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Lineage specification of neuronal precursors in the mouse spinal cord

(neural progenitors/cell plasticity/neuronal differentiation/astrocytes/lineage determination)

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ABSTRACT **We have investigated the differentiation potential of precursor cells within the developing spinal cord of mice and have shown that spinal cord cells from embryonic day 10 specifically give rise to neurons when plated onto an astrocytic monolayer, Ast-1. These neurons had the morphology of motor neurons and >83% expressed the motor neuron markers choline acetyltransferase, peripherin, calcitonin gene-related peptide, and L-14. By comparison, <10% of the neurons arising on monolayers of other neural cell lines or 3T3 fibroblasts had motor neuron characteristics. Cells derived from dorsal, intermediate, and ventral regions of the spinal cord all behaved similarly and gave rise to motor neuron-like cells when plated onto Ast-1. By using cells that expressed the *lacZ* reporter gene, it was shown that >93% of cells present on the Ast-1 monolayers were motor neuron-like. Time-lapse analysis revealed that the precursors on the Ast-1 monolayers gave rise to neurons either directly or following a single cell division. Together, these results indicate that precursors in the murine spinal cord can be induced to differentiate into the motor neuron phenotype by factors produced by Ast-1 cells, suggesting that a similar factor(s) produced by cells akin to Ast-1 may regulate motor neuron differentiation *in vivo*.**

The induction of motor neurons in the spinal cord, one of the first neurons to arise developmentally (1–3), is influenced by epigenetic factors. It has been shown in the chicken that neuronal differentiation (4, 5), and later motor neuron differentiation (6, 7) in the spinal cord, can be regulated by the notochord. Further, conditioned medium from the notochord and floor plate can stimulate motor neuron differentiation in the intermediate zone, a region that does not normally give rise to motor neurons (8). These findings suggest that soluble factors within notochord conditioned medium are responsible for the motor neuron-inducing activity. In addition, these results suggest that precursor cells within the chicken spinal cord are not committed to any particular neuronal pathway. To examine the potential of individual precursors within the spinal cord, and to determine the direct effect of factors on the precursors, we have developed an *in vitro* assay in which the differentiation of individual precursors from the mouse spinal cord could be monitored.

MATERIALS AND METHODS

Cell Culture. All mice used in these experiments were of the CBA/CaWEHI strain except for transgenic mice, which were Bl/6 × DBA/2 hybrids (F_6). The transgenic mice (line H253) contained a *lacZ* gene insertion (9). Mice heterozygous for the transgene were mated and embryos containing the *lacZ* transgene were identified by the 5-bromo-4-chloro-3-indolyl β-D-galactoside histochemistry (10) of biopsied material. Spinal cord cells from embryonic day 10 (E10) to E16 mice, where E0 was the day a vaginal plug was detected, were prepared as described (11, 12).

Astrocytes were derived from E18 spinal cord as described (13); in addition, the cells were passed through 18- to 24-gauge hypodermic needles. Cells (1.5×10^6) were plated into a 25-cm^2 tissue culture flask (Falcon, Becton Dickinson) and passaged three times to eliminate neurons. These cells were plated into wells of a Permonex 8 chamber slide (Lab-Tek, Naperville, IL) and grown to form a confluent monolayer; some cultures were stained for the presence of glial fibrillary acidic protein to confirm the astrocytic nature of the cells.

The NZen 25, NZen 40, 2.3D, and Ast-1 cell lines have been described (14–16). Confluent monolayers of these cell lines or the BALB/c 3T3 cell line were obtained by plating 10^4 cells per well for the NZen 25, NZen 40, 2.3D, or Ast-1 cells or 5×10^3 BALB/c 3T3 cells per well onto Permonex 8 chamber slides and culturing them for 3 days in Dulbecco's modified Eagle medium with 10% (vol/vol) fetal bovine serum (FBS). The 3T3, 2.3D, NZen 25, and NZen 40 monolayers were irradiated (40 Gy) 3 hr prior to plating the spinal cord cells; however, Ast-1 monolayers were not irradiated as their proliferation was contact inhibited. Monolayers were then washed three times in serum-free Monomed medium (CSL, Melbourne, Australia) to remove the FBS. Spinal cord cells were then plated onto the monolayers at 300 cells per well in eight-well chamber slides in Monomed medium and cultured in a humidified incubator at 37°C and 5% CO_2 in air.

