Analysis of Different Promoter Systems for Efficient Transgene Expression in Mouse Embryonic Stem Cell Lines

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Abstract

Mouse embryonic stem (ES) cells are derived from the inner cell mass of the preimplantation embryo and have the developmental capacity to generate all cell types of the body. Combined with efficient genetic manipulation and in vitro differentiation procedures, ES cells are a useful system for the molecular analysis of developmental pathways. We analyzed and compared the transcriptional activities of a cellular polypeptide chain elongation factor 1 alpha (EF), a cellular-virus hybrid (cytomegalo-virus [CMV] immediate early enhancer fused to chicken β-actin [CBA]), and a viral CMV promoter system in two ES cell lines. When transiently transfected, the EF and CBA promoters robustly drove reporter gene expression, while the CMV promoter was inactive. We also demonstrated that the EF and CBA promoters effectively drove gene expression in different stages of cell development: naïve ES cells, embryoid bodies (EBs), and neuronal precursor cells. In contrast, the CMV promoter did not have transcriptional activity in either ES cells or EB but had significant activity once ES cells differentiated into neuronal precursors. Our data show that individual promoters have different abilities to express reporter gene expression in the ES and other cell types tested.

Keywords

Embryonic stem cells; Promoter strength; Transgene expression; In vitro differentiation

Introduction

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of preimplantation mouse embryos \cite{1} and represent embryonic precursor cells that can give rise to any cell type in the embryo \cite{2,3}. This developmental potential of ES cells has provided a powerful system to generate experimental animals with specific genetic alterations via homologous recombination \cite{4,5}. Moreover, ES cells can also be differentiated into many different lineages of cells in vitro \cite{6,7} making them a useful tool to analyze critical steps of early and late events of cell development, which otherwise are difficult to study in experimental animal systems. Since ES cells are easily accessible for genetic modification, they can be used...
to test certain transgene expression during cellular differentiation [8,9]. Another great potential of ES cells is that they may provide an unlimited cell source for transplantation therapies of various brain disorders such as Parkinson’s disease [10–13]. In this potential application, genetic modification of ES cells may be critical to drive their differentiation to the specific cell type(s) that are most desirable for the therapeutic potential [9].

For genetic modification of cells by exogenous transgene expression, different viral and cellular promoters have been used [14–16], and one of the most popular choices has been the cytomegalovirus (CMV) promoter because of its strong activity in most cell lines [17,18]. However, in ES cells, the relative strengths of different promoters have not yet been systematically analyzed. This prompted us to attempt to determine the optimal promoter system(s) for their genetic manipulation. We show that the human polypeptide chain elongation factor 1α promoter (the EF promoter) and the CMV immediate early enhancer fused to chicken β-actin promoter (the CBA promoter) show robust activities in two different ES cell lines, whereas the CMV promoter has only a marginal activity, if any. Additionally, we analyzed the strengths of these promoters in different stages of in vitro differentiated ES cells.

**Materials and Methods**

**Tissue Culture and In Vitro Differentiation of ES Cells**

D3 (wild type) mouse blastocyst-derived ES cell lines were obtained from ATCC (Manassas, VA; http://www.atcc.org/home.cfm), propagated, and maintained as described [11]. J1 ES cell lines were obtained from Dr. En Li at the Massachusetts General Hospital and maintained with the same procedure as for the D3. In brief, undifferentiated ES cells were cultured on gelatin-coated dishes in growth medium consisting of Dulbecco’s modified minimal essential medium (DMEM; Life Technologies; Gaithersburg, MD; http://www.lifetech.com) supplemented with 2 mM glutamine (Life Technologies), 0.001% β-mercaptoethanol (Life Technologies), 1X nonessential amino acids (100X stock from Life Technologies), 10% donor horse serum (Sigma; St. Louis, MO; http://www.sigmaaldrich.com), and human recombinant leukemia inhibitory factor (R&D Systems; Minneapolis, MN; http://www.rndsystems.com) (2,000U/ml). Human 293T cells were maintained in growth medium consisting of DMEM supplemented with 10% fetal bovine serum (FBS) (Hyclone; Logan, UT; http://www.hyclone.com) and antibiotics (Life Technologies).

