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Promoter analysis in ES cell-derived neural cells

Hai-Qing Xian, Kelly Werth, David I. Gottlieb*

Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, MO, USA

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Abstract

Neural cells derived from ES cells in cell culture (ESNCs) have many of the properties of normal neural cells and provide a model of "neurogenesis-in-a-dish." Here we show that ESNCs provide a powerful system for analyzing neural gene transcription. ES cells are transfected with bacterial artificial chromosomes (BACs) containing Olig2, a gene with a key role in neural fate choice. One BAC is modified by recombineering to insert a reporter gene and a gene for selecting stably transfected clones. Another BAC contains a deletion of a suspected Olig2 promoter. Stable transgenic clones of ES cells are isolated, differentiated in culture, and the expression of transgenes is assayed. Differentiated cells dramatically up-regulate transgene expression and a deletion analysis reveals a basal promoter for Olig2. The combination of ESNCs and BAC recombineering will have broad application for analyzing gene transcription in the nervous system and will be applicable to human ES cells. The general approach should also be applicable to the many other cell lineages that can now be derived from mouse and human ES cells in culture.
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Early neural development is under the control of a large repertoire of regulatory genes, many of which are transcription factors. Understanding how these genes are themselves regulated and how they regulate downstream genes remains an important challenge. One approach has been to make transgenic mice carrying potential regulatory regions linked to a reporter and analyze expression in the developing brain. This approach is useful but has basic constraints. Transgenes insert at random and expression is strongly influenced by the chromosome integration site. Consequently, transgenic lineages with the identical insert give widely differing expression patterns necessitating an arbitrary judgment as to which reflects the true specificity of the transgene. Transgenic mice are expensive, making follow-on studies for fine mapping of regulatory regions prohibitive in most cases. Finally, it is often difficult to obtain sufficient cells for biochemical studies of transgenes and constructs based on them. Because of these considerations there is a strong need for an improved system for analyzing regulation of genes that direct neurogenesis.

Here we illustrate a new approach to this problem that incorporates two principles. First, large transgenes are used. Recent advances in BAC engineering make it possible to precisely alter BACs by insertions and deletions generated by homologous recombination [1,2]. BAC transgenes lessen one problem of random transgenes in that expression is less affected by the insertion site than in the case of small transgenes [3,4]. It has even been suggested that many BACs encompass a fully autonomous set of control elements. Accordingly we base our analysis on large transgenes modified to contain reporters, selectable markers, and deletions. The second principle is to harness the power of ES cell-derived neural cells (ESNCs). A variety of methods

* Corresponding author. Fax: +1 314 362 3446.
E-mail address: gottlied@pcg.wustl.edu (D.I. Gottlieb).

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are available to efficiently guide ES cells to differentiate along a neural pathway to create ES (ESNCs) [5–9]. These systems provide the entire series of cells from totipotent ES cells through neural progenitors and onto differentiated neural cells. The basic strategy is to make transgenic clonal ES cells and to measure transgene expression at intervals along the developmental pathway. Thus, many independent transgene insertions can be studied as the cellular environment changes allowing a precise characterization of all clones.

We have applied this approach to analyze the promoter for Olig2, a bHLH transcription factor that plays a key role in generating the oligodendrocyte lineage [10–15]. Olig2 is both spatially and temporally regulated in neural development making its first appearance about E9 and being restricted to ventral domains of the neural tube. The promoter of the Olig2 gene has not been reported in the literature and little is known about *trans*-acting factors that regulate the gene. A 227 kb BAC containing the Olig2 gene has been engineered to allow analysis of expression in transgenic ES cell lines. This region of the chromosome contains elements that allow very significant up-regulation of Olig2 expression. A deletion of a putative promoter confirms that it is active but suggests complexity of promoter structure for the Olig2 gene.

Materials and methods

ES cell lines. ES cells are the mouse RW4 line and its GFP derivative G-Olig2 [16,17].

