Expression of Neuro D1 in Human Normal Pituitaries and Pituitary Adenomas

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Neuro D1 is a basic helix-loop-helix transcription factor expressed in the endocrine cells of pancreas and in a subset of neurons as they undergo terminal differentiation. In the adult pituitary gland, Neuro D1 is expressed in corticotroph cells and contributes to the corticotroph-specific pro-opiomelanocortin (POMC) transcription by interacting with Pituitary homeobox 1 (Ptx 1) transcription factor. In the present study, we investigated the expression of Neuro D1 in human normal pituitaries and different types of human pituitary adenomas using the RT-PCR and immunohistochemical techniques. Using RT-PCR, Neuro D1 mRNA was found to be expressed in ACTH-secreting adenomas ($n = 3$) and 6 of 8 nonfunctioning adenomas. On the other hand, GH-secreting adenomas ($n = 5$) and PRL-secreting adenomas ($n = 3$) were completely negative for Neuro D1 mRNA. Immunohistochemically, Neuro D1 was expressed in all ACTH-secreting adenomas ($n = 10$), and in 14 of 20 nonfunctioning adenomas. In contrast, 3 of 10 PRL-secreting adenomas and 2 of 10 GH-secreting adenomas showed positive Neuro D1 staining in the nuclei. The above results suggest that Neuro D1 contribute to the functional expression and the differentiation of ACTH-secreting adenomas. It also appears from our study that Neuro D1 might play a role in the differentiation of nonfunctioning adenomas, the mechanism of which remains to be further investigated. This is the first study on Neuro D1 in case of human pituitary adenomas.

VOL. 14, NO. 9, P. 892, 2001 Printed in the U.S.A.
Date of acceptance: May 25, 2001.
This work was supported by Grants for Scientific Research (No. 60231445 and No. 90297862) from the Ministry of Education, Science and Culture of Japan.
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KEY WORDS: Neuro D1, Transcription factor, Human pituitary adenoma.

Mod Pathol 2001;14(9):892–899

The basic helix-loop-helix (bHLH) class of transcription factors has been reported to play a pivotal role in tissue-specific determination and differentiation (1, 2). Perhaps the most extensively studied subfamilies of bHLH proteins are those that regulate myogenesis and neurogenesis (3–8). The myogenic bHLH factors, Myo D family, are involved in various steps of myogenesis, and they do have muscle-specific transcription factors (7). The neurogenic bHLH factors, Neuro D family and the related achaete scute transcription factors, regulate activation of genes that commit progenitors to a neural fate and induce terminal neuronal differentiation. Ectopic expression of Neuro D family in Xenopus laevis embryos causes premature differentiation of neuronal precursor (3).

For Neuro D family, three isoforms, Neuro D1, Neuro D2 and Neuro D3, have been isolated (4). Neuro D1 and D2 are initially expressed during embryonic development with persistent expression in the adult nervous system. On the other hand, Neuro D3 is expressed transiently during embryonic day 9.5 and is not detected in the mature nervous system (3, 4, 6). Neuro D family is also expressed in primitive neuroectodermal tumors (9).

The same factor as Neuro D1 was also isolated in the pancreas and was designated as BETA2 (b-cell E-box trans-activator 2), a cell-specific transcription factor of the insulin gene and is expressed in a small subset of endocrine cells of the pancreas (10). Neuro D1-null mice die shortly after birth due to severe neonatal diabetes (11).

In the adult pituitary gland, Neuro D1 is expressed in corticotroph cells and contributes to the corticotroph-specific POMC transcription by interacting with Ptx 1, a homeobox transcription factor (12, 13). There have been no extensive studies on Neuro D1 expression in human pituitary adenomas.

In the present study, we investigated the expression of Neuro D1 mRNA and protein in nontumorous human pituitaries and different types of human pituitary adenomas using the RT-PCR and immunohistochemical techniques in order to clarify the functional correlation with hormone production.

