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Regulated Nuclear Trafficking of the Homeodomain Protein Otx1 in Cortical Neurons

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Otx1 is a homeodomain protein required for axon refinement by layer 5 neurons in developing cerebral cortex. Otx1 localizes to the cytoplasm of progenitor cells in the rat ventricular zone, and remains cytoplasmic as neurons migrate and begin to differentiate. Nuclear translocation occurs during the first week of postnatal life, when layer 5 neurons begin pruning their long-distance axonal projections. Deletion analysis reveals that Otx1 is imported actively into cell nuclei, that the N-terminus of Otx1 is necessary for nuclear import, and that a putative nuclear localization sequence within this domain is sufficient to direct nuclear import in a variety of cell lines. In contrast, GFP–Otx1 fusion proteins that contain the N-terminus are retained in the cytoplasm of cortical progenitor cells, mimicking the distribution of Otx1 *in vivo.* These results suggest that ventricular cells actively sequester Otx1 in the cytoplasm, either by preventing nuclear import or by promoting a balance of export over import signals.

INTRODUCTION

The development of precisely wired neuronal circuits requires that axons grow to appropriate targets and form specific patterns of synaptic connections. In many animals, the emergence of the final adult pattern of connectivity is preceded by the formation of transient "exuberant" connections. A striking example of exuberance in development is seen in layer 5 neurons of the cerebral cortex, which extend axonal connections to multiple subcortical targets and then eliminate a subset of these projections during early postnatal life (Stanfield *et al.,* 1982; O'Leary and Stanfield, 1985; Stanfield and O'Leary, 1985). Although little is known about the mechanisms that control large-scale axonal pruning, recent experiments have revealed that the homeodomain transcription factor Otx1 is required for the elimination of exuberant projections by layer 5 neurons. In mice lacking functional Otx1 protein, layer 5 neurons in visual cortex fail to prune their normally transient axon collaterals in the inferior colliculus and spinal cord. Otx1 is known to bind DNA and regulate gene transcription (Furukawa *et al.,* 1997), suggesting that the requirement for Otx1 in axon refinement is mediated by the transcriptional control of target genes involved in axon elimination. However, during normal development, Otx1 protein is initially localized to the cytoplasm of the layer 5 neurons that form subcortical connections. It is not until the time at which axon pruning begins that Otx1 translocates into cell nuclei. The temporal correlation between the nuclear localization of Otx1 and the period of axon remodeling suggests that pruning may be initiated by the entry of Otx1 into the nucleus.

The regulated translocation of transcription factors, including homeodomain proteins, from the cytoplasm into the nucleus is an increasingly common theme in developmental biology. Transcription factors and other large proteins are transported actively into and out of cell nuclei through the action of importins/karyopherins (reviewed in Jans *et al.,* 2000). These molecules recognize specific targeting signals on intracellular proteins and initiate their transport through the nuclear pore complex, which mediates the exchange of macromolecules through the nuclear envelope. Nuclear import is typically triggered by one or more clusters of basic amino acids known as the nuclear localization signal (NLS), whereas export is regulated by the less well understood nuclear export signal (NES), which is generally composed of nonpolar residues. Whether a protein bearing such a target signal is localized to the cytoplasm or nucleus can be regulated actively: the

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targeting sequence can be masked through protein-protein interactions, or it may be modified directly by phosphorylation. These strategies provide mechanisms by which the transcriptional machinery can respond to changes in the environment or to cell–cell signaling (Turpin *et al.,* 1999).

A well-characterized example of intermolecular target sequence masking is represented by the p65 subunit of the DNA-binding protein NF-κB (RelA), which contains an NLS (Jans *et al.,* 2000). In quiescent lymphocytes, I-κB binds the NLS of NF-κB/RelA, rendering the NLS inaccessible to interactions with importin proteins. During lymphocyte stimulation, I-κB is phosphorylated and degraded (Liou and Baltimore, 1993), thus unmasking the NF-κB NLS and triggering nuclear import. In contrast, the subcellular localization of the yeast transcription factor Pho4 is regulated by direct posttranslational modification (Kaffman *et al.,* 1998; Komeili and O'Shea, 1999; Jans *et al.,* 2000; Komeili and O'Shea, 2000). Under phosphate-rich conditions, Pho4 is phosphorylated at three sites, one within an NLS and two within an NES, altering the affinity of distinct importins for these sequences. Phosphorylation prevents Pho4 from binding the import protein Pse1p and facilitates binding to export proteins, thus triggering the export of Pho4 from the nucleus and its accumulation in the cytoplasm.

To explore the mechanisms that control the subcellular localization of Otx1 during development, we first delineated the timing by which Otx1 accumulates in the nuclei of cortical neurons. We then began to dissect systematically the Otx1 protein to ascertain whether putative NLS sequences could confer nuclear localization in either neuronal or nonneuronal cells. Our results suggest that Otx1 contains a functional NLS within the N-terminus of the homeodomain that directs Otx1 to the nuclei of nonneuronal cells and mature neurons. However, this sequence fails to confer nuclear localization of Otx1 in cortical progenitor cells: Otx1–GFP fusion proteins accumulate in the cytoplasm of ventricular cells, as does Otx1 *in vivo.* Collectively our results are consistent with the possibility that Otx1 can interact with a binding partner that either masks the Otx1 NLS to block nuclear import or favors a balance of export over import signals.

RESULTS

The Subcellular Localization of Otx1 Is Regulated in Vivo

We have reported previously that Otx1 protein is localized to the cytoplasm of early cortical progenitor cells, but is found in the nuclei of postmitotic neurons within the deep layers of the cortical plate during early postnatal life (Weimann *et al.,* 1999). Here we provide a more detailed description of the time course by which Otx1 undergoes nuclear localization.

Cells in the ventricular zone of the developing cerebral wall express Otx1 from the earliest age studied (E13) (Fig. 1). Otx1 protein is present in ventricular cells throughout the period of neurogenesis, but is less obvious at later ages (E17–E19), consistent with previous reports that the expression of *Otx1* mRNA in the ventricular zone is apparently downregulated as development proceeds (Frantz *et al.,* 1994). As reported previously, Otx1 protein appears to be excluded from cell nuclei in ventricular cells and instead exhibits a cytoplasmic localization (Figs. 2A and 2B).

As young postmitotic neurons migrate through the intermediate zone and enter the cortical plate, Otx1 protein is found primarily in neurons destined for the deep layers 5 and 6. Immunolabeling is not apparent in neurons of the upper layers 2–4 at any time during development (Figs. 1 and 3), although transient labeling of the marginal zone (future layer 1) is visible between E17 and P1 (Fig. 1). It is conceivable that some of this labeling is derived from the apical dendrites of deep-layer neurons, which arborize within layer 1 (Chagnac-Amitai *et al.,* 1990; Kasper *et al.,* 1994). Otx1 localizes to the nuclei of deep layer neurons by the end of the first week of postnatal life. At E19, the Otx1 antibody labels dendrites and strongly stains cell bodies, with a subset of cells showing nuclear staining (Figs. 1 and 2C). Shortly after birth, at P1 and P3, many cells show nuclear staining although cytoplasmic label is also apparent (Figs. 1, 3A, and 3B). By P6, most of the staining appears exclusively nuclear (Fig. 1). At P13 little or no cytoplasmic label is apparent; the bulk of the Otx1 protein is localized to the nuclei of neurons in both layer 5 (Fig. 3C) and layer 6 (Fig. 3D), although expression levels are lower in layer 6, as observed previously (Frantz *et al.,* 1994; Weimann *et al.,* 1999). Otx1 retains its nuclear localization in deep layer neurons at all times thereafter, throughout adulthood (not shown). These results reveal that the subcellular localization of Otx1 is regulated in a dynamic temporal and spatial pattern during cortical development.

Otx1 Contains a Basic Sequence That Is Necessary and Sufficient for Nuclear Localization

We first attempted to identify the nuclear localization sequence that directs the import of Otx1 into cell nuclei. The 355 amino acid sequence of Otx1 contains three distinct sequence motifs (Fig. 4A): the homeodomain

