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# Embryonic Stem Cells Express Neuronal Properties *in Vitro*

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**Mouse embryonic stem (ES) cells cultured as aggregates and exposed to retinoic acid are induced to express multiple phenotypes normally associated with neurons. A large percentage of treated aggregates produce a rich neuritic outgrowth. Dissociating the induced aggregates with trypsin and plating the cells as a monolayer results in cultures in which a sizable percentage of the cells have a neuronal appearance. These neuron-like cells express class III β-tubulin and the neurofilament M subunit. Induced cultures express transcripts for neural-associated genes including the neurofilament L subunit, glutamate receptor subunits, the transcription factor Brn-3, and GFAP. Levels of neurofilament L and $GAD_{67}$ and $GAD_{65}$ transcripts rise dramatically upon induction. Physiological studies show that the neuron-like cells generate action potentials and express TTX-sensitive sodium channels, as well as voltage-gated potassium channels and calcium channels. We conclude that a complex system of neuronal gene expression can be activated in cultured ES cells. This system should be favorable for investigating some of the mechanisms that regulate neuronal differentiation.** 

## INTRODUCTION

Embryonic stem (ES) cell lines are clonal cell lines derived from the preimplantation mouse embryo (Evans and Kaufman, 1981; Martin, 1981). ES cells have two properties which, in combination, make them a powerful system for the analysis of mammalian development. First, when injected into the inner cell mass of a host blastocyst, ES cells integrate into the host embryo and populate all cell lineages including the germ line (Bradley *et al.*, 1984; Gossler *et al.*, 1989). In the resulting animal, differentiated tissues are made up of mixtures of host and ES-derived cells. This property of ES cells is termed totipotency. Second, ES cells are able to replicate, without apparent limit, in culture. Even after 250 replications in culture, ES cells remain totipotent (Suda *et al.*, 1987). Because ES cells divide in culture without losing their totipotency, they are ideally suited for cell genetic manipulations. Foreign DNA can be introduced into the ES cell genome either through homologous recombination or random insertion (Thomas and Capecchi, 1987; Friedrich and Soriano, 1991; Skarnes *et al.*, 1992).

ES cells grafted into the inner cell mass of the embryo contribute cells to all parts of the developing nervous system (Gossler *et al.*, 1989). The details of when and how the implanted ES cells join the developing nervous system have not been directly studied. It is assumed that the implanted ES cells go through the same series of developmental stages as normal early embryonic cells. In normal development, the inner cell mass gives rise to the primitive ectoderm, the cell layer from which all cells of the embryo are derived. Through the process of gastrulation, the primitive ectoderm gives rise to the ectoderm, mesoderm, and endoderm. The midline ectoderm, probably under the influence of underlying mesoderm, becomes neuroectoderm, which gives rise to the nervous system (reviewed in Beddington and Smith, 1993; Ruiz i Altaba, 1994). Understanding the mechanisms which regulate the major steps along this pathway is the goal of much current work. One fruitful approach has been to inactivate candidate regulatory genes by homologous recombination in ES cells and observe the effect on neuronal development. Such studies have shown that the Wnt-1 (McMahon and Bradley, 1990; Thomas and Cappechi, 1990) Hox 1.6 (Lufkin *et al.*, 1991; Chisaka *et al.*, 1992; Mark *et al.*, 1993; Carpenter *et al.*, 1993), En-2 (Joyner *et al.*, 1991), and MASH-1 (Guillemot *et al.*, 1993) genes play crucial roles in the early development of the nervous system.

To better define the mechanisms by which these and other regulatory genes work, it would be advantageous to have a system in which the pathway from ES cells to neuronal cells could be reconstituted and studied *in vitro*. Many basic questions about developing neuronal cells cannot be answered with current methods. An *in*

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*vitro* system based on ES cells would have several advantages in addition to the genetic attributes of ES cells already cited. First, large numbers of cells at important steps along the pathway could be isolated. This would allow biochemical analyses which are not possible with the limited number of cells found in the normal embryo to be carried out. Second, the sequential steps of the pathway could be studied in the simplified environment of tissue culture. Here many of the extrinsic factors influencing cells can be controlled and varied systematically. The pathway from ES cells to hematopoietic cells has been reconstituted *in vitro* and provides a concrete example of the power of this approach (Burkert *et al.*, 1991; Schmitt *et al.*, 1991; Simon *et al.*, 1992; Keller *et al.*, 1993; Weiss *et al.*, 1994).

ES cells are capable of differentiating *in vitro* into multiple cell types. To promote differentiation, ES cells are first cultured on a nonadhesive substrate where they grow as multicellular aggregates called embryoid bodies. Embryoid bodies which have been cultured for about 10 days are then plated on an adhesive substrate. Multiple cell types including skeletal muscle-, cardiac-, neuron-, and hematopoietic-like cells differentiate in the outgrowths of the embryoid bodies (Martin, 1981; Mortensen *et al.*, 1991; Sawai *et al.*, 1991; Field *et al.*, 1992; Zhuang *et al.*, 1992). Neuron-like cells are only a small percentage of the total cells and are randomly intermingled with other cell types. The rarity of the neuron-like cells is a major obstacle to meaningful study of the developmental pathway by which the original ES cells differentiate into neuron-like cells. In this paper we have investigated the feasibility of increasing the proportion of cells in ES cell cultures which choose a neuronal fate. Our approach was informed by results with the P19 teratocarcinoma cell line. P19 cells and ES cells share a number of basic features in common. Most importantly, P19 cells, like ES cells, can differentiate along multiple pathways when grafted into the early embryo (Rossant and McBurney, 1982) and *in vitro* (Jones-Villeneuve *et al.*, 1982; Edwards *et al.*, 1983). P19 cells are induced to differentiate along a neuronal pathway by treatment with retinoic acid (RA) (Jones-Villeneuve *et al.*, 1982; Bain *et al.*, 1994). Here we find that RA treatment of ES cell-derived embryoid bodies causes a high proportion of the cells to express multiple neuronal properties. The results support a number of conclusions about the regulatory systems relevant to neuronal differentiation in ES cells and their derivatives.

## MATERIALS AND METHODS

### *Cell Lines*

The D3 ES cell line (Doetschman *et al.*, 1985) was obtained from Dr. S. Korsemeyer. The CCE feeder independent ES cell line (Robertson *et al.*, 1986; Keller *et al.*, 1993) was obtained from Dr. Gordon Keller.

### *Culture Conditions*

*Culture of undifferentiated ES cells.* ES cells were grown on a gelatin-coated tissue culture plastic substrate prepared by coating tissue culture flasks with a 0.1% gelatin solution. Cells were maintained in a medium similar to that described by Robertson (1987). Standard medium consisted of DMEM (high glucose, with 1-glutamine, without pyruvate; GIBCO 11965-043) plus the following: 10% fetal bovine serum, 10% newborn calf serum, and nucleosides stock. Serum lots were not specially selected. Leukemia inhibitory factor (LIF) (GIBCO-ESGRO) at a final concentration of 1000 units/ml and $10^{-4}$ *M* $\beta$-mercaptoethanol were added to standard medium to prevent differentiation of the ES cells. A small percentage of feeder cells from the original stock were present in the D3 cultures.

*Induction of differentiation.* ES cells were subjected to an 8-day induction procedure which consisted of 4 days of culture as aggregates without RA followed by 4 days of culture in the presence of RA. To set up inductions, rapidly growing stock cultures of undifferentiated ES cells were trypsinized with 0.25% trypsin and 1 m*M* EDTA in saline to remove cells from the substratum. The detached cell suspension was triturated to yield a mixture of single cells and small clumps. Cells in this suspension could not be counted accurately. However, harsher dissociation to produce a uniform single cell suspension resulted in loss of ability to form good aggregates at the next stage and so was not done. To standardize cell input for the induction procedure, one-quarter of the cells from a single confluent T25 flask were seeded per 10 ml of medium (standard medium described above) without LIF and $\beta$-mercaptoethanol in a 100-mm-diameter bacteriological (nontissue culture) dish. Whenever a variable such as the presence of RA was to be tested, experimental and control cultures were set up using the suspension from a single flask to ensure that the starting concentration of cells was equal in all cultures. Cell suspensions were cultured for 2 days during which time floating aggregates of cells formed. The medium was changed and culture was continued for an additional 2 days. At this time, the medium was changed and, where indicated, supplemented with $5 \times 10^{-7}$ *M* all-trans retinoic acid. After 2 additional days the medium was exchanged for fresh medium with RA for treated cultures but without RA for controls and culture continued for 2 more days. After the 8-day induction period, aggregates were transferred to tissue culture wells to provide a substrate for cell attachment. About 100 aggregates were seeded into a 35-mm well with 2 ml of

standard medium lacking LIF and β-mercaptoethanol. Culture of these aggregates was continued as indicated in the text. In other experiments aggregates which had been through the 8-day induction period were dissociated with trypsin (0.25% with EDTA; 10 min) and plated on laminin-coated dishes (20 μg per 35-mm dish).

