The green fluorescent protein (GFP) is a widely used reporter in gene expression and protein localization studies. GFP is a stable protein; this property allows its accumulation and easy detection in cells. However, this stability also limits its application in studies that require rapid reporter turnover. We created a destabilized GFP for use in such studies by fusing amino acids 422–461 of the degradation domain of mouse ornithine decarboxylase (MODC) to the C-terminal end of an enhanced variant of GFP (EGFP). The fusion protein, unlike EGFP, was unstable in the presence of cycloheximide and had a fluorescence half-life of 2 h. Western blot analysis indicated that the fluorescence decay of EGFP-MODC-(422–461) was correlated with degradation of the fusion protein. We mutated key amino acids in the PEST sequence of EGFP-MODC-(422–461) and identified several mutants with variable half-lives. The suitability of destabilized EGFP as a transcription reporter was tested by linking it to NFκB binding sequences and monitoring tumor necrosis factor α-mediated NFκB activation. We obtained time course induction and dose response kinetics similar to secreted alkaline phosphatase obtained in transfected cells. This result did not occur when unmodified EGFP was used as the reporter. Because of its autofluorescence, destabilized EGFP can be used to directly correlate gene induction with biochemical change, such as NFκB translocation to the nucleus.

Because of its easily detected green fluorescence, the green fluorescent protein (GFP) from the jellyfish Aequorea victoria is a widely used reporter in studies of gene expression and protein localization (1–4). GFP fluorescence does not require any substrate or cofactor (5); hence it is possible to use it in many species for live cell detection purposes. The fluorescence of GFPs is dependent on the key sequence Ser-Tyr-Gly (amino acids 65–67). This sequence undergoes spontaneous oxidation to form a cyclized chromophore (6). Enhanced GFP (EGFP) contains mutations of Ser to Thr at amino acid 65 and Phe to Leu at position 64 and is encoded by a gene with human-optimized codons (7–9). Crystallographic structures of wild-type GFP and the mutant S65T reveal that the GFP tertiary structure resembles a barrel (10, 11). GFP is a single chain polypeptide of 238 amino acids (12). Most of these amino acids form β sheets that are compacted through an antiparallel structure to form the barrel. An α-helix containing the chromophore is located inside the barrel, which shields it from the external environment. The compact structure makes GFP very stable under a variety of conditions, including treatment with protease (1). The stability of GFP limits its application in some studies, including transcriptional induction studies.

Cellular proteins differ widely in their stabilities. Rapid turnover in proteins is often caused by signals that induce protein degradation. In some cases, the signal is a primary sequence such as the PEST sequence, a sequence possibly correlated with protein degradation (13, 14). In other cases, the signal is a modification such as phosphorylation (15) or a protein-protein interaction (16, 17). Prior to degradation, most short lived proteins need ubiquitin modification, which is catalyzed by a number of ubiquitin modification enzymes (18–21). Only these ubiquitinated proteins are recognized and degraded by a 26 S proteasome.

A few proteins do not require ubiquitin modification for degradation. One such protein is mouse ornithine decarboxylase (MODC) (22–24). MODC is the key enzyme in the biosynthesis of polyamines. This protein is known to be one of the most short lived proteins in mammalian cells, and its half-life is approximately 30 min. In contrast, ornithine decarboxylase of Trypanosoma brucei is quite stable when expressed in mammalian cells (25). Comparison of the primary sequences of these two proteins shows over 60% homology (26); however, MODC has extra sequences at its C terminus that are not needed for ornithine decarboxylase activity (14). This C terminus contains a PEST sequence, and its deletion from MODC prevents its rapid degradation (14). Furthermore, the C-terminal extension is also sufficient for inducing degradation of T. brucei ornithine decarboxylase in vivo, which becomes unstable after fusion with the region (25). Therefore, this region functions as the “degradation domain” of MODC.

The hypothesis that PEST sequences correlate with protein degradation is based on a computer search among short lived proteins (13, 27). Most short lived proteins contain a region enriched with Pro, Glu, Ser, and Thr. This region is often flanked by basic amino acids, Lys, Arg, or His. The role of the PEST sequence in protein degradation has subsequently been demonstrated in several short lived proteins, such as that at the C terminus of mouse ornithine decarboxylase.

