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Concurrent enhancement of transcriptional activity and specificity of a retinal pigment epithelial cell-preferential promoter

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Purpose: To develop a transgene expression system in retinal pigment epithelial cells with the aim of enhancing the transcriptional activity of a weak RPE-specific/preferential promoter.
Methods: The transgene expression system was established by introducing a chimeric transcriptional activator (GAL4-VP16) and its DNA binding sequence and using truncated human and mouse RPE65 promoters in combination with a luciferase reporter gene. Two groups of expression plasmids were constructed for transfection. The group for co-transfection contained two DNA constructs where the reporter and GAL4-VP16 were separately expressed in pLuc and pGV series. The other group, pLuc-GV series, was prepared as single DNA constructs expressing both the reporter and GAL4-VP16. The transcriptional activities of the DNA constructs were assayed by transfection of human RPE cells (RPE51 and D407) and other cell lines (HEK293, COS-1, Hela, HepG2, and F2000).
Results: We found that the transcriptional activity of the human RPE65 promoter was dramatically enhanced 10-13 fold in RPE cells co-transfected with DNA constructs phR65luc and phR65GV when compared to the human RPE65 promoter alone. A comparatively lower, 4-5 fold, increase was observed following transfection with the single DNA construct phR65luc-GV. In RPE cells, when the transcriptional responses to GAL4-VP16 expression were compared between the RPE65 promoter of phR65luc and the minimal promoter of pLuc, the increase in transcriptional activity was about 10 fold higher in phR65luc constructs. Low or non-significant enhancement of promoter activity was observed with these constructs following transfection of the non-RPE cell lines.
Conclusions: Our results indicate that the current transgene expression system dramatically amplifies transcriptional activity of weak and cell-specific/preferential promoters (e.g., the hRPE65 promoter) whilst retaining relative cell specificity.

The retinal pigment epithelium (RPE) is a cell monolayer that performs multiple functions for the maintenance of normal vision. Consequently, malfunctions of RPE cells can cause various ocular diseases such as hereditary retinal degenerations and dystrophies [1,2]. Thus, the RPE layer is an important target tissue for gene therapy. A common approach for gene delivery to specific sites is the use of cell/tissue-specific promoters or cis-acting elements or/and cell/tissue-specific enhancers. When compared to commonly used viral regulators such as the ubiquitously active cytomegalovirus (CMV) promoter and its enhancer, the majority of cell/tissue-specific promoters (particularly in truncated forms) are relatively weak regulators with poor transcriptional activity levels. For example, the RPE65 promoter (R65P), which regulates the expression of RPE65, a major protein specific to the vertebrate RPE [3], displays reasonable cell/tissue-specific activity in RPE cells [4,5] and tissues of transgenic mice [6]. However, its transcriptional activity still remains about one-third that of the SV40 viral promoter [4].

By introducing a chimeric transcriptional activator into a gene expression system (e.g., GAL4-VP16 or VP16-LexA) several groups were able to augment transcription activity of weak cell/tissue-specific promoters [7]. GAL4 is a yeast (*Saccharomyces cerevisiae*) transcriptional factor that activates the transcription of adjacent *GAL1* and *GAL1* genes via its DNA binding domain (aa 1-147) bound specially to four related 17-mer dyad symmetrical sequences in the GAL upstream activating sequence [8]. The herpes simplex virus transcriptional activator VP16 when fused to a heterologous DNA-binding domain, such as GAL4 (aa 1-147), causes the C-terminus of activation domain of VP16 to strongly stimulate transcriptional activity of a reporter gene through numerous 17-mer sites located upstream of the minimal promoter [9,10].

Here, we describe the outcome of combining RPE-preferential promoters of the human RPE65 gene (hR65P) and its mouse counterpart (mR65P) with the chimeric transcriptional activator, GAL4-VP16, and GAL4 DNA binding sequence (5x17-mer, a five-times repeated 17 bp GAL4 binding sequence), with the purpose of developing a new transgene expression system for high transcriptional activity and RPE cell.

