Clonal Cell Lines Produced by Infection of Neocortical Neuroblasts Using Multiple Oncogenes Transduced by Retroviruses

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Toward understanding the early molecular development of neocortical neurons, we report the generation of two clonal murine cell lines derived by sequential oncogenic retroviral infection of neocortical neuronal precursors. The resulting cell lines stably express the telencephalon-specific gene BF-1 and a gene enriched in the neocortical ventricular zone, vzg-1. They also express early neuronal but not glial markers, possess neuron-like processes without chemical synapses by ultrastructure, and appear pyramidal or bipolar with processes resembling apical dendrites, bifurcating and beaded axons, or growth cones. Immortalization appears to have occurred through the expression of oncogene combinations SV40 large T with vras or SV40 large T with both vsrc and vmyc. These cell lines may represent developing neocortical neuroblasts blocked from complete differentiation, and they should be useful in the isolation and analysis of genes involved in early neocortical development.

INTRODUCTION

The neocortex, with its embryologically related telencephalic structures, comprises 6 neuronal layers that arise developmentally from the region immediately opposed to the embryonic ventricles: the transient proliferative layer called the ventricular zone. It is from this zone that cortical neurons are "born" during a discrete period, undergoing their last round of mitosis (Sidman et al., 1959; Angevine and Sidman, 1961) and migrating to their more superficial position as postmitotic, differentiating neurons in the cortical plate. In the mouse, the period of neocortical neurogenesis extends from around Embryonic Day 11 (E11; layer 6) to E17 (Angevine and Sidman, 1961; Caviness, 1982; Derer and Derer, 1990).

Molecular and biochemical analyses of developing neocortical neurons have been hampered by the complexity posed by intact neocortical tissue that consists of nonneural cells (e.g., cells of the endothelium, meninges, and blood) intermixed with multiple types of neurons and glia. This heterogeneity is compounded in the embryonic neocortex by these cell types being themselves at multiple developmental stages ranging from pluripotent cells of the ventricular zone to mature neurons of the subplate (Shatz et al., 1988). Cell lines possessing functional characteristics of developing neocortical neuroblasts could simplify the molecular analysis of their development, just as analogous cell lines have provided many inroads toward understanding the molecular development of the immune system (Ralph, 1979).

Several approaches have been used to produce cell lines from nervous system tissue, including tissue culture of spontaneous tumors (Augusti-Tocco and Sato, 1969), chemical mutagenesis (Schubert et al., 1974), somatic cell hybrids (Klee and Nirenberg, 1974; Greene et al., 1975; MacDermot et al., 1979; Lee et al., 1990), oncogenic transgenes in transgenic mice (Brinster et al., 1984; Messing et al., 1985; Palmiter et al., 1985; Efrat et al., 1988; Hammad et al., 1990; Mellon et al., 1990; Suri et al., 1993), and virally transduced oncogenes (Santoli et al., 1975; Cepko, 1988; Geller and Dubois-Dalcq, 1988; Frederiksen et al., 1988; Bernard et al., 1989; Ryder et al., 1990; Snyder et al., 1992). The majority of the cell lines derived by these means
Neocortical Cell Lines Produced Using Multiple Oncogenes

appear to be either neural crest derivatives or CNS derivatives of glial or pluripotent (neuronal and glial) precursors rather than homogeneously neuronal (see Discussion). None of these cell lines are derived unambiguously from neocortical neuroblasts.

Here we report the development of clonal cell lines from the neocortex. Two different, sequentially delivered retroviruses were used to infect mitotic, cortical neuroblasts. The cell lines express at least two genes that are expressed by cortical neuroblasts in vivo: the transcription factor BF-1 (Tao and Lai, 1992; Xuan et al., 1995) and the putative G-protein-coupled receptor, vzg-1 (Hecht et al., 1995). The cells further possess characteristics consistent with neuroblasts that have committed to becoming neurons but which are blocked from further differentiation.

RESULTS

The cell lines were produced by the sequential delivery of two different oncogenic retroviruses to neuroblasts that were, on the basis of their known birthdate, destined for the superficial layers of the murine neocortex. In vivo infection was followed by culturing of dissociated tissue derived from these infected cortices that allowed the morphological isolation of slowly proliferating, infected neuroblasts from a background of other infected and uninfected cells (“first virus” step). Mechanical isolation of these neuroblasts was followed by reinfection in vitro with a different retrovirus to produce a cell line (“second virus” step). The resulting cell lines were characterized by Northern and Western blots, immunocytochemistry, electron microscopy, and transfection techniques and possess a phenotype consistent with that of neocortical neuroblasts.

In Vivo Infection: First Virus

The telencephalon at known gestational ages was infected in vivo with replication-defective, oncogenic retroviruses. The approach was technically similar to that used for infecting the rodent neocortex in vivo by retroviruses containing the reporter gene lacZ (Walsh et al., 1988; Luskin et al., 1988). A schematic of the method is shown in Fig. 1. Several oncogenic retroviruses were assayed (see Experimental Methods). Cell lines were produced by using two unpublished constructs containing the oncogenes vsrc or vgagmyc (vmyc) in combination with vsrc (Baldacci and R. Jaenisch, unpublished data) and two published viruses containing virus (Dotto et al., 1985) and SV40 large T (Large T; Jat and Sharp, 1986; Jat et al., 1986). The proviral organizations of those viruses that resulted in demonstrable infection are shown in Fig. 2.

