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Concurrent Measurement of Promoter Activity and Transfection Efficiency Using a New Reporter Vector Containing both *Photinus pyralis* and *Renilla reniformis* Luciferase Genes

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Reporter vectors are essential for the quantitative analysis of gene elements that potentially regulate mammalian gene expression (1). These gene elements may be *cis*-acting, such as promoters and enhancers, or *trans*-acting, such as various DNA-binding factors (2). Several kinds of reporter vectors have been developed to study the promoter and/or enhancer activities of genes: chloramphenicol acetyltransferase (CAT)[2] reporter vector, β-galactosidase reporter vector, β-glucuronase, alkaline phosphatase reporter vector, green fluorescent protein reporter vector, and luciferase reporter vector (3–7). Currently, reporter vectors using chemiluminescence-based assays (β-galactosidase reporter vector, β-glucuronase, alkaline phosphatase reporter vector, and luciferase reporter vector) are commonly used in many quantitative analyses of gene elements (8), because the sensitivity of the assays is several orders of magnitude greater than that of conventional colorimetric- or fluorometric-based assays. Of them, luciferase reporter vectors are the most favored reporter vectors for functional analysis of promoters and enhancers of genes, due to their rapid, sensitive, and reproducible assay system (9). In the luciferase assay, luciferin and other components are added to cell extracts, and the production of light from both cell extracts expressing the luciferase gene is measured conveniently by a luminometer or scintillation counter (10).

Although luciferase reporter vectors have been commonly employed in numerous studies, the transfection of only luciferase vector cannot provide normalized values of the activities of gene elements without simultaneous transfection of a second reporter vector to measure the efficiency (11). In this paper, a novel report vector ($pJDL_{cmv}$) was constructed to contain two luciferase genes, *Photinus pyralis* and *Renilla reniformis* luciferases, regulated by two different promoters for the first time, in order to measure simultaneously promoter activity and transfection efficiency. Two promoters of human glutaredoxin and ribonucleotide reductase R2 gene were investigated with this novel reporter vector to verify its appropriateness for simultaneous measurement of promoter activity and transfection efficiency.

Materials and Methods

T4 DNA ligase and restriction enzymes were purchased from Promega (Madison, WI), Chameleon double-stranded site directed mutagenesis kit was from Stratagene (La Jolla, CA), and *Taq* polymerase and deoxynucleotides were from Perkin-Elmer (Norwalk, CT). Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) was used to determine the amount of protein.

Cell culture conditions. HeLa and NT cells were cultured in DMEM supplemented with 10% fetal bovine serum. The cells were grown to 60% confluence for the transfection experiments.

PCR cloning of P. pyralis and R. reniformis luciferase genes. *P. pyralis* and *R. reniformis* luciferase genes were amplified from pGL and pRL vectors (Promega), respectively, by PCR using respective forward and backward primers corresponding to two genes: 5′-TGCTTGGCATTCCGGTACTGTTGG-3′ and 5′-TTTACAATTTGGACTTTCCGCCCTTCTT-3′ for the *Photinus* gene; 5′-CTGCAGAAGTTGGTCGTGAGGCAC-3′ and 5′-TTGTTCATTTTTGAGAACAGC-3′ for the *Renilla* gene (12).

PCR cloning of two DNA fragments containing poly(A) signal for P. pyralis and R. reniformis luciferase genes. Two DNA fragments containing SV40 early and late poly(A) signals were respectively amplified from pEGFP (Clontech) and pRL (Promega) by PCR using forward and

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[2] Abbreviations used: CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; CMV, *cytomegalovirus;* poly(A), polyadenylation.

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backward primers corresponding to two DNA fragments containing the poly(A) signals: 5′-ATCTAGATAACTGATCATAATCAGCC-3′ and 5′-TACGCGTTAAGATACATTGATGAG-3′ for *P. pyralis* luciferase gene; 5′-TAATTCTAGAGCGGCCGCTTCGAGCA-3′ and 5′-CCTGGATCCTTATCGATTTTACCACAT-3′ for *R. reniformis* luciferase gene.

