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## Enhanced expression of GTP cyclohydrolase I in V-1-overexpressing PC12D cells<sup>☆</sup>

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### Abstract

Three of the catecholamine-synthesizing enzymes, i.e., tyrosine hydroxylase (TH), aromatic L-amino acid decarboxylase, and dopamine β-hydroxylase, were earlier shown to be up-regulated in cloned PC12D cells overexpressing V-1, a *cdc10/SWI6* motif-containing protein. GTP cyclohydrolase I (GCH) is the rate-limiting enzyme for the biosynthesis of tetrahydrobiopterin (BH<sub>4</sub>), known as an essential cofactor for TH; and here we found the increased expression of GCH in V-1-overexpressing clones. Both GCH activity and total biopterin content were highly increased in the V-1 clones; whereas the activity of sepiapterin reductase, enzyme in the final step of the BH<sub>4</sub> biosynthesis, was not altered. Biochemical analyses revealed increased levels of GCH protein, mRNA, and transcription in the V-1 clones. Promoter analysis showed increased reporter activity in the construct with 150 bp of the promoter region of the human GCH gene, suggesting the involvement of cAMP-responsive element-mediated transcriptional regulation. © 2002 Elsevier Science (USA). All rights reserved.

**Keywords:** Catecholamine; cAMP-responsive element; GTP cyclohydrolase I; Gene expression; PC12 cells; Tetrahydrobiopterin; Transcription; V-1

Catecholamines (dopamine, norepinephrine, and epinephrine) are neurotransmitters and they play crucial roles in a variety of physiological functions in the brain. Catecholamines are also known to be involved in many neurological and neuropsychiatric diseases such as Parkinson's disease, manic-depressive illness, and schizophrenia. Catecholamines are synthesized from L-tyrosine by the sequential action of four enzymes: tyrosine is converted to DOPA by tyrosine hydroxylase (TH), DOPA to dopamine by aromatic L-amino acid decarboxylase (AADC), dopamine to norepinephrine by

dopamine β-hydroxylase (DBH), and norepinephrine to epinephrine by phenylethanolamine N-methyltransferase. Regulation of the gene expression of catecholamine-synthesizing enzymes is important in brain function under physiological and pathological conditions as well as in the determination of the expression of neurotransmitters during brain development; however the molecular mechanisms of this regulation are poorly understood.

V-1 is a novel protein that may regulate the gene expression of catecholamine-synthesizing enzymes. It was originally identified in the rat cerebellum as one of the proteins the expression of which was transiently increased during the initial stage of postnatal development [1,2]. A high level of the V-1 expression was found to persist in the regions of synaptic plasticity even after the postnatal stage [3]. It is also endogenously expressed in chromaffin cells and overexpression of V-1 in PC12D cells elicited the coordinate up-regulation of the expression of the catecholamine-synthesizing enzymes, i.e., TH, AADC, and DBH, resulting in remarkable

<sup>☆</sup> **Abbreviations:** AADC, aromatic L-amino acid decarboxylase; BH<sub>4</sub>, tetrahydrobiopterin; CRE, cAMP-responsive element; DBH, dopamine β-hydroxylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCH, GTP cyclohydrolase I; PTPS, 6-pyruvoyl tetrahydropterin synthase; SPR, sepiapterin reductase; TH, tyrosine hydroxylase.

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increases in dopamine and norepinephrine contents [4]. The V-1 protein consists of 117 amino acids, ~73% of which represents 2.5 tandem repeats of the cdc10/SWI6 motif, also known as the ankyrin repeat [1]. While a role for the ankyrin repeat in protein–protein interaction has been proposed (reviewed in [5]), no protein has been identified yet as one interacting with the V-1 protein.