*Antibody Staining*

The monoclonal antibody TuJ-1 which recognizes class III β-tubulin, a neuron-specific isoform (Lee *et al.*, 1990; Easter *et al.*, 1993), was obtained from Dr. Anthony Frankfurter. The monoclonal antibody 3H11 which recognizes the M subunit of neurofilaments was obtained from Dr. Gerry Shaw. Cultures were fixed with 2% paraformaldehyde, permeabilized with 0.01% Triton X-100, and exposed to primary antibodies and then to biotinylated anti-mouse IgG. Bound antibody was visualized with the Vectastain ABC system following the manufacturer's directions.

*RT-PCR Assays*

To generate first-strand cDNA, 5 μg of DNase I-digested whole cell RNA from undifferentiated ES cells or from RA-treated, differentiated cells at 7 days postplating was reverse transcribed using random hexamer primers and M-MLV reverse transcriptase following the protocol provided by the enzyme supplier (Gibco BRL). In addition, mock reactions carried out in the absence of reverse transcriptase were also performed for each RNA. After phenol extraction and ethanol precipitation, each cDNA preparation was suspended in 100 μl of $H_2O$.

The oligonucleotide primers and optimized PCR reaction conditions for detection of glutamate receptor mRNAs corresponding to GluR1-4 and GluR6 have been described (Ray and Gottlieb, 1993). Note that the GluR1-4 primers can detect four distinct but closely related mRNAs, each of which encodes a separate glutamate receptor subunit. Therefore, a positive result using these primers indicates that one or more of these mRNAs is present. Oligonucleotide pairs for the detection of Brain-3 (Brn-3), tyrosine hydroxylase (TH), glial fibrillary acidic protein (GFAP), and brain factor 1 (BF-1) mRNAs were based upon published sequences (Goldsborough *et al.*, 1990; Ichikawa *et al.*, 1991; Lewis *et al.*, 1984; Tao and Lai, 1992) as follows: Brn-3, 5′ CGA TCA CGC TCT CGA ACA AC 3′, 5′CGG CGA TGG CGG CGA TCT TC 3′; TH, 5′ TGT CAG AGC AGC CCG AGG TC 3′, 5′ CCA AGA GCA GCC CAT CAA AG 3′; GFAP, 5′ TCG GAG TTG AAA GTT ACA GG 3′, 5′ AGG ATG GTT GTG GAT TCT TC 3′; BF-1, 5′ AAA ACT CGC TGG GCA ACA AC 3′, 5′ CAG GGG TTG AGG GAG TAG GT 3′.

PCR reactions for the detection of GluR1-4 and GluR6 were performed as described (Ray and Gottlieb, 1993), except that the annealing temperature for GluR6 was increased to 55°C. For Brn-3, TH, GFAP, and BF-1, the reactions were carried out using *Taq* DNA polymerase in standard PCR buffer provided by the enzyme manufacturer (Gibco BRL) containing 1.5 m*M* $MgCl_2$. Oligonucleotide primers were present at a concentration of 1 μ*M*. Either 1 or 5 μl of template was used in each reaction. Thirty-five cycles of amplification were performed using a denaturation temperature of 94°C for 30 sec, annealing at 50°C for 30 sec, and primer extension at 72°C for 1 min. One-third of each reaction was loaded onto a 1.5% agarose gel and PCR products were detected by ethidium bromide fluorescence following electrophoresis.

*RNase Protection Assays*

Whole cell RNA was isolated from undifferentiated ES cells or from differentiated cells at 5–7 days postplating as described (Bain and Gottlieb, 1994). RNase protection assays were performed as described (Bain *et al.*, 1993) using 50 μg of whole cell RNA and $5 \times 10^5$ cpm of radiolabeled antisense RNA probe. Probes for $GAD_{67}$ and $GAD_{65}$ have been described (Bain *et al.*, 1993). A 350-bp cDNA probe corresponding to the neurofilament light chain of mouse (Julien *et al.*, 1986) was generated by PCR using the oligonucleotides 5′ GCTGCCAAGGATGAGTCTGA 3′ and 5′ AGCAAGCCAGAAAGCCACTC 3′. This cDNA was inserted into the pBS II SK+ vector (Stratagene) by standard techniques and sequenced to confirm its identity.

*Electrophysiology*

ES cell cultures were perfused at a rate of 1 to 2 ml per min with Tyrode's solution: 150 m*M* NaCl, 4 m*M* KCl, 2 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, 10 m*M* glucose, and 10 m*M* HEPES, adjusted to pH 7.4 with NaOH. Pipettes for whole cell recording were pulled from boralex capillaries and coated with sylgard. The internal solution used to record Na and K currents contained 140 m*M* $KCH_3SO_3$, or 140 m*M* KF, 2.3 m*M* $MgCl_2$, 10 m*M* HEPES, adjusted to pH 7.2 with KOH, 480 μ*M* K-EGTA, and 200 μ*M* Ca-EGTA, prepared as described by Tsien and Pozzan (1989) to give ~70 n*M* free Ca. The open tip resistance ranged from 1 to 5 MOhm. Drug solutions were applied to the cells using an array of microcapillary tubes. The time constant for exchange of the external solution was approximately 30 to 50 msec. Excitatory and inhibitory agonists were dissolved in 160 m*M* NaCl, 2 m*M* $CaCl_2$, 0.5 μ*M* tetrodotoxin (TTX, Sigma), and 10 m*M* HEPES, adjusted to pH 7.4 with NaOH.

Voltage-gated calcium channel current was recorded using barium as the charge carrier (see Bean, 1992). The external solution was 5 m*M* $BaCl_2$, 150 m*M* tetraethyl-

ammonium (TEA) chloride, 2 m*M* $MgCl_2$, 0.1 m*M* EGTA, 1 $\mu M$ TTX, and 10 m*M* HEPES, adjusted to pH 7.4 with TEA hydroxide. For most experiments, pipettes were filled with 100 m*M* CsCl, 10 m*M* EGTA, 1 m*M* ATP (Mg salt), 0.3 m*M* GTP (tris salt), and 40 m*M* HEPES, adjusted to pH 7.4 with CsOH. Similar results were obtained using pipettes filled with 140 m*M* $CsCH_3SO_3$, or CsF, 5 m*M* CsCl, 10 m*M* EGTA, and 10 m*M* HEPES. Current was recorded with an Axopatch 200 amplifier, filtered at 1 to 5 KHz (−3 db, 4 pole Bessel), and digitized at 10 to 20 KHz. Current traces were corrected for leak and capacity using scaled current evoked by an 8- to 10-mV hyperpolarizing step from the holding potential. Partial compensation for series resistance was employed in most experiments. Cells were rejected from quantitative analysis if the voltage error due to residual series resistance was greater than 5 mV, if there was an obvious slow component to the capacity transient, or if there was a sharp "threshold" for activation of inward Na or Ba current. Membrane potentials have been corrected for the junction potential between the internal solution and the Tyrode's solution in which seals were formed. This potential was −10 mV for pipettes containing $CsCH_3SO_3$ while for CsF and CsCl solutions the junction potential was −7 mV. Currents recorded during agonist applications were compressed for storage and analysis by averaging 3 msec of data at 0.1- to 1-sec intervals. Unless otherwise noted, results are expressed as means ± standard error. All experiments were performed at room temperature.

## RESULTS

As mentioned above, inducing ES cells to differentiate by forming embryoid bodies results in heterogeneous cultures in which neuron-like cells comprise only a minor percentage of cells. In trying to develop inducing conditions for neurons we were informed by results with the P19 teratocarcinoma cell line. Simply growing ES cells as aggregates in the presence of RA for 4 days did not result in substantial neuronal induction as it does for P19 cells. However, a variant of this procedure was successful.