Immunohistochemistry and *in Situ* Hybridization Protocols. Cultures were incubated for 3 days and then fixed in either methanol (20 min at −20°C for neurofilament and peripherin staining), 4% paraformaldehyde [4°C for 30 min for Islet 1 (Isl-1) staining], or Zamboni's fixative (17) [4°C for 60 min for choline acetyltransferase (ChAT) or calcitonin gene-related peptide (CGRP) staining] and then washed in mouse tonicity phosphate-buffered saline (MTPBS) with 1% (vol/vol) FBS. The fixed and washed cultures were then incubated with either a rabbit anti-150-kDa neurofilament antibody (Chemicon), diluted 1:400, for 30 min at room temperature; a rabbit anti-peripherin antibody (18), used at 1:200 dilution overnight at 4°C; a rabbit anti-chicken ChAT antibody (19), applied at 1:500 dilution, overnight at 4°C; a polyclonal anti-CGRP antibody (Amersham) applied at 1:100 dilution, overnight at 4°C; or an anti-Isl-1 antibody (20) applied at 1:500 dilution, overnight at room temperature. All antibodies were diluted in MTPBS/1% (vol/vol) FBS. Immunoperoxidase detection was

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Abbreviations: CGRP, calcitonin gene-related peptide; ChAT, choline acetyltransferase; E, embryonic day; Isl-1, Islet 1.

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performed with a Vectastain kit and a Vectastain Elite kit (Vector Laboratories) for Isl-1.

Sense and antisense cRNA were synthesized *in vitro* from linearized pGEM plasmids using T7 and SP6 polymerases and a transcription system kit (Promega) according to the manufacturer's instructions with additional modifications (21). Staining was visualized using diaminobenzidine and cultures were temporarily coverslipped for counting. Following counting of the RL-14.5-positive cells, the coverslip was removed in water and the cultures were stained as described above with the anti-150-kDa neurofilament antibody. The number of neurofilament-positive cells was then counted and expressed as a percentage of L-14-positive neurons.

RESULTS

Spinal Cord Precursor Cells Differentiate into Neurons, Characteristic of Motor Neurons, When Plated Directly onto Ast-1 Monolayers. We previously reported that leukemia inhibitory factor promoted neuronal differentiation of individual precursor cells in the spinal cord when plated onto BALB/c 3T3 fibroblast monolayers (12). In preliminary studies, we screened a number of immortalized neural lines (NZen 25, NZen 40, 2.3D, and Ast-1) and found that Ast-1, which had previously been shown to influence neuronal differentiation of precursors in the developing brain (16, 22), stimulated a large number of neurons to arise from spinal cord precursor cells. In the present experiments, we have studied the effect of Ast-1 on determining neuronal cell fate in the developing spinal cord. When 300 E10 spinal cord cells were plated onto monolayers of the Ast-1 cell line we found that about 280 neurofilament-expressing neurons arose after 3 days—equivalent to 93% of the cells initially plated (Fig. 1). In comparison, when the spinal cord cells were plated at low cell density onto BALB/c 3T3 fibroblasts, far fewer neurons arose—at day 3, equivalent to <10% of the starting population (Fig. 1). The neurons that arose on the Ast-1 monolayers were larger and had a different morphology than those that arose on the 3T3 monolayers: multipolar, compared to the spherical, bipolar neurons on the 3T3 monolayers (compare Fig. 2*a* with 2*b*). Overall, the morphology of the neurons arising on the Ast-1 monolayers resembled purified, cultured motor neurons (23).