Tetrahydrobiopterin (BH<sub>4</sub>) synthesis is essential for catecholamine biosynthesis, because BH<sub>4</sub> is a cofactor for TH. BH<sub>4</sub> is synthesized from GTP in mammals via three enzymatic reactions: GTP cyclohydrolase I (GCH) catalyzes the formation of D-erythro-7,8-dihydroneopterin triphosphate from GTP; and this intermediate is further metabolized by 6-pyruvoyltetrahydropterin synthase (PTPS) and then finally converted to BH<sub>4</sub> by sepiapterin reductase (SPR). GCH is the rate-limiting enzyme for the de novo synthesis of BH<sub>4</sub>. Recently, we found that alteration of the BH<sub>4</sub> content regulates catecholamine and serotonin biosyntheses differently [6].

The GCH enzymatic activity and gene expression are increased in response to various stimuli, whereas those of PTPS and SPR are relatively unaffected (reviewed in [7]). It has been suggested that stimuli that increase expression of the TH gene can often enhance the GCH expression [8–10]. However, the molecular mechanism for co-induction of TH and GCH is unclear.

In this study, we examined BH<sub>4</sub> synthesis in the V-1-overexpressing PC12D cell clones to know whether the biosynthesis of BH<sub>4</sub>, like that of the catecholamine-synthesizing enzymes, was up-regulated in the V-1 clonal cells. We found the enhanced expression of GCH and the elevated level of biopterin content in the V-1 clones. In these cells, GCH enzymatic activity, protein level, and mRNA level were all elevated. We further demonstrated that GCH promoter activity was enhanced, indicating GCH gene transcription.

## Materials and methods

**Cell culture.** Two PC12D clones stably and highly expressing V-1, named V1-46 and V1-69, and two vector control clones, termed C-7 and C-9, were established, as described previously [4]. Parental PC12D cells were cultured in DMEM containing 10% horse serum and 5% fetal bovine serum. The stable clones were cultured in medium containing 280 µg/ml G418 (GIBCO).

**Preparation of cell lysates.** Cells were washed three times and suspended in ice-cold phosphate-buffered saline and then pelleted in a microcentrifuge at 300g for 3 min. For the preparation of cell lysates for the measurement of enzymatic activities and immunoblotting of GCH and SPR proteins, the cell pellet was suspended in 20 mM Tris–HCl buffer (pH 7.5) containing 100 mM KCl, 1 mM EDTA, 1 mM DTT, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM PMSF. The cell suspension was sonicated and the sonicate was centrifuged at 1500g for 10 min; and then the supernatant was immediately collected as the cell lysate. Protein concentration was determined by the Bradford [11] method, with bovine γ-globulin used as the standard. The cell lysate was stored at –80°C in small aliquots until assayed.

**Measurement of GCH activity.** GCH activity was assayed as described previously with a slight modification [12]. The incubation mixture (total volume of 100 µl) contained 100 mM Tris–HCl (pH 8.0), 300 mM KCl, 2.5 mM EDTA, 10% glycerol, and 1 mM GTP as substrate. The reaction was carried out at 37°C for 1 h. The product, D-erythro-7,8-dihydroneopterin triphosphate, was oxidized by iodine solution and dephosphorylated with alkaline phosphatase to neopterin. Neopterin was separated by HPLC on a reverse-phase HPLC column and detected fluorometrically.

**Measurement of SPR activity.** SPR activity was assayed as described previously with a slight modification [13]. The incubation mixture (total volume of 50 µl) contained 100 mM potassium phosphate buffer (pH 6.4), 0.1 mM NADPH, and 0.15 mM sepiapterin as substrate. The reaction was carried out at 37°C for 1 h. Then the reaction was terminated by the addition of iodine solution and kept at room temperature in the dark to oxidize the product, 7,8-dihydrobiopterin, to biopterin. Biopterin was separated and detected by the same method described above for neopterin.

**Measurement of intracellular BH<sub>4</sub> content.** The amount of BH<sub>4</sub> in the cell lysate was measured as biopterin by HPLC with fluorescence detection after iodine oxidation as described above.

**Measurement of TH activity.** TH activity was determined based on the measurement of L-DOPA formed from L-tyrosine by HPLC with electrochemical detection as described previously with a slight modification [14]. The incubation mixture (200 µl) consisted of 200 mM sodium acetate (pH 6.0), 1 mM BH<sub>4</sub>, 0.1 M 2-mercaptoethanol, and 0.2 mg/ml catalase. The reaction was carried out at 37°C for 10 min in air.