To assess the susceptibility of cortical neurons to retroviral infection, cultures were established from brains of embryos infected with vsrc or vsrc–vmyc virus between E12 and 16. DNA was isolated after 8–12 months of continuous culture and Southern blots were hybridized with a src-specific probe to detect proviral copies (Fig. 3). Single proviral copies were detected in each culture, indicating that cortical cells are susceptible to infection with oncogenic retroviruses in vivo which can result in the establishment of long-term cultures.

The appearance of infected cultures is shown in Fig. 4. After 1 week of growth, the cultures formed a mono-
sulted in a robust proliferation of cells: first-virus Large T followed by second-virus \( \text{vras} \) (Fig. 5B) and first-virus Large T followed by second-virus \( \text{vsrc} \)–\( \text{vmyc} \) (not shown). A cell line that was established from colonies derived from those shown in Fig. 5B is shown in Fig. 5C (referred to as cell line TR for the oncogenes Large T and \( \text{vras} \)), and a cell line resulting from second-virus \( \text{vsrc} \)–\( \text{vmyc} \) infection is shown in Fig. 5D (referred to as cell line TSM). Cell line TSM can be distinguished from cell line TR by its more elaborate processes.

These cell lines were derived by consecutive infection with two different retroviruses, suggesting that expression of two or more different oncogenes was involved in causing immortalization. To assess whether each cell line was initially derived from a single cell infected with the viruses, Southern blot analyses were performed to detect proviral copies. Figure 6 shows that each cell line contained a single copy of each expected provirus, demonstrating that the cell lines were indeed clonally derived. Expression of the respective oncogenes was demonstrated by Northern analysis revealing the presence of the expected oncogene transcripts in each cell line (Fig. 7A). The results described in Figs. 6 and 7A are consistent with those found in other studies.

layer of flat, phase-dark cells (probably glia) on top of which could be found phase-bright cells having the appearance of immature neurons. The phase-bright cells proliferated slowly with an estimated doubling time of several days or longer. Slowly proliferating cells were not observed in control flasks (Fig. 4A) and were tentatively identified as infected neuroblasts. Both Large T and \( \text{vsrc} \)–\( \text{vmyc} \) viral infections produced similar-appearing cells that were not seen without infection nor with infection by the \( \text{vsrc} \) vector alone (\( \text{vras} \) was not assayed \textit{in vivo}).

While phase-bright cells were observed in all cultures derived by infection with either Large T or \( \text{vsrc} \)–\( \text{vmyc} \) (see Table 1), E16 was chosen as an age for producing cell lines because it marks the latter stage of cortical neurogenesis beyond which glia but not neurons are generated. This was the most stringent case for the production of cell lines with a neuronal phenotype since both neurons and glia would be expected to result from a precursors cell line derived from this age.

**In Vitro Infection: Second Virus**

Slowly proliferating cells produced after \textit{in vivo} infection with the Large T virus were physically isolated as colonies and transferred to a new tissue culture dish (Fig. 5A). These cells were then infected with a second virus containing a different oncogene(s). Two conditions resulted in a robust proliferation of cells: first-virus Large T followed by second-virus \( \text{vras} \) (Fig. 5B) and first-virus Large T followed by second-virus \( \text{vsrc} \)–\( \text{vmyc} \) (not shown). A cell line that was established from colonies derived from those shown in Fig. 5B is shown in Fig. 5C (referred to as cell line TR for the oncogenes Large T and \( \text{vras} \)), and a cell line resulting from second-virus \( \text{vsrc} \)–\( \text{vmyc} \) infection is shown in Fig. 5D (referred to as cell line TSM). Cell line TSM can be distinguished from cell line TR by its more elaborate processes.

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FIG. 4. Appearance of cells observed in primary culture using phase microscopy after in vivo (first virus) infection. A src-v-src (similar results were obtained with a large T virus) was used in all cases. (A) Uninfected control after 2 months of continuous culture in which the primary culture medium is changed every 3 days. (B) Same field as in (A) after 3 days of continuous culture and (C) after 6 days of continuous culture as a monolayer. Cells have flattened out and can be physically isolated. (D) Morphology of cells which resemble neuroblasts in being phase bright, bipolar, and having a high nucleus-to-cytoplasm ratio. Calibration bar, 50 μm.
with the hypothesis that the combined expression of the transduced oncogenes led to establishment of the permanently growing cell lines.