METHODS

Molecular cloning of hR65P and mR65P: The promoter regions used contained necessary promoter elements including a TATA box, a transcription start site, and positive elements such as Oct-1 and E-box sites for RPE-specific expression [5,6]. Meanwhile, limited promoter sizes were chosen considering that the optimal distance between the GAL4 DNA

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binding sequence and the transcription start site of a promoter ranges from -80 to -1,300 upstream of a reporter gene [10,11]. Therefore, promoter fragments of hR65P (-677 to +45) and the mouse counterpart mR65P (-655 to +31) were prepared and amplified by PCR from human genomic DNA (Promega, Madison, WI) and mouse (129/svJ) genomic DNA, respectively. Amplification was performed using primer pairs 5'-AGC AGA CAG GCA TTA GTG AC-3', 5'-GTT CAG GAT CCA GAG TTC TG-3' for hR65P (Genbank accession number U20476) and 5'-AGC AAT GGT GAA GAC AGT GAT-3', 5'-TTC TGG TAC CAG CTG TAG GA-3' for mR65P (Genbank accession number AF271297). PCR products were then subcloned into the pGEM-T Easy vector (Promega) and the resultant plasmids termed pGEM-hR65 and pGEM-mR65, respectively.

Construction of plasmid DNAs: The system utilizes a truncated GAL4 DNA binding domain and an N-terminal portion of the VP16 activation domain to make a fusion protein, GAL4-VP16. The relevant DNA regions of the GAL4, VP16, and GAL4 DNA binding sequence (5x17-mer) were obtained from the Checkmate™ Mammalian Two-Hybrid System (Promega). The parental plasmids pBIND, pACT, and pG5luc, respectively, consist of GAL4 (aa 1-147), VP16 (aa 411-456) DNAs and a firefly luciferase reporter gene (*luc*) linked with the 5x17-mer sequence located upstream of the reporter gene. The reporter gene in pG5luc, termed pMluc here, is controlled by a major late promoter of adenovirus which is a minimal promoter containing only a TATA box and a transcription initiation site.

Construction of GAL4-VP16 DNA was accomplished by excision of a *Bgl* II-*Eco* RI GAL4 DNA fragment from pBIND and subcloning it upstream of VP16 DNA with the same restriction sites as in pACT. The resultant plasmid was termed pCMV-GV, where GAL4-VP16 is controlled by the CMV immediate-early enhancer/promoter (CMVP). An SV40 nuclear location sequence was linked to the 5' end of GAL4-VP16 DNA even though GAL4 itself contains a nuclear localization signal in its DNA binding region [8]. To construct the pLuc series, the minimal promoter in pMluc was replaced with hR65Pand mR65P. A *Spe*I-*Nco*I promoter fragment was excised from pGEM-hR65 and introduced upstream of the luciferase gene in pMluc replacing the minimal promoter. Other promoter replacements with mR65P were carried out using the same strategy. The resultant plasmids were termed phR65luc and pmR65luc, respectively. pCMVluc, in which the reporter gene is controlled by CMVP, was generated by the introduction of *Bgl*II-*Hind*III CMVP DNA fragment of pACT upstream of the reporter gene of pMluc with the same restriction sites. Construction of the pGV series was achieved by excising a *Pst*I-*Bgl*II fragment of hR65P (-449 to 17) from pGEM-hR65, and subcloning the fragment upstream of GAL4 DNA in pBIND in place of CMVP, resulting in an intermediate plasmid termed pG1. mR65P PCR products were prepared with adapters containing *Bgl*II and *Pst*I sites and inserted upstream of GAL4 DNA in pG1 and the intermediate plasmid was termed pG2. Finally, two *Bgl*II-*EcoR*I fragments, containing hR65P- and mR65P-GAL4 DNAs excised from pG1 and pG2, correspondingly, were subcloned upstream of VP16

