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General transcriptional repression by polyglutamine disease proteins is not directly linked to the presence of inclusion bodies

Masataka Hoshino,[a] Kazuhiko Tagawa,[a] Tomohiro Okuda,[a] and Hitoshi Okazawa[a,b,c,*]

[a] *Department of Molecular Therapeutics, Tokyo Metropolitan Institute for Neuroscience, 2-6, Musashi-dai, Fuchu, Tokyo 183-8526, Japan*
[b] *Department of Neuropathology, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8510, Japan*
[c] *PRESTO, Japan Science and Technology Corporation (JST), 4-1-8, Hon-machi, Kawagoe, Saitama 332-0012, Japan*

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Abstract

By using direct immunocytochemistry of BrU incorporated to RNA in the nuclei, we evaluated the effect of mutant huntingtin and ataxin-1 on general transcription in primary cortical and cerebellar neurons. Our quantitative analyses clearly showed that these mutant polyglutamine disease proteins repress general transcription. In addition, we found that general transcription level was almost similar in inclusion body-positive and -negative neurons. The result suggests that presence of inclusion body is not essential for repressing general transcription in contrast to its reported role for suppressing specific gene transcription in the polyglutamine disease pathology.

Various molecular pathologies including proteasomal dysfunction (see review [1]), endoplasmic reticulum stress [2,3], abnormal phosphorylation [4,5], and calcium homeostasis dysregulation [6] have been implicated in polygutamine diseases. Among these hypotheses, a number of results have suggested that nuclear transport of abnormal proteins (for example, [7,8]) and resultant perturbation of nuclear functions are essential for neuronal death or dysfunction in models of polyglutamine diseases (see review [9]). Especially, dysfunctions of transcription and splicing have been implicated in the polyglutamine disease pathology.

It was reported that specific genes are repressed at transcription level by mutant protein [10]. However, the result naturally did not exclude the possibility that transcription is generally repressed in neurons of polyglutamine (polyQ) diseases. The latter hypothesis is possible because many groups have suggested a number of transcription factors affecting expression of various genes are involved in the polyglutamine disease pathology (see review [9]) and because some factors involved in general transcription are known to interact with mutant disease proteins [11,12]. However, no direct proof that repression of general transcription occurs in the polyglutamine disease pathology has been made.

Inclusion body formation is clearly a pathological hallmark of polyglutamine disease. The nature of inclusion body was once believed to be firm and irreversible aggregates. However, recent analyses have shown that abnormal proteins could shuttle between inclusion bodies and nucleoplasm at least in a part of inclusions [13–15]. These data showed that inclusion body formation is divided into multiple steps and the characters of resultant inclusion bodies are also heterogeneous. These notions are closely linked to a question arisen recently as to which step of mutant protein is really toxic for neurons. In correlation with transcription, it is not yet settled whether inclusion body is essential for transcriptional repression or not.

To explore these questions, we conducted adenovirus vector-mediated expression of huntingtin (htt), the disease protein of Huntington's disease, and of ataxin-1 (AT-1), the disease protein of spinocerebellar ataxia type 1, in primary neurons. To estimate the level of general transcription, we quantified the uptake of BrU into the nucleus and compared the values of inclusion-positive neurons and those of inclusion-negative neurons.

* Corresponding author. Fax: +81-42-321-8678.
E-mail address: okazawa-tky@umin.ac.jp (H. Okazawa).

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We found that general transcription is repressed by mutant htt and AT-1. Surprisingly, our results also indicated that general transcription is not affected by the presence of inclusion bodies. These results support that HDAC inhibitors, a group of general transcription upregulators, promotes survival of neurons expressing mutant proteins [16–18] and suggest that the presence of inclusion is not essential for repression of general transcription.

Materials and methods

Antibodies. Primary antibodies were used as follows. Anti-AT-1 goat polyclonal antibody (H-21, Santa Cruz) and anti-htt goat polyclonal antibody (N-18, Santa Cruz) were diluted at 1:100 in phosphate-buffered saline (pH 7.5) with 0.5% skimmed milk. Anti-BrdU mouse monoclonal antibody (Sigma–Aldrich) was diluted at 1:200 in PBS with 5% normal goat serum. As secondary antibody, anti-mouse IgG goat polyclonal antibody coupled to Cy3 (Jackson ImmunoResearch) diluted at 1:1000, and anti-goat IgG donkey polyclonal antibody coupled to Alexa fluor 488 (Molecular Probes) diluted at 1:1000 were used. Immunostaining with anti-polyglutamine protein antibody and that with anti-BrdU antibody were performed in tandem.