Construction of novel reporter vectors (pJDL$_{basic}$ and pJDL$_{cmv}$) containing both P. pyralis and R. reniformis luciferase genes. The backbone of the novel reporter vector was obtained by PCR-amplifying pUC18 using two primers (5′-TGGCACTTTTCGGGGAAATGTG-3′ and 5′-ATCTGTCGAGCCATGTGAGCCAAAA-3′), and ligating a short DNA fragment containing numerous cloning sites (*Xho*I, *Not*I, *Bst*XI, *Sac*I, *Kpn*I, *Hin*dIII, *Mlu*I, *Sma*I, *Pst*I, and *Bam*HI) to the amplified DNA. Using these cloning sites, *P. pyralis* and *R. reniformis* luciferase genes and their poly(A) signals were cloned to construct a novel reporter vector (pJDL$_{basic}$). Briefly, the *P. pyralis* luciferase gene was blunt-ligated to a DNA fragment with early SV40 poly(A) signal, and the ligated product was ligated to the constructed vector using *Hin*dIII and *Mlu*I sites. *R. reniformis* luciferase was ligated to the amplified late SV40 poly(A) signal DNA fragment using *Xba*I, and the ligated DNA fragment was finally ligated by the use of *Pst*I and *Bam*HI sites to the vector containing the *P. pyralis* luciferase gene and early SV40 poly(A) DNA fragment. The vector containing *P. pyralis* and *R. renifor* luciferase genes was nominated pJDL$_{basic}$. pJDL$_{cmv}$ was constructed by ligating PCR-amplified *cytomegalovirus* (CMV) immediate early promoter to the upstream of the *Renilla* luciferase gene using *Sma*I site.

Determination of promoter activity and transfection efficiency using a novel reporter vector (pJDL$_{cmv}$). A series of reporter vectors was prepared by inserting fragments of various lengths of the promoter region of the human R2 protein gene immediately upstream of the *Photinus* luciferase gene of pJDL$_{cmv}$. Genomic DNA fragments of human glutaredoxin and human ribonucleotide reductase R2 genes were amplified by PCR using oligonucleotides, corresponding to the 5′-ends and 3′-ends of the isolated genes. For human glutaredoxin gene, the 5′-ends were 5′-TATGCTTGGATTCCTCTTA, 5′-ATTTCATCTGTAGTTT, 5′- AAAATGCTGTGAGAGCAAC, and 5′-ATGGAAATGTCTTCGAAGC (13). The 3′-end for human glutaredoxin was 5′-GAGCCCGACCCAGCCAGTT (13). For the human reductase R2 gene, the 5′-ends were 5′-CTTTTTTTCTTCTCTTTTTAA, 5′-TAGTTTGAAGGTTTACAAAG, 5′-GACCACCCCGCCAAAATGT, 5′-GCTGGAGGAGGTGCTTTCGGGAGGC, and 5′-GGGGCAAGCGCAGCCAAT (14). The 3′-end for human ribonucleotide reductase R2 subunit was 5′-GCTGGAGTGAGGGGTCGC (14). Amplified DNA fragments were ligated upstream of the *Photinus* luciferase gene in pJDL$_{cmv}$ reporter vector. For transfections, HeLa and NT cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were grown to approximately 60% confluence in 60-mm petri dishes. The constructed reporter vectors were transfected into the cells with calcium phosphate (15). After transfection, the cells were incubated for an additional 48 h, harvested, and lysed. Prior to measurment of luciferase activity, the amount of protein in the samples was determined as described above. The efficiency of transfection and promoter activity was determined in a Promega TD-20/20 luminometer with the dual luciferase reporter assay system performed according to the manufacture's protocol (Promega).