To further characterize the phenotype of the neurons arising on Ast-1 monolayers, the neurons were examined by immunohistochemistry for the expression of a number of markers that, in the spinal cord, are either exclusively or predominantly restricted to motor neurons: ChAT (24–26), CGRP (27), the intermediate filament peripherin (18), and the homeobox-containing gene *Isl-1* (20). In addition, *in situ* hybridization was carried out to detect mRNA for the lectin L-14, which has also been shown to be expressed preferentially by all motor neurons (28). All of these markers were expressed in neurons arising on the Ast-1 monolayer (Fig. 3). Whereas >83% expressed ChAT, CGRP, and peripherin and were also labeled with L-14 antisense probes, <4% of the neurons expressed *Isl-1* (Fig. 4). In comparison, only 9.5% ± 3.7% of the neurons arising on 3T3 fibroblast monolayers expressed ChAT. Thus, Ast-1 appears not only to promote the differentiation of a large number of neurons but also to favor the production of neurons with many of the characteristics of motor neurons.

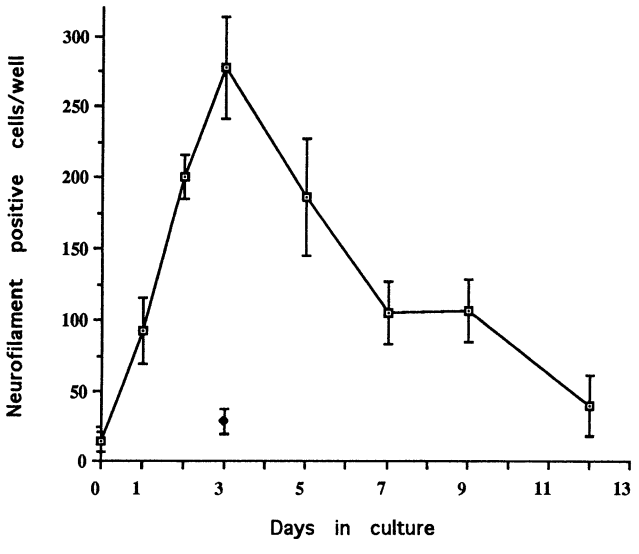

FIG. 1. Time course of the appearance of neurofilament-positive neurons after plating E10 spinal cord cells onto the Ast-1 monolayer. The total number of immunoperoxidase-stained neurons in each well was counted and the mean ± SD for each time point is shown ($n = 4$).

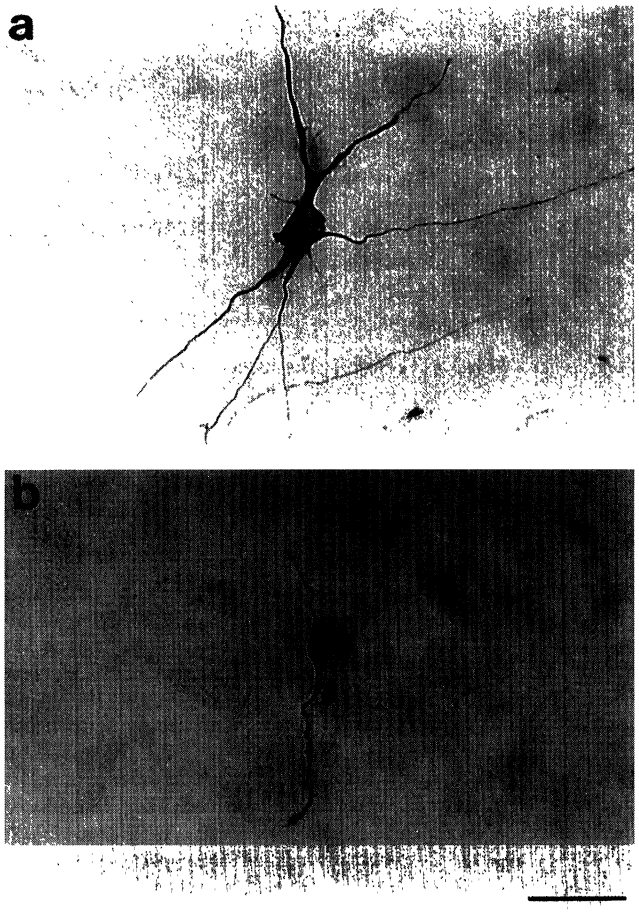

FIG. 2. Neurons arising on Ast-1 (*a*) or 3T3 (*b*) monolayers, stained by immunohistochemistry for the expression of neurofilament. Neurons that arose on the Ast-1 monolayers had larger cell soma and more processes than neurons arising on the 3T3 monolayers. (Bar = 50 μm.)