Table 1. DNA constructs used in the transgene expression system

Name	Promoter regulating the reporter gene (luc)/GAL4-VP16 (GV)	Comments
Double DNA pLuc series		
pMluc (pG5luc)	TATA-luc	
pCMVluc	CMVP-luc	
phR65luc	hR65P-luc	Transfection or co-transfection with pGV series
pmR65luc	mR65P-luc	
Double DNA pGV series		
pCMVGV	CMVP-GV	
phR65GV	hR65P-GV	Co-transfection with pLuc series
pmR65GV	mR65P-GV	
Single DNA pLuc-GV series		
pMluc-CMVGV	TATA-luc CMVP-GV	
pMluc-hR65GV	TATA-luc hR65P-GV	
pMluc-mR65GV	TATA-luc mR65P-GV	Transfection with a pLuc-GV DNA
phR65luc-GV	hR65P-luc hR65P-GV	
pmR65luc-GV	mR65P-luc mR65P-GV	

pLuc series contain the 5x17-mer GAL4 binding site and the luciferase reporter gene (*luc*) regulated by the different promoters; pGV series contain GAL4-VP16 DNA regulated by CMVP or R65P (Figure 1A). pLuc-GV series combine *luc*, the 5x17-mer and GAL4-VP16 DNAs in the same construct (Figure 1B). There are two types of single DNAs according to the regulation of the reporter gene: the minimal promoter (TATA) or the RPE65 promoter (R65P).

DNA in pACT exclusive of CMVP, and the resultant plasmids termed phR65GVand pmR65GV.

To generate single DNA constructs, where the reporter gene is regulated by the minimal promoter and GAL4-VP16 is regulated by hR65P or mR65P, pMluc, and phR65GV were digested with *Alw* NI, then ligated to generate a single plasmid construct, pMluc-hR65GV. pMluc-mR65GV was prepared in the same manner. phR65luc-GV and pmR65luc-GV, in which both the reporter and GAL4-VP16 are regulated by hR65P or mR65P, correspondingly, were generated using a similar strategy, except that phR65luc and pmR65luc were used. All single plasmid expression vectors were constructed with promoter orientation of head to head for optimal activity.

Cell culture: Primary human RPE cells, RPE51 (isolated from a 51-year-old donor), an RPE cell line D407 (a gift from Dr Richard C. Hunt, University of South Carolina School of Medicine, Columbia, SC), and human fibroblast cells, F2000 (Flow Laboratories, Rickmansworth, Herts, UK) and a human kidney cell line, HEK293, obtained from the American Type Culture Collection (ATCC, Rockville, MD), were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.). A human cervix carcinoma cell line, Hela (ATCC), a human hepatoma cell line, HepG2 (ATCC), and a monkey kidney cell line, COS-1 (ATCC) were grown in minimum essential medium (Life Technologies, Inc.). Cell culture media were supplemented with 1% (v/v) penicillin/streptomycin and 10% (v/v) fetal bovine serum (FBS). Cells ($5x10^2$ cells/ml) were seeded in 24-well plates and maintained at 37 °C with 5%CO_2.

Transfection and Luciferase assays: Cells at 50% confluency were transiently transfected using FuGene 6 Transfection Reagent (Roche, Indianapolis, IN). For transfection, 1 µg or 2 µg of plasmid DNA mixed with 1.5 µl or 3 µl of FuGene 6 diluted in DMEM, respectively, were used per well according to the manufacturer's specification. For co-transfection, a 1:1 molar ratio of the reporter vector (pLuc series) and the activator vector (pGV series) was used. All transfected cells were incubated for 40 h before being analyzed for luciferase activity.

Firefly luciferase activity was determined using the Luciferase Assay System (Promega) and TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Briefly, transfected cells were rinsed with PBS (pH 7.4) then incubated with lysis buffer (100 µl/15 mm^2) for several minutes before being harvested and centrifuged. A 20 µl volume of cell lysate was assayed by the addition of 100 µl of luciferase assay reagent using a 2 s delay time and 10 s integration time.