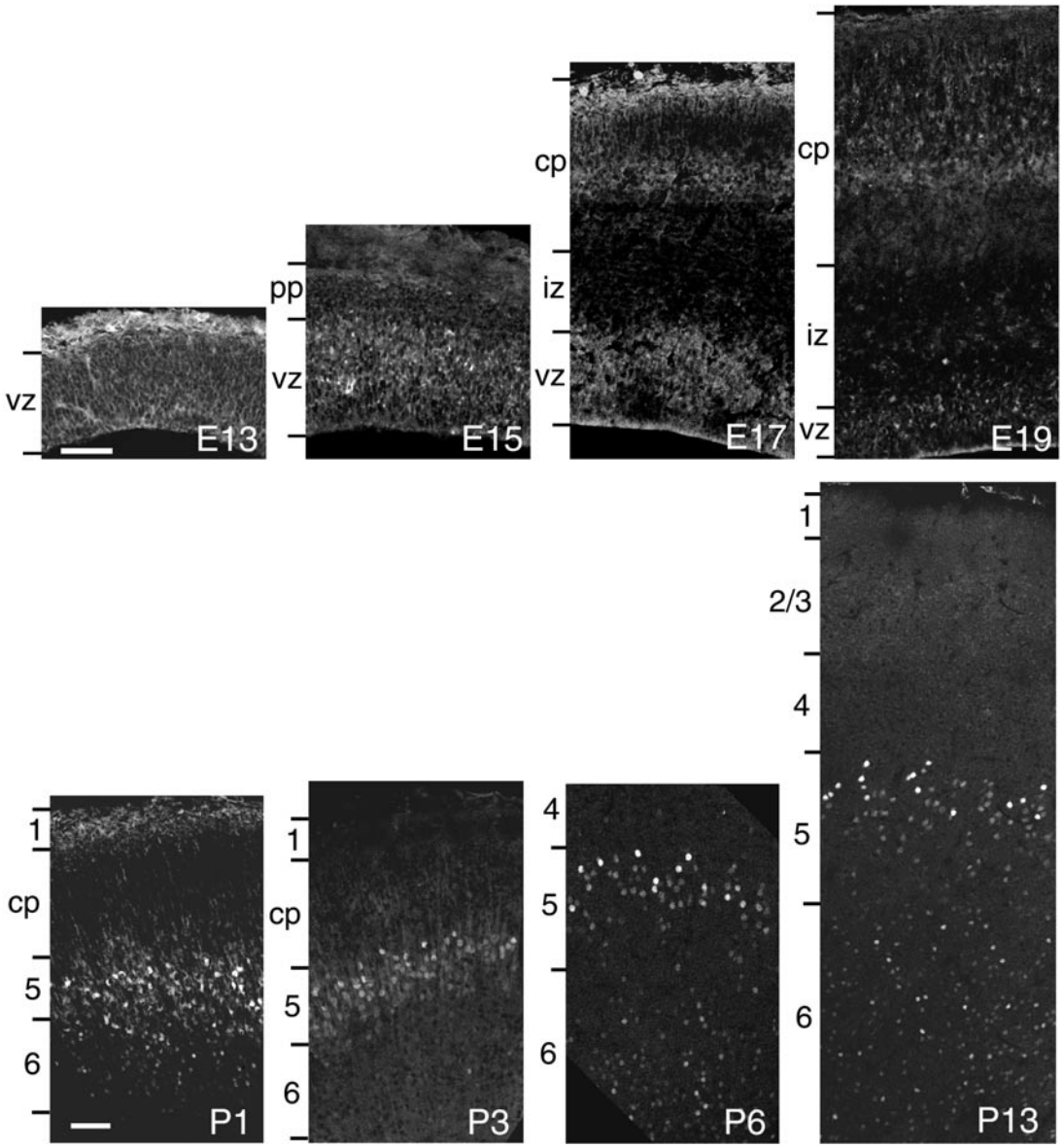

FIG. 1. Distribution of Otx1 protein in the developing rat cortex during embryonic and postnatal development. Otx1 was revealed by immunostaining with the 5F5 antibody. The expression of Otx1 goes through 4 phases during development: (1) E13–E15: Otx1 expression is high in the ventricular zone (vz) during early embryonic development. (2) E17–E19: At late embryonic stages, expression is apparent in cells of the newly formed cortical plate (cp). There is still some staining in the ventricular zone. (3) P1–P3: Otx1 expression is largely restricted to a band of deep layer cells in the cortex, where the protein is found in both the cytoplasm and nucleus. (4) P6 and older: Otx1 expression is confined to the nuclei of a subset of neurons in layers 5 and 6. Abbreviations: vz, ventricular zone; pp, preplate; cp, cortical plate; iz, intermediate zone; the numbers 1–6 denote cortical layers 1–6. Scale bars, 50 μm.

(amino acids 37–97), a histidine-rich region (amino acids 255–302, containing 24 His residues), and a pair of terminal repeats (amino acids 316–351). The primary amino acid sequence of Otx1 does not contain a consensus NLS (PKKKRKV, from SV40 large T antigen: (Mattaj and Englmeier, 1998). However, a stretch of highly basic amino acids found at the beginning of the homeodomain (amino acids 36–42: **RKQRRER**, asterisk in Fig. 4A) might potentially function as an NLS. Nuclear localization sequences are quite variable but are generally composed of basic residues (Jans *et al.*, 2000), and in homeodomain proteins an NLS is often found within or adjacent to the homeodomain itself (Abu-Shaar *et al.*, 1999; Berthelsen *et al.*, 1999; Bryan and

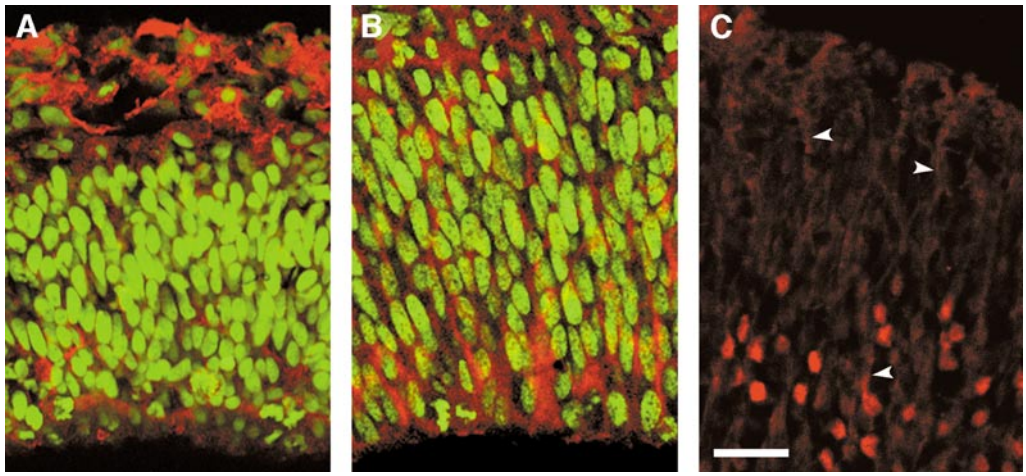

FIG. 2. Subcellular localization of Otx1 protein during embryonic development. High-power confocal images of Otx1 staining (red) in the (A) E13 ventricular zone, (B) E15 ventricular zone, and (C) E19 cortical plate. Syto-11 (green) was used to reveal the nuclei of cells in (A) and (B). (A, B) Otx1 is cytoplasmic in ventricular zone cells at E13 and E15. (C) At E19, Otx1 is present in both the cytoplasm and nuclei of cells in the cortical plate. Immunolabel is also detectable in the apical dendrites (arrowheads) of these neurons. Scale bar, 25 μm.

Morasso, 2000; Fei and Hughes, 2000; Parker *et al.*, 2000). An NLS can also assume a bipartite structure, with two stretches of basic amino acids separated by a spacer of 10–12 amino acids (Jans *et al.*, 2000). In addition to the basic sequence at the start of the homeodomain, Otx1 contains a possible bipartite NLS at the N-terminus of the homeodomain between amino acids 87 and 111: **KNRRAK**CRQQQQSGNGT**KSRPVKKK** (diamonds in Fig. 4A).

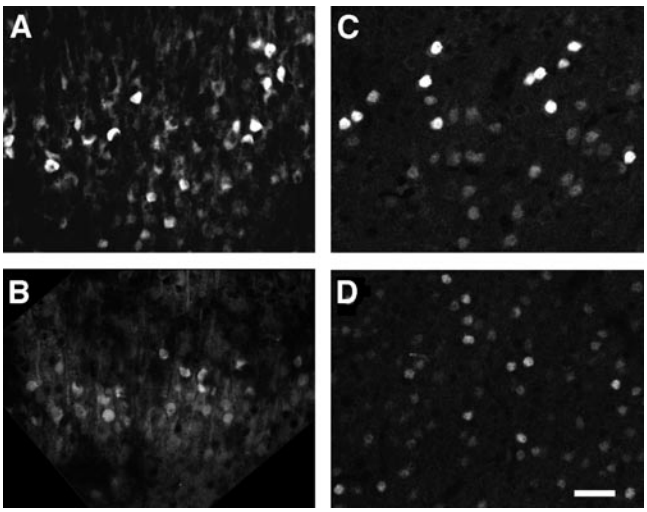

FIG. 3. Subcellular localization of Otx1 protein during postnatal development. High-power confocal images of Otx1 staining in the deep layers of the cortical plate at (A) P1, (B) P3, (C) P13 (layer 5), and (D) P13 (layer 6). (A, B) Deep layer neurons show Otx1 staining in both the cytoplasm and nucleus during early postnatal life. Immunostaining of apical dendrites is particularly noticeable. (C, D) In the more mature neurons in layers 5 and 6 of P13 rats, Otx1 protein is concentrated in cell nuclei. Scale bar, 25 μm.

To localize the sequences in Otx1 that are required for nuclear localization, we generated a series of six *Otx1* deletion constructs encoding fusions between fragments of *Otx1* and the *aequorin* Green Fluorescent Protein (GFP) to track the subcellular localization of each fusion protein (Fig. 4). We also generated a fusion between *GFP* and full-length *Otx1*. Three of the deletion constructs encode proteins in which an N-terminal domain of Otx1 (including the homeodomain) is deleted: ΔN97 (containing amino acids 98–355), ΔN250 (amino acids 251–355), and ΔN309 (amino acids 310–355). The remaining three constructs encode proteins containing truncations at the C-terminus: ΔC98 (containing amino acids 1–97), ΔC254 (amino acids 1–253), and ΔC310 (amino acids 1–309). Each of the latter three constructs includes the RKQRRER sequence at the C-terminus of the homeodomain that might function as an NLS. ΔC98 disrupts the putative bipartite sequence contained at the N-terminus of the homeodomain.