MATERIALS AND METHODS

Tissues Studied

Tissues of the pituitary adenomas were obtained at the time of transsphenoidal surgery. Tumor types were determined on the basis of clinical and biochemical findings before surgery, from morphological and immunohistochemical data (14). Human pituitaries from patients without endocrinologic abnormalities were obtained from autopsy cases performed within 4 hours after death.

5 human normal pituitaries and 50 pituitary adenomas were studied; the latter included 10 GH-secreting adenomas, 10 PRL-secreting adenomas, 10 ACTH-secreting adenomas, 20 non-functioning adenomas (10 of them were immunohistochemically positive for gonadotropin subunit). The tissues were submitted to the following RT-PCR and immunohistochemical studies.

RT-PCR

The following oligonucleotide primers for Neuro D1 were synthesized on the basis of published sequences: up-stream 5'-AGT CCG CCT TAC GGT ACC ATG-3' and down stream 5'-GAC AGT CAC TGT AAG CAC AG-3' which generate a RT-PCR product of 447 bp (15). Total RNA extraction was performed by the single-step method (TRIzol reagent kit, Life Technologies) from 19 cases of adenomas (16). DNAase was used to allow for exclusion of genomic DNA contaminations.

First-strand complementary DNA (cDNA) was prepared from total RNA by using a first strand synthesis kit (Ready-To-Go kit, Pharmacia Biotech Inc, USA). The RT reaction was performed at 37°C for 60 min in a final volume of 33 μL with 5 μg total RNA, each deoxyribonucleotide (dATP, dCTP, dGTP and dTTP), Murine Reverse Transcriptase, RNA-guard(porcine), and *Not*I-d(T)18 primer. The PCR was performed in 100-μL final reaction volumes containing 5 μL RT reaction product as template DNA, corresponding to cDNA synthesized from 500 ng total RNA, 1 $\times$ PCR buffer, 1.5 mmol/L MgCl2, 0.2 mmol/L of dNTP mix, 300 ng of each sense and antisense primer for Neuro D1, and 2.5U amplitaq gold DNA polymerase (Perkin-Elmer). Programmable temperature cycling (Perkin-Elmer/GeneAmp PCR system 9700, Norwalk, CT, USA) was performed with the following cycle profile: 95°C for 5 min, followed by 94°C for 1 min, 55°C for 1 min. After the last cycle, the elongation step was done at 72°C for 10 min.

A 8-μL aliquot of PCR product was analyzed by gel electrophoresis, using a 2% agarose gel, and was stained with ethidium bromide. DNA marker (DNA molecular weight marker XIII, BOEHRINGE MANNHEIM) was used as the standard.

Immunohistochemical Examination

The tissues were routinely fixed in 10% formalin or 4% paraformaldehyde for 8 to 24 hours and embedded in paraffin. To detect the expression of Neuro D1 in the tissues, we used goat polyclonal antibody, Neuro D (N-19), which was produced against the amino terminal part of Neuro D1 (Santa Cruz, CA). The anti-anterior pituitary hormone monoclonal antibodies and their dilutions were as follows, anti-human GH (1:800), anti-human PRL (1:600), and anti-human ACTH (1:800) monoclonal antibodies (DAKO Corp., Carpinteria, CA); anti-human follicle-stimulating hormone β (FSHβ) (1:200), anti-human luteinizing hormone β (LHβ) (1:200), and anti-human TSHβ (1:200) monoclonal antibodies (Immunotech, Marseille, France); anti-α subunit of glycoprotein (α-su) (1:100) monoclonal antibody (Chemicon, Temecula, CA).

Immunohistochemical study was performed by the ABC method (17). In the normal human pituitary glands and pituitary adenomas, a double immunostaining method was applied to identify the relationship between Neuro D1 immunoreactivity and anterior pituitary hormones, as reported previously (18). After visualizing the immunoreactivity of the Neuro D1 by 3,3'-3,3'-diaminobenzidine, the antibodies were removed by rinsing the sections in a glycine/HCl acid buffer, pH 2.2, for 2 hours. Then the tissues were incubated with anterior pituitary hormone antibodies for 1 hour and followed by the incubation with alkaline phosphatase-conjugated second antibodies (DAKO Corp., Carpinteria, CA) and visualized with fast blue. Control studies were done by the previously described method (18).