**Expression of Neuronal Markers**

Cell lines TR and TSM were examined for markers expressed by telencephalic neuroblasts or that are expressed relatively early in neuronal development (see Discussion for the choice of the examined markers). Northern blot analyses revealed the presence of BF-1 and vzig-1 transcripts in both cell lines (Fig. 7A). Other surveyed genes that were not expressed by the lines included members of the dlx, otx, and pax 6 gene families (data not shown). Neuronal gene transcripts for the low-affinity nerve growth factor receptor and 68-kDa neurofilament gene were also present. Western blot analyses (Fig. 7B) demonstrated the presence of the 68-kDa neurofilament protein (NF 68) and neuron-specific enolase (NSE), but not glial fibrillary acidic protein (GFAP).

Based on the clonality of TR and TSM by Southern blot analyses (Fig. 6) and the expression of neurofilaments, we examined the degree of phenotypic homogeneity of TR and TSM by immunocytochemistry using antibodies against neurofilaments, as shown in Fig. 8. Cell lines TR (Figs. 8A–8C) and TSM (Figs. 8D–8F) showed homogeneous immunostaining with antibodies against neurofilaments (shown for monoclonal antibodies SMI 31, SMI 33, and Ig controls, see Table 2). To address further the presence of neurofilaments and to examine the morphology of TR and TSM in more detail, an ultrastructural analysis was pursued. The presence of parallel filaments that resemble neurofilaments along with parallel arrangements of microtubules is consistent with the neuronal processes observed in the CNS (Peters et al., 1976) as shown in Fig. 9A. Other elements visible

### TABLE 1

**Summary of Experimental Procedures**

<table>
<thead>
<tr>
<th>Experiment age</th>
<th>1st virus oncogene</th>
<th>No. of experiments</th>
<th>No. of injected embryos</th>
<th>Phase-bright cells?</th>
<th>Longest 1st-virus culture period (months)</th>
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<td>1</td>
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<td>Yes</td>
<td>2</td>
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<tr>
<td>E10 Large T</td>
<td>1 6</td>
<td>Yes</td>
<td>2</td>
<td></td>
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<tr>
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<tr>
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<tr>
<td>E13</td>
<td>Large T</td>
<td>6</td>
<td>16</td>
<td>Yes</td>
<td>3</td>
</tr>
<tr>
<td>E13 vsrc</td>
<td>2 18</td>
<td>No</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E14</td>
<td>vsrc–vmyc</td>
<td>4</td>
<td>22</td>
<td>Yes</td>
<td>6</td>
</tr>
<tr>
<td>E14 Large T</td>
<td>4 34</td>
<td>Yes</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E15</td>
<td>vsrc–vmyc</td>
<td>4</td>
<td>20</td>
<td>Yes</td>
<td>11</td>
</tr>
<tr>
<td>E15 Large T</td>
<td>2 13</td>
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<td>2</td>
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<tr>
<td>E15 vsrc</td>
<td>2 18</td>
<td>No</td>
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<tr>
<td>E16</td>
<td>vsrc–vmyc</td>
<td>7</td>
<td>33'</td>
<td>Yes</td>
<td>12</td>
</tr>
<tr>
<td>E16 Large T</td>
<td>6 20'</td>
<td>Yes</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E16 No infection</td>
<td>2 0</td>
<td>No</td>
<td>10</td>
<td></td>
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</table>

* Two animals injected as fetuses were born and followed for 12 months without gross evidence of brain tumor formation.

![FIG. 5. In vitro (second virus) infection with a second, different oncogenic retrovirus results in rapid cell proliferation and the establishment of cell lines. A colony similar to those shown in Fig. 4C derived from the in vivo (first virus) infection of Large T at E16 was picked and transferred to a 6-well tissue culture plate that had been coated with 3 μg/cm² Cell Tak. In the absence of further intervention, the colonies slowly dispersed but did not significantly expand. Further use of growth factors, lectins, and chemical mitogens did not allow the production of cell lines. When a duplicate culture was instead reinjected with a retrovirus that expressed either vras or vsrc–vmyc, a massive proliferation resulted as shown in B (shown for a vras second infection). The cell lines that were derived from cultures similar to that shown in B are shown in C (vras as the second virus resulting in a cell line called TR) and D (vsrc–vmyc as the second virus resulting in a cell line called TSM). Note the phase-bright appearance of the cells (e.g., in C and D) along with the presence of extended, sometimes beaded processes (e.g., D). Calibration bar, 50 μm (shown in B for A and B, shown in D for C and D).](image-url)
in axonal initial segments or dendrites (i.e., microtubules, mitochondria, and ribosomes) were also present. A transverse section through a smaller diameter process revealed the characteristic array of parallel microtubules observed in axons (Peters et al., 1976). We have also observed puncta adherens between cells (not shown) but have not observed any clear examples of chemical synapses in ultrastructural surveys of three independent cultures of each line.