Results and Discussion

Reporter vectors are designed for rapid and convenient functional analysis of genetic regulatory elements such as promoters and enhancers. The constructed pJDL$_{cmv}$ can be utilized for analyzing promoters and enhancers sensitively and reproducibly by measuring *P. pyralis* (firefly) luciferase activity. The vector is also able to measure the efficiency of transfection while analyzing genetic regulatory elements. Commonly, two different reporter vectors are simultaneously transfected to cells to measure both the promoter activity and the efficiency of transfection. This method has several drawbacks: (1) at least two different reporter vectors must be prepared to determine the promoter activities normalized with transfection efficiency, (2) the characteristics of individual vectors may influence their respective efficiency of transfection, and (3) different transfection methods may have a deviant effect on the transfection efficiency of the individual reporter vectors employed in the experiments.

To curtail duplication and prevent the appearance of unforeseen attributes in characterizing genetic regulatory elements, a novel reporter vector (pJDL$_{cmv}$) was constructed by cloning two different luciferase genes (*P. pyralis* and *R. reniformis*), the activities of which can be rapidly and sensitively measured by the dual luciferase reporter assay system (Promega). As shown in Fig. 1, pJDL$_{cmv}$ reporter vector contains a high copy number prokaryotic replication origin for maintenance in *Escherichia coli,* and an ampicillin resistance gene for selection. The reporter vector contains the *P. pyralis* luciferase gene (*luc*) followed by the SV40 early gene intron and polyadenylation signal. The poly(A) site limits background transcription from spurious promoters in the vector. For cloning of gene elements, the vector has multiple cloning sites (*Xho*I, *Not*I, *Bst*XI, *Sac*I, *Kpn*I, and *Hin*dIII) generated upstream of the *P. pyralis* luciferase gene. The sites for *Not*I and *Bst*XI restriction enzymes were especially incorporated for