Western Blot Analysis

To confirm the specificity of the anti-Neuro D1 polyclonal antibody, Neuro D (N-19), Western blot analysis for Neuro D1 protein in ACTH-secreting adenomas was performed. ACTH-secreting adenomas which were immunohistochemically positive for Neuro D1 protein were homogenized in ice-cold 50 mM Tris-hydrochloric acid, pH 7.5, containing 2 mM ethylene glycol tetraacetic acid, 1 mM dithiothreitol, and 0.001% leupeptin. Each tissue homogenate was centrifuged at 100,000 μg at 4°C for 1

hour; the supernatants were used for the electrophoresis. The concentration of each sample was measured, and 15 μg of each sample was applied to polyacrylamide gel. After the microphoresis, each sample was transferred to a nitrocellulose membrane. For Western blotting of Neuro D1 protein, Neuro D (N-19) antibody (1:2000 dilution) and subsequently horseradish peroxidase (HRP)-labeled anti goat immunoglobulin (1:400 dilution in Tween 20-phosphate-buffered saline) were applied to each lane as the primary and secondary antibodies, respectively. Immunoreactivity for Neuro D1 protein was visualized by incubation with 3,3'-diaminobenzidine for 1.5 min, which resulted in a brown color.

In Situ Hybridization

The 3'-end biotinylated oligonucleotide probes for Neuro D1 were synthesized according to the published sequence (3, 19). The Neuro D1 antisense probe had the following sequence: 5'-CGC TGC AGG ATA GTG CAT GGT A-3'. The ISH procedure was performed as previously described (20, 21). In brief, after deparaffinization, the sections were treated with 25 ng/mL proteinase K (Boeringer Mannheim) at 37°C for 30 min, followed by hydrochloride treatment, acetylation, and then prehybridization. Thereafter, the sections were hybridized with 3'-biotinylated Neuro D1 probes at 43°C for 18 hrs. After hybridization, the hybridization signals were detected with streptavidin-biotin-alkaline phosphatase using nitroblue tetrazolium-bromochloroindolyl phosphate (NBT-BCIP; Life Technologies). Control experiments were carried out using sense probes that had complementary sequence to the antisense probe.

RESULTS

RT-PCR Analysis

A total of 19 pituitary adenomas were examined for RT-PCR of Neuro D1 mRNA, and the results are shown in Figure 1 and summarized in Table 1. Analysis of Neuro D1 mRNA demonstrated that the expected 447-bp PCR product was detected by ethidium bromide staining in all 3 ACTH-secreting adenomas and 6 of 8 non-functioning adenomas (3 of 4 gonadotropin subunit positive adenomas). On the other hand, Neuro D1 mRNA was not detected in all 5 GH-secreting adenomas or in all 3 PRL-secreting adenomas.

Immunohistochemical Analysis

In five non-tumorous pituitary glands, Neuro D1 protein was expressed in the nucleus of anterior pituitary cells (Fig. 2A). By double staining with

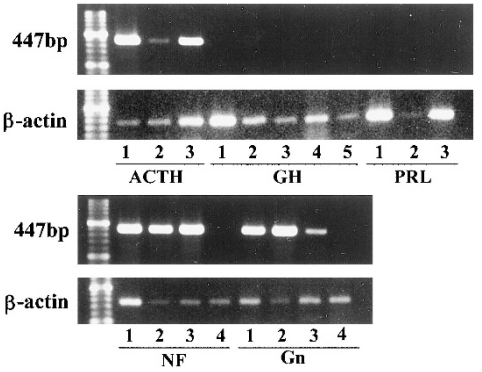

FIGURE 1. RT-PCR for Neuro D1 mRNA of 19 human pituitary adenomas, containing three ACTH-secreting adenomas (ACTH 1–3), five GH-secreting adenomas (GH 1–5), three PRL-secreting adenomas (PRL 1–3), four non-functioning adenomas (NF 1–4), and four gonadotropin-subunit-positive adenomas (Gn 1–4). Analysis of Neuro D1 mRNA demonstrated that the expected 447-bp PCR product was detected in all 3 ACTH-secreting adenomas and 6 of 8 non-functioning adenomas (3 of 4 gonadotropin subunit positive adenomas). On the other hand, Neuro D1 mRNA was not detected in all 5 GH-secreting adenomas or in all 3 PRL-secreting adenomas.

anterior pituitary hormones, Neuro D1 immunoreactivity was mainly localized in corticotroph cells.