ES cells were differentiated as embryoid bodies (EBs) on nonadherent bacterial dishes for 4 days in growth medium consisting of DMEM supplemented with 2 mM glutamine (Life Technologies), 0.001% β-mercaptoethanol (Life Technologies), 1X non-essential amino acids (100X stock from Life Technologies), and 10% FBS (Hyclone). The EBs were then plated onto adhesive tissue culture surfaces. After 24 hours in culture, selection of nestin-positive cells was initiated by replacing the medium with serum-free ITSFn medium [19]. After 10 days of selection, nestin-positive cells were expanded, which involved dissociating the cells by trypsinization and subsequent plating on polyornithine-coated coverslips in N2 medium [20] supplemented with laminin (1 μg/ml) and basic fibroblast growth factor (bFGF) (10 ng/ml). For transfection of EBs, cells were trypsinized after 4 days of suspension culture and plated on tissue culture plates for 12 hours prior to transfection. For transfection of nestin-positive cells, cells were transfected after 2 days of expansion in the presence of bFGF.

**Plasmid Construction**

Internal ribosome entry site-humanized renilla green fluorescent protein (pIRES-hrGFP) was purchased from Stratagene (La Jolla, CA; http://www.stratagene.com). For constructing pEF-hrGFP, the EF promoter was amplified by polymerase chain reaction (PCR) from pTracer-CMV2 (Invitrogen; Carlsbad, CA; http://www.invitrogen.com) using oligonucleotide primers.
containing an NsiI or NotI linker for each end. PCR products were then digested with NsiI and NotI, and ligated into the NsiI and NotI sites of pIRES-hrGFP.

For cloning pCBA-hrGFP, a SalI-EcoRI fragment containing the CBA promoter with the CMV enhancers was excised from pCX-EGFP (a gift from Dr. M. Okabe and Dr. J. Miyazaki at Osaka University; Osaka, Japan) and ligated into the NsiI and EcoRI sites of pIRES-hrGFP. All constructs were confirmed by restriction digestion and sequencing analysis.

**Transient Transfection Assay**

Each hrGFP-expressing construct was transfected along with the pGL3 internal control vector (Promega; Madison, WI; http://www.promega.com), which contains the Simian virus (SV40) enhancer/promoter and the luciferase gene, into the different cell lines using the lipofectamine plus technique (Life Technologies) according to the manufacturer’s protocol. The pGL3 vector was chosen as internal control for transfection efficiencies since reasonable levels of luciferase expression could be obtained in all cell lines transfected. Cells were harvested 36 hours after transfection, lysed in 200 μl lysis buffer (Promega), and assayed for GFP fluorescence using a Fluoro Count Fluorometer (Packard Instrument Company; Meriden, CT; http://www.packardbioscience.com). To correct for differences in transfection efficiencies and to normalize the GFP fluorescence intensity, luciferase activity was detected from the same lysate using a luciferase assay kit (Promega) according to the manufacturer’s protocol. Analysis of variance and Fisher’s post hoc analysis were performed using Statview Software (SAS Institute Inc.; Cary, NC).

**Fluorescence Microscopy**

Thirty-six hours after transfection, cells were fixed in 4% paraformaldehyde, mounted on glass slides using Gel mount (Biomeda Corp.; Foster City, CA; http://www.biomeda.com), and analyzed under fluorescence microscopy. Pictures were taken using a Spot camera (RT Diagnostic Instruments; Sterling Heights, MI).

**Results**

**The EF and CBA Promoters Can Efficiently Drive Gene Expression in ES Cell Lines, Whereas the CMV Promoter is Transcriptionally Inactive**

To optimize transgene expression in ES cells, we generated hrGFP-expressing constructs under the control of three different promoters, the CMV, the EF, and the CBA promoters [21]. Each promoter was subcloned into the pIRES-hrGFP vector (Stratagene) (Fig. 1), and constructs were confirmed by restriction digestion and sequencing analysis. Two different ES cell lines, D3 and J1, were then transfected with these three constructs and analyzed for GFP expression by fluorescent microscopy 36 hours after transfection. As shown in Figure 2, the CMV promoter was unable to drive any detectable level of hrGFP gene expression in either J1 or D3 ES cells, indicating its transcriptional inactivity in those cell lines. In contrast, the EF and CBA promoters drove robust expression of the hrGFP gene, demonstrating that individual promoters have different promoter activities when used in ES cells. These results were duplicated in three additional experiments using separate batches of reporter plasmids.