In this study, we fused the degradation domain of mouse ornithine decarboxylase to the C terminus of EGFP. The degradation domain of MODC dramatically decreased the half-life of EGFP in mammalian cells to 2 h. We mutated key amino acids in the PEST sequence of the fusion protein and identified several mutants with different half-lives. Furthermore, linkage
Destabilized EGFP as a Transcription Reporter

EGFP

EGFP-MODC(422–461)

FIG. 1. The fluorescence stabilities of EGFP and EGFP-MODC-(422–461) in the presence of CHX were examined with a fluorescence microscope. CHO K1 Tet-off cells were transfected with vectors expressing these two proteins. After 24 h, the transfected cells were treated with 100 µg/ml CHX for 0, 1, 2, and 3 h. The fluorescence stabilities of these two proteins were examined with a fluorescence microscope.

of the fusion protein to the binding sequences of the transcription factor NFκB allowed detection of TNFα-mediated NFκB induction in HEK293 cells. The use of destabilized EGFP (dEGFP) as a transcription reporter makes gene induction study possible in real time with living cells. The correlation of induction with the other biochemical changes such as nuclear translocation was also possible.

EXPERIMENTAL PROCEDURES

The cDNAs encoding EGFP and the C terminus of MODC were amplified with Pfu DNA polymerase (Stratagene, Inc., La Jolla, CA). EGFP was amplified with primers that incorporated a SacII recognition sequence at the 5’ end and a HindIII site at the 3’ end. The stop codon of EGFP was deleted to make a continuous open reading frame with the C terminus of MODC. The C terminus of MODC was amplified with primers that incorporated a HindIII recognition sequence at the 5’ end and an EcoRI recognition sequence at the 3’ end. The amplified polymerase chain reaction products were ligated at the HindIII site, and the fusion was cloned into the pTRE expression vector for use in the tetracycline (Tc)-regulated expression system (28). Using this strategy, we made EGFP-MODC-(422–461) and its mutants. Key amino acids of the PEST sequence in the fusion protein were mutated to Ala. The mutants were made using a homology extension procedure (33). The mutations in-

The construct DNAs were purified with Qiagen columns and transfected into CHO K1 Tet-off cells (CLONTECH Laboratories, Inc., Palo Alto, CA) for degradation studies. CHO K1 Tet-off cells stably express a fusion protein of the tet repressor and the herpes simplex virus VP16 (tetracycline-controlled transactivator) and thus can be used for Tc-regulated expression of genes cloned into the pTRE vector (28) (CLONTECH Laboratories, Inc.). Tetracycline-controlled transactivator initiates transcription by binding to a modified cytomegalovirus promoter with Tc repressor binding elements in the pTRE vector. This binding can be blocked by Tc, and hence the expression can be controlled by the level of Tc in the medium. These DNAs were introduced into these cells using CLONfectin (CLONTECH Laboratories, Inc.). After 24 h, transfected cells were subject to functional analyses as described below.

To examine the fluorescence intensity of EGFP or EGFP-MODC-(422–461), the cells were cultured on coverslips. After transfection, the cells were incubated at 37 °C for 24 h and then fixed with 4% paraformaldehyde for 30 min. The coverslips were mounted on a glass slide and examined under a Zeiss Axioskop model 50 fluorescence microscope. To determine protein turnover, the cells were treated with cycloheximide

(ChX) at a final concentration of 100 µg/ml for varying times before paraformaldehyde fixation.

The transfected cells with or without CHX treatment were collected by EDTA treatment, and the cell pellets were resuspended in 0.5 ml of PBS. The cell suspensions were then analyzed for fluorescence intensity using a FACs caliber flow cytometer (Becton Dickinson, Inc., San Jose, CA). EGFP was excited at 488 nm, and emission was detected using a 510/20 bandpass filter.

The transfected cells with or without CHX treatment were collected in PBS, and cell lysates were prepared by sonication. Proteins were separated by SDS gel electrophoresis, transferred onto a membrane, and EGFP and EGFP-MODC fusion proteins were detected using a monoclonal antibody against GFP (CLONTECH Laboratories, Inc.). The detection was visualized with the Western Exposure chemiluminescent detection kit (CLONTECH Laboratories, Inc.).