**Production of anti-SPR antibody.** A recombinant human SPR was expressed in *Escherichia coli* and purified as previously described [15] and a rabbit polyclonal antibody was then raised against the purified human SPR. For immunization, a solution containing the purified recombinant protein (1 mg/ml) was emulsified with Freund's complete adjuvant. A dose of 0.5 mg of the protein in Freund's incomplete adjuvant was then given subcutaneously as a booster injection at 2-week intervals until a suitable titer had been achieved.

**Immunoblot analysis.** A polyclonal antibody against GCH was raised, as described previously [12]. In the experiments on GCH and SPR, the cell lysate was separated by SDS–PAGE (12% polyacrylamide gel) and transferred to a PVDF membrane (Bio-Rad). Proteins were visualized with ECL plus (Amersham Biosciences).

**Quantitative real-time PCR analysis.** Total RNA was isolated from each clone by using the TRIZOL reagent (GIBCO). The total RNA was subjected to reverse transcription by using Superscript II (GIBCO). Analysis of GCH transcripts by quantitative real-time PCR was performed on a LightCycler using a LightCycler-FastStart DNA Master SYBR Green I Kit (Roche). MgCl<sub>2</sub> was added to a final concentration of 4 mM, and two oligonucleotide primers, AG-CATCACCTGGTCCCATTTG (forward) and TTCCACAATCCTG GCAAGTTTG (reverse), were added to a final concentration of 500 nM each. In parallel, we analyzed the 18S rRNA as an internal control for normalization. Real-time PCR of 18S rRNA was performed on an ABI PRISM 7700 using TaqMan Universal PCR Master Mix (Applied Biosystems).

**Isolation of mRNA and Northern blot analysis.** Cells were harvested and mRNA was partially purified by oligo(dT)–cellulose affinity chromatography using a Quick-prep mRNA Purification Kit (Amersham Biosciences) following manufacturer's protocol. About 30–50% of the purified preparation was expected to be mRNA. The preparations of stable PC12D clones (10 µg of RNA) were electrophoresed in a 1% agarose gel, transferred to Hybond N<sup>+</sup> membrane (Amersham Biosciences) by capillary diffusion, and then hybridized with <sup>32</sup>P-labeled mouse GCH cDNA [16] or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. Radioactivity was visualized by using a BAS-1000 Bio-imaging analyzer (Fuji Photo Film, Tokyo).

**Reporter plasmids.** We previously isolated the human GCH gene [17] and cloned a fragment containing 8.1 kb of its 5'-flanking

promoter region into pBluescript plasmid (hGCHpro8.1-pBS). To generate hGCHpro5.2-Luc containing the region –5236 bp from the transcriptional start site [18] to the translational start site upstream of the firefly luciferase cDNA, we double digested hGCHpro8.1-pBS with *EcoRV* and *NcoI* and then inserted the fragment between *SmaI* and *NcoI* sites of PGV-B2. To generate hGCHpro0.45-Luc containing –452 bp of the GCH 5'-flanking region, we double digested hGCHpro8.1-pBS with *HindIII* and *NcoI* and inserted the fragment between the same sites of PGV-B2. hGCHpro0.15-Luc containing –149 bp of the GCH 5'-flanking region was selected from deletion constructs of hGCHpro0.45-Luc, which were generated by digesting with *HindIII* and *KpnI* and deleting with exonuclease III and mung bean nuclease.

**DNA transfection and luciferase assay.** Seapansy luciferase vector, pRL-CMV (Toyook, Tokyo), was used as an internal control to normalize for variations in transfection efficiency. Cells were transfected by lipofection using LipofectAMINE 2000 (GIBCO) according to manufacturer's instructions of the manufacturer. One day prior to transfection, the cells were plated in 24-well plates and transfected at ~80% confluence with 0.75  $\mu$ g firefly reporter plasmids and 0.05  $\mu$ g pRL-CMV per well. At 48 h after transfection, the cells were harvested and assayed for firefly and seapansy luciferase activities by using a PicaGene Dual Luciferase Assay Kit (Toyook, Tokyo).