### *RA Stimulates the Appearance of Cells with Neuron-like Morphology in ES Cultures*

Stock undifferentiated D3 ES cells were grown on a gelatin substrate in the presence of LIF and $\beta$-mercaptoethanol, conditions which are known to suppress differentiation (Williams *et al.*, 1988). To ensure that experiments were done on genetically normal and developmentally totipotent ES cells, an isolate of the D3 line which had been used to create chimeric mice with germ line transmission was used (Veis *et al.*, 1993; Roberts *et al.*, 1994). To ensure that cells were still totipotent when inductions were done, inductions were performed within 10 passages of thawing the canonical stocks.

Undifferentiated ES cells were cultured in nonadhesive dishes in standard medium. Under these conditions the ES cells do not attach to the dish surface and readily form floating aggregates. During the first 2 days, aggregates are small with irregular outlines. By Day 4, aggregates are larger and are mostly spherical; cells visible at the surface of the aggregates appear healthy. The most successful neuronal inductions were obtained by culturing these 4-day aggregates with $5 \times 10^{-7}$ *M* all-trans RA for 4 additional days. The concentration of RA was chosen on the basis of results with P19 cells (Jones-Villeneuve *et al.*, 1982); the effect of other concentrations was not investigated. This protocol is referred to as 4−/4+ treatment. For controls, 4-day aggregates were cultured for 4 additional days in the absence of RA; these were referred to as 4−/4− aggregates.

Induced cells from either the 4−/4+ or 4−/4− protocols were plated as intact aggregates on tissue culture substratum in standard medium lacking RA. Within a day, most aggregates attach to the surface and are surrounded by a thin ring of flat cells which adhere tightly to the surface. The 4−/4+ and 4−/4− aggregates are indistinguishable in appearance. Over the next several days the rings of flat cells surrounding the aggregates enlarge and many aggregates begin to flatten and spread on the bottom of the culture dish. By 3 days postplating, there is a noticeable difference between 4−/4+ and 4−/4− aggregates. Many of the 4−/4+ aggregates give off extensive outgrowths of neurites. In contrast, far fewer 4−/4− aggregates have neuritic outgrowths. Neuritic outgrowths become more complex over the next 3 days and the difference between the 4−/4+ and 4−/4− aggregates remains dramatic. Figure 1 shows a typical 4−/4+ aggregate 6 days after plating. Neurite outgrowth occurs at many points along the perimeter of the aggregate. Higher power pictures show that the neuritic outgrowth is very extensive, forming a dense, tangled collection of neurites. It also shows that many cells with a neuronal appearance are found in these outgrowths. To document that RA treatment increases the extent of neurite outgrowth, counts were made of the number of aggregates with and without neurite outgrowth (Table 1). These observations show that, for the D3 cell line, 7.8 times as many of the 4−/4+ aggregates had neurite outgrowths when compared to 4−/4− aggregates. Similar results were obtained with the CCE feeder independent ES cell line (see below). Here 6.8-fold as many of the 4−/4+ aggregates had neurite outgrowths as compared to the 4−/4− ones.

The above results strongly support the conclusion that ES cells can be induced to express a neuronal mor-

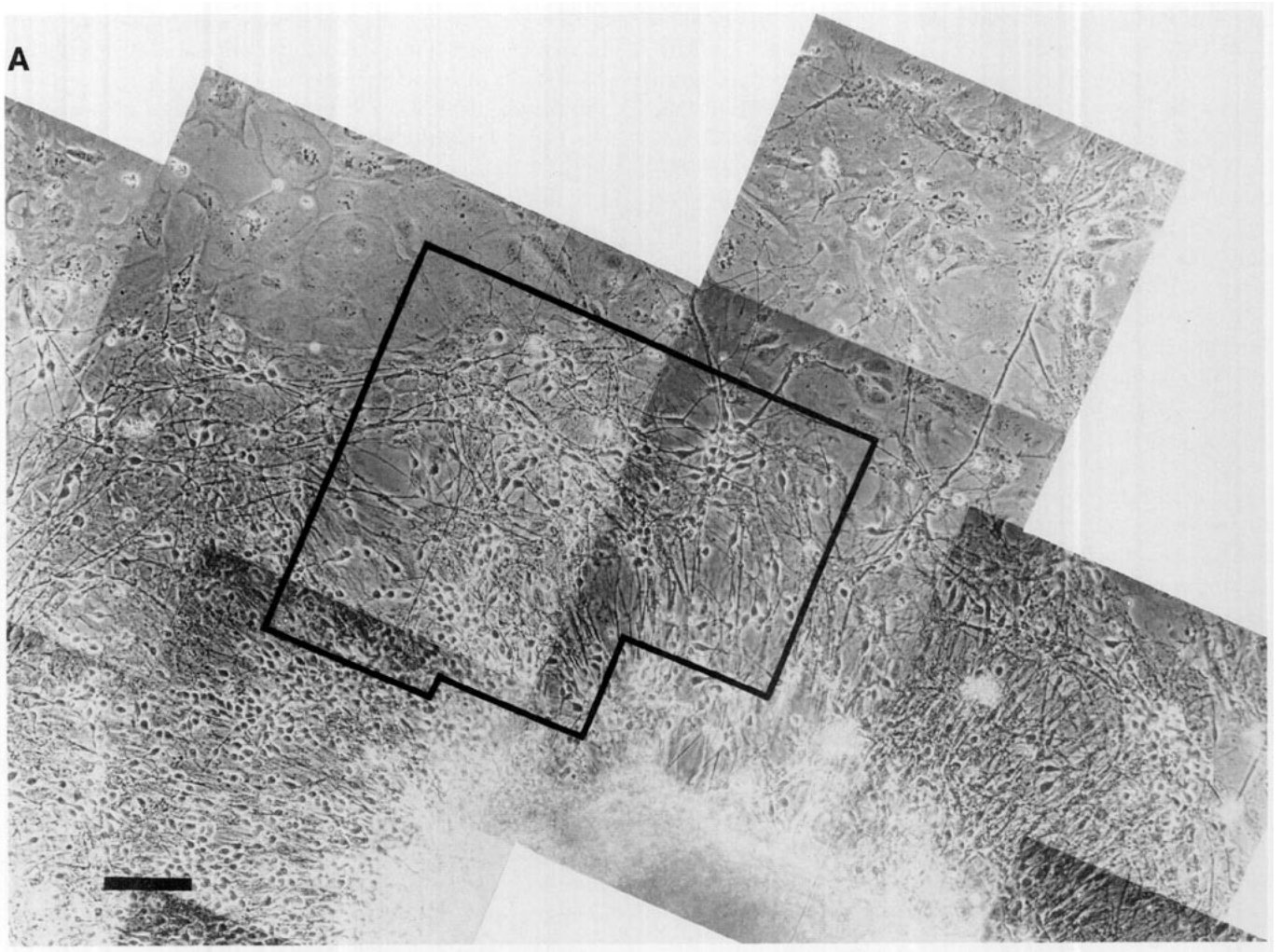



FIG. 1. Extensive neuritic outgrowth from RA-treated aggregates. D3 ES cells were induced by the 4−/4+ protocol and then plated on a tissue culture substrate. A living culture was photographed with phase-contrast optics 6 days after plating. (A) The halo of neurites from a single aggregate is displayed. The aggregate is just visible at the bottom. Emerging from the aggregate are multiple neurites and neuronal cells. Bar, 100 μm. (B) The area delineated by the box in A is shown at higher magnification. Note the dense profusion of neuritic processes. Bar, 50 μm.

phology *in vitro*. However, they are subject to two concerns. The original D3 stocks we utilized contained feeder cells which had been irradiated to prevent cell division. The numbers of these cells decreased dramatically with passage. However, a small number of what appeared to be feeder cells were present in all of the stock cultures used in these experiments. Although very unlikely, it is possible that this minority of feeder cells made a contribution to the results of our experiments. A second concern was that these results might be valid for D3 cells but not for other ES cell lines. We addressed both concerns by repeating the experiments with a feeder independent subline of the CCE line of ES cells. This subline has the capability to differentiate into cells of the hematopoietic lineage (Keller *et al.*, 1993) and therefore has extensive developmental potential. However, it has not been demonstrated to form germ line chimeras and therefore might not be totipotent. As described above, the CCE cells responded in the same way as D3 cells to induction with the 4−/4+ protocol (see Table 1). Since no feeder cells were present, this observation shows that feeder cells are not necessary for the response to RA. They also show that RA stimulates neurite formation in a second ES cell line.