RESULTS

Establishment of transgene expression system: To study the enhancement of transcriptional activation and regulation of cell-specific gene expression in RPE cells, a transgene expression system mediated by GAL4-VP16 was established. The system includes two groups using either single or double DNA constructs to facilitate transgene expression in transfection (Table 1). In the first group, two constructs were produced with one carrying a 5x17-mer DNA binding sequence in front of a variety of promoters and *luc* gene (pLuc) and another carrying the transcriptional activator GAL4-VP16 under the control of CMVP, hR65P, or mR65P (pGV). In this system, following transient co-transfection of pLuc and pGV, GAL4-VP16 expression activates transcription of *luc* gene in pLuc

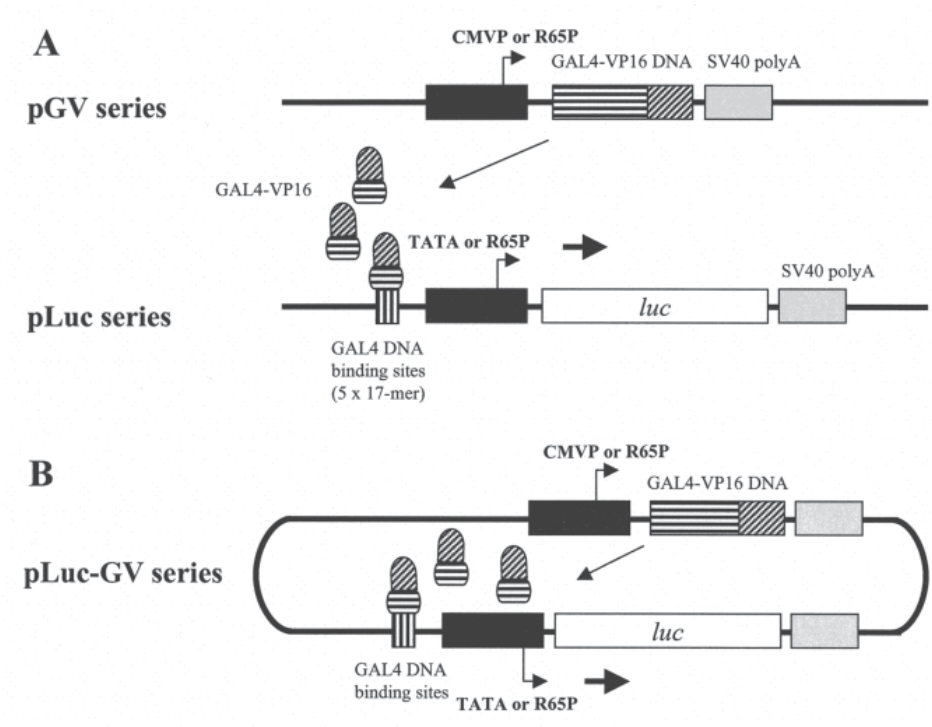

Figure 1. Schematic illustration of the transgene expression system. Various promoters, the minimal promoter (TATA), the cytomegalovirus immediate-early enhancer/promoter (CMVP), and the RPE65 promoter (R65P) were used in the system. **A**: The luciferase reporter and chimeric transcriptional activator GAL4-VP16 were produced separately in DNA constructs, pLuc, and pGV series. pLuc, where *luc* is regulated by TATA, CMVP, or R65P, presents the promoter activity in transfected cells; pGV, where GAL4-VP16 is regulated by CMVP or R65P, is used for co-transfection with pLuc series, correspondingly. **B**: The reporter and GAL4-VP16 are expressed in single DNA constructs, pLuc-GV series. pLuc-GV includes two types of constructs, depending upon whether *luc* is controlled by TATA or R65P. The chimeric activator GAL4-VP16 regulated by CMVP or R65P is produced and subsequently provokes *luc* expression (thick arrow) by binding the 5x17-mer sequence located upstream of the 5' end of TATA or R65P.

via the 5x17-mer DNA binding sequence (Figure 1A). In the second group, GAL4-VP16 DNA and *luc* with the 5x17-mer DNA binding site were constructed into a single plasmid vector (pLuc-GV) to provide an optimal molar ratio of the transcriptional activator and reporter protein (Figure 1B).

