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Role of Cyclins in Neuronal Differentiation of Immortalized Hippocampal Cells

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The proto-oncogene cyclin D1 and the neuron-specific cyclins p35 and p39 are expressed during brain maturation. To investigate the role of these cyclins in neuronal differentiation, we used a conditionally immortalized rat hippocampal cell line, H19-7, that expresses cyclin-dependent kinases 4 and 5 (cdk4 and -5). Cyclin D1, which activates cdk4 and binds but does not activate cdk5, was increased upon differentiation of the H19-7 cells. However, microinjection of either sense or antisense cyclin D1 cDNA or anti-cyclin D1 antibodies had no effect on morphological differentiation of the cells. On the other hand, neurite outgrowth was stimulated by expression of p35 or p39, both of which activate cdk5. A dominant-negative mutant of cdk5 blocked both p35- and p39-induced neurite extension as well as basic fibroblast growth factor (bFGF)-induced neuronal differentiation. However, of these cyclins, only antisense p39 prevented bFGF-induced neurite outgrowth. These studies indicate that cyclin D1 is neither necessary nor sufficient for morphological differentiation, that p35 is sufficient but not required, and that p39 is both necessary and sufficient for neurite outgrowth in the hippocampal cells. Taken together, these results represent the first demonstration of a specific role for p39 in neuronal differentiation, implicate the cyclin-activated kinase cdk5 in this process, and indicate that p39 is able to mediate neurite outgrowth in the presence or absence of cyclin D1.

Cyclins, which trigger signaling cascades by activating cyclin-dependent kinases (cdks), have been implicated in cell growth, differentiation, and cell death (1, 8, 14, 16, 34, 46, 48, 49, 56, 58). Cyclin D1, a proto-oncogene that is overexpressed in a variety of tumors (31, 43), is expressed predominantly in the late G_1 phase of proliferating cells and has been shown to be a key regulator of G_1-S-phase progression in mammalian cells (10, 27, 28, 32, 40, 56). As an activator of cdks, cyclin D1 forms a complex primarily with cdk4 or cdk6 depending on the cell type (2, 26, 30, 57). Several studies indicate that cyclin D1-cdk4 facilitates entry into S phase in mitogen-responsive cells by inactivating pRb through phosphorylation (4, 12, 17, 25, 33). Since pRb binds transcriptional factors such as E2F, preventing initiation of DNA synthesis, inactivation of pRb by the cyclin D1-activated kinase alleviates this inhibition. Cells microinjected during the G_1 interval with cyclin D1 antisense vector or with neutralizing antibodies to the cyclin D1 protein are inhibited from entering S phase (1, 59). Thus, cyclin D1 is considered both necessary and rate limiting for G_1 progression. However, in some cases such as mammary epithelial cells (10), overexpression of cyclin D1 cDNA prolongs the S phase and inhibits growth.

Besides its critical role in cell cycle control, cyclin D1 may also mediate differentiation. Cyclin D1 levels increase at the onset of differentiation of HL-60 myeloid cells (14) and C2C12 muscle cells (16) as well as PC12 cells (48, 53, 54, 58), and expression of cyclin D1 has been suggested to be a key step in the differentiation of PC12 cells (58). In vivo, cyclin D1 expression in neuronal cells increases during rat brain maturation (48, 49) and reduced cyclin D1 expression correlates with neural developmental delay induced by nutritional deprivation (44). The function of cyclin D1 during neuronal differentiation is not clear, since cyclin D1 can interact with proteins other than cdks, including DMP1 (13) and PCNA (38), and cyclin D1 has effects on muscle gene expression that are independent of pRB phosphorylation (47). Disruption of the cyclin D1 gene in mice revealed neurological defects such as abnormal limb reflex similar to those observed by targeted disruption of genes important in neuronal development or function (46). Despite the well-documented importance of cyclin D1 in growth, the lack of cyclin D1 did not cause lethality. Thus, the possibility arises that the absence of functional cyclin D1 during development may be compensated by other related cyclins. Further, these results suggest that, if cyclin D1 plays a role in mediating neuronal differentiation, it is unlikely to be the only mechanism.

More recently, a neuron-specific cyclin termed p35 has been identified and characterized (15, 22, 51). This cyclin belongs to a family distinct from that of the growth-modulatory cyclins D, E, A, and B, which activate cdk2, cdk4, cdk6, and cdc2, depending on the particular cyclin. Unlike cyclin D1, which forms a complex with cdk5 both in vivo (57) and in vitro (20) without any known activity, p35 specifically activates cdk5 (51). p35 is expressed exclusively in postmitotic neurons of the central nervous system, its temporal pattern of expression parallels the development of cortical neurons, and it is required for neurite outgrowth in cultured cortical neurons (34). Consistent with these observations, mice lacking p35 exhibit abnormal cortical lamination which is probably due to altered neuronal migration (3). At least one other protein related to p35 that is able to activate cdk5 in vitro has recently been described (50). This protein, p39, which is slightly larger than p35 (39 kDa), was cloned from a human hippocampal library and has 57% amino acid identity with p35. Although p39 has been found to be expressed in the rat cerebrum and cerebellum and was enriched in the CA1 to CA3 regions of the hippocampus, no

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functional role for p39 in neuronal differentiation has been described.

To investigate the role of cyclin D1, p35, or p39 in neuronal differentiation, we used a rat hippocampal cell line, H19-7, immortalized with simian virus 40 (SV40) temperature-sensitive large T antigen as a model system (7). At 39°C, the temperature at which large T is inactivated, treatment by basic fibroblast growth factor (bFGF) or activated Raf induces differentiation. The differentiated hippocampal cells are resistant to mitogenic stimulation by serum or epidermal growth factor, express neuronal markers such as neuron filament and brain type II sodium channels, and display action potentials (5, 6). Upon transplantation into rat brains, cells that have been similarly immortalized by temperature-sensitive large T antigen exhibit region-specific neuronal differentiation (41, 55).

Our studies indicate that cyclin D1 expression is neither necessary nor sufficient for morphological differentiation. Furthermore, while both neuron-specific cyclins p35 and p39 can induce neurite outgrowth, our results suggest that cdk5 in association with p39 is the key mediator of hippocampal H19-7 neuronal differentiation.

MATERIALS AND METHODS

Cell culture. Rat E17 hippocampal H19-7 cells were immortalized with SV40 temperature-sensitive T antigen as previously described (7). ΔRaf-1:ER cells, generated as previously described (19), were derivatives of H19-7 cells stably transfected with a plasmid expressing an oncogenic human Raf-1 protein fused to the ligand binding domain of the estrogen receptor.

H19-7 and ΔRAF-1:ER cells were grown at 33°C, the permissive temperature at which large T is functional, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). H19-7 cells were selected by 200 μg of G418 per ml, and ΔRaf-1:ER cells were selected by 100 μg of hygromycin per ml.

Differentiation. For differentiation, H19-7 cells were shifted to 39°C to eliminate expression of functional SV40 large T antigen, incubated for 2 days in N2 serum-free medium (5), and then treated with bFGF (6 to 10 ng/ml). After 2 days of incubation in N2 medium at 39°C, no expression is observed from genes that are transiently induced in response to release of large T-associated proteins, such as p53, after the temperature shift. While ΔRAF-1:ER cells could also be incubated in N2 medium for 2 days prior to differentiation, this protocol resulted in a higher background of spontaneously differentiated cells. Therefore, ΔRaf-1:ER cells were incubated for only 1 day at 39°C in N2 serum-free medium and differentiated upon activation of Raf-1 by estradiol (10 nM or 1 μM). Finally, it is also possible to differentiate both cell types by treating them with bFGF (H19-7 cells) or estradiol (ΔRAF-1:ER cells) immediately after the temperature shift. This protocol was used in the microinjection experiments to maximize the expression of the injected cDNAs.

Cyclin D1, p35, and p39 expression plasmids. The 1.1-kb human cyclin D1 cDNA containing the entire coding sequence, which was derived from the plasmid pPL-8 (a gift from A Arnold) (32), was cloned into the *Eco*RI site of the Bujard vector pUHD10-3 in a sense or antisense orientation. pUHD15-1, containing the tetracycline-suppressible transactivator of pUHD10-3, was provided by H. Bujard (9, 42). Sense and antisense p35 and two dominant-negative mutants of cdk5 (N144 and T33) were generously provided by Li-Huei Tsai and were generated as previously described (34). N144 and T33 are kinase-inactive mutants of cdk5 that bind p35 more strongly than the wild-type cdk5. p39 was cloned by PCR with ID-proof polymerase (ID Lab, London, Ontario, Canada) from a human fetal brain library (Clontech, Palo Alto, Calif.) and epitope tagged with a hemagglutinin (HA) epitope inserted after the AUG start site. The fragments were subcloned at the *Eco*RI site of the pCR3.1 mammalian expression vector (Invitrogen, Carlsbad, Calif.) in both sense and the antisense orientations.