Results from plating intact aggregates show that there is extensive neuronal differentiation in 4−/4+ aggregates after they are plated on a permissive substrate. In order to gain a more detailed picture of the induced cells we performed experiments in which the aggregates were trypsinized prior to plating. Preliminary experiments (data not shown) showed that coating the substrate with laminin gave improved cultures. When 4−/4+ aggregates are trypsinized and plated, the majority of cells quickly adhere to the laminin-coated substrate.

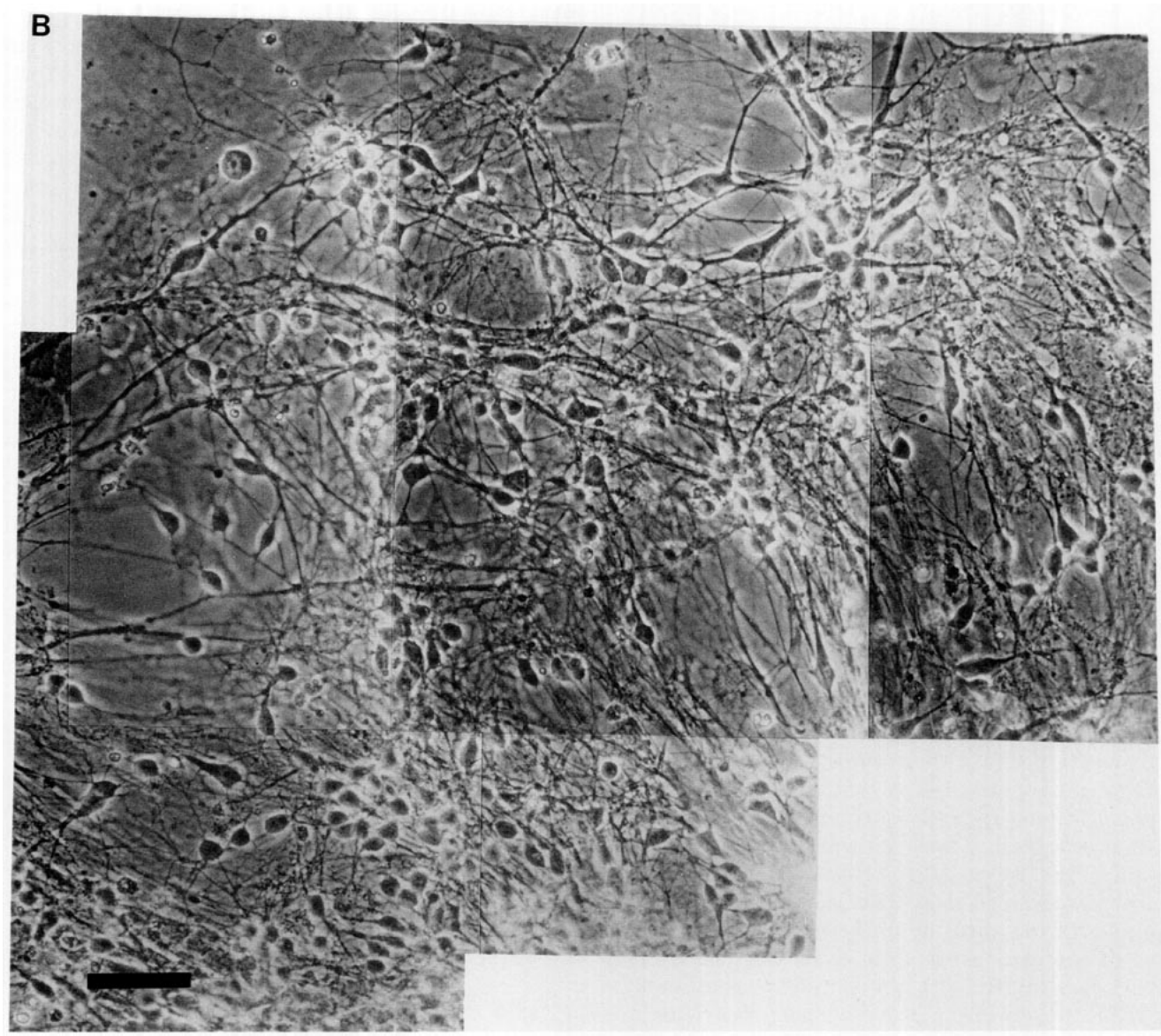



FIG. 1—Continued

By 2 days the culture consists of a population of flat cells adhering tightly to the substrate and a population of neuron-like cells. The neuron-like cells have small cell bodies with several long neurites emerging from them. Most of the neuron-like cells lie on top of the flat cells. Counts of the number of neuron-like cells relative to flat cells in two experiments show that the neuron-like cells comprise about 38% of the population on Day 2 (Table 2). In contrast 4−/4− cultures show almost no neurons. Over the next 3 days the flat cells divide to form a confluent monolayer of cells. The neuron-like cells remain on top of this layer. These cells have large cell bodies and form an extensive network of neurites (Fig. 2). Cultures from 4−/4− aggregates consist mostly of flat cells with only a very few neurons (Fig. 2). These experiments with dispersed cell cultures confirm that RA has a strong action in inducing cells with a neuronal appearance in ES cell aggregates. They also demonstrate that after induction with RA a very substantial fraction of the cells can differentiate along the neuronal path.

### *Expression of Neuronal Proteins by Neuron-like Cells*

In order to determine if the neuron-like cells and processes contain neuron-associated proteins, cultures were stained with antibodies to class III β-tubulin (TuJ-1) and NF-M (3H11). Expression of these proteins is highly restricted to neurons (Lee *et al.*, 1990; Harris *et*

TABLE 1
RETINOIC ACID STIMULATES NEURITE FORMATION BY AGGREGATES OF ES CELLS

| Experiment | 4−/4+ | 4−/4− |
|---|---|---|
| 1 | 87 ± 9 (72) | 20 ± 10 (69) |
| 2 | 90 ± 4.3 (98) | 9 ± 3 (97) |
| | Mean = 88.5 | Mean = 14.5 |
| 3 | 66 ± 5.6 (55) | 7 ± 7.5 (54) |
| 4 | 82 ± 3.2 (88) | 12 ± 3.2 (81) |
| | Mean = 74 | Mean = 9.5 |

*Note.* Experiments 1 and 2 were performed with the feeder independent CCE line of ES cells. Experiments 3 and 4 were performed with D3 ES cells. In each experiment 4−/4+ or 4−/4− aggregates were plated in 35-mm tissue culture plastic wells and allowed to grow for 3–5 days before scoring. Triplicate wells for each experiment are scored. Scoring was done by systematically traversing the wells and scoring each aggregate. Occasionally several aggregates were in apposition; these were not scored to avoid overestimating those with neurites. Aggregates were scored positive if neurites were visible. The percentages of aggregates that are positive along with the standard deviation are given. Total aggregates scored in treated and control wells are given in parentheses.

*al.*, 1991). Aggregates of the CCE ES cell line were induced by the 4−/4+ procedure and then plated as intact aggregates. Seven days after plating, the cultures were fixed and stained (Fig. 3). In some areas of the cultures aggregates had flattened so that neuron-like cells occurred either singly or in small clumps. Most of the neuron-like cells stained with the antibody to class III β-tubulin (Figs. 3A and 3B). Neurites emerging from clumps also stained (Fig. 3E). Many single cells also stained with the antibody to NF-M (Figs. 3C and 3D). Some of the neuron-like cells either did not stain or stained very faintly with this antibody. This could be due either to expression of NF-M being restricted to a subset of the neuron-like cells or to lower sensitivity of the antibody. Neurites emerging from clumps stained intensely with antibody to NF-M (Fig. 3F). Staining with both antibodies is highly specific as judged by the absence of staining of the background cells and by absence of staining with normal mouse serum (data not shown). Identical results have been obtained with cultures derived from the D3 line (data not shown). We conclude that, in this system, cells and neurites which appear neuronal also express two proteins usually associated with neurons.

### *Expression of Neural Genes in Differentiated ES Cultures*

In order to characterize this pathway further, we examined the expression of neural genes in ES cells before and after differentiation. In the first set of experiments, RNA isolated from undifferentiated ES cells and from 4−/4+ aggregates which had been plated and maintained in culture for 7 days was analyzed by RT-PCR (Fig. 4). Expression of the transcription factor Brn-3 which is selectively expressed in the CNS (He *et al.*, 1989) was detected in both undifferentiated and differentiated cells. In contrast, transcripts for tyrosine hydroxylase, glutamate receptor subunits of the GluR1-4 and GluR6 classes, and GFAP were detected only in the differentiated ES cells. While our PCR assays were not quantitative, the results suggest that the expression of at least some neural genes increases following differentiation *in vitro*. Interestingly, transcripts of the transcription factor BF-1, which is expressed selectively in anterior regions of the nervous system (Tao and Lai, 1992), were not detected in either undifferentiated or differentiated cells suggesting that some neuronal genes are not induced under these conditions.