**Statistics.** Student's *t* test was used for statistical evaluations. A level of  $P < 0.05$  was accepted as statistically significant.

## Results

### Increased levels of GCH activity and BH<sub>4</sub> content in V-1-overexpressing PC12D cells

We first measured the TH activity (Fig. 1A) and confirmed that TH activity in V-1-overexpressing clones (V1-46 and V1-69) was significantly higher than that in control clones (C-7 and C-9), as described previously [4]. Because BH<sub>4</sub> is an important regulator of TH activity and GCH is the enzyme in the first and rate-limiting step for BH<sub>4</sub> biosynthesis, we assayed the GCH activity and BH<sub>4</sub> content in the V-1 clones. The GCH activity in the V-1-overexpressing clones was increased 8- to 9-fold compared with that in the control clones (Fig. 1B). Reflecting the elevated GCH activity, the BH<sub>4</sub> content in the V-1 clones was 5- to 6-fold higher than that in the control clones (Fig. 1C). In contrast, the activity of SPR, the enzyme in the final step of the BH<sub>4</sub> biosynthesis, remained unchanged (Fig. 1D).

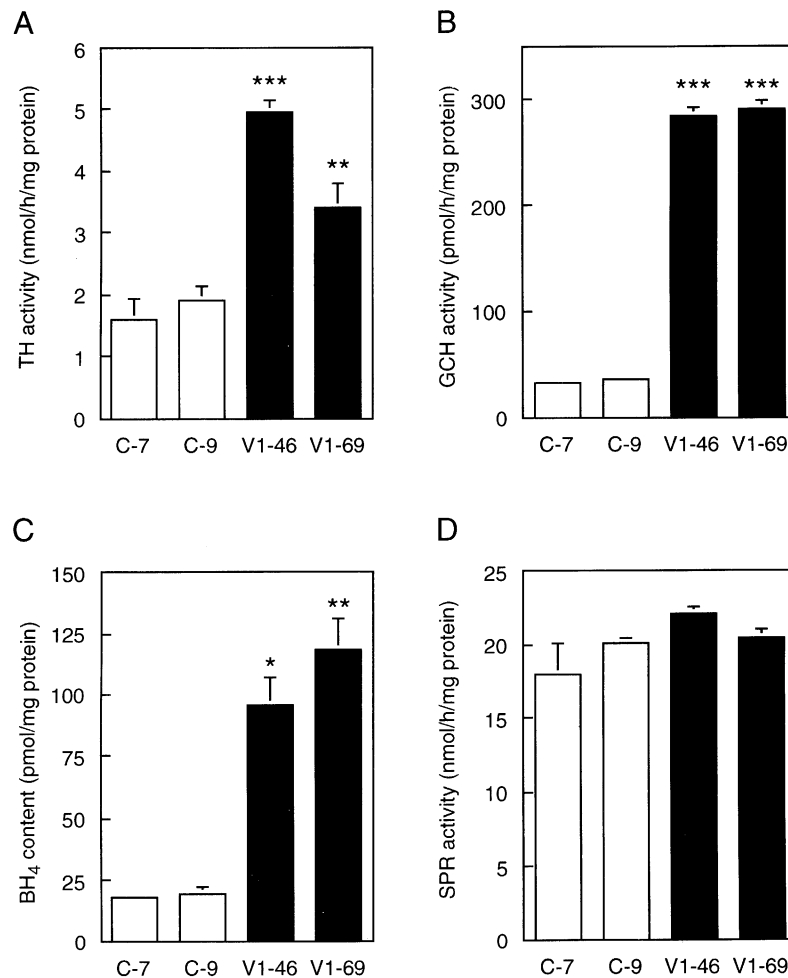


Fig. 1. Increased levels of GCH activity and BH<sub>4</sub> content in V-1-overexpressing PC12D cell clones. Enzymatic activities of TH (A), GCH (B) and SPR (D), and cellular contents of BH<sub>4</sub> (C) in cell lysates prepared from V-1-overexpressing clones (V1-46 and V1-69; closed bars) and control clones (C-7 and C-9; open bars) were measured as described in "Materials and methods". Data are the means  $\pm$  SD values from three independent experiments. Values of  $p$  were calculated based on either value of the control clones: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