**Cell Line Morphologies**

The morphologies of cell lines TR and TSM after immunostaining for neurofilaments were examined for features consistent with those observed in Golgi studies of the developing neocortex in mice and sheep (Cajal, 1960; Kobayashi et al., 1964; Astrom, 1967). These features included (1) simple pyramidal or bipolar (fusiform) morphologies, (2) growth cones, (3) growing but immature apical processes, and (4) small-diameter, axon-like processes extending long distances, bifurcating, and at times showing small varicosities or “beads” (e.g., see Chun and Shatz, 1989b). Examples of these identified morphologies are shown for cell line TSM in Fig. 10. In addition to fairly large-diameter processes (e.g., Figs. 9A, 10A, and 10B), finer axon-like processes could be found coursing...
TABLE 2
Antibodies and Antisera: Dilutions and Sources

<table>
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<tr>
<th>Antibody/antiserum</th>
<th>Concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse monoclonal Map2-3 (α-Map2)</td>
<td>1:1000 (cytochem)</td>
<td>Gift from Dr. R. Vallee, Worcester Foundation</td>
</tr>
<tr>
<td>Mouse IgG1 and IgG2b controls</td>
<td>1:2000 (Western)</td>
<td>Gift from Dr. I Weissman, Stanford University (see also Chun and Shatz, 1989a,b)</td>
</tr>
<tr>
<td>Mouse monoclonal (α-68-kDa neofilament)</td>
<td>1:200 (cytochem)</td>
<td>Sigma Chemical Co., St. Louis, MO; Cat. No. N5139</td>
</tr>
<tr>
<td>Mouse monoclonal SMI 31 (α-phosphorylated neofilaments, 200 kDa and 150 kDa)</td>
<td>1:4000 (cytochem)</td>
<td>Sternberger Monoclonals, Inc., Baltimore, MD; Cat. No. SMI 31</td>
</tr>
<tr>
<td>Mouse monoclonal SMI 33 (α-neofilaments, 200 kDa and 150 kDa)</td>
<td>1:4000 (cytochem)</td>
<td>Cat. No. SMI 33</td>
</tr>
<tr>
<td>Rabbit α-GFAP</td>
<td>1:200 (cytochem)</td>
<td>Sigma, Cat. No. G9269</td>
</tr>
<tr>
<td>Rabbit anti-NSE (α-neuron-specific enolase)</td>
<td>1:2000 (cytochem)</td>
<td>Incstar Corp., Stillwater, MN; Cat. No. 22521</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>1:200–1:8000</td>
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</tr>
</tbody>
</table>

through the culture (Figs. 10C and 10D), generally locating in focal planes above the bottom of the coverslip. Some of the processes extended for hundreds of micrometers (Fig. 10C). In addition, clear bifurcations of the processes could be observed (Fig. 10D, arrows), and some fine processes were also beaded (Fig. 10D, small arrows). Growth cones were also observed growing on the bottom of the coverslip (Figs. 10E and 10F; arrows).

Expression of Neuron-Specific Transfected Reporter Genes

The presence of neurofilaments (Julien et al., 1987) suggested that both cell lines could express neuron-specific genes. To determine whether a neuron-specific exogenous promoter could be faithfully expressed within TR and TSM, constructs were made that contained a 1.6-kb genomic fragment 5' to the NF 68 coding sequence comprising a putative murine neurofilament promoter (Lewis and Cowan, 1986) driving the reporter genes CAT or lacZ. Control constructs were driven by the ubiquitously expressed Moloney 5'-LTR. These constructs were transfected into 3T3 fibroblasts or cell lines TR and TSM. The construct NF–CAT was not expressed in 3T3 fibroblasts (Fig. 11A, 3T3 N lane, compared to LTR–CAT, 3T3 L lane), but was expressed in both TR and TSM. To examine the morphology of the cells that expressed the NF constructs as revealed by β-gal histochemistry, TR (Figs. 11B and 11C) and TSM (Fig. 11D) were transfected with NF-lacZ and the cultures processed for β-gal histochemistry. The labeled cells revealed by lacZ varied from rounded, bipolar appearing to pyramidal-like. Cell lines TR and TSM are thus easily transf ectable and appear to have at least some of the factors necessary for neuron-specific transcription.

DISCUSSION

This study documents two new clonal cell lines produced by a novel strategy of sequentially delivering two different oncogenic retroviruses. They molecularly and morphologically resemble neocortical neuroblasts that have been blocked from further differentiation.

Comparison with Other Retroviral Approaches to Neural Cell Line Production

The use of oncogenic retroviruses in deriving CNS cell lines has generally produced pluripotent or glial cell lines (Cepko, 1988; Geller and Dubois-Dalcq, 1988; Frederiksen et al., 1988; Bernard et al., 1989; Ryder et
al., 1990; Snyder et al., 1992). The paradigm has been to infect primary cultures of grossly dissected postnatal rodent brain (except for Bernard et al., who infected E10 brain), and infected cells were identified by their drug resistance. Since the developing brain consists of a large number of different cell types that include the neural components of blast cells of different lineages along with nonneural, mitotic cells (e.g., endothelium, macrophages, meningeal cells), a large variety of mitotic cells can become infected, which may explain the prevalence in some studies of cell lines that did not express neural markers after retroviral infection of primary cultures (Cepko, 1989). Since G418 drug selection was used in these studies, the resulting cultures likely consisted of (1) all cells that were infected and that expressed the drug resistance (neo) gene and (2) cells that divided rapidly. For example, using a published postinfection selection of 2 days of unselected growth followed by 21 days of G418-selected growth (Jat and Sharp, 1986; Frederiksen et al., 1988), an initially infected cell with a doubling time of 1 day would consist of $2^2$ ($8.4 \times 10^6$) cells compared to a cell with a doubling time of 7 days that would produce $2^7$ cells. Moreover, uninfected cells that could serve as a supporting feeder layer for less robust infected cells would have been eliminated by drug selection.