In order to study expression of neuronal genes during the *in vitro* differentiation of ES cells in more detail, levels of transcripts of the neurofilament light chain (NF-L) and $GAD_{67}$ and $GAD_{65}$ genes were measured with ribonuclease protection assays before and after induction of differentiation. NF-L is an intermediate filament expressed mostly in neurons while GAD is the enzyme which synthesizes γ-aminobutyric acid (GABA) and is localized to inhibitory neurons. There is a substantial amount of NF-L expression even in undifferentiated ES cells (Fig. 5). Expression of other neuronal genes by undifferentiated ES cells has been observed

TABLE 2
NEURONAL DIFFERENTIATION IN DISPERSED CULTURES FOLLOWING RETINOIC ACID TREATMENT

| Experiment | 4−/4+ % neuron-like (total counted) | 4−/4− % neuron-like (total counted) |
|---|---|---|
| 1 | 37.2 (483) | 0 (358) |
| | 40.7 (505) | 0 (289) |
| | 41.4 (760) | 0.4 (266) |
| | Mean = 39.7 ± 2.2 | 0.13 ± .23 |
| 2 | 37.6 (1142) | 0 (391) |
| | 36.8 (709) | 0.2 (347) |
| | 40.1 (776) | 0 (319) |
| | Mean = 38.1 ± 1.7 | 0.06 ± .11 |

*Note.* Cells were induced by the 4−/4+ or 4−/4− procedure. After induction they were trypsinized and the cell suspension was plated on a laminin substrate. Two days after plating the cells were scored. A 40× objective was used in all experiments. Usually fields were chosen sequentially. Occasionally there were clumps of cells or bare spots which made scoring impossible. These fields were bypassed. Cells were scored as either flat cells or neuron-like cells. Each line is the result of counting 10 fields. The percentage of neuron-like cells is given as well as the total cells counted.

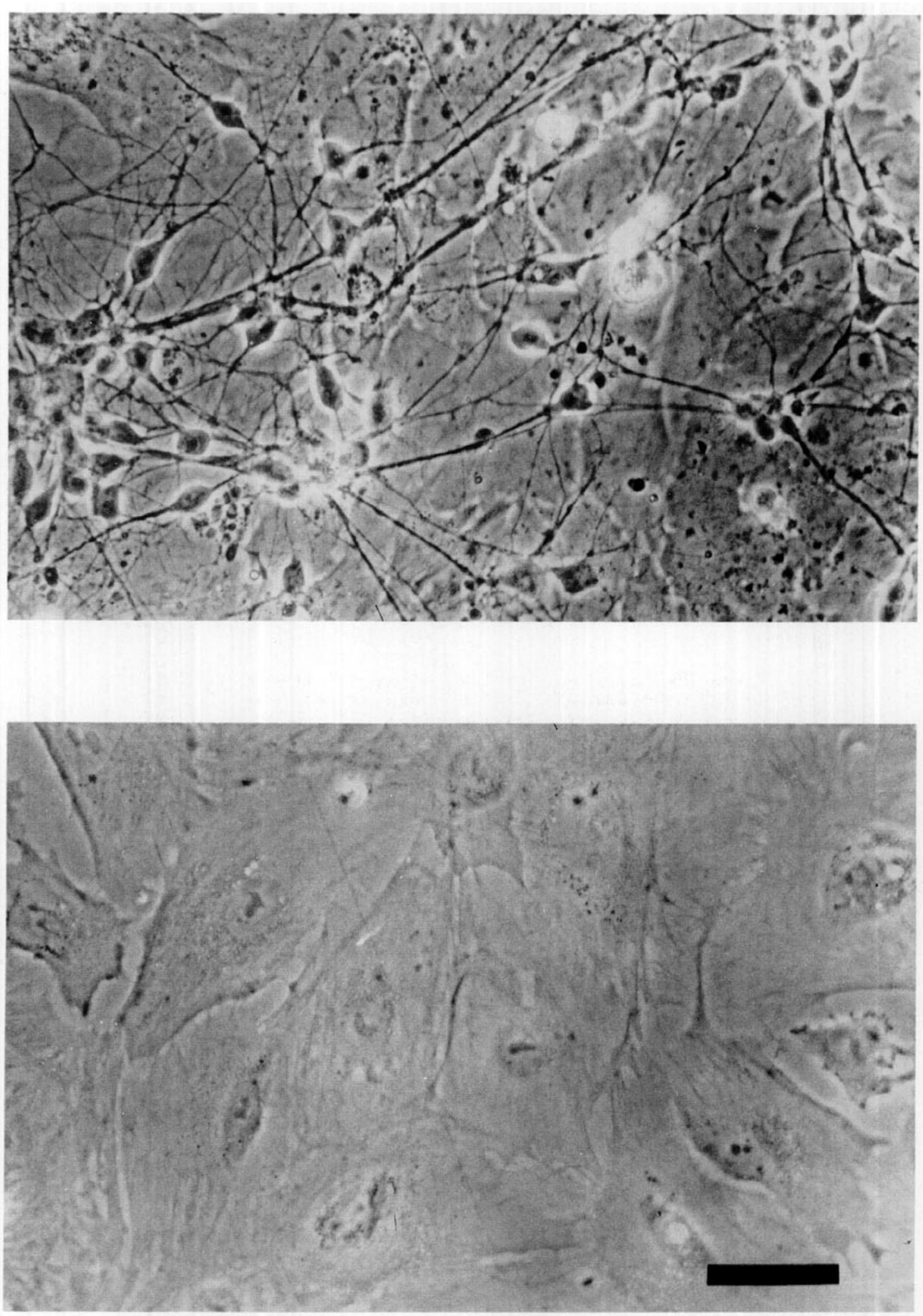
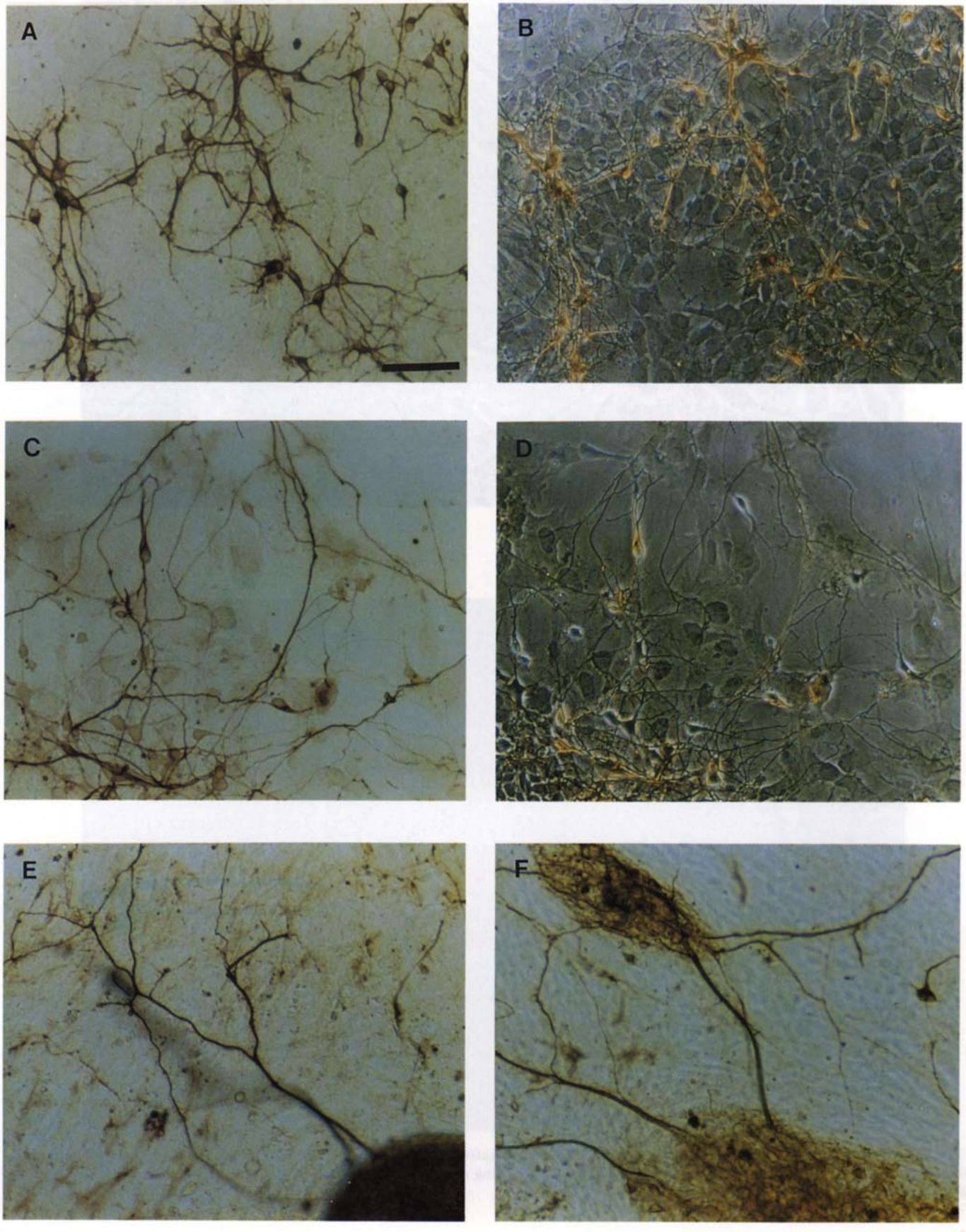