Motor Neuron-Like Cells Are Generated from the Majority of Precursor Cells and Not by the Continuous Expansion of a Small Precursor Subpopulation. Although our experiments demonstrate that motor neurons were the predominant neuronal phenotype arising on the Ast-1 monolayers, it was unclear as to what proportion of the precursor population gave rise to motor neurons, since nonneuronal cells could not be identified on the Ast-1 monolayer. Thus, we used cells from a transgenic mouse that expresses the *lacZ* reporter gene in all cell types (9), enabling the visualization of all cell types present, by assessing the cultures for β-galactosidase activity. After 3 days in culture, we assessed the number of β-galactosidase-positive cells expressing either neurofilament or ChAT to determine the proportion of cells that differentiated into

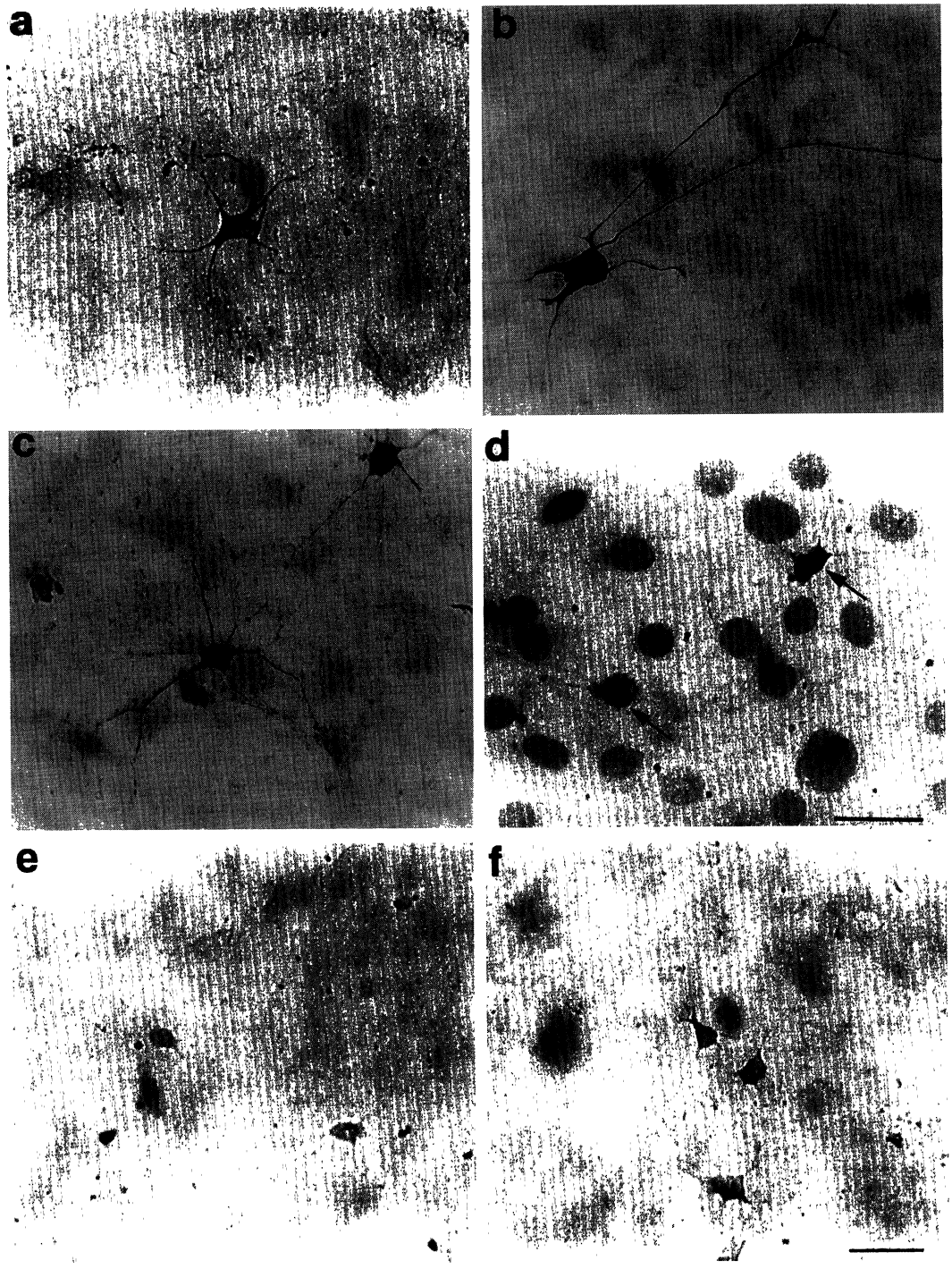

FIG. 3. Morphology and antigenic phenotype of neurons arising from E10 spinal cord cells cultured on Ast-1 monolayers for 3–4 days. Neurons had large polygonal cell somas with multiple processes (*a–f*) and stained positively for ChAT (*a*), peripherin (*b*), and CGRP (*c*); however, only some neurons expressed *Isl-1* (*d*, arrows). *In situ* hybridization of neurons arising on the Ast-1 monolayers labeled with either sense (*e*) or antisense (*f*) probes detected the specific expression of L-14 (*f*). (Bar = 50 μm.)