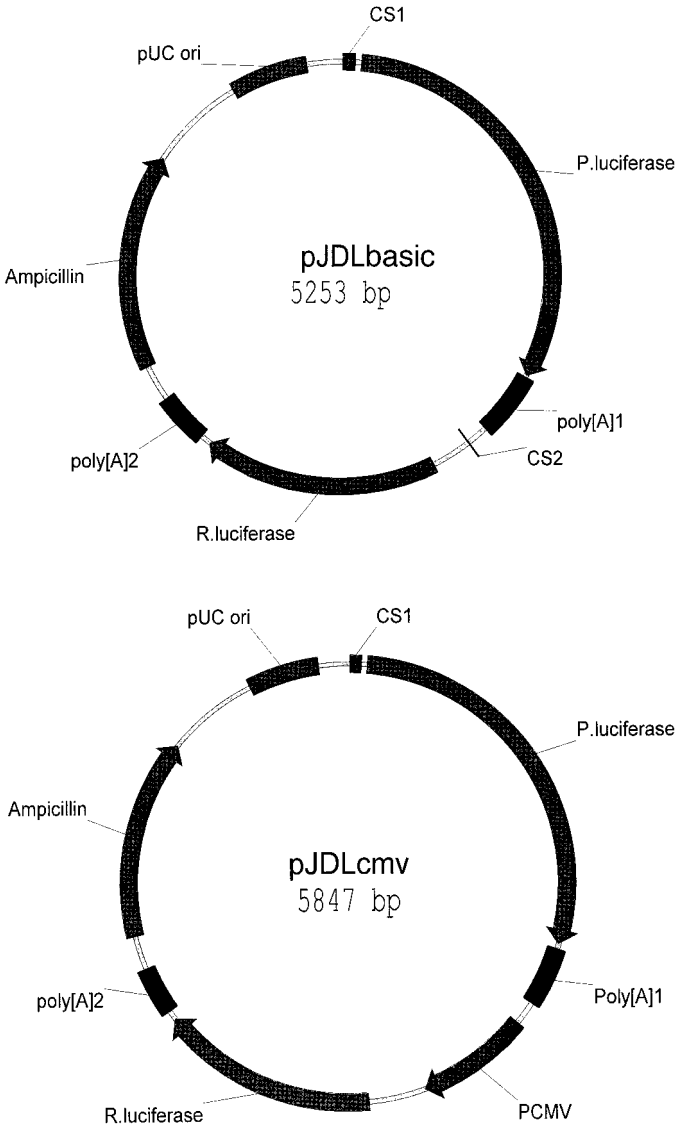

FIG. 1. $pJDL_{basic}$ and $pJDL_{cmv}$ reporter vectors. pJDL reporter vectors contain two *Photninus pyralis* and *Renilla reniformis* luciferase genes. In the $pJDL_{basic}$ reporter vector, two CS (*Xho*I, *Not*I, *Bst*XI, *Sac*I, *Kpn*I, and *Hin*dIII for CS1; *Sma*I and *Pst*I for CS2) are located upstream of *P. pyralis* and *R. reniformis* luciferase genes, respectively, to accommodate various gene regulatory elements. $pJDL_{cmv}$ reporter vector is derived from $pJDL_{basic}$ by inserting human *cytomegalovirus* (CMV) immediate early promoter upstream of *R. reniformis* luciferase gene (*Rluc*). P. luciferase, *P. pyralis* luciferase; R. luciferase, *R. reniformis* luciferase; poly[A]1; the SV40 early gene intron and polyadenylation signal; poly[A]2; the SV40 late gene intron and polyadenylation signal; AmpicillinR, ampicillin-resistant gene; pUC ori, prokaryotic replication origin. The vectors are available through USDA Technology Transfer Office (TTO) at Beltsville, Maryland.

their expandability. Human *cytomegalovirus* immediate early promoter was also inserted in the vector upstream of *R. reniformis* luciferase gene (*Rluc*) to monitor transfection efficiency in many types of mammalian cells. Since CMV promoter has a strong promoter activity, a DNA fragment containing SV40 late gene intron and polyadenylation signal was inserted downstream of the *R. reniformis* luciferase gene to keep spurious background transcription from CMV promoter minimized. To determine CMV promoter activity, the ratio of the *Renilla* luciferase activities in $pJDL_{basic}$ and $pJDL_{cmv}$ was measured in HeLa cells. The luciferase activity of HeLa cells transfected with $pJDL_{cmv}$ is at least 100-fold greater than that of the cells with $pJDL_{basic}$ (data not shown). The integrity of the constructed $pJDL_{basic}$ and $pJDL_{cmv}$ was verified by digesting with several restriction enzymes (data not shown). Also, the vectors were completely sequenced, and their sequences were submitted to GenBank (Accession No. AF 311601).

Promoter activities of the 5′-flanking regions of human glutaredoxin and human ribonucleotide reductase genes were measured using $pJDL_{cmv}$ reporter vector. The 5′-flanking regions of human glutaredoxin and ribonucleotide reductase genes were used to measure their promoter activities because the promoters of these two genes had been previously characterized (13, 14). Genomic DNA fragments were amplified from the genes by PCR and ligated into the reporter vector as described under Materials and Methods. Four different chimeric $pJDL_{cmv}$ reporter vectors (labeled A1–A4, Fig. 2A) from glutaredoxin genes and five different chimeric $pJDL_{cmv}$ reporter vectors (labeled B1–B5, Fig. 2B) were constructed from the human ribonucleotide reductase R2 gene, respectively. The constructed vectors were transfected with calcium phosphate into HeLa cells and NT-2 cells. The dual-luciferase reporter assay system was used to determine promoter activity and transfection efficiency. In the human glutaredoxin gene, four different promoter regions were investigated using HeLa cells and NT-2 cells, and the $pJDL_{cmv}$ reporter vector (Fig. 2A). The highest activity was found in the fragment −823 to +1 bp [A2]. This activity was 30-fold higher than that of controls, but was one-third of the activity observed with the fragment −463 to +1 bp. This fragment of the promoter contains a TATA-like box, and several consensus sequences for transcriptional factors (AP-1, CCAAT-box binding protein, and GATA-1). The data are in line with those of the previous report (13).