Primary neuron culture. Cerebral cortex tissues were isolated from E17 Wistar rat embryos and cerebellar tissues were isolated from P7 Wistar rat pups, minced by razors, and treated with 0.25% trypsin (Gibco) in phosphate-buffered saline (pH 7.5) at 37 °C for 20 min with shaking gently every 5 min. After stopping the reaction by DMEM containing 50% fetal bovine serum, DNase I (Boehringer Mannheim) was added to the solution at the concentration of 100 μg/ml, and tissues were dissociated gently by pipetting with blue tips. Cells filtered by nylon mesh (FALCON, pore size 70 mm) were collected by centrifugation, re-suspended in DMEM supplemented with 20 mM glucose, 16 mM sodium bicarbonate, 4 mM L-glutamine, 25 μg/ml gentamicin, and 10% fetal bovine serum, and then plated on 6-well dishes (Corning) coated by poly-D-lysine (Sigma) at 1.7×10^6 cells/well. For cerebellar neurons, KCl was added to the medium at 25 mM final concentration. Twelve hours after plating, cytosine arabinoside was added to the culture medium at 4 μM final concentration to prevent glial cell contamination.

Construction of adenovirus vectors. Recombinant adenoviruses for expression of htt proteins containing 20 or 111 polyglutamine repeats (AxCAhtt20 and AxCAhtt111, respectively) and AT-1 proteins containing 30 or 82 polyglutamine repeats (AxCAAT1-30 and AxCAAT1-82, respectively) were constructed by subcloning normal/mutant htt exon 1 cDNA [19] or normal/mutant AT-1 cDNA [20] into the *Swa*I site of a cosmid vector, pAxCAwt (Takara). These cosmids were transfected into 293 cells, in which adenovirus containing the insert was generated by recombination with the fragmented virus DNA. These steps were basically performed according to the protocol of Adenovirus Expression Vector Kit (Takara). The viruses were amplified two or three times in 293 cells and working stocks of adenoviruses were prepared by destructing 293 cells by sonication three days after transfection. Primary neuronal cells were infected at a multiplicity of infection (MOI) of 100.

BrU treatment and immunofluorescence microscopy. For in situ detection of transcription, primary neuronal cells three days after infection were incubated for 3 h at 33 °C in culture medium containing 20 mM BrU (Tokyo-Kasei), washed briefly with PBS, fixed, and processed for immunostaining. By subtracting BrU, we determined the condition as described above that does not produce background stains with anti-BrdU antibody. Double-labeling immunofluorescence experiments were performed by separate sequential incubations of each primary antibody followed by incubation with its specific secondary antibody. All incubations were at room temperature for 1 h. The digital images were collected by Living Cell Imaging system of microscopy, IX71 (Olympus) and CCD camera, ORCA-ER (HAMA- MATSU). Saved images were analyzed with quantitative analysis software, AQUACOSMOS (Hamamatsu) as described previously [21]. In brief, we demarcate the nucleus on RC view, summate signals within the drawing on IF view, and subtracting background signals from the sum.

Microarray analysis. The microarray analysis was conducted using 14K rat cDNA microarray chip (Agilent Technologies) according to the manufacturer's protocol. Approximately 50 μg of total RNA samples was prepared from one dish of each primary culture neuron 2 days after infection as described in RT-PCR analysis of Materials and methods. The total RNA was converted to fluorescently labeled cDNA by either Cy-3- or Cy-5-dCTP (Perkin–Elmer/NEM) and oligo(dT) primer using Fluorescent Direct Label Kit (Agilent Technologies). The labeled cDNA reactions for each microarray hybridization were combined and then purified using QIAquick PCR Purification Kit (Qiagen). The purified and labeled cDNA was concentrated by drying the solution under vacuum until dry. The labeled cDNA was resuspended and Deposition Control Targets (Qiagen Operon), Mouse Cot-1 DNA (Invitrogen), and 2× hybridization buffer (Agilent Technologies) were added. The microarray was covered by glass coverslips with spacer, 24 × 30 mm No.4 (Matsunami Glass). The hybridization mixture filled the entire surface beneath the coverslip. The microarray was incubated at 65 °C for approximately 17 h using CHIBIO (Hitachi Soft Engineering). It was washed by 0.5× SSC–0.01% SDS for 5 min at RT and then by 0.06× SSC for 2 min at RT. It was dried by centrifugation at 400*g* for 2 min at RT. After hybridization and washing, Cy-3 and Cy-5 signals were scanned by CRIIBIO and data analysis was conducted by using DNASIS Array (Hitachi Soft Engineering).