motor neurons. We found that 93% ± 1.7% of the β-galactosidase-positive cells were positive for neurofilament and ChAT and had a motor neuron morphology (Fig. 5*a*); the remaining β-galactosidase-positive cells had a nonneuronal morphology (Fig. 5*b*). These results establish that the vast majority of surviving spinal cord cells selectively differentiated into neurons of a single lineage with motor neuron-like characteristics.

To directly examine the developmental fate of individual cells in culture we followed a cohort of cells using time-lapse videomicroscopy. We found that of 16 cells monitored, 6 died during the first 18 hr of culture, 4 differentiated into neurons without intervening cell division, 5 divided once and both progeny differentiated into neurons, and 1 cell gave rise to 3 neurons; in total 17 neurons were generated. This experiment demonstrated that the majority of cells plated either differentiated into neurons directly or underwent one cell division, with all subsequent progeny differentiating into neurons.

Precursor Cells from All Regions of the Spinal Cord Differentiate into Motor Neurons on Ast-1 Monolayers. The preceding results suggested that cells from all regions of the spinal cord have the capacity to differentiate into motor

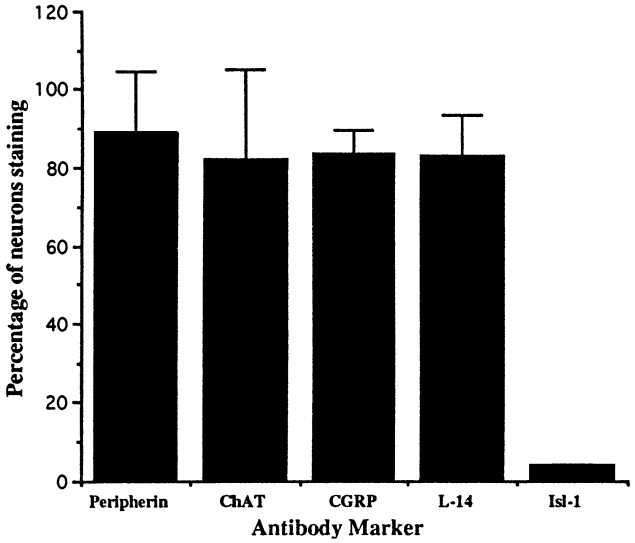

FIG. 4. Percentage of neurons (defined as neurofilament-positive) arising on Ast-1 monolayers staining positively for the various motor neuron markers shown in Fig. 4. Each point is the mean ± SD of the mean (n = 4).