A total of 50 pituitary adenomas immunohistochemically exhibited their hormonal expression (14) (Table 1). Of 20 non-functioning adenomas that expressed one or more of FSHβ, LHβ, and α-SU, 10 were subclassified as gonadotropin subunit-positive adenomas. The incidence of Neuro D1 immunoreactivity was highest in ACTH-secreting adenomas (100%) and non-functioning adenomas (70%) (Fig 2, B–C). This data correlated with those of RT-PCR. In contrast, Neuro D1 expression was not detected in most (8 of 10) GH-secreting adenomas immunohistochemically, and only 2 of them (20%) were positive for Neuro D1. Five of 10 cases were negative for Neuro D1 mRNA by RT-PCR, and one of two Neuro D1 immunoreactive cases was negative by RT-PCR (Table 1, Case 11). In addition, most cases of (7 of 10) PRL-secreting adenomas did not exhibit Neuro D1 immunoreactivity, and only 3 of them (30%) were immunohistochemically positive for Neuro D1. One of these three cases was examined by RT-PCR and was negative for Neuro D1 mRNA (Table 1, Case 22).

The specificity of Neuro D1 antibody was confirmed by the absorption method and Western blot analysis. Using Western blot analysis, the Neuro D1 protein was detected as a band of about 50 kDa in ACTH-secreting adenomas (Fig. 3).

In Situ Hybridization

Hybridization signal for Neuro D1 mRNA was detected in cytoplasm of ACTH-secreting adenoma cells (Fig. 4, A–B). On the other hand, PRL-secreting

TABLE 1. Results of Immunohistochemical Analysis for Anterior Pituitary Hormones and Neuro D1 in 50 Pituitary Adenomas, and RT-PCR Analysis of Neuro D1 in the 19 Pituitary Adenomas