To make a TNFα-responding vector, 4 copies of NFκB binding sequence were cloned into the pSEAP2 vector with the herpes simplex virus thymidine kinase promoter, making pNFκB-SEAP. The SEAP gene was then replaced with the EGFP or dEGFP (EGFP-MODC-(422–461)) gene, making pNFκB-EGFP and pNFκB-dEGFP. These constructs were transfected into HEK293 cells. Twenty-four hours after transfection, the cells were collected with 0.1 µg/ml recombinant human TNFα. The medium or the cells were collected for SEAP assaying (CLONTECH Laboratories, Inc.) or flow cytometric analysis.

To examine both NFκB translocation and NFκB-mediated induction of dEGFP, 293 cells were transfected with pNFκB-dEGFP. After TNF treatment, the transfected cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature and rinsed with PBS three times. The fixed cells were then permeabilized with a blocking solution consisting of 4% bovine serum albumin in PBS containing 0.1% Triton X-100 for 1 h. The cells were incubated with 1.250 diluted polyclonal antibodies against NFκB p65 (Upstate Biotechnology, Lake Placid, NY) in blocking solution for 2 h. After washing with PBS three times, the cells were incubated with 1.250 diluted rhodamine-labeled anti-rabbit IgG (Boehringer Mannheim) for 45 min in PBS containing 4% bovine serum albumin. After rinsing three times with PBS, the stained cells were mounted in Citifluor (Ted Pella, Inc., Redding, CA).

RESULTS

The C terminus of MODC, containing amino acids 422–461, induces T. brucei ornithine decarboxylase degradation in mammalian cells (31). This fragment contains a PEST sequence at amino acids 423–449. To determine whether the PEST domain can also induce EGFP degradation, we appended it to the
C-terminal end of EGFP to make EGFP-MODC-(422–461). This fusion construct was expressed from a Tc-regulated expression vector, pTRE. The fluorescence intensity of the fusion protein was examined under a fluorescence microscope after it was transiently expressed in CHO K1 Tet-off cells. Fig. 1 shows that the fluorescence intensity of EGFP-MODC-(422–461) is similar to EGFP with no difference in the intracellular distribution. Next, we tested the ability of the C terminus of MODC to induce EGFP degradation in vivo. The construct was first transiently transfected into CHO K1 Tet-off cells. Twenty-four hours after transfection, the cells were treated with 100 μg/ml CHX for 0, 1, 2, and 3 h, and the change in fluorescence intensity of the transfected cells was examined by fluorescence microscopy. The fluorescence intensity of the fusion protein in the cells gradually decreased as CHX treatment was extended (Fig. 1) indicating that the fusion protein is unstable. After a 3-h treatment with CHX, fluorescence intensity had decreased by more than 50%. The results suggest that the half-life of the fusion protein is less than 3 h. In contrast, we did not observe a significant change in the fluorescence intensity in the EGFP-transfected cells (Fig. 1). To determine the half-life of the EGFP-MODC fusion protein more accurately, we used flow cytometry to measure the change in fluorescence of cells expressing the fusion protein. Half of the untreated cells maintained fluorescence after a 2-h treatment (Fig. 2). Therefore, we conclude that the half-life of the fusion protein is 2 h. EGFP-transfected cells still had more than 90% of fluorescence relative to untreated cells during the treatment.

To examine the correlation between the half-life of the EGFP fusion protein and its fluorescence, the EGFP and EGFP-MODC-(422–461) transfected cells used for flow cytometry were also used for Western blot analysis with a monoclonal antibody against GFP. As shown in Fig. 3, the antibody was able to detect EGFP and the fusion protein. No detectable change in the level of EGFP was found. However, the EGFP-MODC-(422–461) fusion protein at 31 kDa was unstable, as measured by a decline following CHX treatment. Half of the fusion protein was degraded within 2 h of CHX treatment. We conclude that the half-life of the EGFP-MODC-(422–461) fusion protein is approximately 1–2 h. Our results indicate that the fluorescence decay of the fusion protein correlates with its protein degradation. The fusion protein was designated

**FIG. 2.** A, flow cytometric analysis of the fluorescence stabilities of EGFP and EGFP-MODC-(422–461). CHO K1 Tet-off cells were transfected with the two constructs used in Fig. 1. After 24 h, the transfected cells were treated with 100 μg/ml CHX for 0, 1, 2, and 3 h. The treated cells were collected with EDTA, and 10,000 cells were subjected to FACS analysis. B, the percentage of the fluorescent cells was plotted. Solid circles, EGFP; open circles, EGFP-MODC-(422–461).