Each of the *GFP–Otx1* fusion constructs was transfected into 3T3, 293T, PC12, or P19 cells using calcium phosphate transfection methods. Data are shown for 3T3 cells (Figs. 4 and 6) and 293T cells (Fig. 8); similar results were observed using the other two cell lines (data not shown). Transfection of *GFP* alone into 3T3 cells results in labeling of the entire cell, including both cytoplasm and nucleus (Fig. 4B). The presence of GFP in

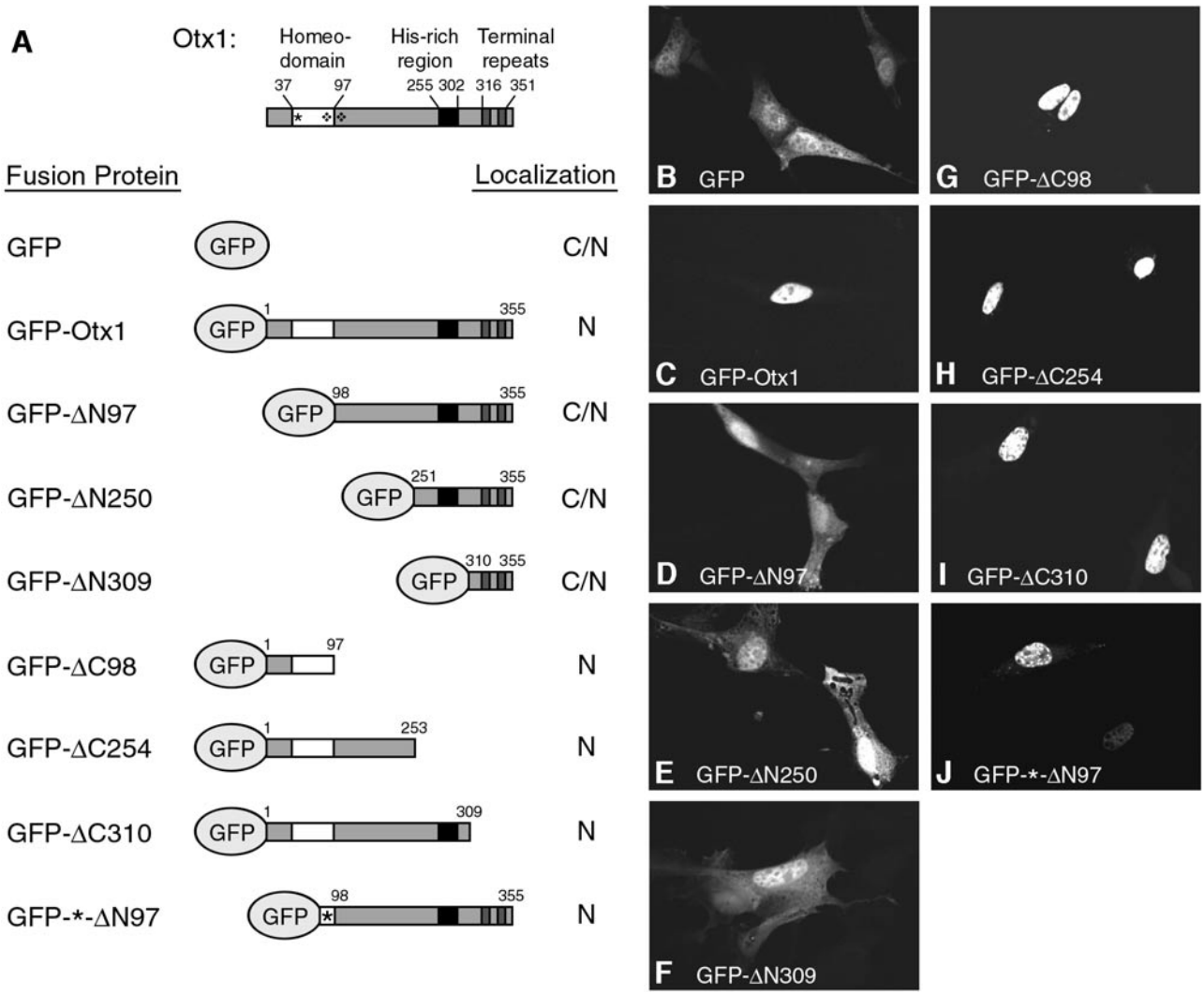

FIG. 4. Deletion analysis of Otx1 protein. Full-length and truncated *Otx1* coding sequence were fused with *GFP* coding sequences in a *GFP* expression vector (*pEGFP-C2*) and were transfected into 3T3 cells. (A) Otx1 contains an N-terminal homeodomain (aa 37–97), a Histidine-rich region (aa 255–302), and two so-called Otx repeats (aa 316–351) at the C-terminus. A also depicts the various fusion constructs that were transfected into 3T3 cells and summarizes the distribution of GFP fluorescence in the cytoplasm (C) or nuclei (N) of the transfected cells. (B) Transfection of *GFP* alone into 3T3 cells results in labeling of both the cytoplasm and nucleus. (C–J) Two patterns of distribution of GFP–Otx1 fusion proteins were observed: Fusion proteins containing the N-terminal portion of Otx1 (aa 1–97), including the homeodomain, or containing the RKQRRER sequence (the putative Otx1 NLS, labeled as *), localize to the nuclei of 3T3 cells (C, G–J). In contrast, fusion proteins that lack either the N-terminal domain of Otx1 or the RKQRRER sequence are distributed throughout the cytoplasm and nuclei of 3T3 cells (D–F).

the nucleus is due presumably to passive diffusion through nuclear pores. The nuclear pore complex forms a channel of ~9 nm in effective diameter through which relatively small proteins (molecular weights <20–30 kDa) can diffuse quickly and through which even larger proteins (up to ~60 kDa) can move slowly (Gorlich and Kutay, 1999). The low molecular weight of GFP (26 kDa) is likely to enable its free diffusion into cell nuclei.

In contrast to GFP, the GFP–Otx1 fusion protein (predicted molecular weight ~68 kDa) was found exclusively in cell nuclei (Fig. 4C), consistent with the presence of a domain within Otx1 that promotes its active import into nuclei and / or its retention in this organelle. (Fusions in which GFP was attached to Otx1 at its C-terminus yielded identical results; data not shown.) Indeed, each of the GFP–Otx1 fusion proteins that contained the N-terminal homeodomain (GFP–ΔC98, –ΔC254, and –ΔC310: Figs. 4G–4I) were also localized exclusively to cell nuclei. In contrast, each fusion protein in which the N-terminus, including the homeodo-

main, was deleted (GFP–ΔN97, –ΔN250, and –ΔN309: Figs. 4D–4F) adopted a distribution identical to that of GFP alone, with protein visible in both the cytoplasm and the nucleus. These results are consistent with the hypothesis that the RKQRRER sequence within the homeodomain plays an important role in nuclear localization. They suggest further that the putative bipartite sequence at amino acids 87–111 is not required for nuclear localization, because this sequence is disrupted in GFP–ΔC98 yet the fusion protein clearly resides in the nucleus.

To ascertain directly whether the RKQRRER sequence is sufficient to confer nuclear localization, we generated the construct *GFP–*–ΔN97,* in which GFP is fused to the RKQRRER sequence and to the Δ*N97* C-terminal fragment of *Otx1*. Although GFP–ΔN97 was found in the cytoplasm and nucleus of 3T3 cells (Fig. 4C), addition of the RKQRRER sequence was sufficient to confer a nuclear localization to the resulting fusion protein (Fig. 4J). Collectively these data demonstrate that a sequence contained within the N-terminal domain of Otx1 is required for the nuclear localization of GFP–Otx1 fusion proteins in both nonneuronal (3T3) and neuronal (PC12, P19) cell lines and that the basic sequence RKQRRER at the N-terminus of the homeodomain is sufficient to confer nuclear localization. This sequence can therefore function as an NLS.

Active Import of GFP–Otx1 into Cell Nuclei

The experiments discussed above suggest but do not prove that GFP–Otx1 fusion proteins, which contain an NLS are transported actively into cell nuclei, and those that lack an NLS are not. Because the size of GFP–Otx1 (~68 kDa) is at the upper limit for possible diffusion through the nuclear pore complex, there is a small possibility that the fusion protein enters cell nuclei by diffusion. Furthermore, fusion proteins lacking the homeodomain (GFP–ΔC fusions) were found in both the cytoplasm and nuclei of 3T3 cells, suggesting either that these small proteins diffuse into cell nuclei or that they contain a nuclear localization activity. To test these possibilities, we generated larger fusion proteins to preclude passive diffusion into nuclei. These proteins were created by attaching a second *GFP* moiety onto the C-terminus of the *GFP–Otx1*, *GFP–ΔN97,* and *GFP–ΔC254* constructs. Western analysis revealed that each of the resulting fusion proteins had a molecular weight of at least 85 kDa (Fig. 5, lanes 3–5).

Transfection of *GFP–Otx1–GFP* into 3T3 cells generated a fusion protein of ~95 kDa molecular weight that localized exclusively to cell nuclei (Fig. 6B), identical to the distribution of GFP–Otx1 (Fig. 6A). The GFP–

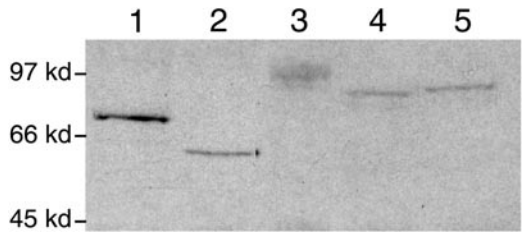

FIG. 5. Western analysis of 3T3 cells transfected by *GFP–Otx1* fusion constructs. Lane 1, GFP–Otx1 (~68 kDa); Lane 2, GFP–ΔN97 (~58 kDa); Lane 3, GFP–Otx1–GFP (95 kDa); Lane 4, GFP–ΔN97–GFP (~85 kDa); Lane 5, GFP–ΔC254–GFP (~86 kDa). The size of fusion proteins deduced from Western analysis agrees with the calculated molecular weight (in parenthesis after each construct). The three fusion proteins in Lanes 3–5 have molecular weights in excess of 80 kDa and thus should not be able to enter cell nuclei by diffusion.

