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CMV promotor activity during ES cell differentiation: Potential insight into embryonic stem cell differentiation

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Abstract

The activity of the $P_{CMV\ IE}$ promoter was studied during the differentiation of ES cells into neurons. In order to do this, stable embryonic stem (ES) cell lines that express enhanced green fluorescent protein (EGFP) under the control of $P_{CMV\ IE}$ were created and these ES cells were differentiated by aggregation of cells in the presence of retinoic acid (RA). Based on our observations that the activity of $P_{CMV\ IE}$ was highest in undifferentiated cells, and that cell–cell interaction and addition of RA that lead to enhanced cell proliferation also increased expression from $P_{CMV\ IE}$, we hypothesized that the activity of $P_{CMV\ IE}$ was positively regulated in cycling cells. However, when analysis was done at the single cell level it was found that BrdU label and EGFP expression were not correlated. EGFP expression was found to be down-regulated in many cells that were BrdU positive and conversely there were significant numbers of BrdU negative cells that were EGFP positive. Further, $P_{CMV\ IE}$ activity was not observed in cells that were nestin positive or in differentiated neurons, but $P_{CMV\ IE}$ was active in cells with a fibroblast-like morphology. Finally, several proteins present in undifferentiated ES cells were found to bind to regulatory regions of $P_{CMV\ IE}$. These were absent when cells were aggregated in the presence of RA. The above results have implications for expression of transgenes in ES cells as well as providing new insight into the mechanism of lineage restriction.

Keywords: CMV; Embryonic stem cells; Transgene expression

1. Introduction

Mouse embryonic stem (ES) cells have served as a valuable tool for understanding neural differentiation pathways (Gottlieb, 2002). ES cells are usually maintained on a fibroblast feeder layer although several groups now have feeder-independent ES cell lines that are maintained only in the presence of Leukemia Inhibitory Factor (LIF). ES cells can be made to differentiate into cells of the neuroectodermal lineage in several ways. These include the use of factors produced by stromal cells (Kawasaki et al., 2000), treatment with vasoactive intestinal peptide (VIP) (Cazillis et al., 2004), aggregation and exposure to retinoic acid (RA) (Gottlieb and Huettner, 1999), and also specific selection of cells of neuroepithelial lineage by treatment with cytokines such as basic fibroblast growth factor (bFGF) (Lee et al., 2000).

Transgene expression has proven to be an important tool in understanding the biology of stem cells and the factors that affect their differentiation into different cell types. The most important aspect of transgene expression is the promoter selected under which the transgene will be expressed, as this will determine the results observed and how they are interpreted. There are two main types of studies that make use of transgene expression. In one, the transgene is used mainly as a marker to track the ES cells, in both in vivo and in vitro mixed culture conditions. As an interesting example of this use of transgene is the study in which one transgene was used to mark ES cells and a different transgene was used to mark neurospheres in a mixed in vitro culture. In this study, the ability of ES cells to induce neurospheres to transdifferentiate into mesodermal lineage was examined (Denham et al., 2005). In some cases, the gene

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of interest is expressed under a ubiquitous promoter in order to check how the sustained expression of the transgene during the process of differentiation influences the type of cells the ES cells will differentiate into (Bahrami et al., 2005). In these contexts, it is assumed that the expression of the transgene is on during the process of differentiation. However, the ideal ubiquitous promoter that would give invariable high levels of expression in all tissues has been difficult to find (Shmerling et al., 2005). In this context, it is important to characterize the expression of a promoter during different stages of ES cell differentiation to understand how a given transgene will be expressed.

The cytomegalovirus immediate early gene enhancer/promoter ($P_{CMV\ IE}$) is commonly used in a variety of mammalian cell lines to express heterologous genes (Kim et al., 2002). A number of recent papers have examined the activity of the $P_{CMV\ IE}$ in both undifferentiated ES cells and during the differentiation of ES cells into neurons and have reported varied results. While Ward and Stern (2002) showed that $P_{CMV\ IE}$ was active in undifferentiated ES cells using a transient transfection assay, an earlier study by Chung et al. (2002) showed that $P_{CMV\ IE}$ was not transcriptionally active in either undifferentiated ES cells or in embryoid bodies. A third study by Zeng et al. (2003) using stable ES cell lines in which differentiation was induced by stromal cells showed that $P_{CMV\ IE}$ was active in undifferentiated ES cells and in nestin positive neuroepithelial precursor cells but not in tyrosine-hydroxylase (TH) positive neurons. More recently, a study by Kawabata et al. (2005) compared different adenovirus vectors to express a transgene and found that the CMV promoter was inactive in mouse ES cells.