Protein extraction and Western blot analysis. Cells were washed with cold phosphate-buffered saline (PBS) and lysed on ice for 5 min with 200 to 300 μl of 1% Triton-based buffer (20 mM Tris [pH 7.9], 137 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 1-μg/ml aprotinin, 20 μM leupeptin, 1 mM Na_3VO_4, 1 mM EGTA, 10 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM β-glycerophosphate [pH 7.4], 10-mg/ml *p*-nitrophenylphosphate). A 10- to 20-μg amount of total cell lysates was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins on the gels were transferred onto nitrocellulose membranes. The blots were incubated for 2 h with primary antibody (1-μg/ml anti-cyclin D1 antibody sc-92 [Santa Cruz]), 0.5-μg/ml anti-cyclin D1 antibody DCS6 [Neo markers, Fremont, Calif.], 1:500 dilution of anti-HA antibody 12CA5, 1-μg/ml anti-cdk4 antibody [Santa Cruz], and 1-μg/ml anti-cdk5 antibody [Santa Cruz]). The blots were then washed with 0.1% Tween 20 in Tris-buffered saline (TBS), incubated with peroxidase-conjugated secondary antibody, and visualized by enhanced chemiluminescence (ECL). The samples were quantitated by optical density scanning of the films.

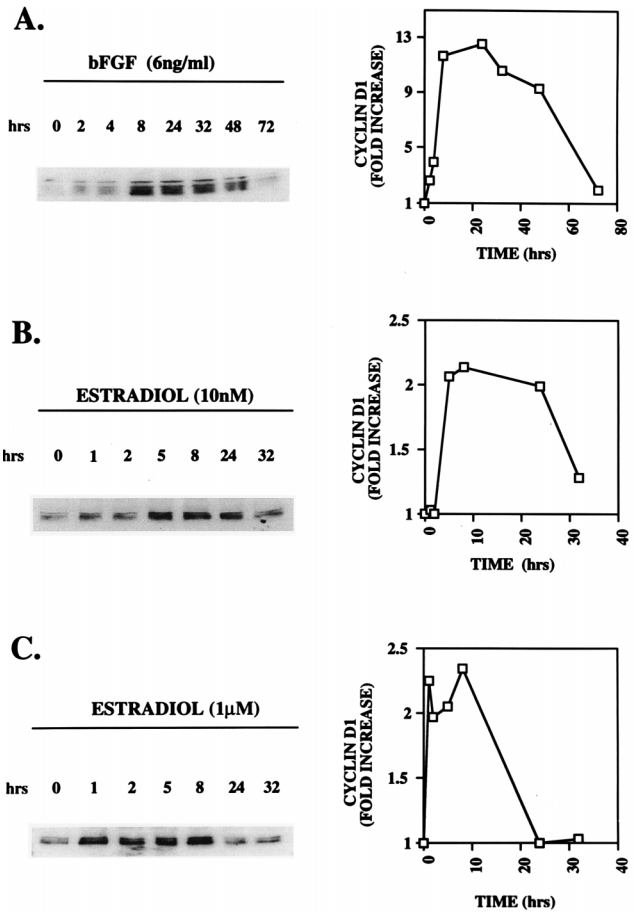

FIG. 1. Cyclin D1 induction during neuronal differentiation of H19-7 and ΔRaf-1:ER cells. (A) H19-7 cells incubated in N2 medium for 2 days at 39°C and treated with bFGF at 6 ng/ml for differentiation; (B and C) ΔRAF-1:ER cells incubated in N2 medium at 39°C for 1 day and treated with 10 nM (B) or 1 μM (C) estradiol for differentiation. Proteins were extracted at the indicated time points with 200 μl of lysis buffer. A 20-μg amount of total cell lysate of each sample was resolved by SDS-PAGE. Western blot analysis with anti-cyclin D1 antibody was carried out as described in Materials and Methods. Gels, ECL films illustrating Western blot analysis of cyclin D1 induction; graphs, time course of cyclin D1 expression. Samples were quantitated by optical density scanning of the films. These data are representative of ≥4 independent experiments. It should be noted that the scales of the *x* and *y* axes are different in the three graphs.

Immunocytochemistry. Cells on 1-mg/ml polylysine-coated coverslips were washed with PBS once for 5 min, fixed with 10% buffered formaldehyde for 10 min, washed with PBS once for 5 min, permeabilized with 0.2% Triton X-100 for 5 min, and washed with PBS three times for 5 min. After blocking of nonspecific binding with 10% goat serum in PBS for 30 min, the cells were incubated with primary antibody (2 μg of anti-cyclin D1 antibody DCS6 per ml, 1:5 dilution of anti-p35 antibody 2D12 [34], and 1:500 anti-HA antibody 12CA5) for 1 h, washed with PBS three times for 10 min, incubated with fluorescein isothiocyanate (FITC)- or Texas red-conjugated goat anti-mouse immunoglobulin G (IgG; Cappel, Westchester, Pa.) for 1 h, and washed with PBS three times for 10 min. Finally, the cells were mounted on a slide and visualized by fluorescence microscopy.

For bromodeoxyuridine (BrdU) immunocytochemistry, NRK cells were fixed with 70% ethanol for 20 min, rinsed with H_2O, treated with 2 M HCl for 10 min, neutralized with 0.1 M borate buffer (pH 9), and then washed twice with PBS for 5 min prior to Triton permeabilization. Anti-BrdU antibody (Oncogene Science, Cambridge, Mass.) was used at 10 μg/ml.

Microinjection. All of the plasmids used for microinjection were purified twice by equilibrium centrifugation in C_sCl-ethidium bromide gradients. A total of 10^4 cells were seeded onto 1-mg/ml polylysine-coated coverslips and grown at 33°C for 2 days before injection. Anti-cyclin D1 antibody DCS6 (0.5 mg/ml) or rabbit