FIG. 2. Dispersed cell cultures derived from 4−/4+ and 4−/4− aggregates. D3 cells were induced by either the 4−/4+ or the 4−/4− protocol, dissociated with trypsin, and plated on laminin. Five days after plating cultures were photographed. Top, culture from 4−/4+ aggregates. Bottom, culture from 4−/4− aggregates. Bar, 50 μm.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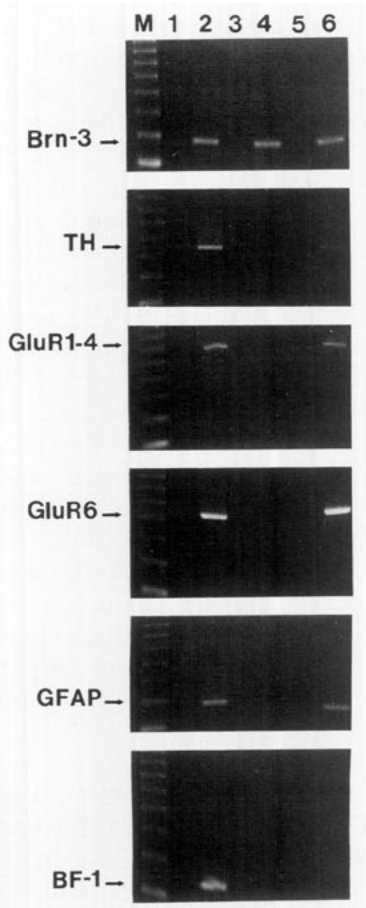


FIG. 4. PCR assays of gene expression. The expression of several neuronal genes in undifferentiated ES cells and in RA-treated, differentiated cells was assessed by RT-PCR assays. A negative control (distilled water added as template; Lane 1) and positive control (adult mouse brain cDNA added as template; Lane 2) were included. In addition, mock reverse-transcribed RNA prepared from undifferentiated ES cells (Lane 3) and differentiated cells (Lane 5) was also amplified. Finally, cDNA from undifferentiated (Lane 4) and differentiated (Lane 6) ES cells was amplified. Molecular weight markers (123-bp ladder) covering the range of 123 to 984 bp are in Lane M. The sizes of the correct PCR products are Brn-3 (228 bp); TH (412 bp); GluR1-4 (749 bp); GluR6 (577 bp); GFAP (234 bp); BF-1 (175 bp).

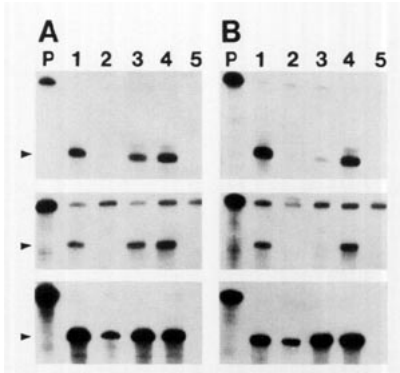


FIG. 5. Ribonuclease protection assays. The expression of $GAD_{67}$ (upper panels), $GAD_{65}$ (middle panels), and neurofilament light chain (lower panels) RNAs was measured using RNase protection assays. (A) Aggregate cultures. As a positive control, whole cell RNA prepared from adult mouse brain (5 $\mu$g) was assayed (Lane 1). Whole cell RNA (50 $\mu$g) from undifferentiated ES cells (Lane 2), cells differentiated in the absence of retinoic acid at 7 days postplating (4−/4− cells plated as aggregates) (Lane 3), and retinoic acid-treated, differentiated cells at 7 days postplating (4−/4+ cells plated as aggregates) (Lane 4) was assayed. To control for nonspecific hybridization, yeast tRNA (50 $\mu$g) was also tested (Lane 5). The radiolabeled, undigested probes are also shown (Lane P). (B) Dispersed cultures. Lanes are the same as for part A except for Lane 3 (50 $\mu$g RNA from 4−/4− cells dispersed and plated on laminin) and Lane 4 (50 $\mu$g RNA from 4−/4+ cells dispersed and plated on laminin).

(Alouani, 1993; Suzuki *et al.*, 1990). Levels of all three transcripts are strongly increased by our induction conditions. To our surprise, the levels of all three are upregulated regardless of whether RA is present in the induction as long as the cells are plated as aggregates after induction (Fig. 5A). If cells are dispersed after induction only the cultures from RA-treated (4−/4+) aggregates express the GAD genes (Fig. 5B). Remarkably, expression of the NF-L gene is increased in dispersed cultures from both 4−/4+ and 4−/4− inductions even though the latter have almost no neuron-like cells. The results demonstrate a complex system of regulation in these cells.

### *Electrophysiological Properties of Differentiated ES Cells*

The electrophysiological properties of ES cells were studied in dispersed cultures prepared from 4−/4+ aggregates that were dissociated with trypsin and plated on a laminin-coated substrate. Whole cell recordings were obtained from 45 cells between 1 and 11 days after plating. Throughout this period, cells with neuron-like morphology had relatively low resting conductance at holding potentials from −70 to −100 mV. Input resistance was highest during the first 5 to 6 days ($R_{in}$ = 2.4 ± 0.4 GOhm; cell capacitance = 9.0 ± 0.5 pF, $n$ = 24). More mature cells had somewhat higher capacitance and

FIG. 3. Neuron-like cells express class III $\beta$-tubulin and NF-M proteins. ES cells of the CCE line were induced by the 4−/4+ protocol and plated as intact aggregates on a tissue culture substratum. Seven days after plating, the cultures were fixed and stained with antibodies. (A and B) Isolated neurons stained with antibody to class III $\beta$-tubulin in brightfield (A) and phase (B). (C and D) Isolated neurons stained with antibody to NF-M in brightfield (C) and phase (D). (E) Neurites emerging from an aggregate stained with antibody to class III $\beta$-tubulin. (F) Neurites emerging from an aggregate stained with antibody to NF-M. Bar in A, 100 $\mu$m. All other panels are at the same magnification.