Our results are surprising, since the CMV promoter is one of the most popular promoters and has been used to overexpress foreign genes in many different cell lines. To confirm the promoter activity of our CMV construct, we also transfected the individual reporter constructs into 293T cells as a control. In contrast to its weak activity in ES cells, the CMV promoter showed a robust activity in 293T cells. The CBA and EF promoters also drove robust expression of hrGFP in 293T cells (Fig. 2).
To compare the strengths of the different promoters more quantitatively, we transfected the two ES cell lines with each hrGFP construct along with an SV40-luciferase construct as a transfection control. Thirty-six hours after transfection, cell lysates were prepared and assayed for GFP expression as well as luciferase activity. Levels of GFP expression were normalized to the luciferase activity, and the relative units are summarized in Figure 3. In J1 and D3 cells, gene expression from the EF promoter was two to three times stronger than from the CBA promoter, whereas the CMV promoter activity was less than 1% compared with that of the CBA promoter. In contrast, the CMV promoter was more than two times stronger than the CBA promoter in 293T cells, indicating a cell specificity of the CMV promoter activity. In all assays, the EF promoter had the strongest activity, suggesting that this promoter is an optimal choice for genetic manipulation and transgene expression in ES cells.

The EF and CBA Promoters are Active Throughout the Different Stages of In Vitro ES Cell Differentiation, Whereas the Viral CMV Promoter Function is Cell Stage-Specific

To test the function of transgenic gene products in genetically modified ES cells, it is important to achieve stable gene expression during different stages of cell differentiation. Thus, we wished to analyze the transcriptional activities of the three promoters during different stages of in vitro differentiated ES cells. Toward this end, ES cells were first differentiated into EBs in which the three primitive germ layers become induced [7], followed by selection of neuronal precursors using minimal media [19]. Each of the three hrGFP-expressing constructs, driven by the CMV, EF, or CBA promoter, was transfected into either undifferentiated D3 cells, D3 cells after EB formation, or D3 cells after selection of neuronal precursors in minimal media. Thirty-six hours after transfection, cells were fixed, mounted, and analyzed for GFP expression using fluorescent microscopy. Whereas the EF and CBA promoters displayed strong activities in ES, EB, and neuronal precursors (Fig. 4), the CMV promoter had no detectable activity in either ES or EB stages. However, the CMV promoter exhibited prominent activity once ES cells were differentiated into neuronal precursors (Fig. 4). These results were duplicated in two independent experiments.

We then quantified the promoter activities in EB cells. After EB formation, cells were dissociated, plated, and transfected with each hrGFP construct along with the luciferase construct as internal control. Thirty-six hours after transfection, levels of GFP expression were determined as described above (Fig. 3) and the relative units are shown in Figure 5. As was the case in undifferentiated D3 cells (Fig. 3), the EF promoter was more than two times stronger than the CBA promoter, whereas the CMV promoter activity was about 6% of that of the CBA promoter. Finally, in neuronal precursors, we could not quantify the promoter activities due to very inefficient transfection, although we tested several different methods available (Fig. 4 and data not shown).

Discussion

Based on their high developmental potential, ES cells are a useful tool for the study of the molecular steps of cellular differentiation [7] and can serve as a potential cell source for transplantation [10,11,12]. Thus, efficient genetic manipulation of ES cells is of great importance, and several groups have previously characterized different promoters in ES cells [22,23]. However, a direct comparison of viral with cellular promoters in naïve as well as in differentiated ES cell lines has not yet been reported. Moreover, preliminary observations in our studies unexpectedly revealed that the CMV promoter was very inefficient for transgene expression in ES cells. We, therefore, systematically analyzed the transcriptional activities of several promoter systems, both in naïve and differentiated ES cells using transient transfection assays. We found that the EF and CBA promoters can robustly drive transgene expression in both D3 and J1 ES cell lines. In sharp contrast, the CMV promoter failed to drive expression
of a reporter gene in the same cell lines. Surprisingly, comparison of normalized promoter activities demonstrated that the CMV promoter activity was less than 1% of the EF promoter activity. Thus, even though the CMV promoter shows robust activity in many cell lines [17, 18], its promoter activity is limited in ES cells.

At present, it is not known why the CMV promoter is inactive in ES cells. One likely explanation is that ES cells may express only a limited number of genes while maintaining a multipotential developmental capacity [24,25]. ES cells may not express some transcriptional (co-)factors necessary for the CMV promoter activity and thus cannot support its function. In contrast, ES cells may have all components necessary for full transcriptional activity of cellular promoters, such as the EF or the β-actin promoter, which may function more ubiquitously.