neurons under the influence of the Ast-1 cell line, whereas *in vivo*, motor neurons are only derived from the ventral region

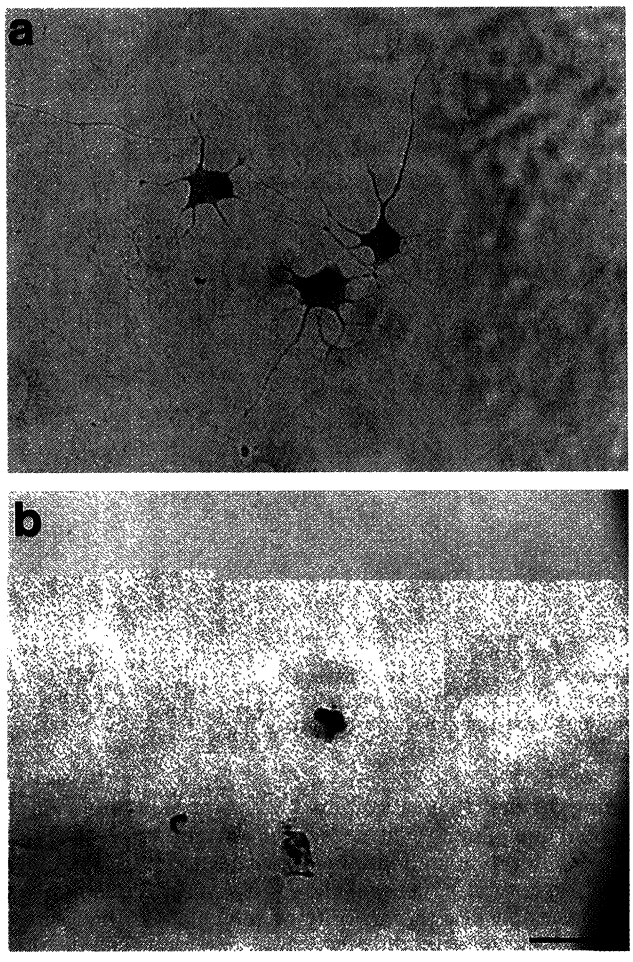

FIG. 5. Coexpression of β-galactosidase (blue) and neurofilament (brown) in motor neuron-like cells (*a*) arising on the Ast-1 monolayers after 3 days in culture. The β-galactosidase-positive cells that did not express neurofilament were of indeterminate morphology (*b*). (Bar = 50 μm.)

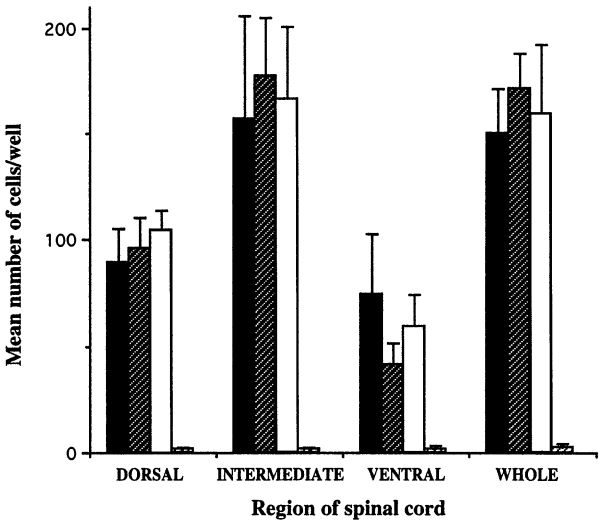

FIG. 6. Cells expressing either neurofilament (■), CGRP (▨), ChAT (□), or *Isl-1* (▨) in cultures of dorsal, intermediate, ventral, or whole spinal cord. Each point is the mean ± SD (n = 4).

of the spinal cord (1, 3). To investigate this further, we subdivided the neural tubes into dorsal, intermediate, and ventral segments prior to dissociating and plating cells. Neurons generated from all regions had a motor neuron morphology and all expressed CGRP, ChAT, and neurofilament (Fig. 6). It was also found that more neurons were generated from the dorsal and intermediate regions than from the ventral region (Fig. 6). As previously observed for the whole cord, the percentage of neurons expressing *Isl-1* was <4% regardless of the anatomical origin of the precursors.