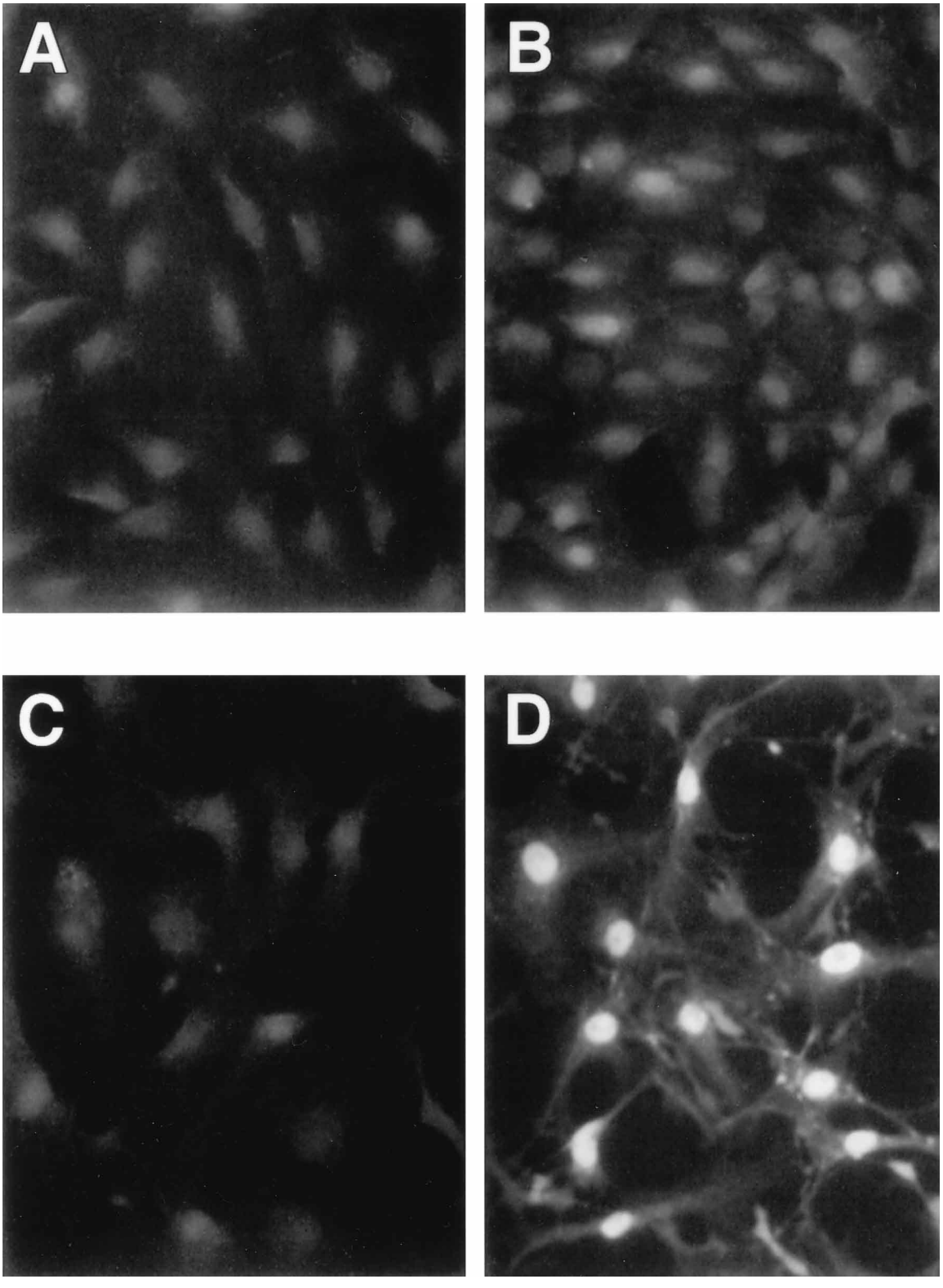

FIG. 2. Cyclin D1 was expressed in the nuclei of H19-7 cells. H19-7 cells (2×10^4) were plated on polylysine-coated coverslips and treated as indicated below. Immunocytochemistry with anti-cyclin D1 antibody was performed as described in Materials and Methods. (A) Cells incubated in N2 medium at 33°C for 48 h; (B) proliferating cells at 33°C in 10% FBS; (C) cells incubated in N2 medium at 39°C for 48 h; (D) cells incubated in N2 medium at 39°C for 48 h and then treated with bFGF for 24 h. These data are representative of two independent experiments.

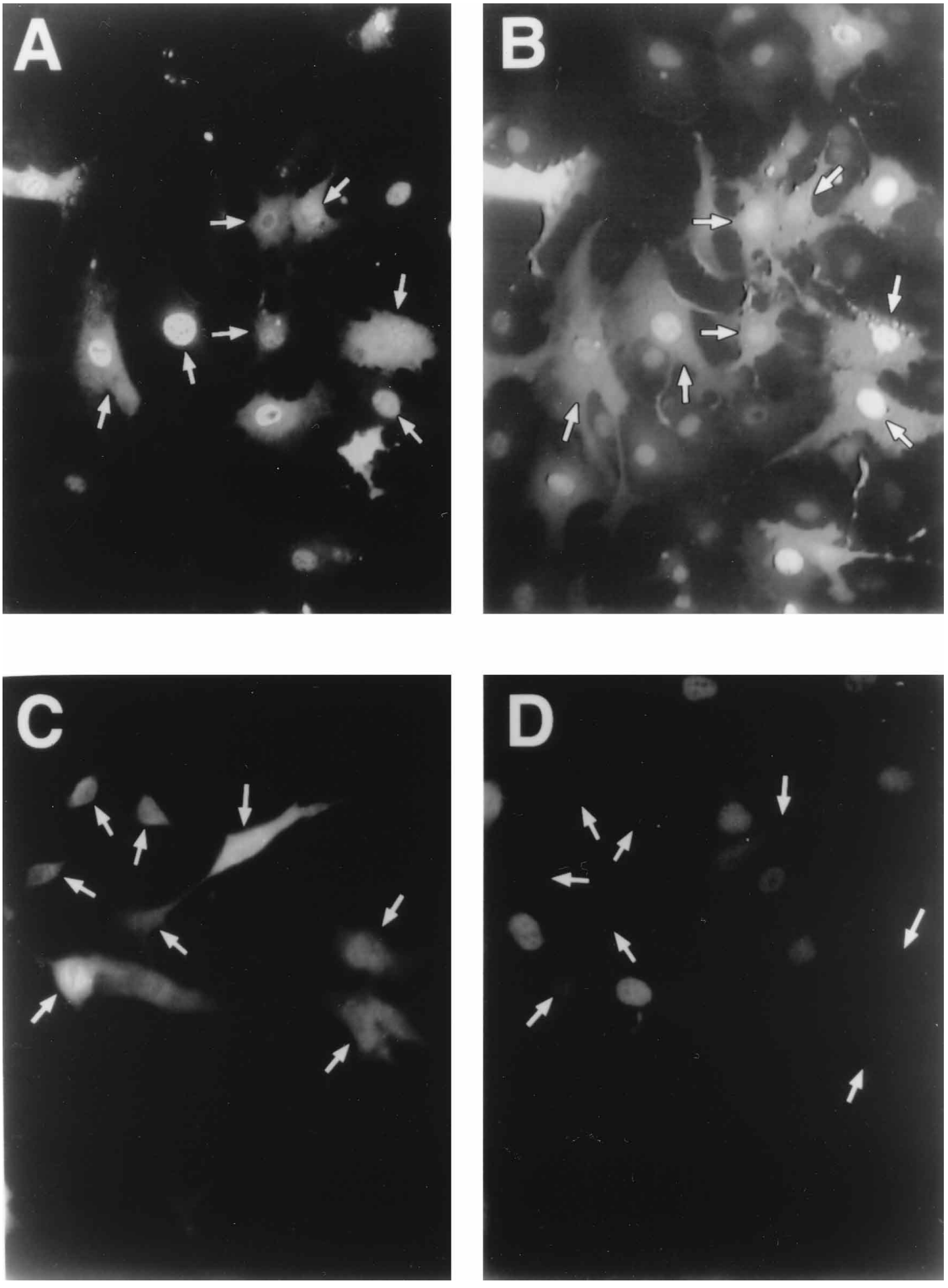

FIG. 3. Cyclin D1 expression was enhanced by injection of sense cyclin D1 cDNA and suppressed by injection of antisense cyclin D1 cDNA. ΔRAF-1:ER cells (10^4) were grown on polylysine-coated coverslips in DMEM with 10% FBS at 33°C for 2 days before injection. Cells were coinjected with dextran rhodamine (0.5 μg/ml), pUHD 15-1 (vector expressing the transactivator [0.25 μg/ml]), and pUHD 10-3 (containing either sense or antisense cyclin D1 cDNA [0.25 μg/ml]). Injected cells were left at 33°C in 10% FBS overnight. Cells injected with sense cyclin D1 were shifted to N2 medium at 39°C for 24 h (A and B). Cells injected with antisense cyclin D1 were shifted to N2 at 39°C and treated with 1 μM estradiol for 8 h (C and D). The cells were fixed and analyzed for expression of cyclin D1 by immunocytochemistry with the anti-cyclin D1 antibody DCS6 and a FITC-conjugated secondary antibody as described in Materials and Methods. (A) Rhodamine-labeled cells coinjected with sense cyclin D1 cDNA; (B) immunostaining of cyclin D1 in cells from panel A; (C) rhodamine-labeled cells coinjected with antisense cyclin D1 cDNA; (D) immunostaining of cyclin D1 in cells from panel B. Arrows, representative injected cells. These data are representative of two independent experiments.

anti-mouse control IgG (0.5 mg/ml; Zymed Laboratories, Inc.) was coinjected with 0.5% dextran rhodamine (Molecular Probes, Inc., Eugene, Oreg.) into cell nuclei by using the Eppendorf transjector-micromanipulator system. cDNA expression vectors for sense or antisense p35 (0.5 μg/ml), p39 (0.5 μg/ml), and cyclin D1 (0.5 μg/ml) or dominant-negative forms of cdk5 (N144 and T33 [0.5 μg/ml]) were coinjected with a plasmid expressing the Green Lantern (Life Technologies, Grand Island, N.Y.) green fluorescence protein (GFP [0.25 μg/ml]). Cells were recovered in 10% FBS at 33°C overnight before being shifted to 39°C in N2 serum-free medium and were treated with differentiating agents.

Cells containing dextran rhodamine or expressing GFP were counted as injected cells. Differentiated cells were defined as cells with refractile cell bodies extending at least two processes, one of which had to be longer than the diameter of the cell body.

Transfection of COS cells. COS cells (3×10^5) were plated in 60-mm plates 1 day before transfection. The cells were transfected with TransIT-LT1 (Mirus, Madison, Wis.) according to the manufacturer's instructions. The TransIT-LT1 reagent was used at a 4:1 ratio to total DNA, and the amount of total DNA was adjusted to 6 μg with pCR3.1 control vector. The cells were washed with opti-MEM serum-free medium (GIBCO, Grand Island, N.Y.) twice and incubated with 1.5 ml of mixed transfection solution in opti-MEM for 6 h. After washing once with PBS, the cells were incubated in DMEM with 10% FBS for 1 day and then harvested. Transfection efficiency was monitored by cotransfection with a plasmid (1 μg) expressing GFP.

RESULTS

Cyclin D1 protein expression was induced during neuronal differentiation by bFGF or activated Raf. To understand the role of cyclin D1 in neuronal differentiation, we studied the expression of cyclin D1 in an immortalized rat hippocampal cell line, H19-7, expressing a temperature-sensitive large T antigen (7). H19-7 cells that were shifted to 39°C for 2 days in N2 medium to inactivate large T antigen and then treated with bFGF for 2 days exhibited a differentiated phenotype. Under these conditions, cyclin D1 protein expression increased more than 10-fold and reached a peak after 8 h of bFGF treatment (Fig. 1A).