ES cell culture. Undifferentiated ES cells and G-Olig2 ES cells were cultured as described previously [5,16]. For the experiments shown in Fig. 1, undifferentiated ES cells were scraped off the bottom of flasks, triturated into small clumps by pipetting and then cultured for two days in M-DFK5 medium. M-DFK5 medium consists of DMEM/F12 supplemented with 1× non-essential amino acids, 1× nucleosides, 0.1 mM mercaptoethanol, 50 μg/ml transferrin, 5 μg/ml insulin, 30 nM Na-selenite, and 5% knockout serum replacement, and is based on a medium previously described [9].

These 2-day-old EBs were plated in a 96-well plate at approximately 20 EBs per well in the same medium in the presence of 2 μM RA and/or 300 μM Shh-N (R&D Systems) for six days; this is referred to as the 2−/6+ protocol. For the experiments in Fig. 3 expanded ES cell clones were scraped off the bottom and partially dissociated by pipetting, and then cultured for six days in M-DFK5 medium in the presence of 2 μM RA and the Shh agonist Hh-Ag 1.4 (Curis) at 30 nM substituted for Shh. This abbreviated procedure gives equivalent differentiation to the 2−/6+ protocol.

Gene expression profiling. Total RNA was made from cultured cells using the RNAwiz kit (Ambion). cDNA was synthesized with reverse transcriptase (Retroscript, Ambion). Individual PCR products were amplified using primers shown in Table 1. Thermal cycling was carried out with a 1 min denaturation step at 95 °C, followed by 35 cycles of 15 s at 95 °C, 30 s at 68 °C, and 45 s at 72 °C.

Engineered BAC clones. All BAC experiments used mouse genomic BAC clone RP23-256L6 obtained from the Children's Hospital of Oakland Research Institute (CHORI) or its derivatives. Engineered versions were produced by recombineering using the lambda red system [1,2]. In brief, cells with BAC RP23-256L6 were transfected with the mini lambda defective phage and integrants were selected by picking tet resistant clones; all subsequent steps were done in the mini-tet background. For luciferase insertion the luciferase cDNA of pGL3 was amplified by PCR with primers containing 50–60 bp of homology to the Olig2 gene (Table 2). This linear construct was transfected into cells with the BAC and mini lambda following established procedures [1]. Recombinants were selected by PCR with primers for detecting the novel junction and clones with the correct junction were verified by sequencing. To provide selection in ES cells, a neo cassette was inserted by an additional round of recombination. We utilized the PGK-EM7-Neo cassette from pL452 [2] which was amplified with primers having 50 bp of homology to Olig2 and 20 bp to the neo cassette (Table 2).

The PCR product was digested with *Dpn*I for 2 h, gel purified (Qiagen), and transfected into BAC containing cells by standard procedures. Neo-resistant clones were analyzed by junctional PCR and Southern blots to identify correctly targeted BACs. BAC DNA was isolated using ProPrepMini Kit (Biotech Support Group).

The deletion in BAC *Olig2.luc.neo. Δ1* was constructed using an oligonucleotide flanking the deletion with 40 bp of homology on each side (Table 2). Deleted clones were identified by screening successive pools of bacterial clones with PCR. The deletion was mapped by sequencing the PCR products spanning the deletion.

Transfection of ES cells. All BAC DNA was linearized at a site within the vector with PlSceI. DNA (20 μg) was electroporated into 1×10^7 RW4 undifferentiated ES cells. Electroporation was done in a Bio-Rad Gene Pulser set for 0.23 kV and 960 μF using a 0.4 cm gap cuvette. Electroporated cells were transferred to a 100 mm gelatinized dish with 10 ml complete medium (CM) (DMEM + 10% FBS, 10% NBCS, and nucleoside supplement) with 1000 U/ml LIF and 0.1 mM β-mercaptoethanol to keep cells undifferentiated. Selection was done with 250 μg/ml neomycin. Eight-day-old colonies were picked, transferred to a 96-well gelatinized plate, and allowed to expand in complete medium with LIF and β-mercaptoethanol. Expanded clones were split into two wells of a 24-well array and cultured to expand further. One well was differentiated (see above) and the other harvested as undifferentiated ES cells.

