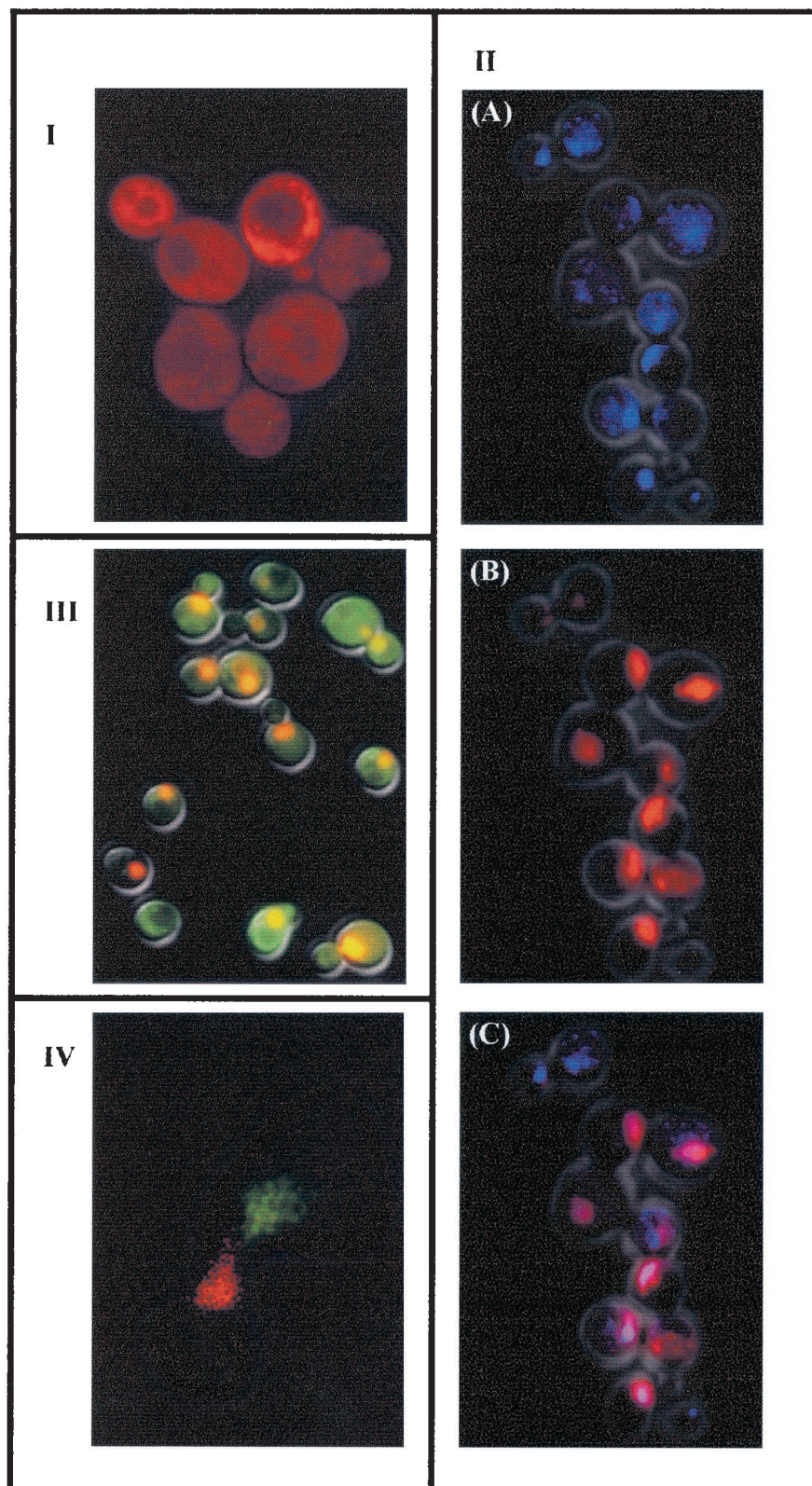


FIG. 1. Fluorescent microscopy of *S. cerevisiae* cells: I, transformed with the pR1 vector; II, harboring the vector pUR36NLS, showing DNA counterstained with DAPI in blue (A), red fluorescence of DsRed-NLS in red (B), and the overlap of both colors (colocalization) in violet (C); III, harboring the vectors pUR36NLS and pUG34 (IV). Confocal laser scanning microscopy of *S. cerevisiae* cells during mating. The different mating types are expressing yEGFP3-NLS or DsRed-NLS. The fluorescences of both yEGFP3-NLS and yEGFP3 are coded in green, and those of DsRed-NLS and DsRed are coded in red.

is an alternative to those techniques that modify cell structure. Moreover, it allows studying dynamic processes in vivo. However, the slow maturation of DsRed (1, 9) can limit its use under the control of an inducible promoter. If we take all of the results together, DsRed should be envisaged more as an addition tool than as a substitute for yEGFP3 in expression studies.

In conclusion, *DsRed*, together with *yEGFP3*, can be used to study competition between different strains or yeast species in confined environments. This methodology also emerges as a powerful technique to investigate fusion events of organelles during mating. In fact, we are using this technique to assess whether nuclear fusion occurs during the conjugation of *Zygosaccharomyces bailii* prior to sporulation, since the production of spores in this species does not seem to be a product of meiosis (14).