A smaller but reproducible increase in cyclin D1 protein was also observed when ΔRaf-1:ER cells were differentiated by sustained activation of Raf. ΔRaf-1:ER cells express an oncogenic Raf kinase fused to the ligand binding domain of the estrogen receptor (7, 19). These cells undergo differentiation by the activation of the ΔRaf-1:ER upon binding of estradiol. The time course of differentiation in response to Raf varies with the potency of the stimulus. Thus, 10 nM estradiol differentiates cells in 1.5 to 2 days, and 1 μM estradiol differentiates the cells in 1 day. At both estradiol concentrations, cyclin D1 protein was induced by more than twofold during differentiation (Fig. 1B and C). However, treatment of ΔRaf-1:ER cells with a low level (10 nM) of estradiol induced maximum cyclin D1 expression after 5 h (Fig. 1B), whereas a high level (1 μM) of estradiol stimulated cyclin D1 expression as early as 1 h after treatment (Fig. 1C). These results indicate that the relative time of cyclin D1 induction correlates with the relative time of morphological differentiation.

The induced cyclin D1 is localized primarily in the nucleus. Translocation of cyclin D1 into the nucleus, where it can act in a complex with cdks to phosphorylate transcriptional regulators, is required for the G_1-to-S transition in many cell types (1). In order to determine the major location of cyclin D1 in differentiating cells, H19-7 cells were immunostained with anti-cyclin D1 antibody. FITC-conjugated secondary antibody was used to visualize the protein by fluorescence microscopy. The results indicated that cyclin D1 induced during differentiation is localized primarily in the nucleus (Fig. 2D).

Overexpression of cyclin D1 does not promote neuronal differentiation. Because the temporal pattern of cyclin D1 induction correlated very well with neuronal differentiation, we determined whether cyclin D1 alone could mediate neuronal differentiation. Two lines of evidence indicate that cyclin D1 itself is not sufficient for morphological differentiation of H19-7 cells. SV40 large T antigen is a suppresser of cyclin D1 expression (39). At the permissive temperature, 33°C, cyclin D1 expression was partially suppressed by large T antigen (Fig. 2A and B). Upon inactivation of large T antigen by a temperature shift to 39°C, a transient increase in cyclin D1 protein expression (>10-fold) that was over by 48 h was observed (Fig. 2C), but this increase in cyclin D1 did not lead to neuronal differentiation (data not shown).

More direct evidence was provided by microinjection of a vector expressing cyclin D1 cDNA into ΔRAF-1:ER cells that were then shifted to 39°C. Overexpression of cyclin D1 protein in the injected cells was observed by immunostaining (Fig. 3A and B). As documented in Table 1, the cells remained undifferentiated. No change in the kinetics or extent of morphological differentiation in the cells injected with the sense cyclin D1 expression vector was observed relative to cells injected with control vector.

Cyclin D1 is not required for neuronal differentiation. Although cyclin D1 expression is not sufficient for differentiation, it might still be required. To investigate its role in this process, we microinjected ΔRAF-1:ER cells with a vector expressing antisense cyclin D1 and determined the effect on Raf-mediated differentiation. As shown in Fig. 3C and D, injection of antisense cyclin D1 dramatically suppressed expression of cyclin D1 protein following differentiation. However, analysis of the cells showed that inhibition of cyclin D1 made no difference in the kinetics or extent of cellular differentiation (Table 1). ΔRAF-1:ER cells were used in these and the following cyclin D1 experiments for purposes of comparison with studies involving microinjection of anti-cyclin D1 antibodies (see below) because these cells differentiate faster in response to 1 μM estradiol and thus avoid potential problems with antibody stability. However, similar results were obtained when H19-7 cells

TABLE 1. Effect of microinjected sense or antisense cyclin D1 cDNA on the differentiation of neuronal cells

Plasmid injection[a]	Treatment[b]	No. of cells[c]	Differentiated cells (%)[d]
Control[e]	No E2		
	16 h	238	14.7 ± 3.5
	24 h	221	16.7 ± 2.4
Antisense cyclin D1	No E2		
	16 h	335	13.5 ± 4.9
	24 h	267	17.1 ± 2.8
Sense cyclin D1	No E2		
	16 h	323	15.6 ± 3.7
	24 h	229	19.5 ± 3.6
Control	1 μM E2		
	16 h	266	55.6 ± 4.8
	24 h	256	63.6 ± 1.7
Antisense cyclin D1	1 μM E2		
	16 h	363	54.1 ± 4.9
	24 h	223	63.2 ± 4.1
Sense cyclin D1	1 μM E2		
	16 h	397	58.5 ± 3.4
	24 h	239	64.8 ± 3.7

[a] ΔRAF-1:ER cells were coinjected with sense or antisense cyclin D1 cDNA in a tetracycline-sensitive vector and the transactivating vector.
[b] Cells were treated with or without estradiol (E2) at the nonpermissive temperature in N2 medium for the indicated times.
[c] Total cell numbers analyzed in four independent experiments.
[d] Means ± standard deviations.
[e] Sense or antisense cyclin D1 cDNA expression vector without the transactivating vector.

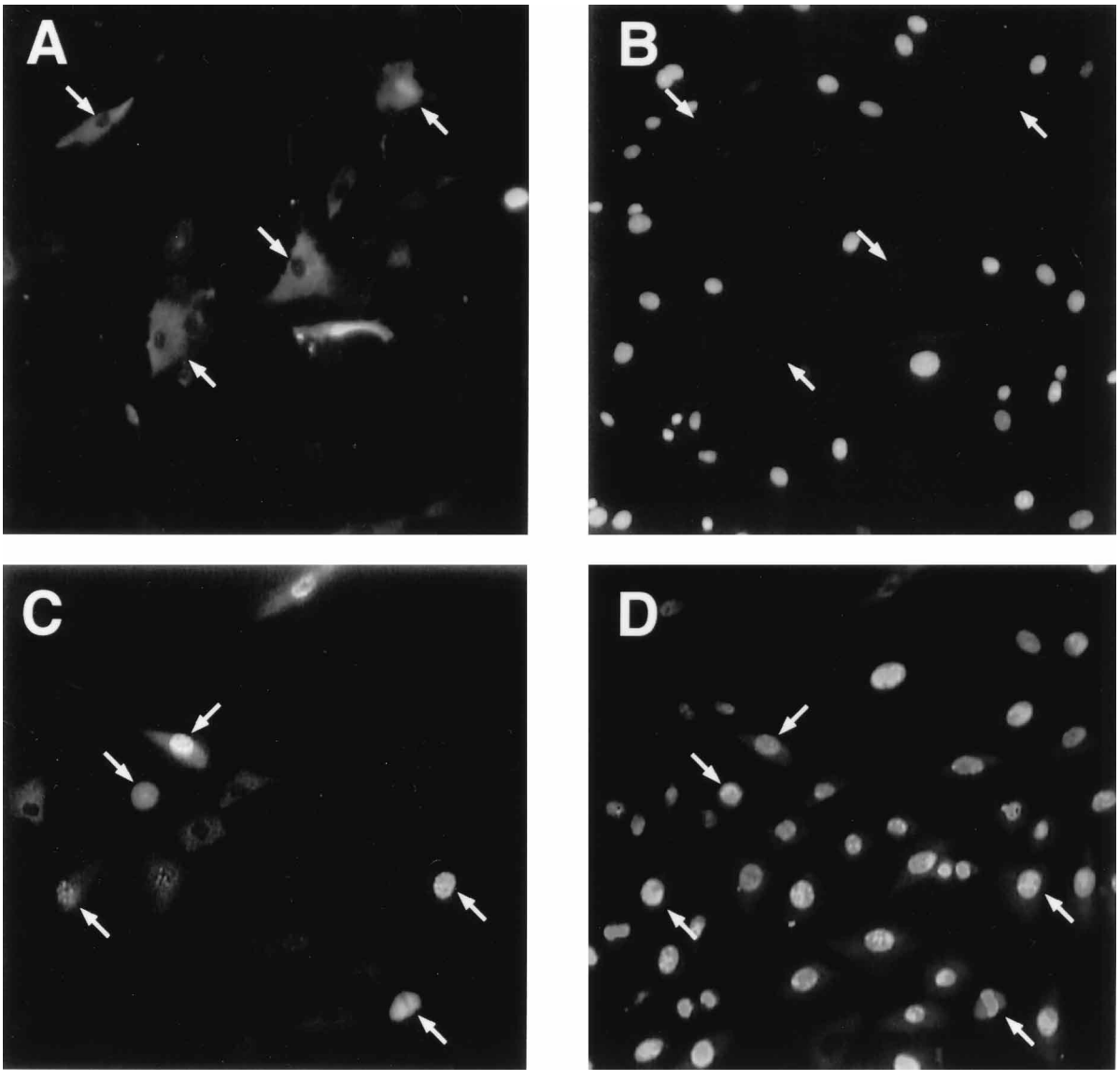

FIG. 4. Microinjection of anti-cyclin D1 antibody DCS6 blocked BrdU incorporation in rat NRK cells. NRK cells were grown on polylysine-coated coverslips in DMEM with 10% FBS at 37°C for 2 days, starved in serum-free DMEM for 2 days, and then coinjected with anti-cyclin D1 antibody DCS6 (0.5 mg/ml) and dextran rhodamine (0.5 mg/ml). After injection, the cells were stimulated with 10% FBS in the presence of 10 μM BrdU and 1 μM fluorodeoxyuridine (FdU) for 16 h. The cells were fixed and analyzed for expression of BrdU by immunocytochemistry with an anti-BrdU antibody and an FITC-conjugated secondary antibody as described in Materials and Methods. (A) Rhodamine-labeled cells coinjected with anti-cyclin D1 antibody; (B) immunostaining of BrdU incorporated into the DNA of cells from panel A; (C) rhodamine-labeled cells coinjected with rabbit anti-mouse control IgG; (D) immunostaining of BrdU incorporated into the DNA of cells from panel C. Arrows, representative injected cells. These data are representative of three independent experiments.

were microinjected with the antisense cyclin D1 cDNA and differentiated by bFGF (data not shown).