**Cell Lines TR and TSM Express Neocortical Markers**

Cell line TSM and particularly TR stably express high levels of the winged helix transcription factor BF-1, a gene that is expressed in the embryonic telencephalon including the neocortex (Tao and Lai, 1992). BF-1 has been shown to be essential for the normal development of the cerebral hemispheres based on studies of a homozygous null mutation in mice (Xuan et al., 1995). BF-1 expression is highest during embryonic life, consistent with TR and TSM showing an immature phenotype. Moreover, the novel gene vzg-1 which shows restricted expression during cortical neurogenesis within the ventricular zone (Hecht et al., 1995) is also expressed by both cell lines. The combined expression of both BF-1 and vzg-1 indicates that TR and TSM were derived from neocortical blasts. This is entirely consistent with the employed infection protocol whereby the predominant mitotic cells susceptible to infection during the first-virus step were mitotic cells within the cortical ventricular zone, particularly neuroblasts destined for cortical layers.

**TR and TSM Express Early Neuronal Markers**

Further consistent with the view that TR and TSM are derived from cortical neuroblasts is their expression of neuronal markers. These include the low-affinity nerve growth factor receptor, neurofilaments, and neuron-specific enolase (but not GFAP). The low-affinity nerve growth factor receptor was used because it is expressed in the developing brain which is composed predominantly of neocortex at comparable developmental periods in the rat (Maisonpierre et al., 1990), thus suggesting an immature neuronal phenotype. Neurofilaments were examined because of the early expression, particularly the 68-kDa variety, in neuroblasts even before their migration from the ventricular zone (Tapscott et al., 1981; Shaw and Weber, 1982; Cochard and Paulin, 1984; Bennet and DiLullo, 1985). Neuron-specific enolase, found in most CNS neurons (Schmechel et al., 1978; Marangos et al., 1980), suggests neuronal maturation beyond a pluripotent state (Schmechel et al., 1980). A marker characteristic of mature neurons, MAP2, was not expressed (data not shown), which is consistent with the inability of these cell lines to differentiate fully. In addition, both TR and TSM allowed expression of transfected reporter gene constructs that were driven by a neuron-specific neurofilament promoter (Lewis and Cowan, 1985, 1986; Julien et al., 1987), indicating the presence of at least some of the trans-acting factors necessary for neuron-specific gene expression.

**TR and TSM Morphologically Resemble Young Cortical Neuroblasts**

By morphology the lines exhibited growth-cone-like extensions and fairly simple morphologies—bipolar or pyramidal—that are similar to the immature neuronal phenotypes observed in the developing cerebral cortex (Cajal, 1960; Kobayashi et al., 1964; Astrom, 1967). By ultrastructural examination, processes containing microtubule and filament arrays like those in CNS neurons were observed. Mature synapses, however, were not ob-

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**FIG. 8.** Cell lines TR and TSM show homogeneous immunostaining for neurofilaments using two different monoclonal antibodies. (A–C) Cell line TR. (D–F) Cell line TSM. A and D are immunostained with the antibody SMI 31 (1:4000), B and E with SMI 33 (1:4000), and C and F with Ig control (1:4000). Immunostaining homogeneously labels all the cells in each culture. Nuclear staining with SMI 31 has been observed by using other similar antibodies against developing brain (Sternberger, 1986). See Table 2 for further details.
FIG. 9. Ultrastructural analysis of cell lines reveals the presence of neuronal processes. In A, a longitudinal section through a cell line process reveals parallel arrays of apparent neurofilaments (arrows pointing right) and microtubules (arrows pointing left) that are reminiscent of an axonal initial segment containing ribosomes and mitochondria (white asterisks, cf. Peters et al., 1976). In B, a transverse section through a fine process reveals the characteristic parallel array of microtubules (note arrows) found in small-diameter axons (shown for cell line TR, but observed in both TR and TSM). Mature synapses were not observed. Calibration bar, 0.2 \( \mu m \).