Diagnosis	Case No. Age/Sex	Immunohistochemistry									RT-PCR Neuro D1 mRNA
		GH	PRL	ACTH	FSHβ	LHβ	TSHβ	α-SU	Neuro D1 F	Neuro D1 I	
Cushing's	1. 47/F	−	−	+++	−	−	−	−	+	++	Positive
	2. 18/F	−	−	+++	−	−	−	−	++	+++	Positive
	3. 44/F	−	−	+++	−	−	−	−	++	+	Positive
	4. 27/M	−	−	+++	−	−	−	−	+++	+	ND
	5. 52/F	−	−	+++	−	−	−	−	++	+	ND
	6. 48/F	−	−	+++	−	−	−	−	++	+++	ND
	7. 53/F	−	−	+++	−	−	−	−	+++	+++	ND
	8. 52/F	−	−	+++	−	−	−	−	+++	+++	ND
	9. 49/F	−	−	+++	−	−	−	−	++	+−	ND
	10. 23/F	−	−	+++	−	−	−	−	+++	+++	ND
Acromegaly	11. 71/F	+++	+++	−	+	−	−	+	+−	++	Negative
	12. 30/M	+++	+++	−	−	−	−	−	−	−	Negative
	13. 62/M	+++	+++	−	−	−	−	−	−	−	Negative
	14. 57/F	+++	++	−	−	−	−	+	−	−	Negative
	15. 45/F	+++	+++	−	−	−	−	+++	−	−	Negative
	16. 27/F	+++	+++	−	−	−	−	+	−	−	ND
	17. 35/F	+++	+++	−	−	−	−	+	++	+	ND
	18. 47/M	+++	+++	−	−	−	−	+	−	−	ND
	19. 23/M	+++	+++	−	−	−	−	+	−	−	ND
	20. 23/M	++	++	−	−	−	−	−	−	−	ND
PRL-secreting adenoma	21. 19/M	−	+	−	−	−	−	−	−	−	Negative
	22. 63/F	−	+++	−	−	−	−	−	++	++	Negative
	23. 21/F	−	+++	−	−	−	−	−	−	−	Negative
	24. 23/F	−	+++	−	−	−	−	−	++	+++	ND
	25. 19/F	−	+++	−	−	−	−	−	−	−	ND
	26. 31/M	−	+++	−	−	−	−	−	−	−	ND
	27. 69/M	−	+++	−	−	−	−	−	−	−	ND
	28. 28/F	−	+++	−	−	−	−	−	−	−	ND
	29. 24/F	−	+++	−	−	−	−	−	−	−	ND
	30. 23/F	−	+++	−	−	−	−	−	+−	+	ND
Null cell adenoma	31. 64/F	−	−	−	−	−	−	−	+−	+	Positive
	32. 57/F	−	−	−	−	−	−	−	++	+−	Positive
	33. 71/F	−	−	−	−	−	−	−	+++	++	Positive
	34. 16/M	−	−	−	−	−	−	−	+++	++	Negative
	35. 50/F	−	−	−	−	−	−	−	+++	++	ND
	36. 72/F	−	−	−	−	−	−	−	+++	++	ND
	37. 55/F	−	−	−	−	−	−	−	−	−	ND
	38. 37/M	−	−	−	−	−	−	−	+++	++	ND
	39. 42/F	−	−	−	−	−	−	−	−	−	ND
	40. 32/M	−	−	−	−	−	−	−	−	−	ND
Gonadotropin-subunit positive adenoma	41. 29/M	−	−	−	+	+	−	++	++	++	Positive
	42. 43/F	−	−	−	++	−	−	−	++	++	Positive
	43. 62/M	−	−	−	+	+	−	+	+++	++	Positive
	44. 80/M	−	−	−	+++	−	−	+	−	−	Negative
	45. 53/M	−	−	−	++	−	−	+	++	+−	ND
	46. 51/F	−	−	−	+	−	−	+	++	+	ND
	47. 58/M	−	−	−	++	−	−	++	−	−	ND
	48. 64/F	−	−	−	++	+	−	+	−	−	ND
	49. 37/M	−	−	−	+	+	−	+	++	+	ND
	50. 69/M	−	−	−	++	−	−	−	+	+−	ND

Minus sign indicates negative; +−, less than 5%; +, 5 to 20%; ++, 20 to 50%; +++, over 50% of adenoma cells.
Neuro D1 immunoreactivity is estimated by two indexes; frequency and intensity.
F, frequency; I, intensity; ND, not done.

adenomas, including three cases which were immunohistochemically positive for Neuro D1, exhibited no signal for Neuro D1 mRNA (Fig. 4, C–D).

DISCUSSION

Neuro D1, a basic helix-loop-helix transcription factor, is expressed during embryonic development and participate in the terminal differentiation step (1–3). In the adult nervous system, Neuro D1 is expressed in olfactory bulbs, hippocampus, cerebellum and a subset of anterior pituitary gland cells, predominantly corticotroph cells, suggesting a secondary role of Neuro D1 (3, 4, 22).

It has been well known that bHLH transcription factors must form dimers through their HLH do-