To confirm that cyclin D1 is not required for neuronal differentiation, we also microinjected cells with a neutralizing antibody against cyclin D1. Since the presence of the large T antigen in H19-7 cells at the permissive temperature interferes with normal cell cycle regulation, we initially tested the antibody in rat NRK fibroblasts to demonstrate that microinjection of the antibody can prevent cyclin D1 function in rat cells. As shown by BrdU staining to monitor incorporation of this thymidine analog into newly synthesized DNA, microinjection of the anti-cyclin D1 antibody completely blocked NRK cells from entering S phase (Fig. 4). When the antibody to cyclin D1 was injected into ΔRAF-1:ER cells, no significant difference in terms of percentage of differentiation between the anti-cyclin D1 antibody-injected cells and the control antibody-injected cells was observed (Table 2). These results indicate that cyclin D1 is not required for neuronal cell differentiation.

Expression of the cyclin D1- and p35-activated kinases, cdk4 and cdk5. One possible explanation for the lack of cyclin D1 function in H19-7 cells would be lack of expression of the cyclin D1-activated kinase cdk4. To determine whether cdk4 is expressed in these cells, extracts were prepared from bFGF-treated H19-7 cells and analyzed by Western blotting with anti-cdk4 antibody for cdk4 expression during differentiation. As shown in Fig. 5A, the levels of cdk4 remained constant after bFGF treatment.

In contrast to cyclin D1, p35 is found exclusively in postmi-

TABLE 2. Effect of microinjected anti-cyclin D1 neutralizing antibody on the differentiation of neuronal cells

Antibody injected[a]	Treatment[b]	No. of cells (*n*)[c]	Differentiated cells (%)[d]
Control[e]	1 μM E2 (16 h)	331 (6)	49.5 ± 5.2
Anti-cyclin D1[f]	1 μM E2 (16 h)	401 (6)	53.0 ± 4.8
Control	1 μM E2 (24 h)	404 (4)	56.5 ± 4.2
Anti-cyclin D1	1 μM E2 (24 h)	347 (4)	60.3 ± 4.6
Control	10 nM E2 (32 h)	289 (4)	62.8 ± 4.3
Anti-cyclin D1	10 nM E2 (32 h)	232 (4)	64.9 ± 4.0

[a] ΔRAF-1:ER cells were injected as described in Materials and Methods.
[b] Cells were treated with estradiol (E2) at the nonpermissive temperature in N2 medium for the indicated times.
[c] Total cell numbers analyzed in the indicated numbers of independent experiments.
[d] Means ± standard deviations.
[e] Rabbit anti-mouse IgG.
[f] Monoclonal anti-cyclin D1 antibody DCS6.

totic central nervous system neurons, specifically activates cdk5, and has been shown to promote neurite outgrowth in cultured cortical neurons (34). To determine whether the p35-activated kinase is expressed in the immortalized hippocampal cells, extracts from bFGF-treated H19-7 cells were analyzed by Western blotting with antibody against cdk5. As shown in Fig. 5B, cdk5 was expressed at a constant level, independently of differentiation.

p35-cdk5 promotes neurite outgrowth in H19-7 cells. Since cdk5 is expressed in the immortalized hippocampal cells, we determined whether activation of this kinase by p35 can promote neurite outgrowth. Initially, an expression vector containing rat p35 cDNA was microinjected into H19-7 cells, and the cells were assayed for expression of the p35 protein by immunostaining with an anti-p35 antibody. As shown in Fig. 6A and B, p35 expression in cells could be detected following microinjection of a vector containing p35 cDNA. It should be noted that no p35 expression was detected in either proliferating or differentiated H19-7 cells by either immunostaining or Western blotting in the absence of the p35 expression vector (data not shown). Morphological analysis of the injected cells after shifting them to 39°C under differentiating conditions indicated that p35 is capable of promoting neurite outgrowth (Fig. 6C; Table 3), presumably through activation of cdk5. Comicroinjection of cells with vectors expressing both sense and antisense p35 cDNA blocked neurite outgrowth, indicating that p35 expression is responsible (Fig. 6D; Table 3).

To determine whether p35 promotes neurite outgrowth in these cells through activation of cdk5, H19-7 cells were either injected with N144, a vector expressing a dominant-negative form of cdk5 (34) alone (Fig. 6E), or coinjected with the p35 expression vector and N144 (Fig. 6F). As shown in Fig. 6E and F and Table 3, p35-mediated neurite induction is blocked by expression of the dominant-negative cdk5 protein. These results suggest that p35-activated cdk5 can promote neurite outgrowth in immortalized hippocampal neurons.

Differentiation of H19-7 cells requires activated cdk5 but not p35. Although we did not detect p35 in H19-7 cells, this result could be due to the insensitivity of our assay. To confirm that p35 is not mediating differentiation of the H19-7 cells, vectors expressing antisense p35 were microinjected into H19-7 cells, and the effect on bFGF-mediated differentiation was monitored. As shown in Fig. 7A and B and Table 4, antisense p35 has no effect on the kinetics or extent of H19-7 differentiation. However, both of the dominant-negative mutants of cdk5 inhibited neurite outgrowth in the presence of bFGF (Fig. 7C and D; Table 4). Thus, cdk5, the kinase regulated by p35, is required in bFGF-induced neuronal differentiation in H19-7 cells, but another cyclin is responsible for its activation.

p39-cdk5 promotes neurite outgrowth in H19-7 cells. Since a related cyclin, p39, is expressed in the hippocampus and has been shown to activate cdk5 in vitro (50), we determined whether activation of cdk5 by p39 can also promote neurite outgrowth in H19-7 cells. Initially, we cloned p39 by PCR from a human fetal brain library, and introduced an HA epitope tag at the N terminus of the p39 cDNA by site-directed mutagenesis. To check the integrity of the construct, the vector pCR3.1 expressing the HA-tagged human p39 was transfected into COS cells, and the expression of p39 was monitored by immunoblotting with an anti-HA antibody. As shown in Fig. 8A, p39 was expressed, and the expression could be suppressed by cotransfection with antisense p39. The vector expressing HA-p39 was then microinjected into H19-7 cells, and the cells were assayed for expression of the p39 protein by immunostaining with an anti-HA antibody. As shown in Fig. 8B, HA-p39 expression could also be detected in microinjected H19-7 cells and was inhibited by coinjection with the antisense expression vector (data not shown). Morphological analysis of the injected cells after shifting them to 39°C under differentiating conditions indicated that p39, like p35, is capable of promoting neurite outgrowth (Fig. 9A and B; Table 5). Comicroinjection of cells with vectors expressing both sense and antisense p39

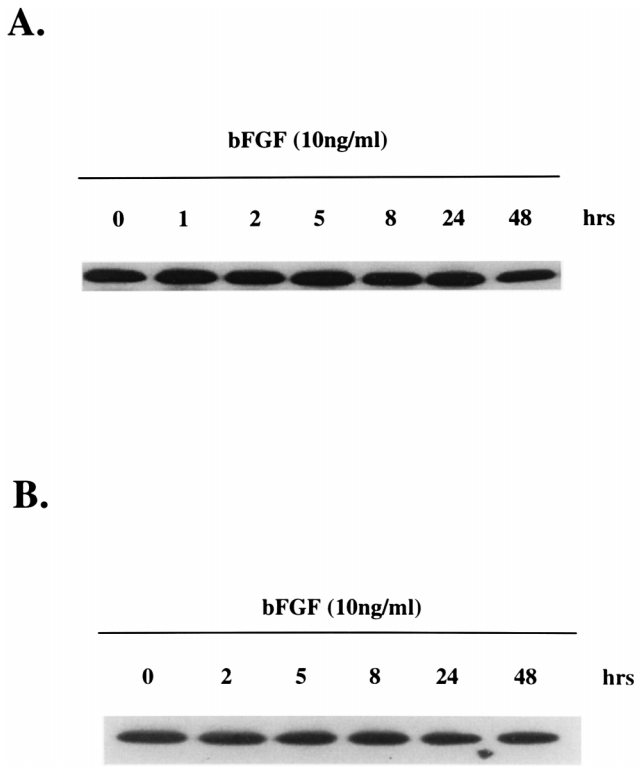

FIG. 5. Expression of cdk4 and cdk5 protein was independent of neuronal differentiation. H19-7 cells were incubated in N2 medium at 39°C for 2 days and treated with bFGF (10 ng/ml) to induce differentiation. The cells were lysed with 1% Triton X-100-based buffer at the indicated time points. A 20-μg amount of the total cell lysates were resolved by SDS-10% PAGE. Western blot analysis was performed with an anti-cdk4 antibody (A) or anti-cdk5 antibody (B). These data are representative of two independent experiments.