The use of ES cell lines to study the molecular mechanisms of cellular differentiation includes transgene expression techniques, which require stable promoter activities during the different stages of cell development. In this study, we demonstrated that the EF and the CBA promoter showed stable activity in different stages of cell differentiation, including neuronal precursors. This is consistent with the findings that both the EF and the CBA promoter are active throughout development and adulthood in transgenic animals, although there are some variations in the levels of expression in different tissues and developmental stages [26,27].

Surprisingly, we observed that the CMV promoter became active upon differentiation of ES cells to neuronal precursor cells, suggesting that this promoter may be useful when attempting to express transgenes only at later stages of differentiation or in maturated cell populations. Consistent with this notion, another group found weak gene expression using CMV-promoter-containing adenoviruses in transduced ES cells, but infection of ES-cell-derived cardiac myocytes caused strong CMV-driven gene expression [28]. Although it is possible that this low expression from the CMV-promoter-containing adenoviruses in ES cells may be due to low infection rates, our study suggests that it is likely based on the weak or inactive CMV promoter activity. In summary, our work shows that different promoters exhibit significantly different transcriptional activities in ES cells and further suggests that the EF or CBA promoters are superior to the CMV promoter for efficient transgene expression in ES cells.

Summary

Genetic engineering of ES cells requires efficient promoter systems, which ensure potent and stable expression of exogenous genes. The transcriptional activities of three different promoters were analyzed in two ES cell lines revealing robust GFP reporter gene expression from the EF and CBA promoters in both undifferentiated ES cells and neuronal precursors. In contrast, the CMV promoter only drove gene expression in the differentiated neural precursor cells, demonstrating differential promoter activities in ES cells and during ES cell development.

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References


Figure 1. Plasmid map of pIRES/hrGFP, which was used for the expression of hrGFP under the control of three different promoter candidates CBA, CMV, and EF subcloned into the promoter site (for details see Materials and Methods)
MCS = multiple cloning site; HA = hemagglutinin tag; IRES = internal ribosome entry site.
Figure 2. Activities of the EF, CBA, and CMV promoters in D3 and J1 ES and 293T cells
Each hrGFP-expressing plasmid driven by one of the three different promoters was transiently transfected into the cell lines described. Thirty-six hours after transfection, cells were fixed, mounted, and analyzed for GFP expression using fluorescence microscopy.
Figure 3. Quantification of promoter strength in D3 and J1 ES and 293T cells
Each cell line was transfected with the individual hrGFP-expressing construct along with the luciferase-expressing plasmid pGL3 to control for transfection efficiency. The pGL3 vector drove reasonable levels of luciferase expression under the control of SV40 promoter in all cell lines transfected. Thirty-six hours after transfection, cell lysates were prepared and assayed for GFP expression and luciferase activity. Levels of GFP expression were normalized to the luciferase activity, and the relative units were calculated with the activity of the CBA promoter set to 100. Each promoter data set represents n = 4 from two independent experiments in the case of D3 and J1 cells, and n = 3 for 293T cells. Analysis of variance results were: for D3 cells, $F_{3,11} = 96.257$, $p < 0.005$; for J1 cells, $F_{3,11} = 62.972$, $p < 0.005$; for 293T cells, $F_{3,7} = 48.117$, $p < 0.005$. Post hoc analysis was performed with a significance level of 5%. * indicates that the activity of EF was significantly greater than that of CBA and CMV. # indicates that the activity of CBA was significantly greater than that of CMV. § indicates that the activity of CMV was significantly greater than that of CBA.
ES cells were differentiated into EB followed by further selection of neuronal precursors as described elsewhere [21] and in \textbf{Materials and Methods}. Undifferentiated ES, EB, and neuronal precursor cells were transfected with each of the hrGFP-expressing constructs driven by one of the three promoters (CBA, CMV, and EF). Thirty-six hours after transfection, cells were fixed, mounted, and analyzed for GFP expression using fluorescence microscopy.
Figure 5. Quantification of promoter strength in EB cells
Thirty-six hours after transfection, cell lysates were prepared and assayed for GFP expression and luciferase activity. Levels of GFP expression were normalized to the luciferase activity, and the relative units were calculated with the activity of the CBA promoter set to 100. Each promoter data set represents n = 3. The result of the analysis of variance was $F_{3,7} = 13.148$, $p < 0.005$. Post hoc analysis was performed with significance level of 5%, and * indicates that the activity of EF was significantly greater than that of CBA and CMV.