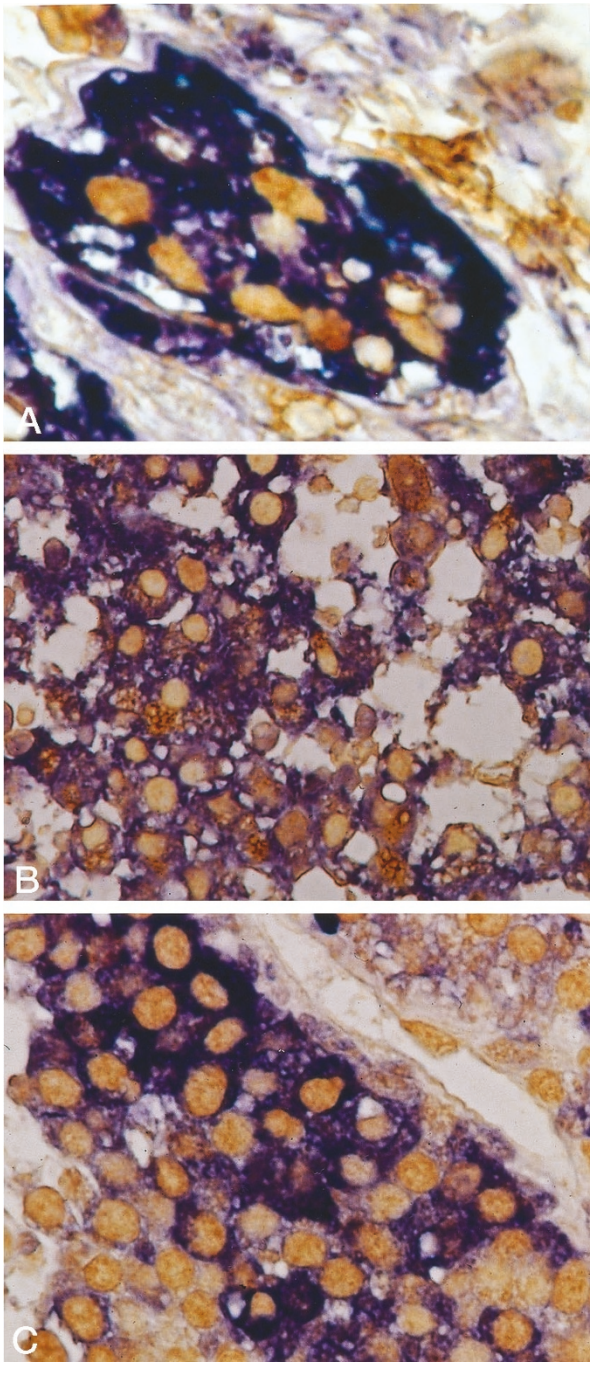

FIGURE 2. **A**, Immunohistochemistry for Neuro D1 protein in human normal pituitary. Expression of Neuro D1 protein is observed in the nuclei of anterior pituitary cells in a brown color. Double immunohistochemical staining indicates that Neuro D1 immunoreactivity (brown) is mainly co-localized in ACTH-immunoreactive cells (blue) (original magnification 200). **B**, Immunohistochemical detection of Neuro D1 protein in ACTH-secreting adenomas. Immunoreactivity for Neuro D1 protein is observed in the nuclei of adenoma cells and co-localized with ACTH in the positive cells (original magnification ×200). **C**, Immunohistochemical detection of Neuro D1 protein in gonadotropin-subunit positive adenomas. Immunoreactivity for Neuro D1 protein is observed in the nuclei of adenoma cells and co-localized with FSH-β in the positive cells (original magnification ×200).

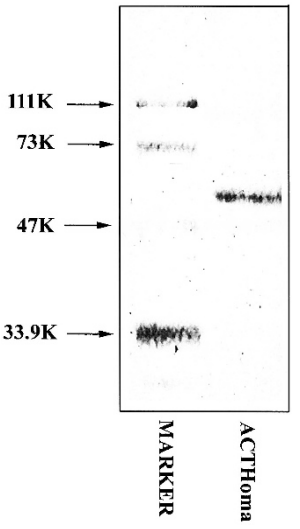

FIGURE 3. Western blotting for Neuro D1 protein in ACTH-secreting adenoma. The Neuro D1 antibody, Neuro D(N-19), detected a band at 50 kDa.

mains in order to bind DNA and this protein-DNA interactions depend on the basic region (23, 24). Neuro D1 complex, forming heterodimers with various ubiquitous bHLH factors, specifically binds to one E box (CANNTG) of the POMC promoter and activates transcription of the POMC gene (13). Neuro D1 complex exhibits transcriptional synergism with Ptx 1, a bicoid-related homeodomain protein, which seems to be a general regulator of pituitary specific transcription and also activates the POMC gene transcription (25–27). So the interaction between Neuro D1 and Ptx 1 may contribute to cell-specific transcription of POMC gene and corticotroph cell differentiation during pituitary ontogeny (12, 13, 26).