Luciferase assays. Cells were washed with PBS and 1× cell lysis buffer (Promega) was added and incubated for 5 min at room temperature. Cell lysates from approximately 1×10^5 cells were transferred to a 96-well white plate (LumiNunc). One hundred microliters of Luciferase Assay Reagent (Promega) was added and the luminescence was read on a Perkin–Elmer Wallac 1420 Luminometer. All assays were internally calibrated with a standardized firefly luciferase solution to ensure uniformity between experiments done on different days.

Protein assay. Five microliters of cell lysate of each sample was added to 200 μl Bio-Rad protein assay reagent (Bio-Rad Laboratories). The protein concentration was measured according to the manufacturer's instructions.

Results

Olig2 gene expression in neurally differentiated ES cells

An ES cell line with GFP knocked in to the Olig2 gene(termed G-Olig2) has been constructed and allows visualization of Olig2 expression via GFP fluorescence [16,17]. Using a standard neural induction protocol, about 2% of cells become GFP (that is Olig2) positive [16]. A more lengthy protocol for obtaining pure Olig2-expressing cells [17] is not suited to high-throughput experiments. Therefore, we sought conditions for robust Olig2 gene expression in a simple protocol and used

Table 1
Primers for gene-specific PCR

Name	Sequence (5′–3′)
Olig2	Forward: AGCCAACACCAGCGCCCAGAGCCAGGTTCTCCT Reverse: GATGACTTGAAGCCACCGCCGCCCAGTTTGTCC
Olig1	Forward: AGA TGT ACT ATG CGA TTT CCC AGG CGC G Reverse: GTT CAG GTC CTG CAT GCG CTT CCG CTC
Nkx2.2	Forward: CTT TCT ACG ACA GCA GCG ACA ACC CCT ACA CTC Reverse: CAT ACC TTT CTC CGC CCG GGC ACG TTT CAT CTT
Nkx6.1	Forward: TTGGCAGGACCAGAGAGAGCACGCTTGG Reverse: CCTCCGACGCGTGCAGCAGGAGGC
Isl1	Forward: GTG TTG GTT GCG GCA ATC AAA TTC ACG AC Reverse: CCG CGC TGG ATG CAA GGG ACT GAG AG
Isl2	Forward: TGCACTCCAGAGCGACCTGGACCAAC Reverse: GTC TCC AGT CTC AGC GGT GCA GGC
Lim3	Forward: GGACAGTATTTCCGCAATATGAAGCGCTC Reverse: CTG AAG GAC AAG GCT CAA GTT GGT GTC T
Hb9	Forward: TCC GAC TGC TCA TCT GAG GAC GAC TC Reverse: GTCAGGCAGGAGCCACTCCTAGAAAGGGT
Nanog	Forward: AGGACTTTCTGCAGCCTTACGTACAGTTGC Reverse: CTCTTCTGGAGTGTCTGAAGACAGCTACAG
Oct3	Forward: ACGAGAAGAGTATGAGGCTACAGGACACCT Reverse: CTCCTGATCAACAGCATCACTGAGCTTCTT
HNF4	Forward: GTC ATT GTT GCT AAC ACG ATG CCC TCT CAC Reverse: GAC TGG TCC CTC GTG TCA CAT CTT CTT TGC
Brachyury	Forward: TAC ATG TCC TAT AAT CAT GTT CTA CAG C Reverse: GAC ATT AGA GGT GTA CCT TGT GTC TCA CTA
Nestin	Forward: GAG GAG CTG GCC AGG CGC CTA TGC GAA GTG Reverse: TGC AAG CGA GAG TTC TCA GCC TCC AGC AGA
Sox10	Forward: ACG GCG AGG CGG ACG ATG ACA AGT TC Reverse: TCC CGT TCT TCC GCC GCC GAG GTT G