### Up-regulation of GCH protein and mRNA in the V-1 clones

As shown in Fig. 2A, Western blot analysis revealed that the amount of GCH protein was much increased in the V-1 clones, paralleling the increase in the enzymatic activity. The level of SPR protein was unaffected (Fig. 2A).

Next, we determined the GCH mRNA level by the quantitative real-time PCR method. We also quantified

18S rRNA as an internal control for normalization. The amount of GCH mRNA relative to that of 18S rRNA in the V-1 clones was increased 3- to 5-fold compared with that for the parental PC12D cells, whereas the vector control clones and parental cells were at the same level (Fig. 2B).

Because two transcripts for GCH (about 1.2 and 3.0 kb) were reported to exist in the rat adrenal medulla and PC12 cells [19], we performed a Northern blot analysis. It turned out that both sizes of GCH mRNAs

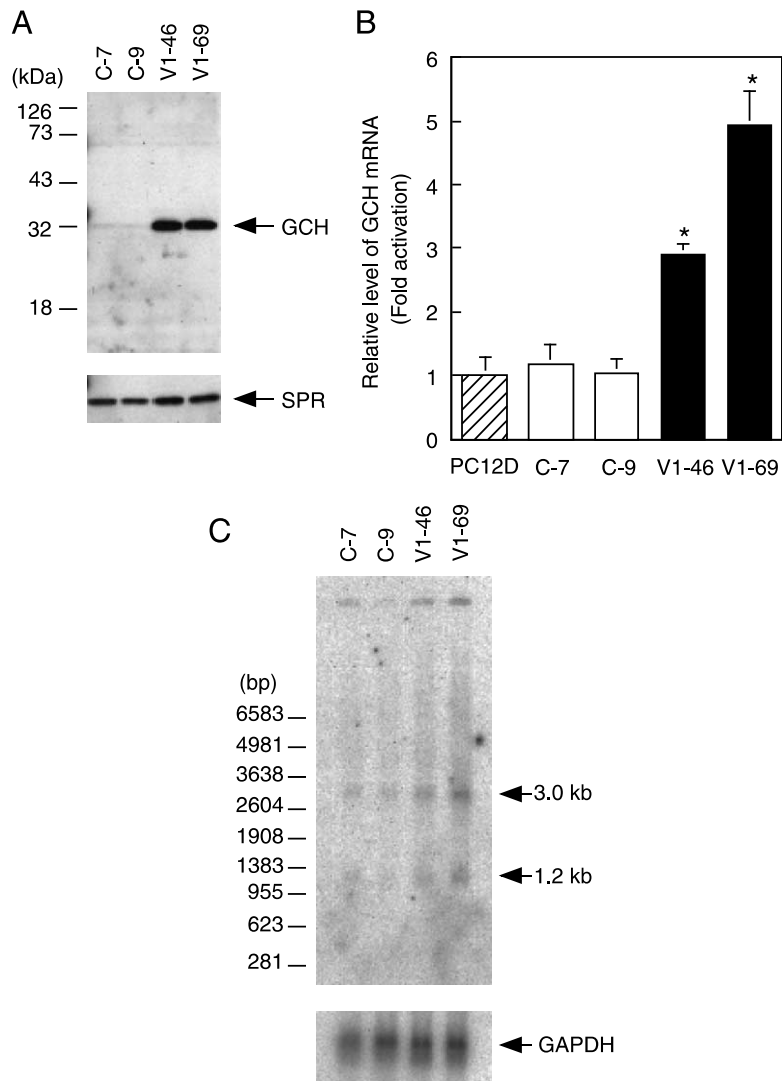


Fig. 2. Up-regulation of GCH protein and mRNA levels in the V-1 overexpressing clones. Analyses by Western blotting (A), real-time quantitative PCR (B), and Northern blotting (C) were performed as described in “Materials and methods”. A, Western blotting was performed by using anti-GCH (upper panel) and anti-SPR (lower panel) antibodies as described in “Materials and methods”. Fifteen microgram amounts of cell lysate protein of the V-1-overexpressing clones and control clones were used. The position of GCH is indicated by the arrow. B, levels of GCH mRNA in the V-1-overexpressing clones (closed bars) and those in the control clones (open bars) are expressed as fold activation relative to those in the parental PC12D cells (hatched bar). 