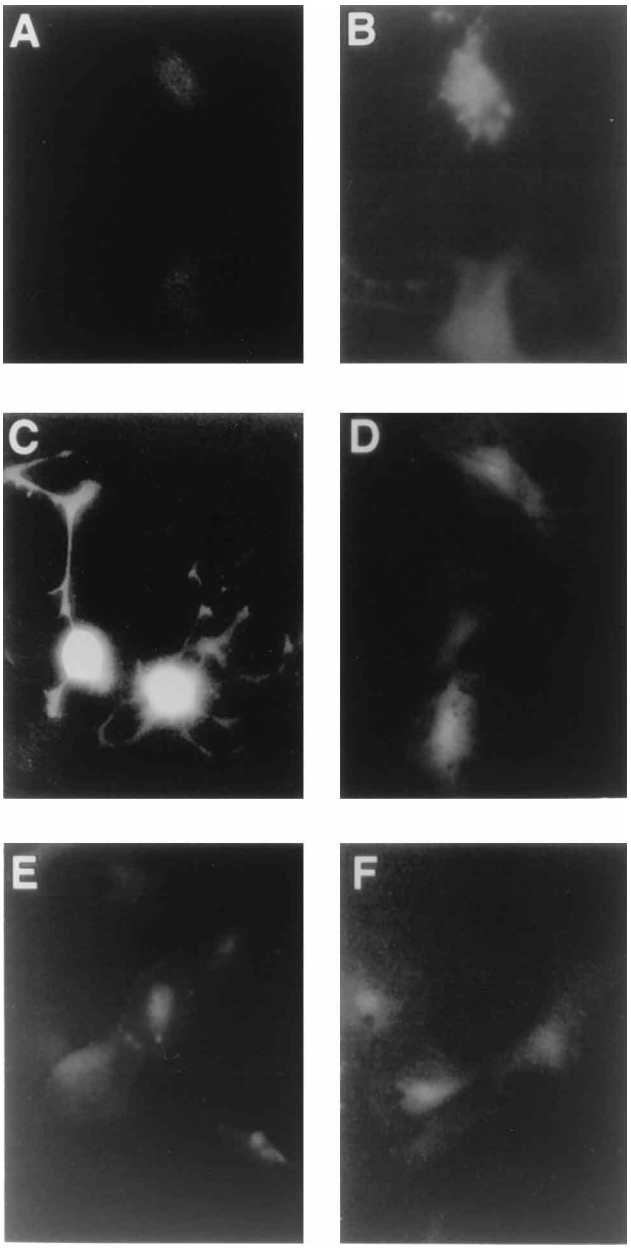

FIG. 6. p35 induced neuronal differentiation can be blocked by a dominant-negative mutant of cdk5. H19-7 cells (10^4) were grown on polylysine-coated coverslips in DMEM with 10% FBS at 33°C for 2 days before injection. The cells were coinjected with expression plasmids for GFP and the vectors described below. The cells were kept in DMEM with 10% FBS at 33°C overnight after the injection and then either directly assayed (A and B) or shifted to 39°C in N2 medium for 24 h (C, D, E, and F). (A) GFP-labeled cells coinjected with p35 cDNA (33°C); (B) immunostaining of p35 in cells from panel A; (C) GFP-labeled cells coinjected with p35 cDNA (39°C); (D) GFP-labeled cells coinjected with sense p35 plus antisense p35 (39°C); (E) GFP-labeled cells coinjected with N144, a dominant-negative mutant of cdk5 (39°C); (F) GFP-labeled cells coinjected with p35 plus N144 (39°C).

cDNA blocked neurite outgrowth, indicating that p39 expression is responsible (Fig. 9C; Table 5).

To determine whether p39 promotes neurite outgrowth in these cells through activation of cdk5, H19-7 cells were coinjected with the p39 expression vector and N144. As shown in Fig. 9D and Table 5, p39-mediated neurite induction is blocked by expression of the dominant-negative cdk5 protein. These results suggest that p39-activated cdk5 can promote neurite outgrowth in the immortalized hippocampal neuronal cells.

Differentiation of H19-7 cells requires p39. Analysis of H19-7 cDNA by PCR with primers based on the human p39 cDNA sequence generated a fragment with a size comparable to that of full-length p39 which specifically hybridized to human p39 cDNA (data not shown). To determine whether p39 might be the endogenous activator of cdk5 in H19-7 cells, vectors expressing antisense p39 were microinjected into H19-7 cells, and the effect on bFGF-mediated differentiation was monitored. As shown in Fig. 9E and F, and Table 5, antisense p39 significantly inhibited H19-7 differentiation.

DISCUSSION

Biological processes leading to neuronal differentiation are a complex series of events. Terminally differentiated neurons are arrested in the G_0 phase, and some key regulators of the cell cycle are found to be downregulated (11, 37, 58). Surprisingly, one of the most important cyclins in the late G_1 phase, cyclin D1, was found to increase during neuronal cell differentiation (54, 58). To determine whether this cell cycle regulator is required for neuronal differentiation, we studied the role of cyclin D1 in the differentiation of conditionally immortalized rat hippocampal neuronal cells (H19-7). Although we found that cyclin D1 protein was induced and that the induction correlated with cell differentiation stimulated either by bFGF or by activated Raf-1, overexpression or suppression of cyclin D1 had no effect on neuronal differentiation in our system. In contrast, expression of the neuron-specific cyclins p35 and p39 did induce neurite outgrowth via activation of cdk5 in H19-7 cells. However, our results suggest that cdk5 mediates the formation of neurites in differentiating H19-7 cells specifically through p39.

Although a role for neuron-specific kinases in neuronal differentiation is not surprising, it is possible that when other cyclin-activated kinases are expressed in the same cells, they might also share some common substrate specificities. However, in H19-7 cells, cyclin D1 is not able to induce neurite outgrowth even though cdk4 is expressed. Since the cyclin inhibitor p21 is also induced during neuronal differentiation of H19-7 cells (data not shown), it is possible that the cyclin

TABLE 3. p35 promotes neuronal differentiation

Plasmid injected[a]	No. of cells[b]	Differentiated cells (%)[c]
Control[d]	119	16.8 ± 1.7
Cyclin D1	139	16.0 ± 1.3
Control[e]	119	19.3 ± 5.4
p35	144	48.3 ± 3.9
AS p35	108	16.0 ± 2.1
N144	98[f]	15.6 ± 4.4[g]
p35+N144	136[f]	14.5 ± 0.5[g]
p35+AS p35	76[f]	13.5 ± 5.9[g]

[a] H19-7 cells were coinjected with the indicated plasmids and the GFP expression plasmid. The cells were shifted to the nonpermissive temperature in N2 medium for 16 h.
[b] Total cell numbers analyzed in three independent experiments (except where indicated).
[c] Means ± standard deviations (except where indicated).
[d] Sense cyclin D1 expression vector without the transactivating vector.
[e] pCMV vector.
[f] Total cell numbers analyzed in two independent experiments.
[g] Mean ± range.