Table 2
Primers used in recombineering

Purpose	Sequence (5′–3′)
Insert luciferase	Forward: TTCGGAAGGTTGAAAAAAGAAGGATCATTCGAGAGCTTAGATCATCCCTGGGGCCATGGAAG ACGCCAAAAACATAAAG Reverse: TCCGCGCTGCTCCGCAGCCCTTGGCTCTCCAGGACGCACCCCCGCTGGCCGGCTTACACGGCGA TCTTTCCG
Insert PGK-Neo	Forward: CCACCACAGCTGCAGCTACTACTGGGATCTCAGAAGTAAGGCTTTGCTTC CGCTCTAGAACTA GTGGATC Reverse: CCTGGACACCAGTCTTGATCTTGGGATAGCTAAGCCTGGACCAAGTTAGTCAGCCCAATTCCGA TCATATT
Delete 2 kb	AACCTGGGCGCTCGGATGCTCCGGGCAGTCACACTAGCCTTCCACCCAGCTATAAAAACCAGCGGAACC CCGAAAGGTGT

the G-Olig2 cell line as a tool. We discovered that simultaneous treatment with RA and sonic hedgehog (Shh) induces vigorous expression of Olig2 in a relatively brief period (Fig. 1A), with approximately 33% of the cells becoming GFP+ in six days. A similar result has been found with non-transgenic ES cells (Xian, unpublished data). While it is likely that this variant protocol induces cells to follow a meaningful pathway of neural differentiation, it is conceivable that it simply induces Olig2 gene expression in an inappropriate context. To show Olig2 expression signifies appropriate differentiation, we measured the expression of a panel of genes that mark major fate choices in early development in sorted GFP+ cells (Fig. 1B). Two genes for pluripotent cells, nanog and Oct 3, are not expressed. Neither are HNF4 and brachyury, markers for early endoderm and mesoderm, showing that induced cells are neither pluripotent, endoderm nor mesoderm. As expected, not only Olig2 but Olig1 is