With this in mind the present study examines the activity of the $P_{CMV\ IE}$ during ES cell differentiation into neurons using a novel method of differentiation. This was done for several reasons. First, it enabled us to study stage-specific expression from $P_{CMV\ IE}$ during the differentiation of ES cells. In particular, since it was shown that $P_{CMV\ IE}$ is active in nestin positive cells but not in TH positive cells we wanted to investigate whether down-regulation of the promoter occurs in cells that become post-mitotic at any stage during the progression from undifferentiated cells to a differentiated cell. Second, we wanted to define a stage during the differentiation of ES cells that is marked by the down-regulation of the $P_{CMV\ IE}$ and begin characterization of proteins that may be involved in the shift from a stem cell to a precursor cell. Third, it enabled us to determine whether the activity of the $P_{CMV\ IE}$ observed using the RA differentiation protocol would be similar to that observed with the stromal cell induced differentiation. This would tell us whether the regulation of $P_{CMV\ IE}$ reflects a common molecular event that results in changes in gene expression that accompany neuronal differentiation or whether the changes reflect aspects that are specific to a particular pathway of differentiation.

The $P_{CMV\ IE}$ region is well characterized and provides a convenient starting point to begin analysis of the proteins that may be regulated during differentiation of ES cells. The *cis*- regulatory sequences in this region have been shown to contain binding sites for several important transcription factors. The $P_{CMV\ IE}$ has five cyclic AMP-response elements (CRE), four NF-Kappa B sites, one AP-1 site and serum response elements (Meier and Stinski, 1996). We reasoned that, if the down-regulation of the promoter was correlated with an important stage in the differentiation of ES cells, then we could further investigate the transcriptional factors and signaling pathways that are involved in this switch by using the CMV promoter elements. Identifying some of the transcription factors involved would provide information about the intermediary pathways that regulate lineage decision and cell cycle exit.

We have used the model of ES cell aggregation to stimulate embryoid body formation and RA induction to convert ES cells into cells of the neuroectodermal lineage. In this pathway of ES cell differentiation we first looked to see if cell–cell interaction and RA regulated $P_{CMV\ IE}$ by looking at cells in aggregates in the presence and absence of RA as well as when aggregates were dissociated and plated as single cells onto substrate. We then determined whether down-regulation of $P_{CMV\ IE}$ correlated with cell cycle exit. Second, we looked to see whether down-regulation of $P_{CMV\ IE}$ correlated with the expression of nestin, a marker of neuroectodermal lineage, which is not expressed at high levels in undifferentiated cells. Finally, we looked at two well characterized regions of the CMV promoter, a 19 base pair repeat which contains a consensus CRE binding site and a 21 base pair sequence which has previously been shown to bind proteins which modulate the activity of the CMV promoter in embryonal carcinoma cells (Kothari et al., 1991; Bain et al., 2003).

Our results show that $P_{CMV\ IE}$ is highly active in undifferentiated ES cells and in aggregates. Addition of RA to the aggregates leads to a maintenance of expression from $P_{CMV\ IE}$ and results in an increase in the number of cells that remain in the cell cycle. Interestingly we found that the activity of $P_{CMV\ IE}$ was correlated with cells of specific lineages rather than only being correlated with cycling cells. While nestin positive neuroepithelial precursor cells did not show EGFP expression, fibroblast-like cells expressed EGFP at high levels. Finally, proteins were present in nuclear extract from undifferentiated ES cells that bound to the CRE sequence and a 21 base pair sequence that is important for modulating expression from the $P_{CMV\ IE}$. In clear contrast, nuclear extract from cells that had been differentiated with RA and plated out after disruption of the aggregates did not bind to these regulatory sequences. This result is significant since it implies that these proteins that are absent may play an important role in the conversion of ES cells into cells of the neuroectodermal lineage.

2. Materials and methods

2.1. Cell culture

Undifferentiated (UD) D3 ES cells were maintained in ESM media supplemented with LIF (DMEM with 15% FBS, 4 mM L-Glutamine, 1X NEAA, 1X Pen-Strep, 10^{-4} M β-mercaptoethanol and 10^3 U/ml LIF). Confluent D3 cells (80%) were transfected with the pEGFP–N1 plasmid (BD Biosciences) by electroporation and selection was initiated 24 h later with 0.4 mg/ml G418. Resistant colonies were subcloned to develop stable lines expressing EGFP under the control of $P_{CMV\ IE}$. ES cell differentiation was initiated by aggregating 10^6 D3 cells in 90 cm bacteriological plates for 2 days in the absence of