FIG. 10. The morphology of cell line TSM revealed by neurofilament immunostaining resembles that described for young, superficial neocortical neurons in previous Golgi studies. (A and B) These fusiform/bipolar or pyramidal morphologies are also observed in the developing neocortex. (C and D) Fine processes course through the culture, some for hundreds of micrometers. Some of the processes bifurcate (e.g., D, large arrows) or appear "beaded" (e.g., D, small arrows). (E and F) Growth cones (e.g., arrows). TSM is shown immunostained with anti-neurofilament antibody SMI 33 after 5 days in culture on a Cell-Tak-coated glass coverslip and viewed using Normarski optics. Calibration bars for 50 \( \mu m \) are shown in B for A and B, in D for C–E; for 25 \( \mu m \) in F.
TR and TSM Are Likely Derived from Neocortical Neuroblasts

TR and TSM are likely derived from neocortical neuroblasts, based on the presented lines of evidence. First, the infection protocol made use of neuronal birthdates to target cortical neuroblasts, since these blasts were the only neuronal population within the infected and subsequently dissected neocortical tissues. Second, TR and TSM express BF-1, which is required for proper telencephalic development, and vsg-I, which is expressed in the neocortical ventricular zone. Third, TR and TSM express early neuronal but not glial genes. Fourth, TR and TSM morphologically and ultrastructurally resemble immature cortical neurons. In addition, both TR and TSM also appear to be insensitive to the neurotoxic effects of kainic acid (data not shown), which is further consistent with an immature neuronal phenotype, based on lesion studies in which immature cortical neurons are spared while more mature subplate neurons are killed (Chun and Shatz, 1988a; Ghosh et al., 1990). Taken together, these cell lines display a phenotype that resembles that of young cortical neurons just after commitment to becoming a neuron, through early morphological differentiation in the cortical plate but preceding robust synaptogenesis and MAP2 expression.

These cell lines may be especially helpful in identifying genes represented by low-abundance mRNAs or expressed transiently during normal cortical development. They may further provide a culture system for examining the molecular physiology of channels and receptors that are endogenously or exogenously expressed. Additionally, such clonal cell lines may provide a means for examining the molecular characteristics of a single neuronal type, including those related to a neuron’s genomic organization.

EXPERIMENTAL METHODS

Fetal Surgery

Timed-pregnant, Balb/c mice were anesthetized with either 20 µg/g body weight of metomidate hydrochloride (Hypnodil) for ages E12–E15 or 15 µl/g body weight of Avertin (Hogan et al., 1986) for ages E15 and older. After adequate anesthesia, the surgical field was sterilized, the uterus was accessed by laparotomy using a midline abdominal incision, and the uterine horns were visualized. The cerebral vesicles could be seen through the uterine wall by direct illumination using a fiber optic light source and binocular dissecting scope. Injections were made into the ventricles with a side-bore glass micropipet, and every embryo of a single mother was injected with the same supernatant, volumes ranging from approximately 0.5 µl at E12 to 5 µl at E16. After recording the anatomical location of the injected embryos, the uterus was returned and the incision was closed with surgical staples. Except for cases in which the animals were allowed to be born (see Table 1), embryos were allowed to develop for 1–2 days before harvesting.

Tissue Culture

Culturing tissue from first-virus infection: Embryo isolation and plating of first-virus-infected neocortices. After killing pregnant animals by cervical dislocation, sterile technique was used to isolate embryo heads in a sterile culture dish containing prewarmed iHepes–EDTA (see Media, below). Cerebral vesicles were rapidly removed from the isolated heads and placed in a new dish containing prewarmed Medium I, and the neocortical regions were removed with needle forceps from structures distal to and including the thalamus; special attention was made to remove as much of the meninges as possible. Efforts were also made to avoid the hippo-

FIG. 11. Cell lines TR and TSM but not 3T3 fibroblasts express reporter genes driven by the 68-kDa neurofilament promoter. To examine the usefulness of cell lines TR and TSM for transfection studies, and to determine whether some of the trans-acting factors necessary for neuron-specfic gene expression were present in the cell lines, four reporter gene constructs were made in which the putative promoter for the 68-kDa neurofilament protein, a 1.6-kb HindIII–SmaI genomic fragment upstream to the 68-kDa neurofilament gene (Lewis and Cowan, 1986) was ligated 5’ to either CAT or lacZ within a plasmid vector backbone. These constructs were compared to positive control constructs in which the reporter genes were driven by the Moloney 5’ LTR. In parallel cultures of cells containing Balb/c 3T3 fibroblasts were compared to comparable numbers of cells from cell lines TR and TSM by transient transfection of the construct LTR–CAT (labeled L for each cell line sample); this construct was readily expressed in each cell line. In contrast, the construct NF 68–CAT (labeled N) was not expressed in 3T3 cells (regardless of DNA concentration, data not shown) but was expressed in cell lines TR and TSM. To examine the morphology of the cells expressing the NF 68 promoter, NF 68–lacZ constructs were transfected into cell lines TR and TSM (shown for cell line TR) and processed for β-gal histochemistry. (B) (Calibration bar, 50 µm) The population of lacZ-expressing cells. At higher magnification, the cell indicated by the arrow in B is shown in C. Some processes were partially filled by the β-gal reaction product as shown in D. The transfection efficiency ranged up to several percentage points of the cells present in the final culture, and all techniques examined including CaPO4, lipofection, and electroporation were successful for transfection. Stable transfectants have been produced using CaPO4 (data not shown).
campal formation that lies medially in the cerebral ves-
icle. After dissection, 3–10 neocortices were pooled in a
sterile test tube containing fresh prewarmed Medium I,
and a cell suspension was produced by repetitive pi-
petting. The suspension was diluted with 37°C Medium
I and plated in triplicate in a 250-ml tissue culture flask
(Falcon or Corning). Cells were grown at 37°C with 5%
CO₂. The medium was changed every 3–5 days.