**FIG. 3.** Western blot analysis of protein stabilities of EGFP and EGFP-MODC-(422–461). Cells collected during flow cytometry were used for preparing cell lysates. The cell lysates were subject to SDS gel electrophoresis and transferred onto a membrane. EGFP and the EGFP fusion protein were detected with a monoclonal antibody against GFP.
To evaluate the contribution of PEST amino acids to protein degradation in dEGFP, we mutated Pro, Glu, Ser, and Thr residues of dEGFP, as well as the flanking basic amino acid residues, to Ala. Next, we monitored degradation by the change in fluorescence intensity (Fig. 4). The data for these EGFP-MODC fusion mutants are shown in Table I. We found that the degradation rate was not proportional to the number of PEST residues but was determined by their positions, such as the Glu mutations. Mutation of the Glu residue at amino acid 444 stabilized the protein, but mutation of the Glu residues at amino acids 428, 430, and 431 shortened the half-life; only 20% of the cells fluoresced 2 h after treatment. These results suggested that the Pro, Glu, Ser, and Thr residues of the PEST sequence contribute to protein instability in varying ways.

To determine whether dEGFP can be used as a transcription reporter, we linked it to 4 copies of NFκB binding sequence and the constitutive thymidine kinase promoter. The construct was transiently transfected into HEK293 cells for monitoring TNFα-mediated NFκB activation (Fig. 5). The time course induction with kinetics is similar to SEAP, another commonly used genetic reporter. Induction of both SEAP and dEGFP occurred after 2 h, was maximized at 6 h, and declined after that. The results demonstrate that dEGFP can be used as a reporter to monitor transcription induction. In contrast, unmodified EGFP, when used as the reporter for induction, did not respond.

### Table I

FACS analysis of EGFP, EGFP-MODC-(422–461), and mutations in transfected CHO K1 Tet-off cells

<table>
<thead>
<tr>
<th>Constructs</th>
<th>0 h</th>
<th>Initial</th>
<th>2 h</th>
<th>4 h</th>
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<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
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<tr>
<td>EGFP</td>
<td>100</td>
<td>(63.6)</td>
<td>107</td>
<td>92</td>
</tr>
<tr>
<td>EGFP-MODC-(422–461)</td>
<td>100</td>
<td>(12.6)</td>
<td>52</td>
<td>29</td>
</tr>
<tr>
<td>P426A/P427A</td>
<td>100</td>
<td>(11.5)</td>
<td>39</td>
<td>11</td>
</tr>
<tr>
<td>P438A</td>
<td>100</td>
<td>(34.1)</td>
<td>79</td>
<td>60</td>
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<tr>
<td>E428A/E430A/E431A</td>
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<td>(17.3)</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>E444A</td>
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<td>(12.6)</td>
<td>69</td>
<td>65</td>
</tr>
<tr>
<td>S440A</td>
<td>100</td>
<td>(21.6)</td>
<td>78</td>
<td>66</td>
</tr>
<tr>
<td>S445A</td>
<td>100</td>
<td>(23.5)</td>
<td>29</td>
<td>20</td>
</tr>
<tr>
<td>T436A</td>
<td>100</td>
<td>(46.9)</td>
<td>70</td>
<td>47</td>
</tr>
<tr>
<td>D433A/D434A</td>
<td>100</td>
<td>(11.31)</td>
<td>22</td>
<td>6</td>
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<tr>
<td>D448A</td>
<td>100</td>
<td>(32.6)</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>H423A</td>
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<td>(12.2)</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>R449A/H450A</td>
<td>100</td>
<td>(27.9)</td>
<td>93</td>
<td>86</td>
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</table>
well to TNFα during the treatment. This result might be because of a higher basal level of the stable protein accumulating in the cells. Therefore, unlike dEGFP, which has a rapid turnover, stable EGFP cannot be used as a reporter for the study of transcriptional induction.