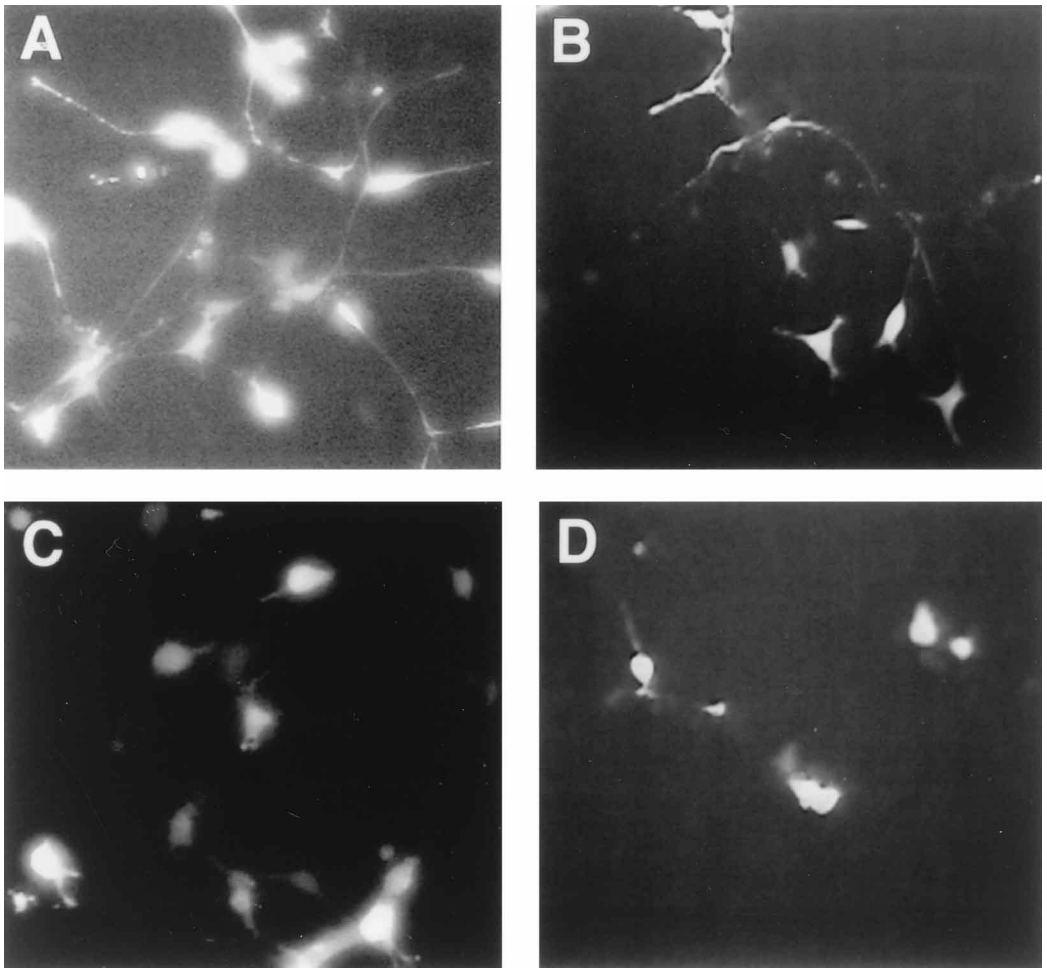

FIG. 7. Dominant-negative mutants of cdk5, but not antisense p35, blocked bFGF-induced neuronal differentiation. H19-7 cells (10^4) were grown on polylysine-coated coverslips in DMEM with 10% FBS at 33°C for 2 days before injection. The cells were coinjected with plasmids expressing GFP and either pCMV control vector (A), antisense p35 (B), N144, a dominant-negative mutant of cdk5 (C), or T33, another dominant negative mutant of cdk5 (D). The cells were kept in DMEM with 10% FBS at 33°C overnight after the injection and then shifted to 39°C in N2 medium and treated with bFGF (10 ng/ml) for 24 h.

D1-cdk4 kinase is inactive. In the most commonly studied neuronal cell system, PC12, cyclin D1 was also found to increase upon differentiation (58). In this case, however, the cyclin D1-associated kinase activity decreased with differentiation, and overexpression of cyclin D1 caused cell arrest in the G_1 phase. Thus, it was proposed that cyclin D1 is required for growth arrest prior to the commitment to differentiation. Since the H19-7 cells are already growth arrested prior to treatment with differentiating agents, the results with PC12 cells are not in conflict with those presented here. In fact, consistent with our results, overexpression of cyclin D1 in PC12 cells did not lead to differentiation, suggesting that cyclin D1 itself does not activate a differentiation signal.

The reason why cyclin D1 expression increases in neurons during rat brain development is not clear. In contrast to p35-cdk5, the cyclin D1-cdk5 complex can associate with the $p27^{kip}$ inhibitor (20). Thus, one possibility is that cyclin D1 competes with p39 or other activating cyclins for cdk5, delaying the onset of neurite outgrowth until sufficient cdk5 activator has been generated. It has been noted previously that p35 can displace cyclin D1 binding to cdk5 (20). The observation that the timing of cyclin D1 expression (both induction and disappearance) correlates with the onset of differentiation is consistent with a role for cyclin D1 as a temporary inactivator of cdk5. However, overexpression of cyclin D1 does not prevent bFGF (and thus p39)-induced neurite outgrowth, suggesting that cyclin D1 may not be an effective competitor of p39. An alternative explanation is that cdk5 becomes refractile to cyclin D1 when endogenous levels of D1 are high. Clearly, more extensive studies

TABLE 4. Dominant-negative cdk5 blocks bFGF-induced neuronal differentiation

Plasmid injected[a]	No. of cells[b]	Differentiated cells (%)[c]
Control[d]	185	57.5 ± 8.3
AS p35	111	51.1 ± 6.4
N144[e]	142	18.7 ± 7.0
T33[f]	166	21.1 ± 8.9

[a] H19-7 cells were coinjected with the indicated plasmids and the GFP expression plasmid. The cells were treated with bFGF at the nonpermissive temperature in N2 medium for 24 h.
[b] Total cell numbers analyzed in three independent experiments.
[c] Means ± standard deviations.
[d] pCMV vector.
[e] Plasmid expressing a dominant-negative form of cdk5.
[f] Plasmid expressing a dominant-negative form of cdk5.

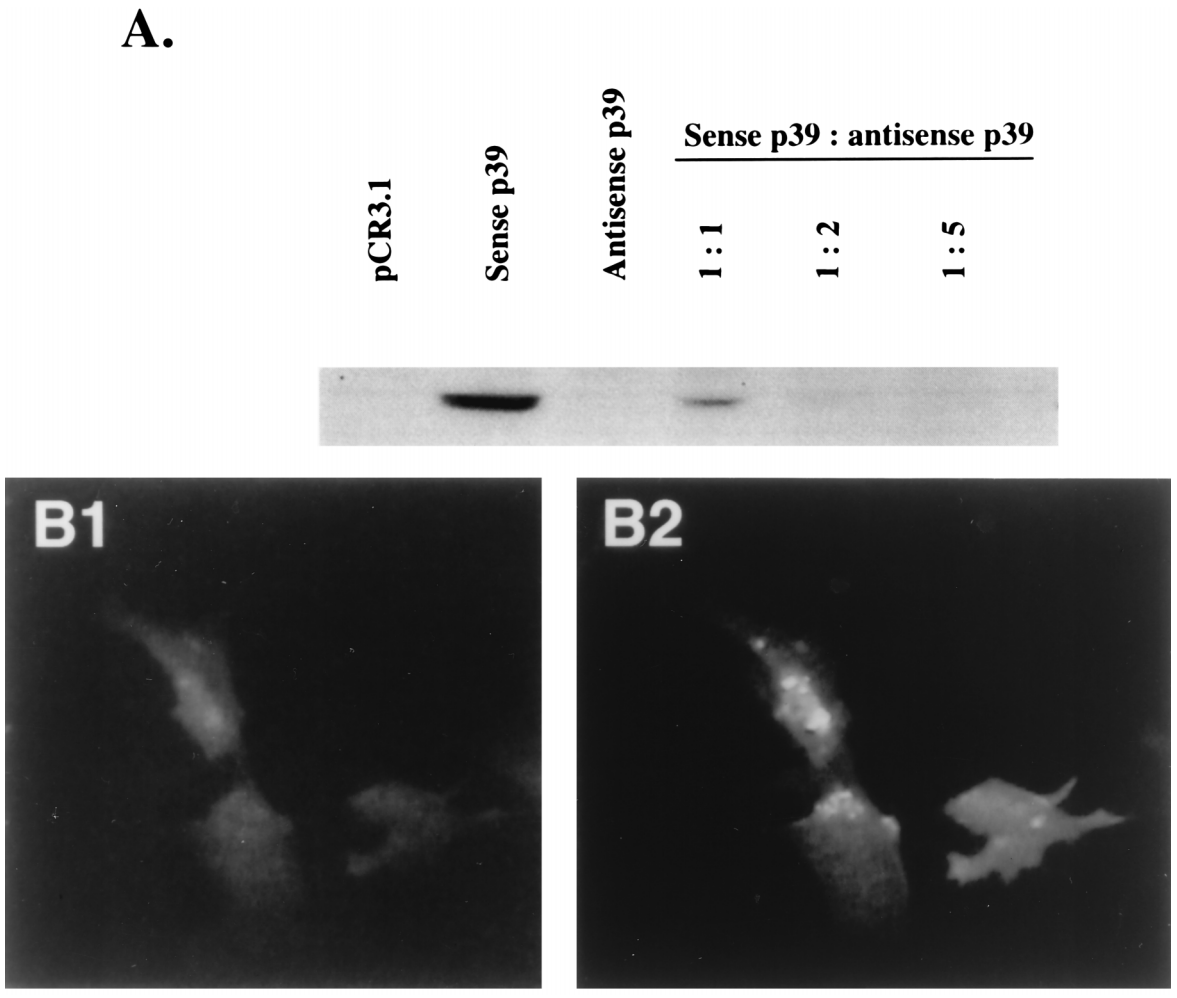

FIG. 8. Human p39 cDNA expression in COS and H19-7 cells. (A) COS cells were transfected with the plasmid as indicated, and 50 mg of the total protein extract was resolved by SDS-PAGE. p39 expression was detected by immunoblotting with anti-HA antibody. (B) H19-7 cells were coinjected with dextran rhodamine and the p39 expression plasmid as described in Materials and Methods. (B1) Rhodamine-labeled cells coinjected with p39 cDNA; (B2) injected cells immunostained for p39 expression by anti-HA primary antibody and FITC-conjugated secondary antibody. These data are representative of two independent experiments.