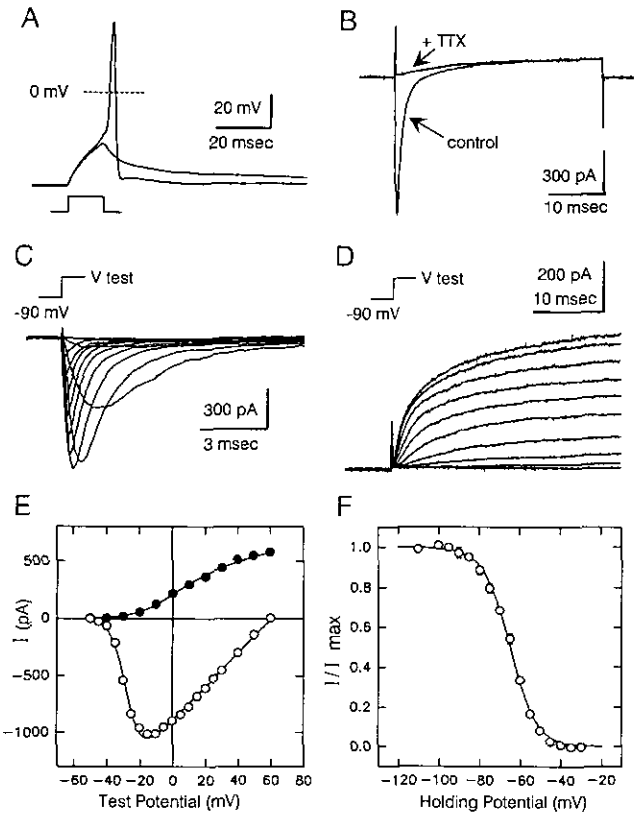


FIG. 6. Current- and voltage-clamp recordings from neuron-like differentiated cells. ES cells were induced by the 4−/4+ protocol and plated on laminin substrate. (A) Depolarizations induced by a 12-msec current step of 30 pA. Two traces are shown superimposed. In one trace, the cell fired an action potential. Internal solution, $KCH_3SO_3$, 10 days after plating. (B) Inward and outward currents recorded in a different cell under voltage clamp. Inward current at the beginning of the voltage-step was completely blocked by 1 $\mu M$ TTX. Internal solution, $KCH_3SO_3$; holding potential, −90 mV, 6 days after plating. (C) TTX-sensitive current recorded for test potentials from −50 to +50 mV. (D) Outward current recorded in the presence of 1 $\mu M$ TTX over the same range of test potentials. Traces shown in B–D are averages of five individual sweeps corrected for leak and capacity. (E) Plots of peak inward (○) and outward (●) current versus test potential, from the experiment shown in B–D. (F) Steady-state inactivation of TTX-sensitive inward current. Peak current recorded during a step to −10 mV is plotted as a fraction of maximum versus the holding potential. The smooth curve is the best fit of $I/I_{max} = 1/(1 + \exp((V_m - V_{1/2})/n))$ with $V_{1/2} = -65$ mV and $n = 6.6$.

lower input resistance ($R_{in} = 0.92 \pm 0.13$ GOhm, $C_{cell} = 19 \pm 2$ pF, 9 to 11 days after plating, $n = 17$; differences are significant at $P < 0.01$) ). By the fifth day after plating, all of the neuron-like cells displayed voltage-gated inward and outward currents when stimulated with a depolarizing voltage step (Fig. 6B). Similar currents were detected as early as 24 hr after dissociation; however, 3 of 16 cells tested within the first 4 days displayed little or no inward current. Action potentials were elicited by injection of depolarizing current in 21 of 24 cells (Fig. 6A). In contrast to the properties of cells with neuronal morphology, recordings from 4 of the flat background cells revealed a high resting input conductance (mean $R_{in} = 95 \pm 33$ MOhm) and little evidence of voltage-gated currents.

For neuron-like cells, the initial spike of inward current recorded under voltage-clamp was completely blocked by 0.5 to 1 $\mu M$ tetrodotoxin (TTX, $n = 30$), which suggests that voltage-gated sodium channels underlie this current. Figure 6C shows the TTX-sensitive current recorded at 11 different test potentials. There was a smooth increase in peak current and rate of inactivation for test potentials from −50 to about −10 mV where the current reached a maximum (see Fig. 6E). During the first 5 to 6 days after plating the maximal sodium current ranged from undetectable to greater than −1000 pA (mean for cells that displayed current, −308 ± 45 pA). More mature cells appeared to express larger currents; however, for many of these cells it was not possible to ensure adequate voltage control. Steady-state inactivation of TTX-sensitive current was examined in three cells; the potential that produced half-inactivation was −63 ± 2 mV (see Fig. 6F). In the presence of TTX, outward current was observed for steps to test potentials of −30 mv and above (Figs. 6D and 6E). Steady-state current at +40 mV ranged from 150 to 1200 pA (mean 504 ± 78 pA, $n = 14$) with pipettes containing $KCH_3SO_3$ or KF. This current is likely to be mediated by voltage-gated potassium channels because internal solutions containing cesium produced a sharp decline in its amplitude during the first few minutes after whole cell recordings were established.

We tested 27 cells for sensitivity to excitatory and inhibitory agonist. By 9 to 10 days after plating, current was elicited in all cells with neuronal morphology by one or more of the following compounds: kainate, NMDA, GABA, and glycine. Most cells at this age were sensitive to all four agonists. At the fifth day after plating 2 of 4 cells responded to at least one out of the four agonists, while 5 cells tested during the first 4 days in dispersed culture gave virtually no response to any of the compounds. The trace in Fig. 7A, recorded at 9 days after plating, shows that currents evoked by GABA or glycine desensitized over a time course of several seconds, while currents activated by kainate and NMDA showed less decay for applications of a few seconds duration. At negative holding potentials the amplitude of currents gated by GABA or glycine was strongly dependent on the composition of the internal solution. Large inward currents were observed only when the internal solution contained a high concentration of chloride. Recordings obtained with fluoride or methanesulfonate as the main internal anion showed little or no current during applications of GABA or glycine at −60 to −80 mV; however, large de-

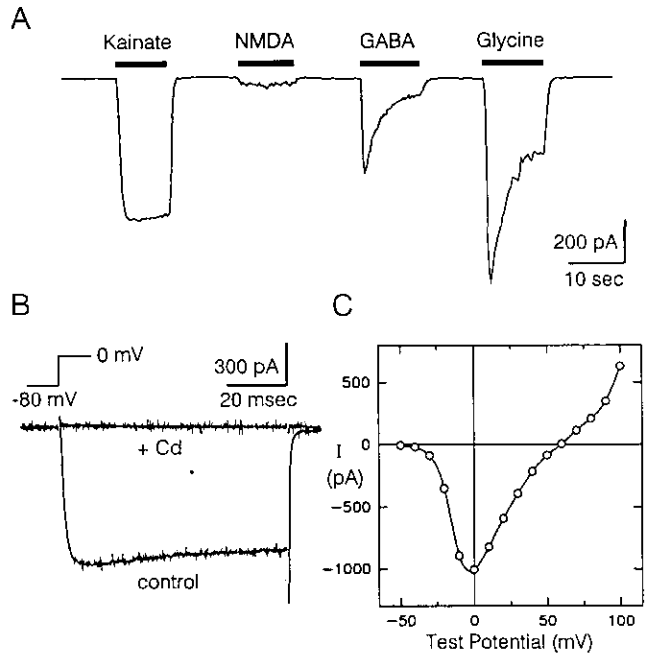


FIG. 7. Agonist-gated currents and voltage-gated calcium channel current in more mature neuron-like cells. (A) Whole cell currents activated by 100 $\mu M$ kainate, 40 $\mu M$ NMDA (with 1 $\mu M$ glycine), 100 $\mu M$ GABA, and 100 $\mu M$ glycine. Internal solution, CsCl; holding potential, −70 mV, 9 days after plating. (B) Currents elicited by a 100-msec step from −80 to 0 mV in control solution (5 m$M$ $BaCl_2$, 150 m$M$ TEA-Cl, 2 m$M$ $MgCl_2$, 0.1 m$M$ EGTA, 1 $\mu M$ TTX, and 10 m$M$ HEPES, pH 7.4, with TEA-OH) and control plus 3 m$M$ $CdCl_2$. Individual traces corrected for leak and capacity current are shown (0.5 msec has been blanked at the beginning and end of the voltage step, filtered at 5 kHz and sampled at 10 kHz). Internal solution, $CsCH_3SO_3$, 11 days after plating. (C) Cd-sensitive current elicited from a holding potential of −80 mV. Peak current is plotted versus test potential.

sensitizing outward currents were observed for these agonists while holding at 0 to +40 mV (data not shown). Taken together, these results suggest that GABA and glycine are activating chloride permeable channels. The currents activated by kainate and NMDA reversed polarity near 0 mV for all of the internal solutions used in this study. The cell shown in Fig. 7A had a relatively modest response to NMDA, but in many cases the current gated by NMDA was comparable in size to that of kainate. At a holding potential of −70 mV, the mean amplitude of kainate current was $-410 \pm 90$ pA ($n = 13$), while the mean amplitude of current gated by NMDA was $-170 \pm 30$ pA ($n = 13$).

Current through voltage-gated calcium channels was recorded using barium as the charge carrier. As shown in Fig. 7B, whole cell barium current was completely blocked by 3 m$M$ cadmium. In all of the cells tested, there was only modest inactivation of barium current during an 80-msec test pulse ($n = 11$, recorded on Days 9 to 11 after plating). Cadmium-sensitive inward current was maximal for test potentials near 0 mV (Fig. 7C). Peak current at this potential ranged in amplitude from −100 to −1.1 nA (mean $-430 \pm 130$ pA, $n = 9$). As previously described (Fenwick *et al.*, 1982; Lee and Tsien, 1982), intracellular cesium was able to carry outward current through calcium channels when cells were depolarized above +50 to +60 mV (Fig. 7C).