In initial experiments, cultures derived from Large T
infection were allowed to grow for up to 1 week followed
by 2 weeks of selection for neomycin resistance using
the antibiotic G418. This selection always resulted in
the growth of large, pleomorphic cells that did not appear
neuronal in being phase dark, flat, and without extensive
processes. When immunostained for neurofilament, the
pleomorphic cells were nonimmunoreactive (not shown).
G418 selection never resulted in slowly growing cells
(morphology shown in Figs. 3B–3D). Since some of the
dissayed viruses did not contain a drug-selectable marker, virus infection, Opti-MEM I (Gibco±BRL) containing 55
μM β-mercaptoethanol, 20 mM glucose, 1% Pen±Strep,
10% heat-inactivated fetal calf serum (fics) (Hyclone, Logan, UT), 10% heat-inactivated horse serum (Hyclone), 1% Pen±Strep, 20 mM additional D-glucose; Medium II for cells immediately after second-
 virus infection, Opti-MEM I (Gibco±BRL) containing 55
μM β-mercaptoethanol, 20 mM glucose, 1% Pen±Strep,
10% fics (Hyclone); Medium III for established cell lines,
Opti-MEM I containing 2.5% fics, 20 mM D-glucose, 55
μM β-mercaptoethanol, 1% v/v penicillin (5000 u/ml),
and streptomycin (500 μg/ml).

**Retrovirus Growth**

Ten recombinant retroviruses were assayed repre-
senting different versions of the four constructs of Fig.
2. In addition, two constructs containing tempera-
ture-sensitive mutations for large T antigen (Frederiksen 
et al., 1988) and vsrc were assayed but did not produce
useful cell lines. Viral supernatant with a titer of ap-
proximately 10⁵ colony-forming units (CFU)/ml on
3T3 fibroblasts was obtained for the viruses used in
this study. A typical injection at E16 was a volume of
5 μl or potentially 500 CFU.

The ψ2 producer cells were grown in DMEM (Gibco±
BRL) containing 10% defined and supplemented, heat-
inactivated bovine calf serum and 1% v/v penicillin
(5000 u/ml) and streptomycin (5000 μg/ml). After
achieving 80% confluence, the medium was changed
and replaced with fresh medium for 3 days. The supernatant
was filtered with a 0.45-μm syringe filter and aliquots
were stored at −70°C. The overall time required to pro-
duce a line, from first-virus infection to second-virus col-
ony expansion and freezing of cell lines was approxi-
mately 2 months.

**Immunocytochemistry**

Cells were grown on sterile circular coverslips coated
with Cell-Tak placed in 24-well Costar (Cambridge, MA)
plates. After growth to the desired density, the medium was removed by aspiration and the cells were immersion-fixed in freshly depolymerized 4% paraformaldehyde, 0.1 M NaH2PO4, for 20 min at 25°C, rinsed in PBS (20 mM NaH2PO4, 150 mM NaCl2, pH 7.4), and blocked in 2.5% BSA/PBS (both with and without 0.3% Triton X-100). A buffer containing 10 mM Tris–HCl–150 mM NaCl was used with the antibody SMI 31 to avoid antibody competition with phosphate in PBS. The antibodies, source, and concentration are shown in Table 2.

Multiple sets of duplicate coverslips were exposed to the primary antibody/serum along with controls. Bound antibody was visualized using avidin–biotin kits (Vector Labs, Burlingame, CA) and horseradish peroxidase histochemistry using 3,3′-diaminobenzidine as the chromogen. Samples were dehydrated, coverslipped, and observed.

**Electron Microscopy**

Cells were split after mechanical dissociation into Falcon 6-well tissue culture plates that had been coated with 3 μg/cm² Cell-Tak (Collaborative Research). After one medium change and 3 days of growth, the medium was carefully removed by aspiration and the cells were immediately fixed at room temperature in a solution containing 2% paraformaldehyde, 2% glutaraldehyde (EM grade), 4 mM CaCl2·2H2O, 0.1 M sodium cacodylate for 1 h. The fixative was then removed and the cells were washed twice, 5 min/wash, with 0.1 M sodium cacodylate with 2 mM CaCl2·2H2O. Cells were then osmicated for 40 min in 2% OsO4 in 0.1 M sodium cacodylate, 2 mM CaCl2·2H2O, dehydrated through graded ethanols to 70% ethanol containing 0.5% uranyl acetate (UA; 12 h, 4°C), then further dehydrated to 100% ethanol, 3:1 ethanol:epon, 1:1 ethanol:epon, 100% epox (placed on a rotating platform for 2 h), then polymerized in a 60°C oven for 24 h. Thin sections were mounted on copper grids and stained with 1% UA followed by 0.2% lead citrate, washed, dried, and observed.