In the human ribonucleotide reductase R2 gene, the highest promoter activity was observed for the fragment −800 to +1 bp [B4], and the activity was as much as 100-fold greater than that of the controls (Fig. 2B). Eighty percent of maximum activity occurred with the fragment −610 to +1 bp [B3]. The fragment −125 to +1 bp [B1] had more than half-maximum activity, approximately 50-fold greater than that of controls, and contained the TATA-like box sequence and three CCAAT box sequences. The data obtained in this study are almost identical to those of the previous study (14).

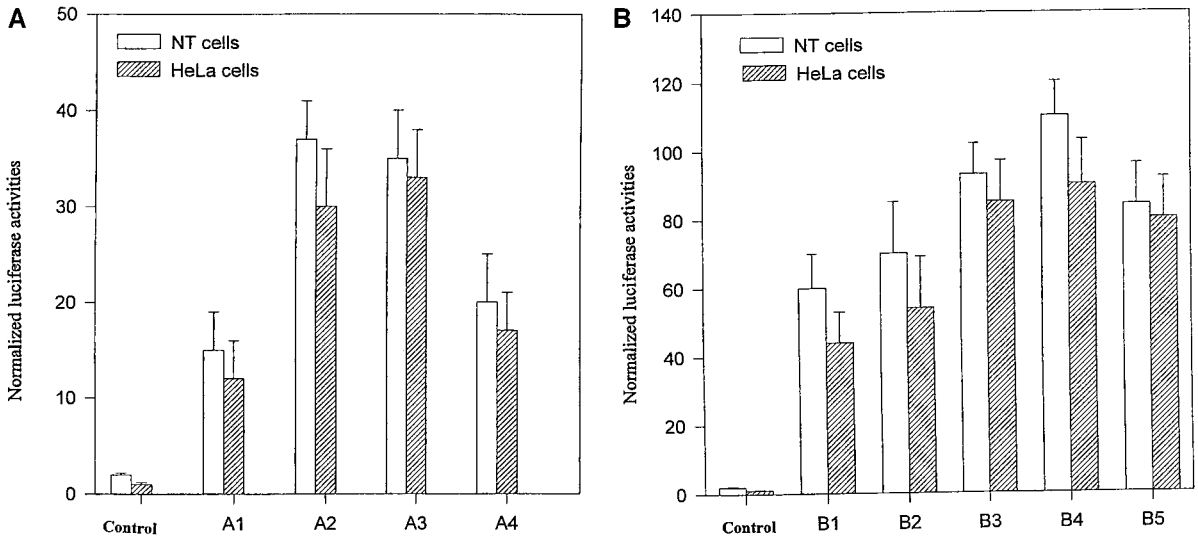

FIG. 2. Promoter activities using chimeric $pJDL_{cmv}$ reporter vectors. Four different DNA fragments (B1–B4) of human glutaredoxin (GRX) promoter (GeneBank Accession No. U40574) were inserted into the $pJDL_{cmv}$ vector for luciferase as described under Materials and Methods. Five different DNA fragments (A1–A5) of human ribonucleotide reductase R2 (RR2) promoter (GeneBank Accession No. AF149206) were inserted into the $pJDL_{cmv}$ vector for luciferase as described under Materials and Methods. Relative luciferase activities of the chimeric $pJDL_{cmv}$ reporter vectors containing promoter constructs (control, A1–A4 and B1–B5) transfected into NT cells or HeLa cells were determined as described above. (A, B) Respective promoter activities from the chimeric vectors containing promoter constructs from human glutaredoxin gene and human ribonucleotide reductase R2 gene. Data points represent the mean ± SD of three or more samples.