Evaluation of the reporter activities in RPE51 cells: To assess transcriptional activity, primary RPE cells (RPE51) at early passage were used. Transient transfection of constructs containing the hR65P or mR65P showed that without additional transcriptional activation mediated by GAL4-VP16, the transcriptional activity of the RPE65 promoters remained low (~ 20% for hR65P and <5% for mR65P) compared to the CMVP (Figure 2A). The lower activity of the mouse counterpart in the human cells indicated species-specificity of the promoter. Activity of the minimal promoter was very low or negative. To investigate if the minimal promoter activity can be greatly enhanced by transcriptional activation via GAL4-VP16, pMluc was co-transfected with members of the pGV series. The reporter gene activities of co-transfection with pMluc and pGV series showed significant increases of the minimal promoter activity (20-100 fold) when compared to the control pMLuc. However, they remained much lower (<30%) than the activity of the CMVP (Figure 2B). There was no significant increase ($p>0.05$) in the co-transfection of pMluc with phR65GV when compared to the activity of hR65P itself (Figure 2A,B). However, dramatic enhancement of transcriptional activity was achieved when phR65GV and phR65luc were co-transfected into RPE51 cells (Figure 2C). There were 3 fold and 13 fold increases in *luc* activity when compared to pCMVluc ($p<0.01$) and phR65luc ($p<0.001$; Figure 2A,C), respectively. These results demonstrate that hR65P, when in the presence of the transcriptional activator GAL4-VP16, produces a remarkably high level of the reporter gene expression in RPE cells. The lower transcriptional activities of the mouse