**Routine Molecular Biologic Techniques**

Standard procedures were used in the following techniques (Ausubel *et al.*, 1991).

**DNA.** Cells were grown in 15-cm tissue culture flasks to high density, washed in iHepes–EDTA, 4°C, collected by scraping, pelleted, and lysed (lysis buffer: 75 mM NaCl, 25 mM EDTA, 10 mM Tris–HCl, pH 8.0, 1% SDS, and 1.25 v/v 10 mg/ml protease K) at 37°C for 6 h, extracted with phenol:chloroform, precipitated with sodium acetate and isopropanol, spooled, washed in 70% EtOH, air-dried, and dissolved in 10:1 TE. Five micrograms of genomic DNA was digested with the appropriate restriction enzymes and size fractionated in 0.8% agarose, blotted in 0.4 M NaOH to Gene Screen plus (NEN, Boston, MA), and hybridized with 32P-labeled random-primed probes derived from full length cDNAs against the oncogene of interest in aqueous hybridization buffer (Church and Gilbert, 1984). After high stringency washing (0.2× SSC, 65°C), blots were autoradiographed on Kodak XAR film.

**RNA.** RNA isolation and Northern blot analyses can be found in Chun *et al.* (1991). Briefly, total RNA was isolated by LiCl–urea precipitation after polytron disruption of the cells, proteinase K–SDS digestion of the resulting pellet, phenol:chloroform extraction followed by sodium acetate–ethanol precipitation. Poly(A)+ RNA was isolated by a single pass through an oligo(dT)-cellulose type 7 column (Pharmacia). RNA was analyzed under denaturing conditions in formaldehyde-containing 1% agarose gels. After blotting of RNA to Hybond-N (Amersham) followed by cross-linking at 1200 mJ, membranes were probed with random-primed probes derived from the full length oncogene cDNAs: the 2.6-kb BamHI fragment (Jat and Sharp, 1986; Jat *et al.*, 1986), the 1.6-kb fragment from BamHI-linkered *vsrc* (Schwartz *et al.*, 1983), a 3-kb fragment from BglII-linkered *vgagmyc* (Land *et al.*, 1983), the 800-bp EcoRI-linkered fragment consisting approximately of the first 800 bp 3′ to the ATG for NGFR (Buck *et al.*, 1987), the 550-bp BglII–SacI fragment for NF 68 (Lewis and Cowan, 1985), the 1600-bp SfiI–Xhol fragment from mouse BF-1 mN3 (Xuan *et al.*, 1995), the full length *vzg-1* coding sequence (Hecht *et al.*, 1995), and the 800-bp BamHI–HindIII fragment for γ-actin (Enoch *et al.*, 1986). Probes for *dlx-2/Tes-1* were produced by HindIII–Xbal digestion of plasmid E289-1, producing a 1.7-kb fragment (gift from D. J. Rubenstein, UCSF). These probes were hybridized in aqueous buffer at 65°C and washed to 0.2× SSC, 65°C.

**Chloramphenicol acetyltransferase assays.** Neuronal cell lines or fibroblast control lines were transiently transfected using DEAE-dextran in 10-cm dishes. After growth for 48 h, the cells were harvested, extracts were isolated, and an aliquot of the extract was incubated with acetyl CoA and [14C]chloramphenicol. Acetylation was identified by thin-layer chromatography.

**Transient transfections.** DEAE-dextran achieved the highest efficiencies. Between 10⁶ and 10⁷ cells were plated on 10-cm dishes and grown to the equivalent of 80% confluence. The cells were then washed in serum-free Optimum I (Gibco–BRL) and overlaid with 10–30 μg of double cesium gradient purified, sterile expression vector (as supercoiled plasmid)/100 mg of DEAE-dextran/
ml TBS (10 mM Tris, 0.15 M NaCl, pH 7.5). The mix was incubated at 37°C for 2–6 h, the overlay was removed, and the cells were shocked with 10% DMSO in PBS (10 mM phosphate, 0.15 M NaCl, pH 7.4) for 1 min at 25°C. The DMSO was removed and the cells were gently washed twice with fresh Opti-MEM I. The cells were then fed with Medium III and assayed at 48–72 h of growth at 37°C.

**β-Galactosidase histochemistry.** Transfected cells were fixed in 2% fresh paraformaldehyde in 20 mM PBS containing 2 mM MgCl₂ for 10 min. The fixative was then removed by aspiration and the culture was washed 3× in PBS–2 mM MgCl₂ and stained in X-gal solution (Turner and Cepko, 1987) containing 35 mM K₃Fe(CN)₆, 35 mM K₄Fe(CN)₆·H₂O, 2 mM MgCl₂ at 37°C for 8–12 h in the dark. The dishes were then rinsed in PBS and observed.

**Western blots.** Procedures are similar to those reported previously (Chun and Shatz, 1988a,b, 1989a). Cell lines were grown to high density, washed in cold PBS, incubated in primary antibody/antiserum, Chun, J. J. M., Schatz, D. G., Oettinger, M. A., Jaenisch, R., and Balti-

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REFERENCES


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