would be required to resolve this issue. The observation that cyclin D1 is expressed in a temporal pattern that correlates with the relative rates of differentiation in our cells raises the possibility that cyclin D1 may be coregulated with other genes such as p39 that are important in differentiation. Alternatively, cyclin D1 may play a role in neuronal function. In this respect, it is intriguing that mice deficient in cyclin D1 do have neurological defects (46). The lack of more extensive impairment may be due to substitution by other cyclins. Thus, it is possible that multiple cyclins may regulate neuronal development and function.

The induction of neurite outgrowth by activated cdk5 is presumably mediated through phosphorylation of cytoskeletal proteins. Although cdk5 is widely distributed, its active form has been found only in the nervous system (21, 29, 45, 52). Among the targets of cdk5 phosphorylation are tau and MAP2, microtubule-associated proteins that have been implicated in neuronal polarization and microtubule rearrangements (18, 23). Furthermore, cdk5 has been shown to phosphorylate the neuron-specific medium and heavy neurofilament proteins (24, 45). The neurofilaments are the major axonal cytoskeletal proteins and thus are required for neurite outgrowth. Phosphor-

TABLE 5. p39 promotes and anti-sense p39 blocks neuronal differentiation

Plasmid injected[a]	Treatment[b]	No. of cells[c]	Differentiated cells (%)[d]
Control[e]	−bFGF (24 h)	174	8.8 ± 1.6
p39	−bFGF (24 h)	142	39.1 ± 7.8
p39+N144	−bFGF (24 h)	101[f]	10.7 ± 2.4[g]
p39+ASp39	−bFGF (24 h)	109[f]	9.73 ± 2.7[g]
Control[e]	+bFGF (24 h)	137	54.6 ± 8.5
ASp39	+bFGF (24 h)	199	18.4 ± 1.6

[a] H19-7 cells were coinjected with the indicated plasmids and the GFP expression plasmid.
[b] Cells were treated with (+) or without (−) bFGF at the nonpermissive temperature in N2 medium.
[c] Total cell numbers analyzed in three independent experiments (except where indicated).
[d] Means ± standard deviations (except where indicated).
[e] pCR 3.1.
[f] Total cell numbers analyzed in two independent experiments.
[g] Mean ± range.

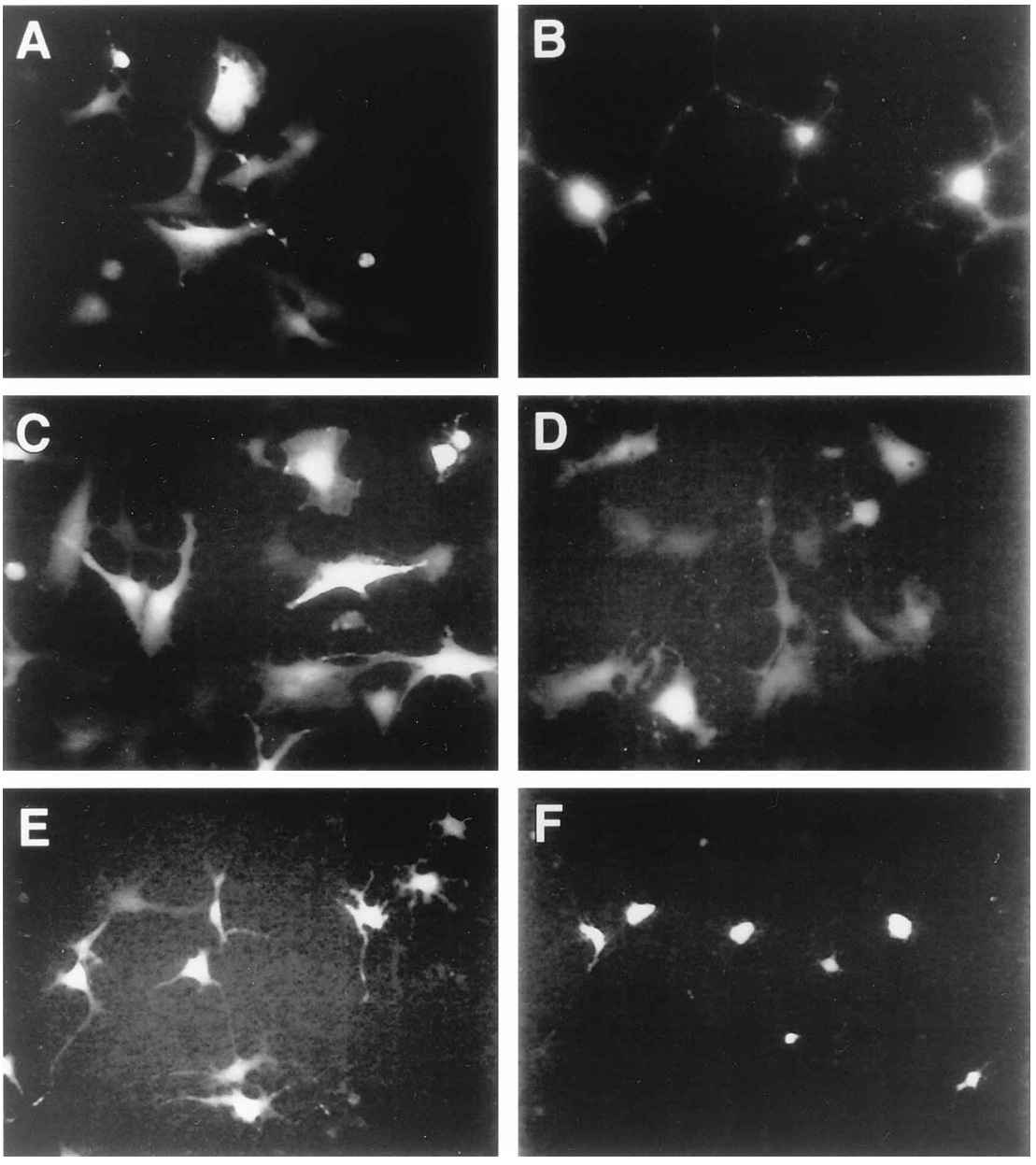

FIG. 9. p39 in association with cdk5 is both sufficient and required for H19-7 cell differentiation. H19-7 cells were coinjected with the indicated plasmids and GFP as described in Materials and Methods. Cell differentiation was monitored by GFP expression after 24 h in N2 medium with or without bFGF. Cells injected with control plasmid pCR3.1 without bFGF (A), with sense p39 without bFGF (B), with sense plus antisense p39 without bFGF (C), with sense p39 plus N144, a dominant-negative mutant of cdk5, without bFGF (D), with control plasmid pCR3.1 plus bFGF (E), and with antisense p39 plus bFGF (F) are shown.

ylation of the neurofilaments is thought to mediate axonal transport of these proteins and to determine their localization (35). A defect in the neurofilament transport process is consistent with some of the pathologies observed in the neurons of cdk5-deficient mice (36).

The results presented here provide the first direct evidence that p39 can regulate neurite outgrowth. The observation that p39 rather than p35 is the cyclin that activates cdk5 in H19-7 cells during differentiation suggests that there may be multiple cdk5-activating cyclins that differ in their localization. This is consistent with results from mice with disrupted p35 genes (3), showing that the defects were a subset of those found in mice with disrupted cdk5 genes (36). For example, the hippocampi in mice lacking p35 were still organized into a laminar structure similar to that for wild-type mice, whereas the hippocampi in mice lacking cdk5 were unable to form the normal three-layered structure. Mice lacking p35 were viable although more subject to lethal seizures, whereas mice lacking cdk5 suffered perinatal lethality. Although the reason for the multiplicity of cdk5 activators is not yet clear, it is likely that they are regulated in a distinct manner which may enable discrete temporal expression, since different regions of the central nervous system develop at different rates. Alternatively, it is possible that p39 and other neuron-specific cyclins have other physiological functions in addition to their role as cdk5 activators.

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