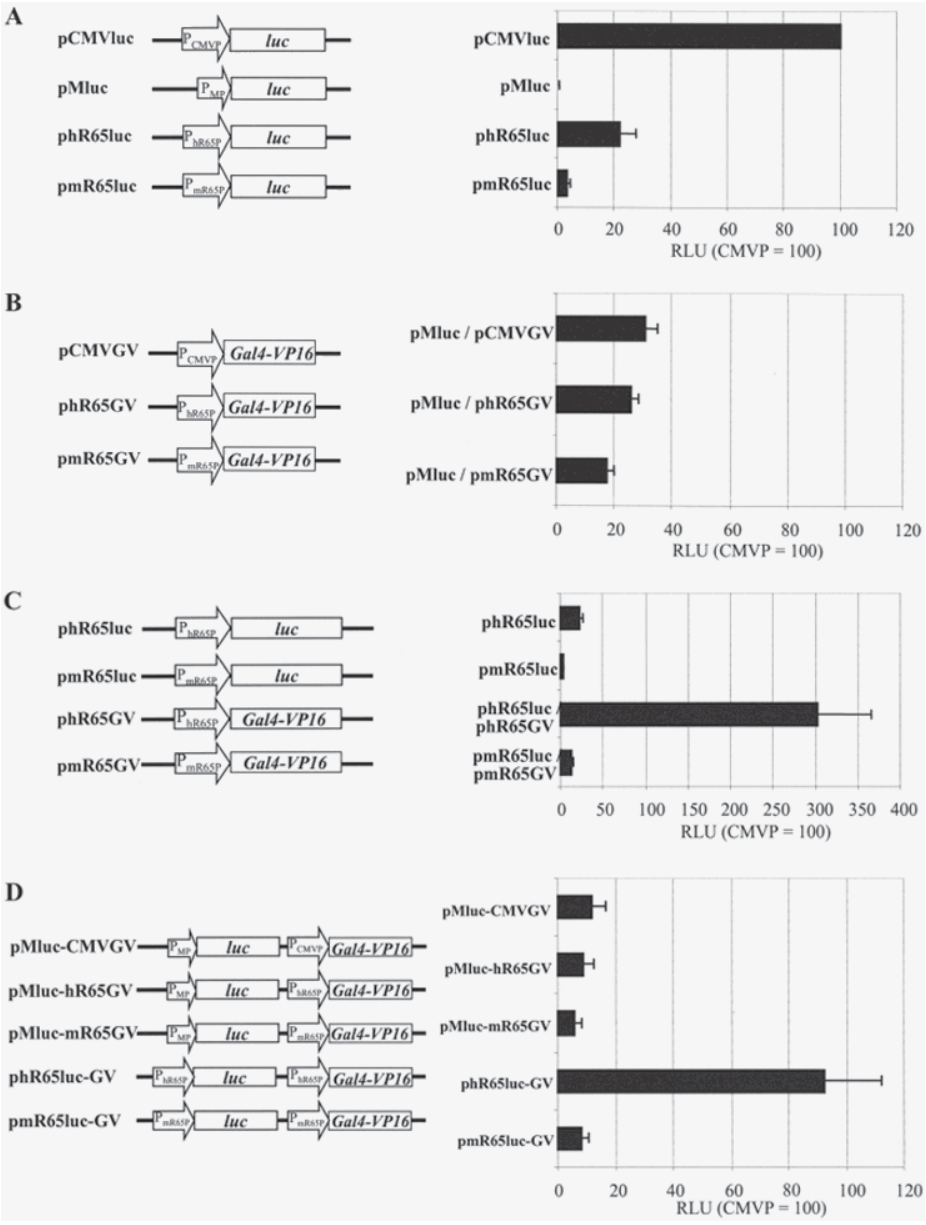

Figure 2. Schematic illustration of DNA constructs and reporter activities. Schematic diagram of DNA constructs in the system and reporter activities of different DNA constructs in RPE51 cells. The cells were transiently transfected or co-transfected with the different DNA constructs and incubated for 40 h prior to luciferase assay. **A**: pLuc constructs and their promoter activities. **B**: pGV constructs and enhancement of the TATA activity in pMluc co-transfection with pGV, correspondingly. **C**: pR65luc, pR65GV constructs and enhancement of the RPE65 promoter activity in pR65luc co-transfection with pR65GV, correspondingly. **D**: pLuc-GV constructs and activities of the single plasmids transfection. The luciferase activities, relative light unit (RLU) readings from the luminometer, were evaluated as fold relative to the activity of pCMVluc (CMVP=100). The error bars represent the standard deviations of triplicate measurements.

promoter constructs (e.g., pmR65luc/pmR65GV) suggest that the strong species specificity of the RPE65 promoter has been retained.

Based on the optimal results of phR65luc and phR65GV, for the second group of DNA constructs (Table 1), a series of single DNA plasmids was prepared, including phR65luc-GV, where both *luc* and GAL4-VP16 expressions were under the control of hR65P. Transfection with the majority of single DNA constructs resulted in a lower reporter gene activity (Figure 2D) when compared to the CMVP (<20% of the CMVP) or to co-transfection of phR65luc and phR65GV (Figure 2C). The highest activity (approximately 92% of CMVP) was obtained from the transfection using the single DNA phR65luc-GV (Figure 2D). The enhancement of transcriptional activity was significantly different (>4 fold) when compared to the activity of hR65P ($p<0.01$; Figure 2A). These results demonstrate that enhanced transgene expression of the system could only be achieved when hR65P drove both the reporter gene and GAL4-VP16 expression, either following co-transfection or single transfection of RPE51 cells.

Interestingly, co-transfection of pGV constructs in RPE51 cells, where GAL4-VP16 is controlled by hR65P, mR65P, or CMVP, in combination with pMluc, respectively, did not result in a strong increase of the minimal promoter activity (Figure 2B). The GAL4-VP16 mediated enhancement of the minimal promoter activity was investigated by performing co-transfection with pMluc/pGV constructs or transfection with the single DNA pMluc-GV (Table 1) in several non-RPE lines including HEK293, COS-1, Hela, HepG2, and F2000. In the majority of the cell lines, the minimal promoter activity remained very low or negative as seen in RPE51 cells (data not shown).