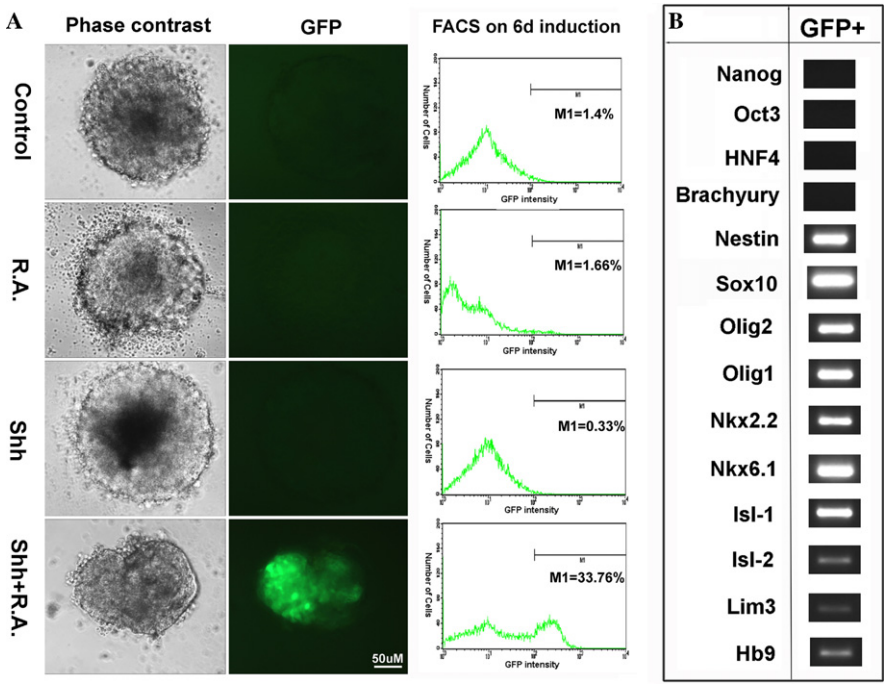

Fig. 1. Induction of ventral neural phenotype in ES cells. (A) Induction of Olig2/GFP by RA and Shh. The G-Olig2 ES cell line was treated as indicated with either standard medium (control), retinoic acid (RA), sonic hedgehog (Shh) or Shh + RA. Typical EBs seen in phase contrast or GFP fluorescence are shown. FACS profiles for each condition are given in the rightmost column; M1 indicates the fraction of intensely positive cells. The combination of RA + Shh induces GFP expression. (B) Expression of lineage-related genes after induction. Induced cultures were sorted and GFP+ cells were profiled by RT-PCR. Genes associated with pre-neural stages are not expressed while those associated with the ventral nervous system are.

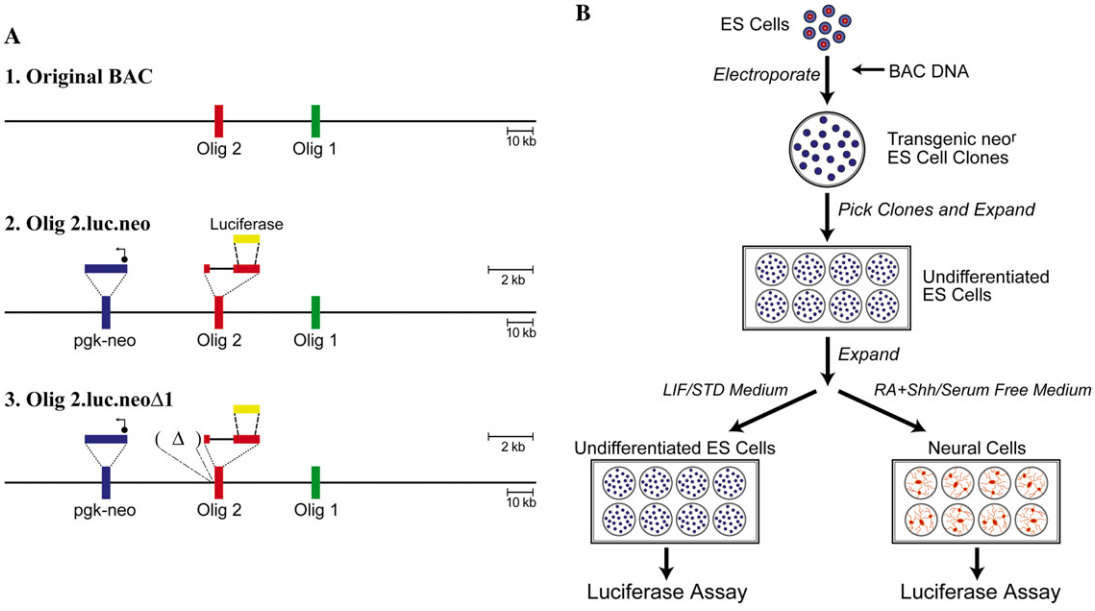

Fig. 2. BAC maps and experimental scheme. (A) BACS. (1) The original BAC (clone RP23-256L6) with the positions of Olig2 and Olig1 genes is shown. Scale is indicated. (2) After two rounds of engineering, luciferase and pgk-neo have been inserted to produce *Olig2.luc.neo*. Scale for the inserts (2 kb) is shown. (3) Diagram of the deletion clone *Olig2.luc.neo. Δ1*. (B) Experimental flowchart. The steps from transfection of ES cells with BACs through measurement of luciferase activity are illustrated. Details in the text.

expressed, as are other markers for the ventral compartment of the nervous system including Nkx6.2, Nkx2.2, Isl-1, Isl-2, Lim 3, and HB9. In conclusion, we have developed a straightforward and convenient induction system based on RA and Shh to differentiate ES cells along a meaningful ventral neural pathway. We have

also shown that omitting the first two days of culture and substituting Hh-Ag 1.4, an Shh agonist, for Shh gives equivalent results (Xian, unpublished data). The method is practical for investigating the expression of BAC transgenes in stably transfected ES cell clones.