Results

To investigate the level of general transcription, we applied direct immunocytochemistry of BrU taken up from the culture medium to RNA. This method was previously used for analysis of general transcription in non-neuronal cells expressing SMN, the causative protein of a motor neuron disease, SMA [22]. We modified the technique and conducted this experiment with primary neurons (Fig. 1). We quantified nuclear intensities of immuno-labeling of anti-BrdU antibody by an Olympus microscope with the software AQUACOSMOS as described previously [21]. Before analyzing neurons expressing mutant polyQ proteins, we tested whether this assay can detect transcriptional upregulation by HDAC inhibitor or not (Fig. 2). A HDAC inhibitor, sodium butyrate, is reported to upregulate transcription in animal models of polyQ diseases (16–18). We could actually detect upregulation of general transcription in a dose-dependent manner (Fig. 2), proving that our BrU-uptake assay is available. We could also detect transcriptional upregulation by a novel synthetic peptide inhibiting interaction between PQBP1 and RNA polymerase II with the BrU-uptake assay (Hoshino et al., in preparation).

Next we constructed adenovirus vectors for expression of mutant htt and AT-1, and infected them into primary neurons prepared from the cerebral cortex of E17 rat embryos and from the cerebellum of P7 rat pups

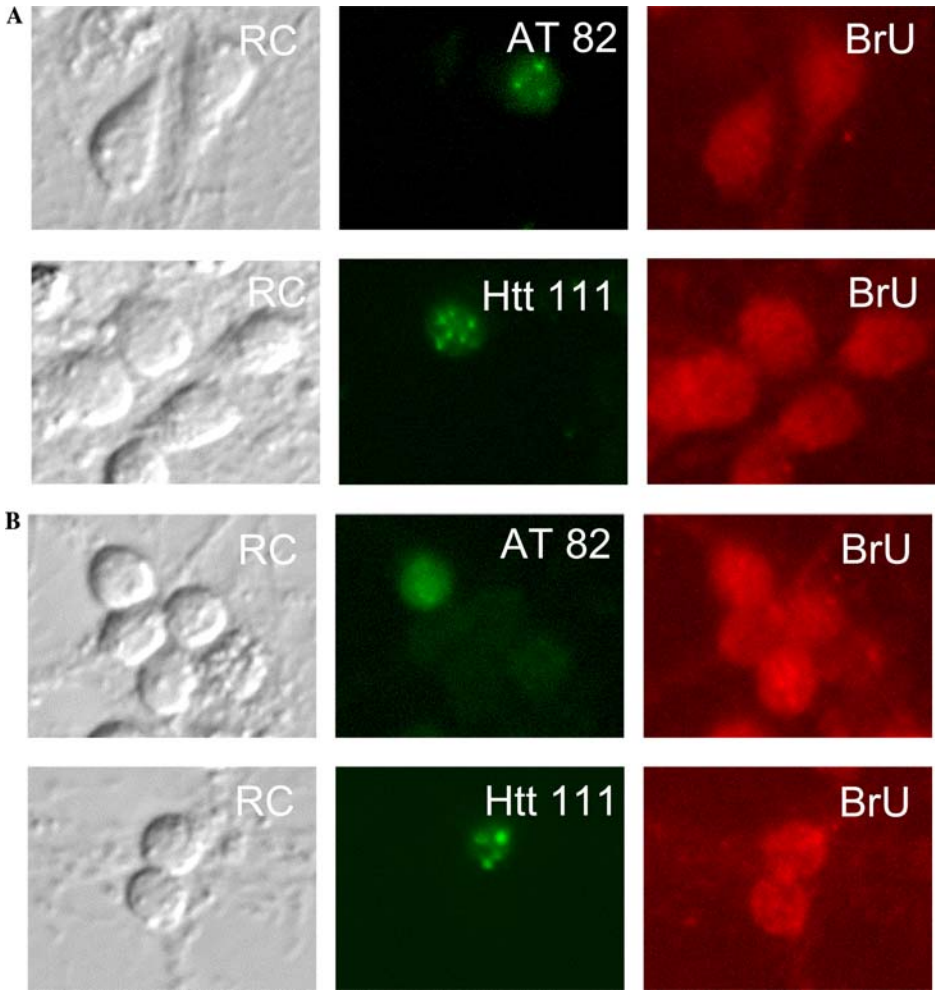

Fig. 1. (A,B) BrU uptake of cortical and cerebellar neurons expressing either mutant AT-1 or htt.