ΔC254–GFP fusion protein (~86 kDa) was also found in nuclei (Fig. 6E), as was the smaller GFP–ΔC254 (Fig. 6D), consistent with the presence of a putative NLS in the Otx1 homeodomain. The GFP–ΔN97–GFP fusion protein (~85 kDa), however, localized exclusively to the cytoplasm of transfected 3T3 cells (Fig. 6C). This result was anticipated because the truncation of the N-terminal of Otx1 (and presumed absence of the NLS), coupled with the absence of diffusion as a route for nuclear entry, should preclude the entry of the fusion protein into the nucleus. These data support the notion that Otx1 is imported actively into cell nuclei, and that an NLS is required for this activity.

GFP–Otx1 Accumulates in the Cytoplasm of Cortical Ventricular Cells But Is Nuclear in Differentiating Neurons

The results described above demonstrate that Otx1 contains a sequence that functions as an NLS and directs the protein into the nuclei of both neuronal and nonneuronal cell lines. However, we have also demonstrated that during normal development, Otx1 protein is found initially in the cytoplasm of ventricular cells and young neurons; only later as deep layer neurons differentiate does the protein eventually translocate into cell nuclei (Figs. 1–3). Why then is Otx1 localized faithfully to the nuclei of 3T3, 293T, PC12, or P19 cells, but found in the cytoplasm of cortical progenitors? One possibility is that the embryonic form of Otx1 lacks the N-terminal sequence required for nuclear localization; however, Western analysis of embryonic vs postnatal brains reveals no difference in the apparent molecular weight of Otx1 (data not shown). It therefore seems more likely that Otx1 is actively sequestered in the

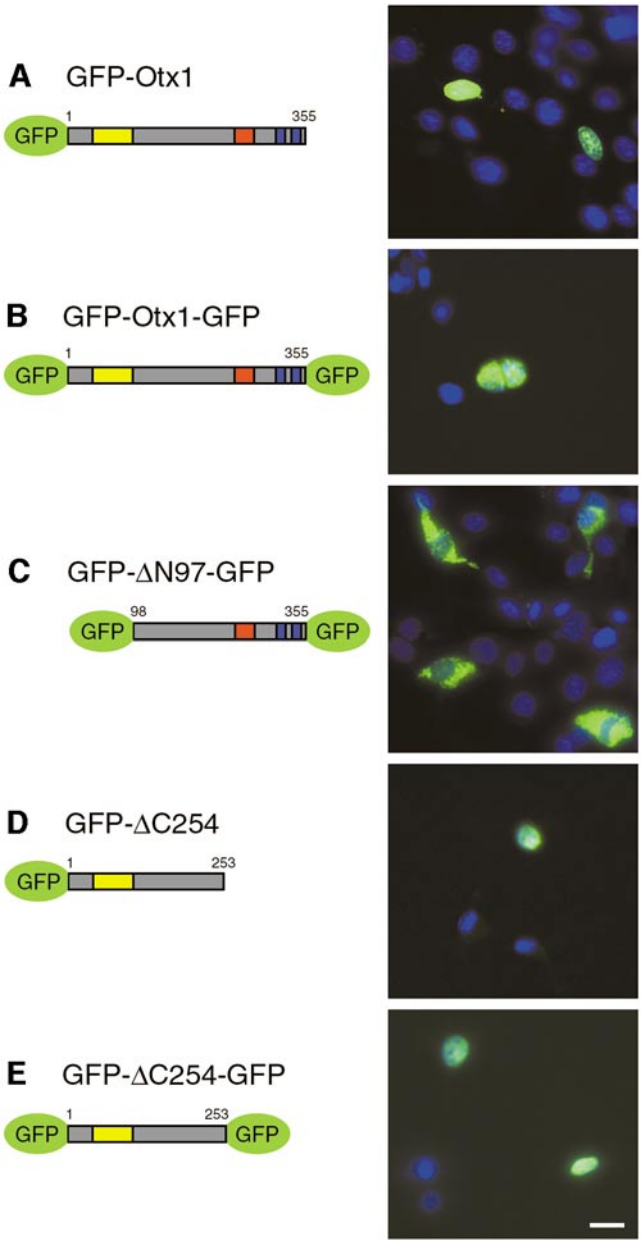

FIG. 6. The N-terminal region of Otx1 is required for the import of large fusion proteins into the nuclei of 3T3 cells. The left panel depicts the different *GFP–Otx1* fusion constructs, and the right panel shows the results of transfecting these constructs into 3T3 cells (green, GFP fluorescence; blue, DAPI nuclear counterstain). Fusion proteins that contain the N-terminal region of Otx1 are localized to cell nuclei (A, B, D, and E). (C) The large (85 kDa) GFP–ΔN97–GFP fusion protein that lacks the the N-terminal region of Otx1 localizes to the cytoplasm of 3T3 cells and appears to be excluded from cell nuclei, presumably due to the absence of the Otx1 NLS. Scale bar, 20 μm.

cytoplasm of cortical ventricular cells, or actively exported from cell nuclei, at early stages of development.

To assess the mechanisms that regulate the translocation of Otx1 from the cytoplasm to the nucleus of cortical cells *in vivo*, we transfected primary cortical cells with *GFP–Otx1* fusion constructs and assessed the subcellular localization of the resultant fusion proteins. Biolistic transformation was used to express the fusion proteins in ventricular cells that occupy a relatively normal tissue environment. Gold particles (0.6 μm) coated with the different cDNA constructs were introduced into living explants of the E14 cerebral wall using a Biolistic "gene gun" (Lo *et al.*, 1994). Visualization of transfected ventricular cells revealed that both GFP–Otx1 and GFP–ΔC98, each of which concentrated in the nuclei of 3T3 cells, exhibited a cytoplasmic distribution in ventricular zone cells (Figs. 7A and 7B). Both fusion proteins contain the N-terminal NLS, yet neither was

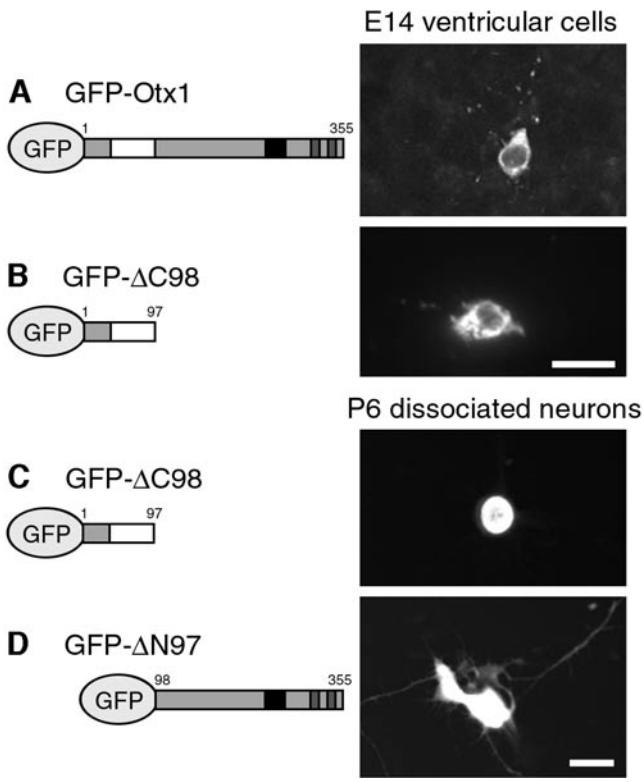

FIG. 7. Distribution of GFP–Otx1 fusion proteins in E14 ventricular cells and P6 neurons. The constructs shown in the left panel were introduced into E14 ventricular cells (A and B) by the Biolistic gene-gun method and into dissociated P6 neurons by calcium phosphate transfection. (A) GFP–Otx1 localizes to the cytoplasm of E14 cortical progenitor cells. (B) Transfection of *GFP–ΔC98* shows that the first 97 aa of Otx1 are sufficient to confer cytoplasmic localization in cortical ventricular cells. (C) In contrast, GFP–ΔC98 localizes to the nuclei of differentiating neurons from the deep layers of P6 brains. (D) The N-terminal domain of Otx1 is required for nuclear localization in differentiating neurons, since the GFP–ΔN97 fusion protein appears in both the nuclei and cytoplasm of transfected neurons, including neurites extending from the cell body. Scale bar, 10 μm.