A BAC containing Olig2 and Olig1, and its engineered derivatives

RP23-256L6 is a BAC with a 227 kb segment of mouse chromosome 16 that includes Olig2 and Olig1. Olig2 is located approximately 100 kb from the left end and Olig1 is 42 kb downstream of Olig2 (see Fig. 2A). The structure of the clone was verified by PCR, Southern blots, and limited sequencing. The extent of the Olig2 gene is given as position 91,660,398–91,663,524 of Ch16 in the May 2004 build of the UCSC compilation of the mouse genome. We conclude that RP23-256L6 represents a valid copy of the genomic region containing Olig2 and Olig1.

BAC RP23-256L6 was modified in three steps by homologous recombination (Fig. 2A) using the lambda red system [1]. First, the firefly luciferase gene was inserted into the coding exon of Olig2 in-frame to provide a reporter for transcription. This insertion simultaneously deleted 990 bp of the Olig2 ORF. Sequencing confirmed that the luciferase was inserted correctly and had not been mutated during construction; restriction mapping showed that rearrangement of the BAC had not occurred during this engineering step. Next, a neomycin cassette for selection of stable BAC integrations in ES cells was inserted 50 kb upstream of the Olig2 gene to yield BAC clone *Olig2.luc.neo*. Transcription of the neo cassette was in the opposite orientation of Olig2 to avoid read-through to luciferase. Southern mapping shows that the insertion was in the planned location and that rearrangements had not occurred. Finally, *Olig2.luc.neo* was further engineered by deleting 2 kb immediately upstream of the Olig2 cap site to produce *Olig2.luc.neo Δ1*.

Expression in stable transgenic clones—undifferentiated ES cells

Olig2.luc.neo was linearized at the Pl-SceI site in the vector, electroporated into wild type ES cells and neo-resistant ES cell colonies selected (Fig. 2B). Although the structure of BACs was not studied directly, it is known that linearized BACs usually integrate into ES cells as intact molecules with one or, at best a few, integrations per cell [18,19]. Each clone was first expanded as undifferentiated cells, split, and then cultured as undifferentiated cells or induced to become neural with RA + Shh. Each culture was assayed for luciferase activity and total protein, and all values were calculated as light units/μg protein; for convenience only the numbers are presented in the remainder of this work. A total of 48 independent clones were assayed and results were expressed as luciferase activity normalized to protein. Thirty-nine of the 48 clones (81%) had activity above background (taken as 200). The average activity was 37,000 and the range of values was from 427 to 143,000. It has been proposed that BAC transgenes in mice derived by conventional transgene technology are expressed in an autonomous manner in which the integration site has relatively little influence [3,4]. To examine this issue in our system, clones were arrayed in rank order of their expression (Fig. 3B). Clones 17–43 (26 clones) make up 66% of the total positive clones and have expression of 15,300–73,600, a range of only 4.8-fold. Thus, about 2/3 of clones that have luciferase activity fall within a range of five of each other. We conclude that most randomly inserted BAC transgenes show a reasonably uniform level of expression but that some clones give highly variable expression. These 'outlier' clones could represent cases where the insertion site dominates the expression of the transgene. Since some BACs insert as partial deletions [18], they could also represent deletions of positive or negative regulatory elements.

Neural differentiation up-regulates expression

The same 48 transgenic clones assayed as undifferentiated ES cells were differentiated with the RA + Shh protocol for four days and assayed for luciferase activity (Fig. 3B; lower panel). The nine clones that had been negative as undifferentiated cells remained negative. Of the 39 clones positive as ES cells, 25/39 or 64% showed significant induction (2× or more). The average of positive clones was 220,545; this is equal to 5.9-fold the population average of undifferentiated cells. Sister cultures of the differentiated cells were cultured for an additional four days (total of eight days differentiation) (Fig. 3A). The average of all clones increased to 1401, 325 or a 6.4× increase over the 4-day differentiation. In summary, eight days of neural induction produce a 38-fold increase in activity averaged across all positive clones. We conclude that the inserted BAC contains at least some of the regulatory elements for turning on the expression of the Olig2 gene in neural cells.