18S rRNA was used for the internal control of normalization. Data are the means  $\pm$  SD values from three or four independent experiments. Values of  $p$  were calculated compared with the values of the parental PC12D cells: \* $p < 0.01$ . C, Partially purified polyA<sup>+</sup> RNA preparation of stable clones (5  $\mu$ g) was used for Northern blotting. An analysis using the mouse GCH cDNA probe is shown in the upper panel and a subsequent reprobings of the blot with the mouse GAPDH cDNA probe is shown in the bottom panel.

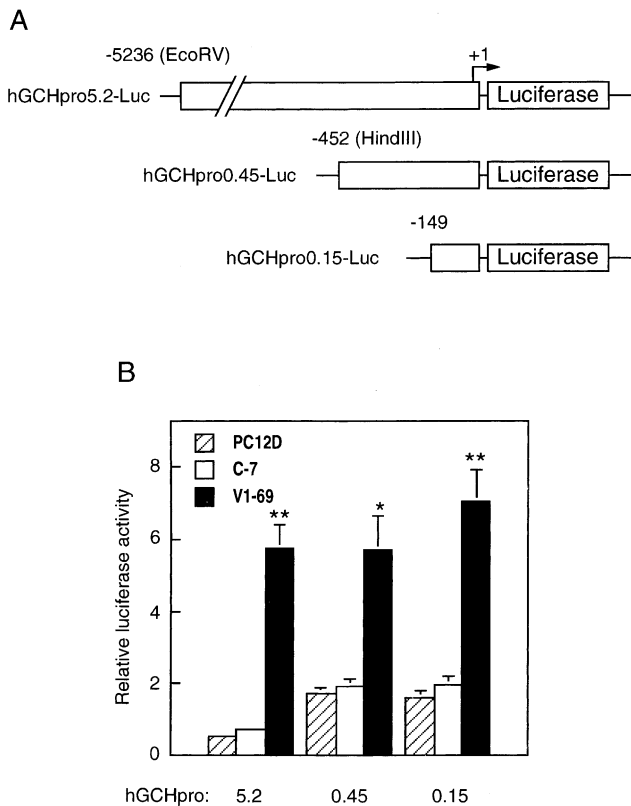


Fig. 3. Enhancement of GCH promoter activity in the V-1 clones. Reporter activities of GCH promoter plasmids (hGCHpro5.2-Luc, hGCHpro0.45-Luc, and hGCHpro0.15-Luc) in the V-1-overexpressing clone (V1-69, closed bars), the control clone (C-7, open bars), and the parental cells (PC12D, hatched bar) were measured as described in "Materials and methods." A seapansy luciferase vector, pRL-CMV, was used as an internal control to normalize for variations in transfection efficiency. Data are the means  $\pm$  SD of triplicate cultures and are representative of those of two other independent experiments. Values of  $p$  were calculated based on the value of either the parental cells or the control clone: \* $p < 0.05$ , \*\* $p < 0.01$ .

in the V-1 clones were increased compared with their levels in the control clones (Fig. 2C).

#### Increased promoter activity of the GCH gene in the V-1-overexpressing clones

To examine the transcription of the GCH gene, we transfected the parental and cloned PC12 cells with plasmid constructs containing 5.2, 0.45, and 0.15 kb of the human GCH 5'-flanking region fused to a luciferase reporter gene. Reporter activities of all constructs relative to the activity of pRL-CMV were significantly increased in the V-1 clone (V1-69) compared with those in the control clone (C-7) and the parental cells (Fig. 3). Similar activation was observed using the other V-1 clone (V1-46, data not shown). These data suggest that the increased level of GCH mRNA in the V-1 clones was mainly due to an increased transcriptional rate.