In the present study, we investigated the transcriptional and translational expressions of Neuro D1 in non-tumorous pituitary glands and various human pituitary adenomas. Using RT-PCR, Neuro D1 mRNA could be detected in all 3 ACTH-secreting adenomas and 6 of 8 non-functioning adenomas. No expression could be detected in all 5 GH-secreting adenomas or all 3 PRL-secreting adenomas. Immunohistochemically, Neuro D1 protein was detected in the nucleus of non-tumorous anterior pituitary cells, and mainly localized in corticotroph cells which process POMC into ACTH, which is in agreement with the *in situ* hybridization results (13). In human pituitary adenomas, the incidence of Neuro D1 immunoreactivity was highest in ACTH-secreting adenomas (100%) and non-functioning adenomas (70%). In contrast, only 2 cases of GH secreting adenomas and 3 cases of PRL secreting adenomas exhibited immunoreactivity to Neuro D1, and most of them were immunohistochemically negative for Neuro D1. Our result is consistent with the data obtained with pituitary derived cell lines, AtT-20 (corticotroph lineage), GH3 (somatolactotroph lineage) and αT3 (gonado-

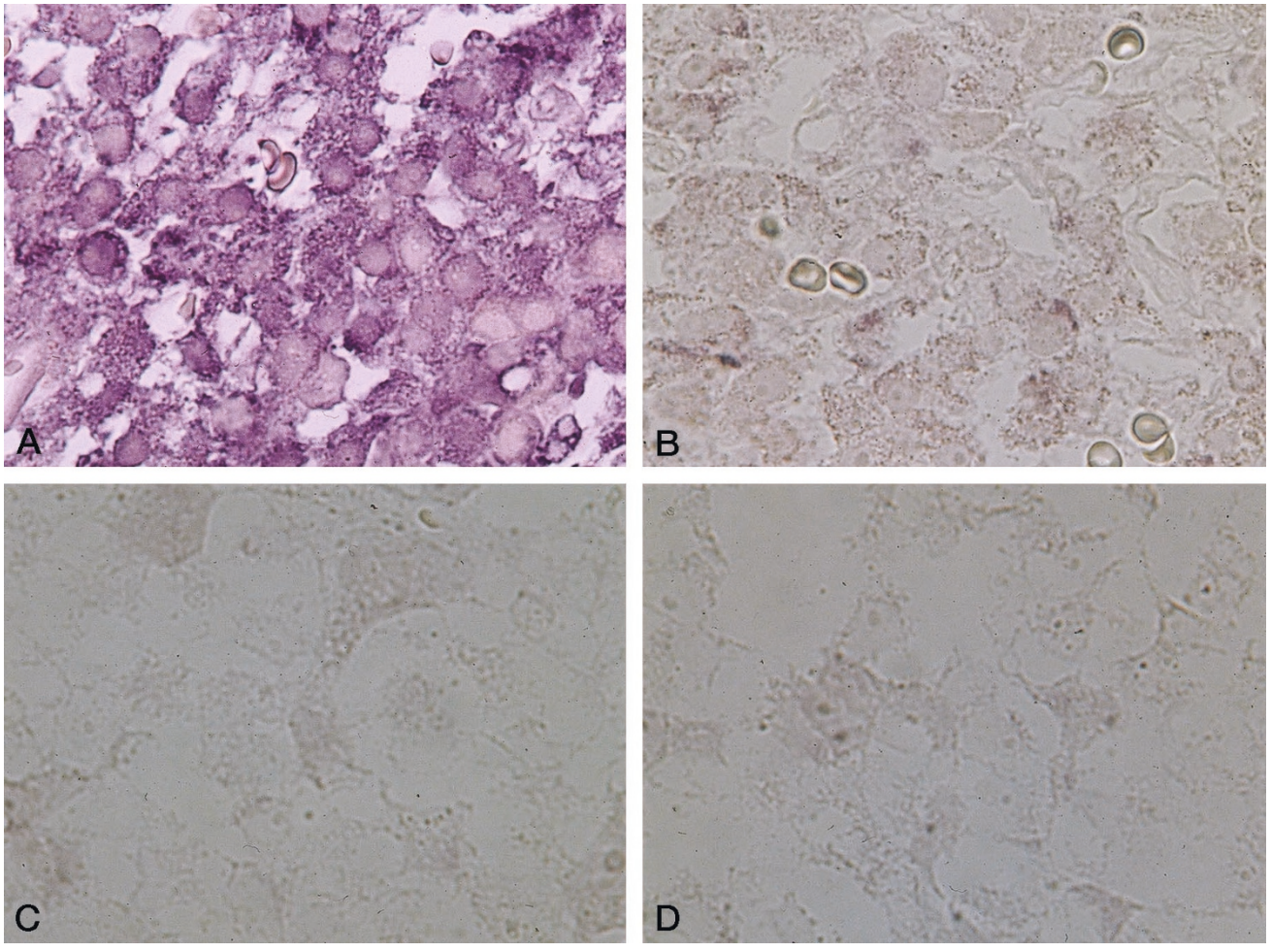

FIGURE 4. Non-radioisotopic *in situ* hybridization performed on paraffin sections of ACTH-secreting adenoma (**A** [AS], **B** [SS]) and PRL-secreting adenoma (**C** [AS], **D** [SS]) both of which are immunohistochemically positive for Neuro D1. Neuro D1 mRNA was detected in ACTH-secreting adenoma. On the other hand, no signal was detected in PRL-secreting adenoma. AS, antisense probe; SS, sense probe.

troph lineage), which showed that Neuro D1 was only detected in AtT-20 and αT3 cell lineage (13). Although occasional cases of GH secreting adenomas and PRL secreting adenomas showed immunohistochemically positive for Neuro D1, the low incidence of immunoreactivity and the absence of Neuro D1 mRNA by RT-PCR and *in situ* hybridization suggest that immunohistochemically positive Neuro D1 may not be a major factor in functional differentiation of both GH secreting adenoma and PRL secreting adenoma, but this remains to be further investigated.