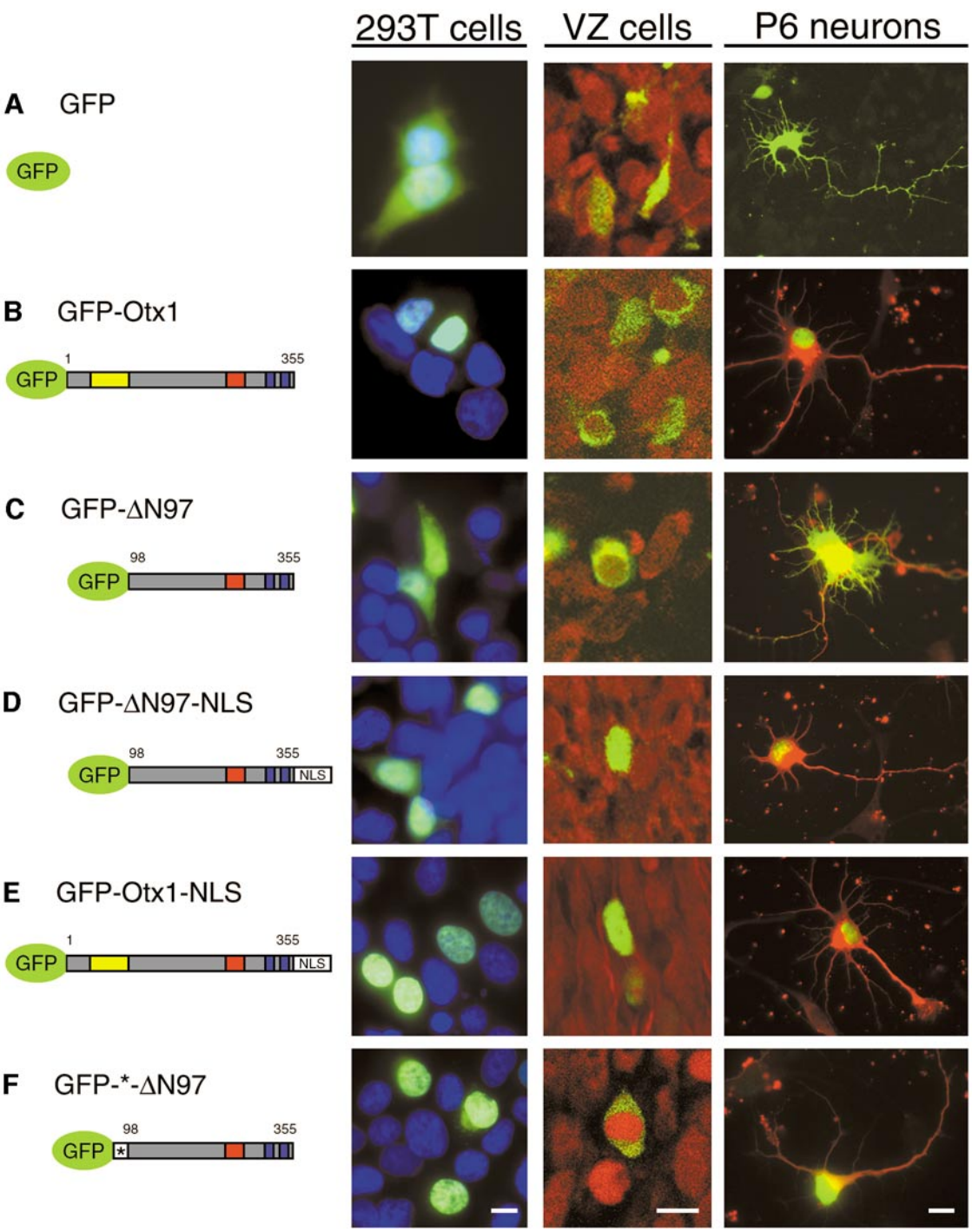

FIG. 8. Distribution of GFP–Otx1 fusion proteins in 293T cells, E14 ventricular cells, and dissociated P6 cortical neurons. The constructs shown in the left panel were introduced into 293T cells using calcium phosphate transfection methods (green, GFP fluorescence; blue, DAPI nuclear counterstain); into the E14 ventricular zone (VZ) by electroporation into intact brains (green, GFP fluorescence; red, propidium iodide nuclear counterstain); or into dissociated P6 neurons by calcium phosphate transfection (green, GFP fluorescence; red, TuJ1 immunostaining, except in panel A where no counterstain was used). (A) GFP is found diffusely in the cytoplasm and nuclei of transfected 293T cells, cortical ventricular cells, and P6 neurons. (B) Fusion proteins of GFP with full-length Otx1 localize to the nuclei of 293T cells and postmitotic cortical neurons, but accumulate in the cytoplasmic rings surrounding the nuclei of most transfected ventricular cells. (C) GFP–ΔN97 fusion proteins are found in the cytoplasm of all three cell types, consistent with the absence of an NLS in this construct. (D) Addition of an SV40 nuclear localization sequence (NLS) to GFP–ΔN97 results in fusion proteins that enter the nuclei of all three cell types, demonstrating that the SV40 NLS is sufficient to direct nuclear import in cortical progenitors and neurons. (E) Addition of the SV40 NLS to GFP–Otx1 is sufficient to confer nuclear localization in most cortical VZ cells. GFP–Otx1–NLS is also nuclear in 293T cells and cortical neurons, as expected. (F) Addition of the putative endogenous NLS sequence, RKQRRER (abbreviated as *) to *GFP–ΔN97* is sufficient to direct the GFP–*–ΔN97 fusion protein into the nuclei of 293T cells and P6 neurons. However, this fusion protein is retained in the cytoplasm of ventricular progenitor cells. Scale bars, 10 μm.

nuclear in its distribution. Transfection of ventricular cells with the *GFP–ΔN97*, *–ΔN250*, or *–ΔN309* fragments also yielded a cytoplasmic distribution of the resultant fusion protein, as expected because none of these constructs contained the N-terminal NLS (data not shown).

In contrast to ventricular progenitor cells, but like normal layer 5 neurons *in vivo*, postmitotic neurons that were obtained from the deep layers of P6 rats, placed in culture, and transfected with the same constructs exhibited quite distinct patterns of protein localization. GFP–Otx1 and GFP–ΔC98 localized to the nuclei of cultured neurons (Figs. 7C and 8B, and data not shown), suggesting that the NLS in Otx1 can and does function to confer nuclear localization in differentiating neurons. The N-terminus of Otx1 is required for nuclear localization: GFP–ΔN97 is distributed throughout the cytoplasm of neurons in culture, including neurites (Figs. 7D and 8C). The fusion protein is also present in neuronal nuclei, presumably by diffusion through the nuclear pore complex as in 3T3 cells (c.f. Fig. 4D).

These data show that the localization of GFP–Otx1 in the cytoplasm of ventricular cells and in the nuclei of differentiating neurons mimics the normal distribution of Otx1 protein *in vivo*. The presence of a functional NLS in Otx1 suggests that its cytoplasmic localization in progenitor cells is regulated either by mechanisms that mask this NLS and prevent nuclear import, or by mechanisms that promote the active export of Otx1 protein from the nucleus.

Addition of the SV40 NLS to GFP–Otx1 Promotes Nuclear Localization in Ventricular Cells

To ascertain whether the cytoplasmic localization of Otx1 is the result of a mechanism that prevents the import of Otx1 into ventricular cell nuclei, or one that promotes its rapid export after nuclear entry, we generated GFP–Otx1 fusions that also contain the NLS from SV40 large T antigen, a well characterized NLS that contains a cluster of basic amino acids (PKKKRKV[132]; Jans *et al.*, 2000). We reasoned that if the endogenous NLS of Otx1 is masked in progenitor cells through inter- or intramolecular interactions, thus preventing nuclear import, addition of the SV40 NLS should result in the entry and localization of the fusion protein in cell nuclei. If, however, Otx1 localization in ventricular cells is normally mediated by an export mechanism, the presence of an additional NLS should fail to alter its cytoplasmic localization due to the rapid extrusion of Otx1 from nuclei.

GFP–Otx1 fusion proteins were expressed in ventricular cells from E14 rat embryos by electroporation of DNA constructs into whole brains (see Experimental Methods) and transfected cells were visualized in brain slices. The same constructs were also introduced into 293T cells or P6 cortical neurons using calcium phosphate transfection. The introduction of GFP alone into 293T cells, cortical ventricular cells, or postmitotic neurons resulted in the detection of fusion proteins in both cell cytoplasm and nuclei (Fig. 8A), as expected from studies in 3T3 cells. When ventricular cells were transfected with *GFP–Otx1*, the GFP label surrounded cell nuclei (Fig. 8B), similar to the pattern seen in dissociated progenitors. In contrast, transfection of 293T cells or in P6 cortical neurons with *GFP–Otx1* resulted in exclusively nuclear labeling (Fig. 8B). Deletion of the N-terminal 97 amino acids of Otx1, including the homeodomain, resulted in cytoplasmic localization of the GFP fusion proteins in all three cell types (Fig. 8C). This result was expected because the *ΔN97* Otx1 construct does not contain a functional NLS.

To ascertain whether the SV40 NLS is sufficient to direct the import of Otx1–GFP fusion proteins into the nuclei of either cortical or 293T cells, we fused the SV40 NLS onto the *GFP–ΔN97* Otx1 construct, which lacks an endogenous NLS. The resulting GFP–ΔN97–NLS fusion protein was found in the nuclei of 293T cells, cortical ventricular cells, and postmitotic neurons (Fig. 8D). Although there was faint labeling of the cytoplasm of 293T cells, the fusion protein was strongly concentrated in the nuclei of ventricular cells and neurons, indicating that the SV40 NLS is sufficient to mediate nuclear localization in cortical progenitors and their progeny.

We then tested the localization of fusion proteins in which the SV40 NLS has been added to the C-terminus of GFP–Otx1. GFP–Otx1–NLS was found in the nuclei of 293T cells and cortical neurons, as expected (Fig. 8E). Strikingly, the SV40 NLS was indeed sufficient to alter the localization of Otx1 in cortical ventricular cells: the fusion protein was localized to the nucleus in most ventricular cells that expressed GFP–Otx1–NLS (Fig. 8E). These results show that ventricular cells are capable of importing full-length Otx1 into their nuclei, and raises the question of why they do not do so normally. One possibility is that the normal localization of Otx1 in the cytoplasm of ventricular cells results from a mechanism in which the import of Otx1 into cell nuclei is blocked. We hypothesize that the NLS of Otx1 may be masked, either through a post-translational modification such as phosphorylation (Komeili and O'Shea, 1999; Komeili and O'Shea, 2000), or by the binding of an interacting protein present in the cytoplasm of ventricular cells, similar to the interaction between NF-κB/RelA and I-κB (Liou and Baltimore, 1993). Addition of a second NLS from SV40 enables the import machinery to

recognize Otx1 as a target for import, despite the presence of the binding partner.