## Discussion

In the present study, we showed an increased BH<sub>4</sub> content and enhanced expression of GCH in the V-1-overexpressing clones of the PC12D cell line. The data indicate that GCH is up-regulated along with three of the catecholamine-synthesizing enzymes, i.e., TH, AADC, and DBH, in the V-1 clones to elevate the BH<sub>4</sub> level.

To characterize the transcriptional machinery acting for the expression of these genes including GCH in the V-1 clones, we performed transient transfection assays with various lengths of the 5'-upstream region of the GCH gene. The results of the promoter analysis showed that 0.15 kb of the GCH promoter region was sufficient for the increased reporter activity both in the V1-69 clone (Fig. 3) and in the V1-46 clone (data not shown). This construct is nearly of the same size as the minimum promoter, which was recently identified as the region contributing to basal and cyclic AMP-induced transcriptional activities and containing a noncanonical CRE [10,20]. Recently, using a reporter construct having the canonical CRE, we demonstrated that the CRE-mediated transcriptional activity was enhanced in the V-1 clones (Suzuki et al., manuscript in preparation). These data suggest that the enhanced expression of GCH may be due to the elevation of the CRE-mediated transcription in the V-1 clones.

Whereas GCH activity and BH<sub>4</sub> content were increased in the V-1 clones, SPR activity and the protein level were little affected (Figs. 1 and 2). Previous reports described the induction of GCH activity by various hormones or cytokines with the constitutive expression of SPR in various cell types [21,22]. Our data confirm that GCH is more highly regulated than SPR and suggest that the regulation of GCH gene expression is closely related to the modulation of the BH<sub>4</sub> content.

Our finding of the increased biopterin content in the V-1 clones suggests that the up-regulation of the BH<sub>4</sub> cofactor contributes to the increased levels of dopamine and norepinephrine [4]. BH<sub>4</sub> is not only essential for TH enzymatic activity but also for the stability of the TH protein [6]. In addition, it was reported that the gene expression of GCH was induced along with that of TH by various stimuli that also prompted elevation of the BH<sub>4</sub> level [8–10]. Therefore, our data also suggest that elevation of the BH<sub>4</sub> level by GCH induction is essential when TH gene expression is induced to increase catecholamine levels. This suggestion is supported by the findings of a previous study in gene therapy showing that triple transduction with TH, AADC, and GCH genes in adeno-associated virus vectors improved the rotational behavior of parkinsonian rats more effectively and resulted in a greater dopamine production in 293 cells than did double transduction with TH and AADC genes [23].

In the V-1-overexpressing cells, the mechanism(s) elevating the TH, AADC, and DBH mRNA levels is still unknown. However, our data showing the enhanced transcription of the GCH gene suggest that any transcriptional machinery could be expected to play key roles in the concordant expression of these enzymes, even though the expression of each enzyme is governed by different mechanisms. In particular, the concordant gene expression of all catecholamine-synthesizing enzymes should be required at the developmental stage at which catecholaminergic neurons arise. Since the expression pattern of V-1 in the brain seems to be closely related to the brain development and neural plasticity [1–3], the present data imply the existence of transcriptional machinery for the concordant gene expression of the catecholamine-synthesizing enzymes including GCH in developing catecholaminergic neurons.

Although V-1 contains 2.5 tandem repeats of the ankyrin repeat, which may serve as an interface for protein–protein interaction [5], no V-1-associating protein has been identified yet. V-1 has no putative DNA binding domain and it likely acts as an adapter protein in the cytosol [4]. To clarify the entire cascade of the V-1 overexpression for the up-regulation of the catecholamine synthesis, we are now investigating proteins interacting with V-1 as well as regulatory proteins that enhance the expression of the catecholamine-synthesizing enzyme genes including GCH.

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