Deletion of basal promoter

We have shown that there is a minimal promoter immediately upstream of the Olig2 cap site (Xian and Gottlieb, unpublished results). To determine if this promoter is functional in BAC transgenes, *Olig2.luc.neo. Δ1* was tested in the same manner as *Olig2.luc.neo*. Forty-eight independent neo-resistant clones were selected, expanded, and analyzed for luciferase activity in undifferentiated ES cells and neural cells (Fig. 3C). In the undifferentiated cells only 22% of the clones had

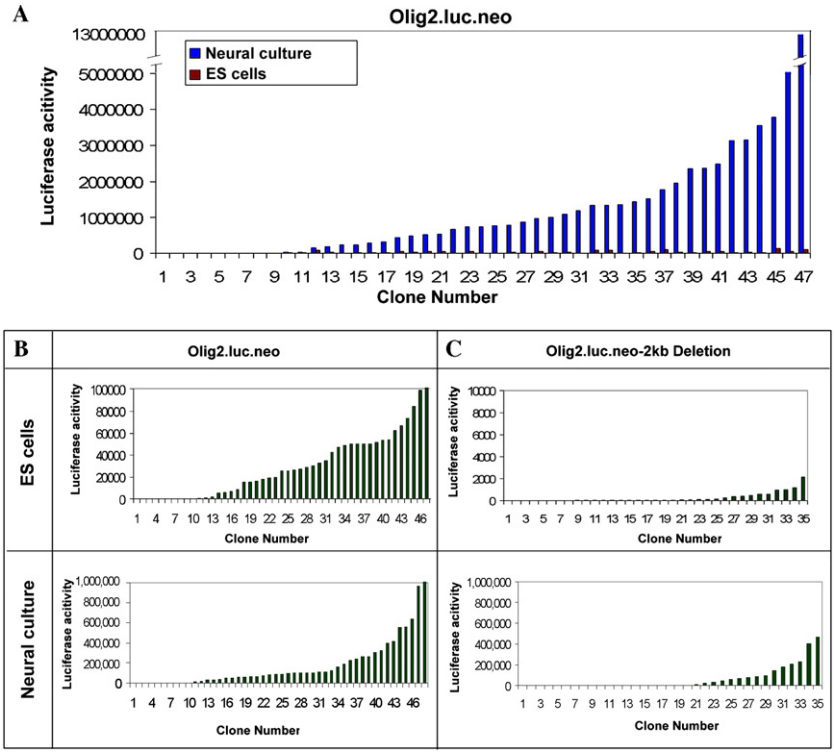

Fig. 3. Gene expression in transgenic clones. (A) Eight day neural differentiation induces luciferase expression in *Olig2.luc.neo* transgenes: all 48 clones were analyzed for luciferase expression in neural cultures differentiated for eight days (blue bars). Clones are presented in rank order for neural cultures. Matching red bars show activity in the same clone at the undifferentiated ES cell stage (not in rank order). Note the large increase in expression after differentiation. (B) Expression at the ES cell stage is presented in rank order for the 48 clones (upper panel) and after four day differentiation (lower panel); note, difference in the scale of *y*-axis. (C) Deletion in *Olig2.luc.neo. Δ1* abolishes expression in ES cells but not neural cells. Clones are arrayed in rank order of expression for ES cells (upper panel) and neural cells (lower panel). Note the low values on the *y*-axis in the upper panel. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

measurable activity but that was extremely low. The average activity was only 209 compared to 31,000 with *Olig2.luc.neo* (Fig. 3B). We conclude that deleting the proximal 2 kb portion of the gene greatly lowers expression in ES cells. Sister cells of the same clones were neurally differentiated and a total of 35 differentiated clones were assayed. Surprisingly, 19/35 or 54% of clones had significant activity, with an average activity of 61,082. While lower than the intact BAC at the same stage of differentiation, this is still considerable. An interesting possibility is that there is an upstream promoter that it is not active at the undifferentiated ES cell stage but is so in neural cells. Experiments to define this promoter by 5′RACE extension are underway.