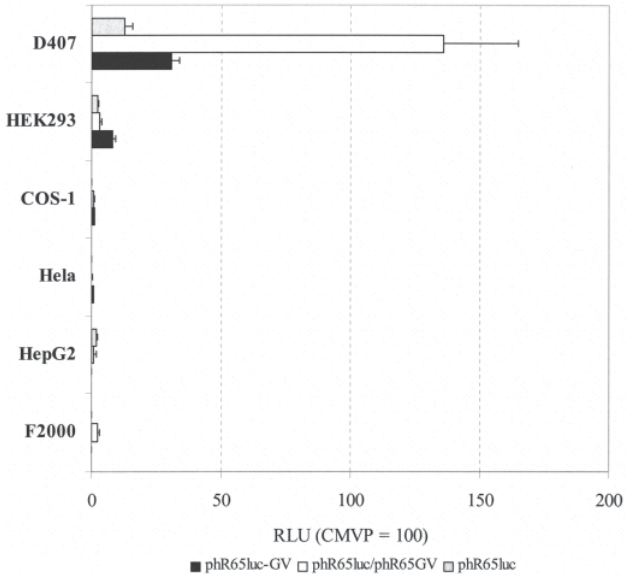

Figure 3. Cell specificity of hR65P construct reporter activity. Comparison of reporter activities of hR65P constructs in RPE cells (D407) and non-RPE cell lines (HEK293, COS-1, Hela, HepG2, and F2000). The transfections and luciferase analysis were performed as Figure 2. For each cell line, RLU readings for activities of phR65luc, phR65luc/phR65GV and phR65luc-GV were individually standardized using pCMVluc construct (CMVP=100). The error bars represent the standard deviations of triplicate measurements.

Cell specificity: Retention of RPE specificity by the RPE65P constructs was determined by transfection of another human RPE cells (D407), human non-RPE cell lines (HEK293, Hela, HepG2 and F2000), and a monkey non-RPE cell line (COS-1) with phR65luc, phR65luc-GV or co-transfected with phR65luc/phR65GV plasmid DNA (Figure 3). Similar to the data from RPE51 cells, phR65luc showed weak transcriptional activity in D407 cells and low or no significant activity in the other cell lines compared to CMVP. The co-transfection of phR65luc with phR65GV demonstrated the highest level of reporter gene expression in the two RPE cultures (Figure 2C and Figure 3). However, the activities of the three constructs in D407 cells were approximately half of the activity measured in RPE51 cells. When compared to the activity of hR65P (Figure 2A), there was a greater than 5 fold increase in the reporter gene activity of phR65luc/phR65GV-transfected cells (Figure 3). In D407 cells, the enhanced activity of the single plasmid construct, phR65luc-GV, doubled when compared to phR65luc ($p<0.001$), but was much lower (less than 5 fold) than the activity detected from the co-transfection with phR65luc/phR65GV ($p<0.01$). Generally, transcription activity for all constructs was lower in D407 cells than in RPE51 cells (Figure 2 and Figure 3). Very low and no significant activities were detected in transfected HEK293, Hela, HepG2, F2000, and COS-1 cells when compared to the CMV promoter activity of pCMVluc (Figure 3). These results indicate that the constructs retained relative RPE specificity in some cell lines whilst maintaining a high level of transgene expression in RPE cells.

DISCUSSION

In this study, we presented an in vitro transgene expression system that utilizes the chimeric transcriptional activator GAL4-VP16 to enhance the weak transcriptional activity of the truncated human and mouse RPE65 promoters in RPE cells. Data obtained suggest that the use of the RPE65 promoters that regulate both the reporter gene and the GAL4-VP16 results in a dramatic enhancement of cell-/tissue specific reporter gene expression whilst maintaining relative RPE cell-specificity.