DISCUSSION

The results of this study suggest that most neural precursors, regardless of their dorso-ventral position in the developing spinal cord, possess the ability to differentiate into neurons with many of the characteristics of motor neurons—properties usually confined to a population of precursors in the ventral region of the spinal cord. It was interesting to note that a larger number of motor neurons was generated from the intermediate zone compared with the ventral or dorsal regions since previous studies in the chicken have shown that motor neurons can be readily induced in the intermediate zone by factors emanating from the floor plate and notochord (8). This implies that the intermediate region contains larger numbers of uncommitted precursors. Overall, our results support the contention of cellular "plasticity" within the developing spinal cord of mammals and are in agreement with previous studies in the chicken that used retroviral tracing studies (29) and transplantation studies (4–7) to address this problem.

The finding that only motor neuron-like neurons develop on the Ast-1 monolayer, rather than a variety of neuronal phenotypes, suggests that Ast-1 cells selectively stimulate the differentiation of a single neuronal lineage. An alternative interpretation, given that motor neurons are the first neurons to arise in the spinal cord (1–3), is that any stimuli that cause neuronal differentiation early will result in motor neuron formation. This seems unlikely, as our previous studies showed that only 10% of neurons generated from E10 spinal cord precursor cells with leukemia inhibitory factor were motor neurons (12).

Surprisingly, only a small percentage of neurons generated in our cultures expressed *Isl-1*, since previous studies suggested that a large proportion of ChAT- and L-14-expressing neurons also expressed *Isl-1* (8, 28). However, we found that <4% of

the motor neurons derived from the whole E10 spinal cord expressed *Isl-1*; thus our results suggest that *Isl-1* expression is not required for all motor neuron differentiation. In support of this idea are recent findings demonstrating that *Isl-1* is not expressed in all motor neurons *in vivo* but is mainly restricted to cells of the median ventral horn (30). Ast-1 may preferentially induce motor neuron differentiation via a pathway independent of *Isl-1*. Alternatively, *Isl-1* expression may be linked to regional or functional specification rather than to lineage specification—a property shared by many other homeobox-containing genes in the nervous system.

Signals from the notochord or floor plate initiate motor neuron differentiation *in vivo* (6, 31) and in explant cultures (8), but because multiple interactions may occur in these assays it cannot be determined whether the signals from these structures act directly on individual precursor cells or via intermediate cells. The finding that motor neuron differentiation occurs in cells that are at some distance from the floor plate (8) supports the idea of an indirect induction of motor neuron differentiation by the floor plate. Motor neuron differentiation occurring on Ast-1 monolayers, however, is most likely to be the result of Ast-1 factor(s) acting directly on the E10 murine precursor, since the low cell density of precursors (300 cells per cm^2) makes it unlikely that paracrine stimulation is involved. Nevertheless, as the floor plate and the Ast-1 cells appear to have the characteristics of an astrocyte precursor (16, 32), it is possible that the Ast-1 and the floor plate cells may produce similar molecules. Little is known about these activities except that soluble factors appear to be involved: conditioned medium derived from the floor plate and notochord induces motor neuron differentiation in ectopic regions of the chicken spinal cord (8). Preliminary studies from our laboratory show that this motor neuron-inducing activity is present in medium conditioned by the Ast-1 cells (unpublished observations). Soluble factors derived from the Ast-1 cell line also stimulate the differentiation of neurons from the cephalic regions of the embryonic neural tube (16) and from precursors within the adult brain (33).

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