The RKQRRER Sequence Directs Nuclear Localization in Postmitotic Neurons and Nonneuronal Cells, But Not Progenitors

According to this model, the endogenous NLS in Otx1 may bind directly to a cytoplasmic protein, which prevents the NLS from interacting with importins. If this were indeed the case, addition of the putative endogenous NLS from Otx1 (RKQRRER) onto GFP–ΔN97 should be sufficient to trigger nuclear import in 293T cells or neurons, but not in cortical progenitors due to the presence of a binding partner. To test this hypothesis, we transfected the *GFP–*–ΔN97* construct into all three cell types and found that although the endogenous NLS does direct the fusion protein into the nuclei of both 293T cells and neurons (Fig. 8F), GFP–*–ΔN97 is retained in the cytoplasm of ventricular progenitors. These data suggest that the sequence RKQRRER functions as an NLS in neurons and that the recognition of this sequence is blocked in ventricular cells. Our results are consistent with the possibility that ventricular cells express a binding partner that interacts directly with the NLS and prevents it from initiating nuclear import. The eventual translocation of Otx1 into cell nuclei could be triggered by the downregulation, degradation, or modification of this putative binding partner during later stages of neuronal differentiation. Further studies will be required to test these hypotheses directly.

The Transcriptional Activation Domain of Otx1 Maps to the C-Terminal Repeat Region

To further define the domains within Otx1 that mediate distinct functions, we identified the sequences required for the transcriptional activation of target genes. A series of *Otx1* deletion constructs were fused to the *LexA* DNA-binding domain (Fig. 9) and introduced into *Leu2* reporter yeast cells, along with a *LacZ* reporter plasmid. The resulting transformants were tested for growth on Leu-minus plates and for β-galactosidase activity (Golemis *et al.,* 1987; Golemis and Khazak, 1997; Fashena *et al.,* 2000). Fusions between LexA and full length Otx1 activated the transcription of reporter genes, as expected. The only other fusion construct that mediated transcriptional activation was that containing amino acids 310–355, the region containing the so-called Otx repeats (Furukawa *et al.,* 1997) (Fig. 9). These results suggest that the transcriptional activation domain of Otx1 maps to the C-terminal repeat region. Similar results have been obtained from deletion analysis of the related protein Crx (Chau *et al.,* 2000; Mitton *et al.,* 2000).

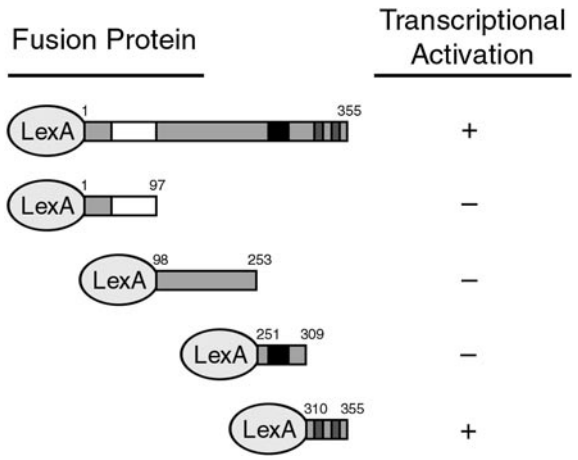

FIG. 9. Mapping the transactivation domain of Otx1. Fusions of *LexA* and full-length or truncated *Otx1* were constructed as shown in the left panel and were tested for transactivation activity in the two-hybrid system (see Experimental Methods). Transactivation of target constructs (+) was only found with fusion proteins that contain amino acids 310–355 of the Otx1 protein.

DISCUSSION

The transcription factor Otx1 is required for axon refinement by neurons in the deep layers of the developing cerebral cortex (Weimann *et al.,* 1999). Here we have presented a more detailed account of the subcellular localization of Otx1 in progenitors and neurons during cortical development, showing that Otx1 is retained in the cytoplasm of progenitor cells and undergoes nuclear translocation during the first week of postnatal life in the rodent, a time that corresponds to the onset of axon remodeling by layer 5 neurons. The introduction of GFP–Otx1 fusion proteins into 3T3 cells, 293T cells, and other cell lines revealed that Otx1 is imported actively into cell nuclei. The N-terminal region of the protein is necessary for nuclear import, and a basic sequence contained within this region is sufficient to direct the import of fusion proteins into cell nuclei, suggesting that this sequence functions as an NLS. All variants of GFP–Otx1 that contain this domain target to the nuclei of both neuronal and nonneuronal cell lines. However, GFP–Otx1 fusion proteins that contain the N-terminal domain are retained in the cytoplasm of cortical progenitor cells, suggesting that these cells actively direct the localization of Otx1 to the cytoplasm rather than the nucleus. A number of possible

mechanisms, including the expression of proteins that bind to Otx1 and modify its subcellular distribution or the phosphorylation of nuclear targeting sequences, could regulate the localization of Otx1 during cortical development.

The Nuclear Localization of Otx1 Correlates with the Onset of Axonal Remodeling by Layer 5 Neurons

Immunostaining of sections through the developing cortex shows that Otx1 undergoes a progressive shift in its localization from the cytoplasm to cell nuclei as cortical development proceeds. In progenitor cells in the ventricular zone of E13 and E15 rat embryos, Otx1 is largely or exclusively cytoplasmic. By early postnatal ages, Otx1 immunoreactivity is visible in both the cytoplasm and the nuclei of differentiating neurons in the deep layers of the cortical plate. By P6 the protein has assumed its adult distribution within a subset of neurons in cortical layers 5 and 6. The timing of Otx1 nuclear translocation correlates closely with the onset of axon remodeling by layer 5 neurons. Layer 5 subcortical projection neurons initially extend axons to a variety of transient targets; between the ages of ~P4–P20 in the rat, these neurons eliminate inappropriate connections through the selective loss of certain axon branches (Stanfield *et al.*, 1982; O'Leary and Stanfield, 1985; Stanfield and O'Leary, 1985). Otx1 is normally expressed by the neurons that form these "exuberant" connections, and in the absence of Otx1 the layer 5 neurons fail to undergo axon pruning, instead retaining their normally transient axonal connections into adulthood (Weimann *et al.*, 1999). Because Otx1 encodes a transcription factor, and because the normal onset of pruning correlates temporally with the import of the protein into the nucleus, we hypothesize that Otx1 regulates the initiation of pruning through a mechanism that involves the transcription of target genes involved in axon elimination. The timing of entry of Otx1 into neuronal nuclei may thus control the onset of axon elimination.

Interestingly, an Otx homolog in sea urchin embryos appears also to undergo a regulated translocation from the cytoplasm to the nucleus, although this is unrelated to axonal connectivity. The sea urchin homolog of Otx (SpOtx) is found in the cytoplasm during early development at the 16-cell stage and only later translocates into cell nuclei (Chuang *et al.*, 1996), where it is thought to activate aboral ectoderm-specific gene expression (Mao *et al.*, 1996; Li *et al.*, 1999). Two-hybrid analysis revealed that a proline-rich region of SpOtx resembling an SH3-binding domain can bind to α-actinin, suggesting that the protein may be anchored to the cytoskeleton prior to nuclear import (Chuang *et al.*, 1996). However, we think it unlikely that the cytoplasmic localization of Otx1 in cortical progenitors can be explained by a similar interaction because the GFP–ΔC98 fusion protein, which localizes to the cytoplasm of ventricular cells, does not contain a comparable proline-rich SH3 domain.

It is increasingly common to encounter transcription factors that translocate between the cytoplasm and nucleus in a dynamic manner. Alterations in the subcellular localization of transcriptional regulators add a readily modifiable control over cellular transcription. For example, the rapid import of NF-κB into the nucleus during lymphocyte stimulation (Liou and Baltimore, 1993) or Pho4 into yeast nuclei during phosphate starvation (Komeili and O'Shea, 2000) enables cells to alter gene expression in rapid response to changing environmental conditions or cell–cell signaling. During development, even homeodomain proteins involved in patterning or cell fate regulation can undergo dynamic and highly regulated changes in subcellular localization. For example, the Extradenticle (Exd) protein in *Drosophila* is present in most cells of the fly embryo but is translocated into the nuclei of only a subset, in response to Wingless and Decapentaplegic signaling (Mann and Abu-Shaar, 1996; Aspland and White, 1997). In each of these cases the presence of a transcription factor in the cytoplasm precludes transcriptional activity yet poises the cell to respond rapidly and efficiently to signals from the environment.

One wonders why the brain goes to the trouble of transcribing *Otx1* mRNA and translating it into protein for several weeks before Otx1 is called into action and enters cell nuclei. Like Exd, Otx1 is present in the cytoplasm of many cells but localizes to the nuclei of just a subset. One possibility is that Otx1 actually does have a role in controlling important cellular functions at times when the protein appears cytoplasmic. Our immunohistochemical observations do not rule out the possibility that Otx1 shuttles actively between the cytoplasm and nucleus, with the balance of the protein residing in the cytoplasm at any given time. Otx1 may therefore regulate transcription at times earlier than the first week of postnatal life. In addition, the homeodomain of Otx1 is most closely related to that of bicoid, a protein that acts not only within cell nuclei to regulate transcription but also in the cytoplasm of the syncytial *Drosophila* embryo to regulate protein translation (Rivera-Pomar *et al.*, 1996). While it is not known whether Otx1 is similarly active in the cytoplasm of vertebrate cells, genetic data do suggest a role for Otx1 at stages prior to nuclear localization in cortex. Two different *Otx1* mutations result in lethality at around

the time of birth when the mutation is present in a C57/Bl6 background strain (Suda *et al.*, 1996; Weimann *et al.*, 1999), and the cerebral cortex of *Otx1* mutants is smaller than that of wild type animals (Acampora *et al.*, 1996), suggesting that Otx1 may be important for early processes that regulate cell proliferation or survival.