In previous studies where weak, ligand-responsive, and tissue-specific promoters were used to regulate the expression of the chimeric transcriptional activator GAL4-VP16, dramatic enhancement of the transcriptional activation of the minimal promoter was only seen following the addition of an exogenous molecule [12-14]. It had also been shown that transcriptional activity was increased by endogenous agents in specific cell types. For example, when GAL4-VP16 was regulated by the carcinoembryonic (CEA) promoter, the augmentation of the synthetic minimal promoter's activity was much higher in CEA-positive cells than in CEA-negative cells [15]. These observations demonstrated that the combination of an inducible cell/tissue-specific promoter driving the chimeric

transcriptional activator and a minimal promoter whose transcriptional activation had been enhanced by the presence of necessary endogenous or exogenous factors (e.g., an inducible agent), yielded much stronger upregulation of a cell-/tissue-specific transgene than could be achieved with a viral promoter alone.

In the current study, we found that there were significant differences in the expression levels of the reporter gene depending on whether its expression was driven by the truncated hRPE65 promoter or the minimal promoter. In the RPE cell lines tested, the hRPE65 promoter produced much higher levels of the reporter gene compared to the minimal promoter. This observation indicated that either endogenous factors necessary for the minimal promoter were absent or not present at sufficient levels to induce expression of the reporter gene, or that in contrast to the minimal promoter the truncated hRPE65 promoter contained regulatory elements indispensable to effect transcription of the reporter gene.

RPE65 protein is highly and preferentially expressed in the RPE in vivo, but it is not expressed in RPE cells in vitro [3,16]. The lack of required endogenous agents, such as transcription factors, in combination with a weak RPE65 promoter, are believed to be the major cause for the low expression levels seen in cultured RPE cell lines. In the current experiments, using the hRPE65 promoter to drive the expression of both the GAL4-VP16 and the reporter gene, high expression levels of the reporter gene were only obtained in low passage RPE51 cells, suggesting that the regulatory elements of the human RPE65 promoter that are necessary for transcriptional enhancement and maintenance of relative cell-specificity are more active in low passage RPE cells (RPE51) than in established RPE cell lines (D407).

The RPE65 protein had also been found in mammalian and amphibian cone photoreceptors [17], indicating that different regulatory mechanisms may exist in different cell types in vivo. These regulatory agents might include unknown alternative promoters within the RPE65 gene which exhibit poor transcriptional activity, but which could be strongly enhanced by a chimeric activator such as GAL4-VP16 or VP16-LexA [12,18]. Considering the poor transcriptional activity of the minimal promoter it is possible that the human RPE65 promoter contains one of these alternative promoter elements.

In our study, the expression of the reporter gene was dramatically upregulated in RPE cells when the human RPE65 promoter was used to control the expression of both the reporter and the transcriptional activator genes. Therefore, the regulatory elements of the human RPE65 promoter were necessary for transcriptional enhancement and maintenance of cell specificity. A similar example of this type of transcriptional enhancement was previously described by Nettelbeck and coworkers [18]. In their study, a weak and endothelial cell (EC)-specific von Willebrand factor-promoter was used to regulate a reporter gene and the chimeric protein VP16-LexA for strong enhancement of the promoter activity while maintaining EC specificity.

Surprisingly, our results showed that expression levels of the reporter gene were consistently higher in co-transfections with the double plasmids than in transfections with the single plasmids. This would indicate that either the transfection efficiencies were consistently higher in co-transfection experiments, due to the smaller size of the plasmids, or some other unknown factors could be acting on the ability of the promoter to effect transcription of the transgene. Further studies are warranted to clarify these important issues as they influence the potential value of chimeric transcriptional activator systems for gene therapy.

In general, these results have shown that in relevant cell types, high and cell/tissue-specific gene expression levels can be achieved by introducing a cell/tissue-specific and functionally active promoter into a gene expression system mediated by a chimeric transcriptional activator.

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