Discussion

Here we present a new system for analyzing the expression of genes involved in the pathway from totipotent to ventral neural cells. This system allows us to test large regions of DNA (~200 kb) for the presence of *cis*-acting sequences that promote expression of the Olig2 genes. Three conclusions may be drawn from the results.

Our first conclusion is that the genomic region included in the BAC illustrated in Fig. 2 contains elements that are sufficient to mediate regulated expression of the Olig2 gene. This is best illustrated in Fig. 3A where expression is measured in a set of transgenic clones at the undifferentiated ES cell stage and in induced neural cultures. Each clone that expresses at all shows a large increase of expression in the neural vs. undifferentiated cell stages. The most reasonable conclusion is that at least some of the elements needed for regulated expression are present in the transfected BAC. There are several important limitations to the analysis. First it is not established how much of the BAC has inserted in any particular clone. All clones contain the neo gene and luciferase, which are separated by 50 kb. While most BACs insert intact [18], a subset of clones may contain BACs with endpoint deletions. Some deletions might give higher expression while others may give lower expression compared to the intact BAC. In future experiments, we plan to place a second selectable marker on the 3′ end of the BAC and perform dual drug selection. In this way, all clones will be guaranteed to contain a much longer expanse of DNA. A second limitation of the present assays is that it may be distorted by cellular

heterogeneity. The RA + Shh induced cultures are not uniformly Olig2 expressing at the cellular level so the population assays used here may underestimate the degree of induction on a cellular basis. We are exploring methods to measure expression at the cellular level. In spite of these current limitations the data in Fig. 3A clearly support the idea that functional and cell specific *cis*-acting sequences are located on the BAC.

It has been claimed that BACs for some genes express in an integration site-independent manner [3,4]. Our data open the possibility that this is not strictly true, at least for BAC transgenes in ES cells. There are a wide range of expression levels among expressing clones and the most likely explanation is that expression is under the influence of the chromosomal region of the insertion. Having noted the variation it should be said that a fairly large number of clones fall within a factor of 10 of each other. While we have not done a head-to-head comparison with a 'mini-gene' transgene, we suspect that the variation with the BAC is substantially less. In future studies, we intend to see if flanking insulator sequences cloned into the insert will diminish the variation.

Finally, the results give insights into the function of the 2 kb upstream of the cap site that was deleted. The deletion greatly lowers the expression in ES cells but leaves substantial expression in neural cells. The most likely explanation is that there is a second promoter upstream of the deletion that is active in neural but not totipotent cells. We are currently attempting to locate this promoter by RACE mapping the ends of transcripts from positive clones.

Although transgenic mice are being used to analyze *cis*-acting elements on neural genes, there remains a strong need for cellular model systems that would provide a more high-throughput approach. We present a system based on ES cell-derived neural cells (ESNCs) that is suitable for analyzing *cis*-regulatory elements of neural expressed genes and have used it to show that the Olig2 gene has a basal promoter near the cap site. The system should be applicable to a wide variety of genes since neural stem cells, multiple types of neurons, and glial cells all appear in ESNC cultures. This system should allow the study of large numbers of genes, a project that would be greatly constrained in mice due to costs. It will also allow sequential rounds of deletions to localize the actual *cis*-acting sequences and prove their role by mutagenesis. Unlike transient transfection, this system is based on integrated DNA and will doubtlessly reveal elements essential for expression in the context of chromatin. An additional consideration is that it is practical to generate about 10^7 ESNCs; thus, the system will support biochemical studies of the relevant transcriptional machinery. For these multiple reasons this system should provide a very useful complement to research on gene regulation using intact mice. Finally, human ES cells readily give rise to ESNCs [20,21] making parallel analysis of human neural genes in human cells feasible.

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