Pituitary ontogeny is governed by a complex myriad of factors, including a number of putative transcription factors, that are expressed at distinct and highly specific phases of pituitary development (28–30). Most pituitary adenomas are monoclonal in origin (31), and recently it has been shown that various transcription factors, such as Pit 1, Prop-1, SF-1, Ptx 1 or Ptx 2, may also participate in the differentiation and functional expression of pituitary adenomas (20, 32–36). Myo D, which converts fibroblast to myoblast, seems to have an ability of determining "the cell fate" (8), and functional similarities between Myo D and Neuro D are suggested. Thus our results suggest that Neuro D1 may participate in the functional differentiation of ACTH secreting adenomas and non-functioning adenomas.

It was recently shown that Ptx 1 is expressed in various types of human pituitary adenomas, including ACTH secreting adenomas, and play an essential role for the differentiation and functional expression of pituitary adenomas (36). The interaction between Neuro D1 and Ptx 1, like their interaction in normal pituitary gland (12, 13), may contribute to the transcription of POMC gene in ACTH secreting adenomas. In addition, the interaction between Neuro D1 and other gonadotroph-specific transcription factors, such as SF-1, Egr-1 and GATA-2, may contribute to the functional expression of gonadotroph adenomas, and further investigations are required to clarify the role of Neuro D1 in non-functioning adenomas.

In summary, Neuro D1 is mainly expressed in ACTH secreting adenomas and non-functioning adenomas. Our results suggest that Neuro D1 con-

tributes to the functional expression and the differentiation of ACTH-secreting adenomas. It also appears from our study that Neuro D1 might contributes to the functional expression and the differentiation of non-functioning adenomas, which require further investigation. This is the first study of Neuro D1 in human pituitary adenomas.

Acknowledgments: *The authors thank Drs. Shigeyuki Tahara, Reiko Kurotani, Kiyoteru Komatsubara, Ichiro Takumi, and Yoshiko Itoh for their technical assistance, and Dr. Johbu Itoh for his photographic assistance.*

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