We do not know what signals or events trigger the translocation of Otx1 from the cytoplasm of cortical progenitors into cell nuclei. It is tempting to speculate that the arrival of the axons of layer 5 neurons at their subcortical targets may provide such a signal, but this has not yet been tested. The possibility that neuronal activity could play a role is also intriguing, since patterned activity clearly plays a role in the remodeling of axonal connections between the retina and thalamus (Shatz, 1996), thalamus and cortex (Chapman *et al.*, 1986), and between cortical neurons with callosally projected axons (Innocenti, 1981). However, it is not yet known whether activity influences either the refinement of exuberant axonal projections from layer 5 neurons or the movement of Otx1 into neuronal nuclei at the onset of pruning.

Otx1 Contains a Functional NLS within Its Homeodomain

In order to regulate the transcription of target genes, transcription factors must enter cell nuclei and gain access to DNA. The size of the nuclear pore complex severely limits the entry of proteins into the nucleus by diffusion, thus most nuclear proteins are imported actively into the nucleus from the cytoplasm, an activity that is generally mediated by an NLS on the protein cargo. Otx1 does not contain a consensus NLS sequence but does contain two domains that could potentially function as an NLS. We identified a possible bipartite NLS sequence located near the C-terminus of the homeodomain; however, our deletion studies suggest that this sequence is not required for nuclear import because the ΔC98–GFP fusion protein (in which the sequence is disrupted) still localizes efficiently to the nuclei of 3T3 cells. However, we found that Otx1 also contains a highly basic sequence at the N-terminus of the homeodomain (RKQRRER) that is sufficient to confer nuclear localization onto GFP–Otx1 fusion proteins in fibroblast cell lines. In the presence of this basic sequence, GFP–Otx1 fusion proteins were concentrated in cell nuclei, whereas in its absence fluorescence was present in both the cytoplasm and nucleus. Addition of a second GFP moiety to such constructs, which increased the molecular weights of the fusion proteins to preclude diffusion through the nuclear pore, resulted in an exclusively cytoplasmic distribution. Finally, transferring the RKQRRER sequence onto the ΔN97 C-terminal fragment of Otx1, which is normally cytoplasmic, proved sufficient to localize the resulting fusion protein to the nuclei of neurons and fibroblast cells. We therefore believe that the RKQRRER sequence serves as a functional NLS for Otx1.

Like Otx1, a number of other homeodomain proteins have an NLS that resides in or near the homeodomain itself. Examples include Lhx3 (Parker *et al.*, 2000), Dlx3 (Bryan and Morasso, 2000), Exd (Abu-Shaar *et al.*, 1999), PBX1 (Berthelsen *et al.*, 1999), and the Otx homolog Crx (Fei and Hughes, 2000). Indeed, the presence of a homeodomain in the DNA binding domain is widely generalizable to other transcription factors including members of the POU (Sock *et al.*, 1996), HMG box (Poulat *et al.*, 1995), HLH (Tapscott *et al.*, 1988), and bZip (Waeber and Habener, 1991) protein families. It is not known why nuclear import and DNA binding are so closely localized on these molecules but it has been speculated to be an evolutionary consequence of exon shuffling (Sock *et al.*, 1996).

Mechanisms Controlling the Subcellular Localization of Otx1 during Development

The shift in the localization of Otx1 from the cytoplasm to the nucleus during cerebral cortical development suggests that the subcellular localization of Otx1 is controlled actively in neurons and their precursors. A number of mechanisms can regulate the localization of transcription factors within eukaryotic cells. One possibility is that phosphorylation of Otx1 regulates its recognition by import or export receptors. Direct phosphorylation of a target recognition site can mask the site and prevent its interactions with importins, as is the case for the NLS of Pho4 (Komeili and O'Shea, 1999, 2000). Phosphorylation can also enhance the affinity of importin or exportin proteins for targeting sequences, as exemplified by the NLS of the *Drosophila* transcription factor Dorsal (Briggs *et al.*, 1998), the NLS of NF-AT (Jans *et al.*, 2000; Komeili and O'Shea, 2000), and the NES of Pho4 (Komeili and O'Shea, 1999, 2000). Our studies have revealed no obvious differences in the apparent molecular weight of Otx1 at embryonic ages, when the protein is cytoplasmic, compared to postnatal ages following nuclear localization (data not shown). We thus have no evidence suggesting that Otx1 is differentially phosphorylated at distinct stages of development. There is, however, a potential phosphorylation site that both overlaps the Otx1 NLS sequence and is contained within the GFP–ΔC98 fusion, which targets to the cytoplasm of ventricular cells. The sequence TPR (amino acids 34–36) overlaps with RKQRRER (amino

acids 36–42) and presents a potential site for Protein Kinase C phosphorylation. Furthermore, the nearby site SQLD (amino acids 48–51) could serve as a site for Casein Kinase II action. All other consensus phosphorylation sites in Otx1 are in C-terminal regions of the protein that are not required for cytoplasmic localization. The possibility that Otx1 is differentially phosphorylated at embryonic vs postnatal ages therefore warrants further direct exploration.

Another mechanism that might regulate the subcellular distribution of Otx1 is the masking of target sequence recognition by protein-protein interactions. The localization of several transcription factors is regulated by binding partners: for example, the binding of I-κB to the NLS of NF-κB prevents nuclear import (Liou and Baltimore, 1993; Jans *et al.*, 2000), and the NES of NF-AT binds to calcineurin when calcium levels are low, thus blocking nuclear export (Komeili and O'Shea, 2000). The intermolecular interactions that regulate localization can be surprisingly complex, as exemplified by the homeodomain protein Extradenticle (Exd), which binds the Meis family member Homothorax (Hth) in cells of the developing *Drosophila* embryo (Rieckhof *et al.*, 1997). Exd contains both NLS and NES sequences. In the absence of Hth, the NES is dominant and Exd is found in the cytoplasm. However, when bound to Hth, the NES of Exd is effectively inactivated, shifting the balance from export to import and resulting in nuclear localization (Abu-Shaar *et al.*, 1999; Affolter *et al.*, 1999; Berthelsen *et al.*, 1999). The additional presence of an NLS in Hth may contribute to the formation of a nuclear complex (Abu-Shaar *et al.*, 1999).

The behavior of Otx1 in cortical neurons is reminiscent of these and other examples of the regulated transport of transcription factors from the cytoplasm to the nucleus. The patterns of localization of Otx1 fusion proteins in cultured cell lines vs cortical ventricular cells is consistent with the possibility that Otx1 binds to a protein that normally sequesters Otx1 in the cytoplasm of progenitors. The simplest explanation is that the putative interacting protein binds directly to the Otx1 NLS and blocks nuclear import in progenitor cells; degradation or loss of the interacting protein in older neurons would result in nuclear localization as the Otx1 NLS is unmasked. Three lines of evidence are consistent with this idea. First, addition of the SV40 NLS to an Otx1–GFP fusion protein confers nuclear localization in ventricular cells, suggesting that the endogenous NLS of Otx1 is blocked or masked in some way. Second, both full-length Otx1–GFP fusions and the smallest N-terminal fragment (ΔC98, which contains the NLS) behaved identically in our transfection assays: both are nuclear in 3T3 cells and other cell lines, as well as in mature neurons, whereas both are cytoplasmic in ventricular cells. These data suggest that the information required for cytoplasmic localization in progenitor cells is contained within the N-terminal 97 amino acids of Otx1. Third, although the RKQRRER sequence confers nuclear localization of GFP–*–ΔN97 in nonneuronal cells and in postmitotic neurons, GFP–*–ΔN97 is found in the cytoplasm of ventricular cells. This result suggests that the RKQRRER sequence itself may be selectively masked or modified in progenitors. Collectively our data are consistent with the hypothesis that a putative binding partner in ventricular cells binds directly to the N-terminal domain of Otx1 and possibly to the NLS itself. This hypothesis remains to be tested directly by searching for possible Otx1 binding partners in ventricular cells.

The role of export mechanisms in controlling the subcellular localization of Otx1 remains to be defined. Otx1 does not contain an obvious NES, such as the canonical sequence LxxLxxLxL or its close variants (Wen *et al.*, 1995; Bogerd *et al.*, 1996; Mattaj and Englmeier, 1998). However, it is possible that Otx1 in ventricular cells might bind to a partner protein that contains an NES and thus promotes a balance of export over import. Precedent for such a mechanism is suggested by the interaction between the protein I-κBα/p65, which contains an NES, binds to the p50/p105 subunit of NF-κB, and localizes this complex to the cytoplasm (Jans *et al.*, 2000). Although I-κBα does bind directly to the NLS of NF-κB p50/p105, crystallographic data suggest that this interaction does not render the NLS inaccessible to importins and raise the possibility that the NES of I-κBα may deliver the complex out of the nucleus (Jans *et al.*, 2000). It is plausible that Otx1 also has a binding partner that contains an NES, and that this domain bears the primary responsibility for localizing Otx1 in the cytoplasm of cortical progenitors and young neurons.

It is also possible that Otx1 contains an atypical NES that competes with the NLS for control of the protein's subcellular localization. Studies of the mechanisms that control nuclear export have lagged behind those examining import, and the NES sequences that have been defined are varied. In addition to the set of four critically spaced leucines that trigger nuclear export by the Crm1 exportin protein (Gorlich and Kutay, 1999), sequences rich in hydrophobic amino acids may trigger export (Bogerd *et al.*, 1996; Abu-Shaar *et al.*, 1999) as can sequences that are quite divergent from the typical leucine-rich motif (Meyer *et al.*, 1996). In considering whether Otx1 contains an atypical NES, it is important to note that both the full length Otx1–GFP fusion and the ΔC98 fusion protein are sequestered in the cyto-

plasm after transfection into progenitor cells (Figs. 7A and 7B). Thus, if an NES within Otx1 contributes to its cytoplasmic localization in young cells, the NES must be contained within the initial 97 amino acids of the protein. This N-terminal domain does contain sequences rich in leucine and other hydrophobic residues (e.g., amino acids 5–30: LKQPPYGMNGLGLAGPAMDLLHPSVG and amino acids 50–58: LDVLEALFA), but whether these sequences signal nuclear export remains to be determined experimentally.