dEGFP, like EGFP, is an autofluorescent protein whose emission does not require any cofactors or substrates. As a result, we were able to use it to examine the correlation between TNFα-mediated transcription induction and NFκB translocation from the cytoplasm to the nucleus. Sixteen hours after transfection with pNFκB-dEGFP, HEK293 cells were treated with TNFα for 0, 0.5, 2, and 6 h and stained with polyclonal antibodies against NFκB p65 and then with a rhodamine-conjugated secondary antibody. As shown in Fig. 6, transcriptional induction was monitored with dEGFP (green) and the translocation process of NFκB with rhodamine (red). NFκB was located in the cytoplasm of HEK293 cells before TNFα treatment. TNFα induced rapid translocation of NFκB from the cytoplasm to the nucleus. This process was nearly complete after 30 min, but dEGFP was not detected during this period. After 2 h, NFκB had accumulated in the nucleus, and the induction of dEGFP lessened. NFκB relocated from the nucleus to the cytoplasm, and the induction of dEGFP was highest after 6 h. The results demonstrate that the translocation of NFκB is transient in response to TNFα and that all of the translocated NFκB was recruited back to the cytoplasm after 6 h. This transient translocation of NFκB resulted in its transient activity in the mediation of transcription induction of dEGFP or SEAP.

DISCUSSION

We destabilized EGFP by appending the degradation domain of MODC to the C terminus of EGFP. The fusion protein is unstable in the presence of CHX. Analysis with flow cytometry indicated that the half-life of dEGFP is approximately 2 h. Western blot analysis indicated that the half-life was 1–2 h. The difference in the half-life by two different methods may have been because of the presence of nonfluorescent premature and mature GFP. Maturation of the EGFP chromophore is post-translational and proceeds with a half-time of about 25 min (7). Fluorescence measurement detects solely mature GFP, but Western blotting detects both premature and mature GFP. Thus, the two methods will result in different half-life determinations. Because we wish to use GFP as a reporter at the fluorescence level rather than at the protein level, its fluorescence half-life is a more important quality. We conclude that the half-life of dEGFP is 2 h.

The C terminus of MODC that induces EGFP degradation in vivo contains a PEST sequence from amino acids 423 to 449. Of these 26 amino acids, 10 are Pro, Glu, Ser, and Thr and 3 are Asp. Mutation analysis indicates at least 1 Pro, Glu, Ser, and Thr is required for protein degradation. However, we found that degradation does not correlate with the number of PEST residues. Some mutants were even more unstable, such as mutant S445A. This resulted in a conflict with those observed in the study of yeast uracil permease where instability is correlated to the total number of Ser residues in the PEST-like sequence (33). Therefore, the PEST hypothesis needs more validation probably by determining the contributions of PEST sequences of different short lived proteins to protein degradation. Because it is easily detected, EGFP is an excellent reporter for studies of protein degradation.

The rapid turnover of dEGFP provides at least three advantages over EGFP. First, the rapid turnover of dEGFP allows its application studies requiring destabilized GFP, such as circa-
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NFκB 5 min after TNF treatment (36). The DNA binding activity of NFκB is believed that the reappearance of IκBα is rapid, and it reappears within 40 min of the treatment. The reappearance of IκBα is also CHX-sensitive, suggesting that it requires new protein synthesis. Because IκBα is the target gene of NFκB, it is believed that the reappearance of IκBα is mediated by NFκB. The new synthesized IκBα does not affect the DNA binding activity of NFκB immediately (36), indicating a window for NFκB to mediate induction of target genes, although the mechanism that underlies interaction of new synthesized IκBα and the nuclear NFκB is unclear. In this study, we demonstrated that the retardation of NFκB in the nucleus is coincident with its induction of the reporter gene dEGFP. Therefore, use of the color protein dEGFP as a transcription reporter allows monitoring of transcription induction more directly and its coincidence with other biochemical change easier.

Acknowledgments—We thank Nhatnam Ngo for making plasmids, Vanessa Gurtu for helping with flow cytometry analysis, Dr. Phillip Coffino for useful discussion, Dr. Valerie Natale and David Gunn for reading the manuscript, and Marion Kerr and Jeff Baughn for preparation of figures.

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Generation of Destabilized Green Fluorescent Protein as a Transcription Reporter
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doi: 10.1074/jbc.273.52.34970

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