Since the proximal promoter region (−125 to +1 bp) of the R2 gene contributed at least 50% of the maximal promoter activity, and the three sequential CCAAT sequences contribute to the activity, $pJDL_{cmv}$ containing this promoter region was used to verify their roles in the promoter activity. Site-directed mutations in each of the three CCAAT sequences or in all three sequences of the human ribonucleotide reductase R2 gene (GeneBank Accession No. AF149206) were performed with the double-stranded site-direct mutagenesis kit (Stratagene). The reporter vectors with the mutations in CCAAT sequences were transfected into HeLa cells. For each individual mutated CCAAT sequence, promoter activity was decreased approximately 20–40% compared to that of the control (data not shown). The data are also similar to those reported in a previous study (14).

Unequivocally, these data indicate that promoter activity and transfection efficiency can be measured simultaneously with the $pJDL_{cmv}$ reporter vector. Also, site-directed mutation can be performed in $pJDL_{cmv}$ reporter vector with the GeneEditor mutagenesis kits. Because all transfection experiments were performed with the calcium phosphate method, similar experiments were performed to verify the aforementioned data by different transfection methods such as DEAE-dextran and cationic lipid transfection agents (15–17). In all three transfection methods, the values of promoter activity normalized with transfection efficiency were similar in HeLa and NT cells (data not shown). Therefore, measuring promoter activity with this novel reporter vector can also eliminate the variation arising from different transfection methods. A great benefit of utilizing this reporter vector is that the time and cost involved in transfections can be reduced by half. For a further application, $pJDL_{basic}$ reporter vector can be used for measuring the relative activity of two different gene elements, because *P. pyralis* and *R. reniformis* luciferase genes can be separately regulated by two gene elements. This application makes it possible to evaluate the promoter activities of two different gene elements at the same time/in the same assay. Therefore, this reporter vector opens the possibility that the relative promoter activity and the promoter activity normalized with transfection efficiency can be determined by measuring two different luciferase activities in an assay with the dual-luciferase reporter assay system.

Conclusion

Both promoter activity and transfection efficiency can be measured at the same time with a new reporter vector ($pJDL_{cmv}$) containing both *P. pyralis* (firefly) and *R. reniformis* luciferase genes. DNA fragments of two promoters from the human glutaredoxin and ribonucleotide reductase R2 subunit were cloned upstream of *P. pyralis* luciferase of $pJDL_{cmv,}$ and these chimeric $pJDL_{cmv}$ reporter vectors were transfected to HeLa and

NT cells to measure simultaneously the promoter activities as well as transfection efficiency. The promoter activities and transfection efficiency were successfully determined by measuring the activities of *P. pyralis* and *R. reniformis* luciferases, respectively, using the dual-luciferase reporter assay system. Therefore, significant time and cost can be saved using this novel reporter vector in both basic and applied transfection studies.

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Immunoprecipitation of Human Telomerase Reverse Transcriptase with Telomerase Activity

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Telomerase is a ribonucleoprotein that elongates telomeres at the end of chromosomes. It is composed of a catalytic protein subunit, telomerase reverse transcriptase (hTERT),[2] a telomerase-associated protein (TEP1), and a stably associated RNA moiety (hTR) which acts as an intrinsic template for the elongation of telomeres (1, 2). *In vitro* reconstitution experiments show that hTERT and hTR may be sufficient for the activity of the enzyme (3). Recent studies of the purification of hTERT protein using peptide affinity chromatography and immunoprecipitation of telomerase activity with specifically designed anti-hTERT and anti-TEP1 antibodies have provided evidence that the phosphorylation status of telomerase is an important posttranslational mechanism in regulating its function (4–6). Nevertheless, further biochemical studies analyzing the regulation of hTERT protein and telomerase activity are needed. Currently, there are only a few commercially available anti-hTERT antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, and Biotrend Chemikalien, Germany) which are not well characterized in independent studies (7). In the conventional

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[2] Abbreviations used: hTERT, telomerase reverse transcriptase; TEP1, telomerase associated protein; TRAP, telomeric repeat amplification protocol; Chaps, 3-[3-cholamidopropyldimethylamino]-1-propanesulfonate; RIPA, radioimmunoprecipitation assay.

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