## DISCUSSION

The results of this study show that ES cells are capable of differentiating in culture into cells expressing multiple phenotypes, each of which is usually associated with neuronal cells. The most efficient induction is obtained by culturing stem cells as aggregates for 4 days and then exposing the aggregates to RA for an additional 4 days. Aggregates induced by this procedure and plated intact on an adhesive substrate produce an extensive neuritic outgrowth resembling those seen in primary cultures of brain cells. When aggregates of induced cells are dissociated to single cells and plated, they result in cultures with two main cell types: flat cells adherent to the background and cells with a neuron-like appearance growing on top of the flat cells. These cultures resemble young primary cultures of neurons. Antibody staining shows that the neuron-like cells derived from ES cells express the M subunit of neurofilaments and class III $\beta$-tubulin protein. Multiple genes coding for proteins associated with basic neuronal functions including transmitter synthesizing enzymes (GAD and TH), transmitter receptor subunits (GluRs), and a cytoskeletal subunit (NF-L) are upregulated in induced cultures. The electrophysiological properties of differentiated ES cells also provide strong evidence for a neuron-like phenotype. Within a few days after plating, virtually all of the cells with neuronal morphology express voltage-gated Na+, K+, and Ca2+ channels as well as functional receptors for a variety of neurotransmitters. Although much further work will be needed to identify the various channel subtypes that are present in the differentiated cells, our initial characterization clearly demonstrates that expression of robust electrical excitability and transmitter sensitivity is not confined to a small subset of cells but is a widespread property. Taken together, the morphological, immunological, molecular, and physiological data argue that at least a subset of the genetic regulatory pathways used in neuronal differentiation is activated by ES cells in culture. It is important to point out that the level of expression of neuronal phenotypes is quite high. About 38% of the cells in cultures made by dissociating 4−/4+ aggregates are neuron-like in their morphology. Steady-state levels of transcripts for both GAD genes rise dramatically with differentiation and in the induced cultures are approximately 10%

of those in the adult mouse brain. Likewise, levels of NF-L transcripts are high.

In spite of these promising observations it would be premature to classify the cells with neuronal appearance in these cultures as neurons. Certain fundamental features of normal neurons such as the ability to form synapses, express a restricted neurotransmitter phenotype, or express the morphology of a well-characterized subset of neurons have not yet been documented. In addition, recent studies of glial cells (Barres, 1991), as well as other cell types, have shown that expression of a variety of neuronal proteins, including voltage- and transmitter-gated channels, is more widespread than was once thought. For all of these reasons, we consider it important to designate the differentiated cells as "neuron-like" cells rather than true neurons.

This study supports three conclusions about the *in vitro* pathway from ES to neuron-like cells. First, neuron-like cells can differentiate from ES cells in an environment other than the intact embryo. ES cells are believed to represent cells of the preimplantation embryo's inner cell mass. In normal development, these cells go on to form a layer of cells called the primitive ectoderm. The primitive ectoderm then gives rise to ectoderm, mesoderm, and endoderm through the process of gastrulation. In lower vertebrates, and presumably in mammals (reviewed in Ruiz i Altaba, 1994), axial mesoderm induces the overlying ectoderm to differentiate into neuroectoderm which gives rise to the nervous system. Although some of the cells in embryoid bodies are arranged in epithelium-like structures, the complex and highly structured series of cellular changes and interactions of the normal embryo does not occur in cultured aggregates of ES cells. Nevertheless, substantial neuronal differentiation takes place. We conclude that there are pathways leading from ES cells to neuron-like cells that do not require the full array of cellular interactions associated with normal early embryogenesis. However, a partial set of the interactions found in the embryo may be occurring in our system. Some of the cells in embryoid bodies derived from the D3 and CCE ES cell lines express the brachyury gene (Keller *et al.*, 1993; Yamada *et al.*, 1994) and hence are probably differentiating along a mesodermal pathway. Therefore some or all of the cells differentiating along a neuronal pathway in our cultures may be in contact with mesoderm-like cells or their secretions. Nevertheless, the results clearly show that certain aspects of neuronal differentiation can take place in a system that is morphologically less structured than the normal embryo. That ectoderm is capable of neuronal differentiation without interacting with mesoderm has been shown in *Drosophila* (Rao *et al.*, 1991). The ability to activate a neuronal pathway in embryoid bodies is consistent with the finding that other major lineages including hematopoietic and cardiac are activated in embryoid bodies (Burkert *et al.*, 1991; Schmitt *et al.*, 1991; Wobus *et al.*, 1991; Simon *et al.*, 1992; Keller *et al.*, 1993; Weiss *et al.*, 1994).

A second conclusion from these studies is that RA can activate a pathway leading to expression of multiple neuronal genes and phenotypes in genetically normal, totipotent early embryonic cells. The presence of RA during induction has two clearly documented effects. Aggregates exposed to RA during induction and then plated as intact aggregates express neurites to a much greater degree than control aggregates. Also if cells from RA-treated aggregates are dissociated and plated, they differentiate into cultures with a high proportion of neuron-like cells. Controls not exposed to RA produce very few neuron-like cells. These results are consistent with evidence emerging from other recent studies. RA promotes the replication and survival of multipotential neural crest precursor cells in culture and leads to a greater number of differentiated neurons (Henion and Weston, 1994). It also stimulates the regeneration of auditory hair cells, which are a highly specialized form of neuron from cochlear epithelium (Lefebre *et al.*, 1993). RA regulates the neurotransmitter phenotype of postmitotic neurons in culture (Berrard *et al.*, 1993) and promotes the differentiation of retinal precursors to photoreceptors in culture (Kelley *et al.*, 1994). The outgrowth of neurites from embryonic amphibian spinal cord is stimulated by RA (Maden and Holder, 1991). Finally, RA induces the differentiation of P19 cells along the neuronal pathway (Jones-Villeneuve *et al.*, 1982).

The mechanisms by which RA exerts its effects is the subject of a large field of investigation. Two classes of receptors which bind RA, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), have been identified. While a detailed analysis has not yet been performed, mRNAs encoding RARs are present in ES cells, and the expression of some of them can be induced by RA treatment (Leroy *et al.*, 1991; Zelent *et al.*, 1991; Kastner *et al.*, 1990). ES cells also express substantial amounts of RXR$\alpha$ and RXR$\beta$ but very low levels of RXR$\gamma$ transcripts (Bain and Gottlieb, 1994). It will be important to determine which of these receptors are active in the pathway leading to neuronal differentiation of ES cells.

The final conclusion of these studies is that the system which controls the expression of neuronal phenotypes in ES cells in culture is complex. There appears to be an uncoupling between expression of neuronal gene transcripts and morphological phenotype. For instance, the NF-L gene is expressed in undifferentiated ES cells and also in the flat nonneuronal cells derived from 4−/4− inductions which are dissociated before plating. Similarly, RA treatment of aggregates is necessary for the

full expression of morphological phenotype when induced cells are plated as aggregates or dispersed cells. However, aggregation alone is enough to induce NF-L and GAD transcripts for cells plated as aggregates. Taken together these results show that RA and cell–cell contact have a major influence on differentiation in this system but that they regulate the expression of neurites, NF-L, and the GAD genes differently.

The relationship of the pathway just described to the normal pathway leading from the inner cell mass to tissues that generate neurons is a major open question. Since ES cells are genetically normal and RA has a proneuronal effect on normal cells, it very likely that some components of this *in vitro* pathway are accurate models of *in vivo* commitment to neuronal differentiation. Recent evidence in *Xenopus* strongly implicates the activin type II receptor as a mediator of signals that suppress neuronal differentiation (Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou *et al.*, 1994). Inhibition of signaling by this receptor leads to neuronal differentiation. In P19 cells there is suggestive evidence for a similar role for activin receptors (Hashimoto *et al.*, 1990, 1992). Activin receptors might have a similar role in neural differentiation in ES cells. Such a role would be evidence for a common feature of neural differentiation shared by mammalian pluripotent cells and the *Xenopus* embryo. On the other hand, it is possible that components of the regulatory system leading to neurons in the normal nervous system are missing or altered in the *in vitro* system. It will be important to learn more about both the similarities and differences between ES derived neuron-like cells and actual neurons.

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