EXPERIMENTAL METHODS

Immunohistochemistry

The 5F5 monoclonal anti-Otx1 antibody (Weimann *et al.,* 1999) was used to immunostain sections through the developing cerebral cortex of rats between the ages of embryonic day (E) 13 and postnatal day (P) 15. Embryonic brains were dissected out and fixed by immersion in 4% paraformaldehyde. Postnatal animals were sacrificed with an overdose of Nembutal and perfused with 4% paraformaldehyde. Brains were then sectioned on a freezing microtome at 25 μm. Sections were mounted onto slides and processed for immunohistochemistry using the Otx1 antibody (1:10) and a Texas red-conjugated secondary antibody. Sections were counterstained with the nuclear dye Syto11 (Chenn and McConnell, 1995). Images of labeled sections were acquired with a BioRad MRC600 confocal microscope and processed with Adobe Photoshop.

Construction of Recombinant Plasmids

DNA fragments encoding either full-length or truncated *Otx1* coding sequence were amplified by PCR using a rat *Otx1* cDNA clone as template (Frantz *et al.,* 1994). Cloning sites (*Eco*RI and *Bam*HI) were introduced by adding the sequence of restriction sites at the 3′ end of PCR primers so that the amplified *Otx1* coding sequences could be cloned in frame into pEGFP-C2 and -N1 plasmids (Clontech Laboratories). All cloning sites were sequenced to assure accuracy. The following primer pairs were used to generate the various *Otx1* coding sequences: full length *Otx1,* oligos 1 and 8; Δ*N97,* oligos 2 and 8; Δ*N250,* oligos 3 and 8; Δ*N309,* oligos 4 and 8; Δ*C98,* oligos 1 and 5; Δ*C254,* oligos 1 and 6; and Δ*C310,* oligos 1 and 7. The oligonucleotide sequences were as follows: Oligo1: 5′-gcacgaattcatgatgtcttacctcaaacaacc-3′; Oligo2: 5′-cgacgaattccagagcgggaatggaacgaaa-3′; Oligo3: 5′-cgacgaattctaccttgcgcccatgcactc-3′; Oligo4: 5′-cgacgaattcctcgccttcaactctgccga-3′; Oligo5: 5′-cgcaggatcctgctgctggcggcacttg-3′; Oligo6: 5′-cgcaggatccgcaaggtaggagctgcagt-3′; Oligo7: 5′-cgcaggatccccagagcctccataaccttg-3′; Oligo8: 5′-cgcaggatccaagacctggaaccaccatga-3′.

To generate larger fusion proteins containing two GFP moieties, the *EGFP* coding sequence was amplified by PCR and the cloning sites *Bam*HI and *Eco*RI were added to the 5′ and 3′ of the *EGFP* coding sequence. The *EGFP* sequence was then cloned into the *GFP–Otx1, GFP–*Δ*N97,* and *GFP–*Δ*C98* plasmids (N1) at *Bgl*II and *Eco*RI sites. The resulting DNA constructs are able to express fusion proteins with two copies of GFP (Fig. 6).

To introduce the SV40 *NLS* sequence into the fusion proteins, two oligonucleotides (5′-gatcctggcggcggtaagaagaagcggaagctgggtctg-3′ and 5′-gatccagacccagcttccgcttcttcttaccgccgccag-3′), which encode the SV40 NLS (GGG**KKKRKV**G), were annealed and then cloned into the *GFP–Otx1, GFP–*Δ*N97,* and *GFP–*Δ*C98* constructs at the *Bam*HI site. Similar methods were used to introduce the basic sequence of Otx1 (RKQRRER) into the *Xho*I–*Eco*RI site in the pEGFP-C2 backbone of the *GFP–*Δ*N97* fusion construct. The two oligonucleotides used were 5′-tcgagtcggaagcagcgacggcagcgcg-3′ and 5′-aattcgcgctcccgtcgctgcttccgac-3′.

Transfection Methods

Standard calcium phosphate transfection methods (Ausubel *et al.,* 1997) were used to introduce DNA constructs into 3T3, P19, PC12, and 293T cells. Transfected cells were incubated overnight in DMEM containing 10% calf serum, fixed with cold 2% paraformaldehyde, and mounted for observation. The transfection efficiency varied between 10–30% for 3T3 cells and 1–10% for PC12 and P19 cells. 293T cells were counterstained using 0.5 μg/ml DAPI (Sigma). Images were acquired using an epifluorescence microscope (Nikon Optiphot) and cooled Digital Camera (Princeton Instruments) and processed using the Open Labs Imaging System (Improvision).

To transfect postmitotic cortical neurons, tissue from the deep layers (layers 4, 5, and 6) of the P6 rat cerebral cortex was dissected out and dissociated into a single-cell suspension (McConnell, 1988). Trypan blue was used to assess the viability of the resulting cells (generally >90%). Cells were plated onto poly-d-lysine and laminin-coated 4-chamber slides at a density of 50,000 cells/cm^2 and incubated overnight in "complete" Neurobasal medium (Gibco-BRL) supplemented with 1× B27 supplement (Gibco-BRL), 0.5% glucose, 1× glutamine (Gibco-BRL), and 1× Penicillin-Streptomycin (Gibco-BRL) overnight. Under these conditions, the majority of cells develop a neuronal morphology. Cells

were then subjected to calcium phosphate transfection, as described above, and cultured for an additional 24 h, at which point they were fixed in 4% paraformaldehyde and analyzed. This method yielded a transfection efficiency of ~1%.

E13/14 progenitor cells were transfected using one of two methods. For experiments in Fig. 7, the Bio-Rad Biolistic gene gun system (Lo *et al.,* 1994) was used to introduce DNA directly into progenitor cells in living explants of the developing cortex. Gold beads (0.6-μm-diameter) were coated with DNA constructs using the Bio-Rad protocol and stored at 4°C before use. The dorsal telencephalon of E13 or E14 rat embryos was dissected into cold HBSS and flattened onto wet filters with the ventricular surface facing up. DNA-coated gold beads were introduced into cells using a Biolistic gene gun (Lo *et al.,* 1994). The explant was then incubated on the floating filter in complete Neurobasal medium for 12–24 h. The tissue was then fixed in cold 2% paraformaldehyde and mounted for observation under a Bio-Rad MRC600 confocal microscope.

For experiments in Fig. 8, ventricular cells were transfected by electroporation of DNA constructs into intact brains using a modification of the methods described by Itasaki *et al.* (1999). The heads of E14 Long–Evans rat embryos were dissected away from the bodies, embedded in 3% low-melting agarose (Gibco-BRL) in magnesium- and calcium-free Dulbecco's phosphate-buffered saline (PBS; Gibco-BRL), and trimmed to a minimal size. DNAs were resuspended in PBS at a concentration of 2 μg/μl with 0.04% Trypan blue immediately before use, and injected into the lateral ventricles using a fine micropipet. A 0.3-mm-thick platinum anode was inserted into the brain at the midline, and 0.3-mm-thick platinum cathode wire (shaped as a loop) was placed on the lateral side of the telencephalon. A Grass 588 stimulator (Grass Medical Instruments) was used to pass five 50-ms pulses of 150 V through the electrodes. The telencephalon was then removed from the head and incubated for 24 hours in complete Neurobasal medium. The tissue was fixed in 4% paraformaldehyde, cryoprotected in 20% sucrose, embedded in Tissue-Tek (Sakura Finetek USA, Inc.), frozen, and sectioned at 15 μm. Nuclei were stained with 0.5 μg/ml propidium iodide (Molecular Probes), coverslipped using Fluoromount-G (Southern Biotechnology Associates, Inc.), and imaged using a Zeiss LSN 510 laser confocal microscope.

Western Analysis

3T3 cells were transfected with various DNA constructs using the methods described above and incubated until the culture became confluent. GFP fluorescence was checked to ensure a significant percentage of cells (>15%) expressed the DNA construct. Cells were then scraped off the slides, transferred to an eppendorf tube, and sonicated in 2× SDS sample buffer. Standard SDS–PAGE methods (Sambrook *et al.,* 1989) were used to run protein samples onto gels; the proteins were then transferred onto nitrocellulose blots and probed with the 5F5 anti-Otx1 antibody (1:7) (Weimann *et al.,* 1999).

Two-Hybrid Methods

The standard yeast two-hybrid system (Golemis *et al.,* 1987; Golemis and Khazak, 1997; Fashena *et al.,* 2000) was used to map the transcriptional activation domain within the Otx1 protein. Briefly, the *Otx1* coding sequence (full-length or truncated, as described in Fig. 9) was cloned into a *pMW103* plasmid in frame with the *LexA* sequences (plasmids were obtained from E. Golemis, Fox Chase Cancer Center). These plasmids were cotransfected with plasmid *pSH18-34* (*LacZ* reporter) into EGY48 (*Leu2* reporter) yeast cells. The resulting transformants were tested for their growth on Leu-minus plates and β-galactosidase activity. The *pSH17-4* plasmid, which encodes the GAL4 activation domain, was used as a positive control and *pRFM1,* which encodes the N-terminus of bicoid, served as a negative control (Golemis *et al.,* 1987).

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