We are especially grateful to Gerda Lamers for technical support with the confocal laser scanning microscopy experiments.

This study was supported by a research grant (contract PRAXIS XXI P/AGR/11135/98). Fernando Rodrigues was a recipient of a fellowship from PRAXIS XXI (Fundação para a Ciência e Tecnologia).

#### REFERENCES

- Baird, G. S., D. A. Zacharias, and R. Y. Tsien. 2000. Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. *Proc. Natl. Acad. Sci. USA* **97**:11984–11989.
- Bullock, W. O., J. M. Fernandez, and J. M. S. Short. 1987. XL1-Blue: a highly efficient plasmid transforming *recA* *Escherichia coli* strain with  $\beta$ -galactosidase selection. *BioTechniques* **4**:376–378.
- Chalfie, M., Y. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher. 1994. Green fluorescent protein as a marker for gene expression. *Science* **263**:802–803.
- Cormack, B. P., G. Bertram, M. Egerton, N. A. Gow, S. Falkow, and A. J. Brown. 1997. Yeast-enhanced green fluorescent protein (yEGFP) a reporter of gene expression in *Candida albicans*. *Microbiology* **143**:303–311.
- Craven, R. A., D. J. Griffiths, K. S. Sheldrick, R. E. Randall, I. M. Hagan, and A. M. Carr. 1998. Vectors for the expression of tagged proteins in *Schizosaccharomyces pombe*. *Gene* **221**:59–68.
- Ehrig, T., D. J. O'Kane, and F. G. Prendergast. 1995. Green-fluorescent protein mutants with altered fluorescence excitation spectra. *FEBS Lett.* **367**:163–166.
- Gietz, R. D., and R. H. Schiestl. 1995. Transforming yeast with DNA. *Methods Mol. Cell Biol.* **5**:255–269.
- Haseloff, J. 1999. GFP variants for multispectral imaging of living cells. *Methods Cell Biol.* **58**:139–151.
- Heikal, A. A., S. T. Hess, G. S. Baird, R. Y. Tsien, and W. W. Webb. 2000. Molecular spectroscopy and dynamics of intrinsically fluorescent proteins: coral red (dsRed) and yellow (Citrine). *Proc. Natl. Acad. Sci. USA* **97**:11996–12001.
- Inune, H., H. Nojima, and H. Okayama. 1990. High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96**:23–28.
- Makkerh, J. P. S., C. Dingwall, and R. A. Laskey. 1996. Comparative mutagenesis of nuclear localization signals reveals the importance of neutral and acidic amino acids. *Curr. Biol.* **6**:1025–1027.
- Mayer, G., H. Launhardt, and T. Munder. 1999. Application of the green fluorescent protein as a reporter for Ace1-based, two-hybrid studies. *BioTechniques* **27**:86–84.
- Miyamoto, Y., N. Imamoto, T. Sekimoto, T. Tachibana, T. Seki, S. Tada, T. Enomoto, and Y. Yoneda. 1997. Differential modes of nuclear localization signal (NLS) recognition by three distinct classes of NLS receptors. *J. Biol. Chem.* **272**:26375–26381.
- Mollapour, M., and P. W. Piper. 2001. Targeted gene deletion in *Zygosaccharomyces bailii*. *Yeast* **18**:173–186.
- Mozy, A. D., J. M. McCaffery, and J. M. Shaw. 2000. Dnm1p GTPase-mediated mitochondrial fission is a multi-step process requiring the novel integral membrane component Fis1p. *J. Cell Biol.* **151**:367–380.
- Niedenthal, R. K., L. Riles, M. Johnston, and J. H. Hegemann. 1996. Green fluorescent protein as a marker for gene expression and subcellular localization in budding yeast. *Yeast* **12**:773–786.
- Pringle, J. R., A. E. Adams, D. G. Drubin, and B. K. Haarer. 1991. Guide to yeast genetics and molecular biology. *Methods Enzymol.* **194**:565–602.
- Rodrigues, F., A.-M. Zeeman, C. Alves, M. J. Sousa, H. Y. Steensma, M. Côte-Real, and C. Leão. 2000. Construction of a genomic library of the food spoilage yeast *Zygosaccharomyces bailii* and isolation of the  $\beta$ -isopropylmalate dehydrogenase gene (*ZbLEU2*). *FEMS Yeast Res.* **1407**:1–5.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1998. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Tsien, R. Y. 1998. The green fluorescent protein. *Annu. Rev. Biochem.* **67**:509–544.
- Tsien, R. Y. 1999. Rosy down for fluorescent proteins. *Nat. Biotechnol.* **17**:954–957.
- von Arnim, A. G., X. W. Deng, and M. G. Stacey. 1998. Cloning vectors for the expression of green fluorescent protein fusion proteins in transgenic plants. *Gene* **221**:35–43.
- Wildt, S., and U. Deuschle. 1999. cobA, a red fluorescent transcriptional reporter for *Escherichia coli*, yeast, and mammalian cells. *Nat. Biotechnol.* **17**:1175–1178.
- Zonneveld, B. J. M. 1986. Cheap and simple yeast media. *J. Microbiol. Methods* **4**:287–291.