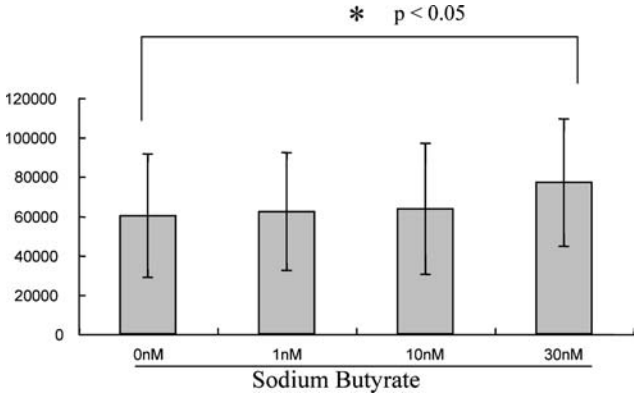

Fig. 2. To examine sensitivity of the BrU uptake-mediated transcription assay, we observed the effect of sodium butyrate on general transcription in cortical neurons. Twenty four hours after addition of sodium butyrate, BrU was added to the medium. BrU assay was performed after further 3 h. We could observe statistical significant increase of BrU uptake (t test) in a concentration-dependent manner.

as described previously [21]. We analyzed these neurons 3 days after infection when neurons do not show decline in the natural survival ratio (Tagawa et al., unpublished observation). We have observed that htt111 shows mild but statistically significant increase of cell death after day 4 while AT82 does not significantly increase cell death from day 2 to 4 (Tagawa and Hoshino, unpublished observation). Before day 2, we could not exclude non-specific effect of adenovirus infection. Therefore, we selected day 3 for the analysis. We added BrU to the culture medium for a short time and then stained neurons simultaneously with anti-BrdU antibody and with anti-polyglutamine disease protein antibody (Fig. 1). We quantified nuclear intensities of immuno-labeling of anti-BrdU antibody by an Olympus microscope with the software AQUACOSMOS as mentioned above [21], and compared the intensities among mock vector-, normal protein expressing vector-, and mutant protein expressing vector-infected cells (Fig. 3). In cortical neurons, mutant AT-1 and htt repressed BrU signals when compared to normal AT-1 and htt, respectively. The results clearly indicated that these mutant proteins repress transcription in general. Interestingly, normal htt tends to up-regulate transcription although we could not confirm it statistically. In cerebellar neurons, the effects

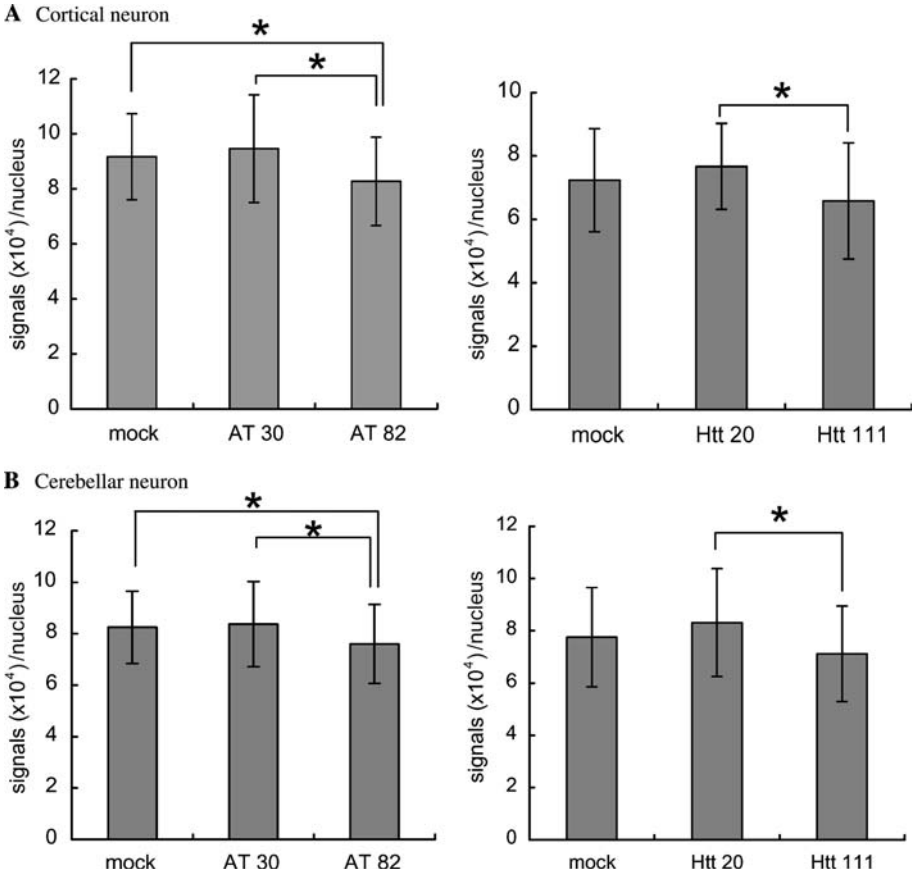

Fig. 3. Comparison of BrU uptake among neurons infected by mock-infected (mock), normal protein-expressing (AT30 or Htt20), and mutant protein expressing neurons (AT82 or Ht111). Signals in a nucleus were automatically summated by demarcating the nucleus on the digital image with AQUACOSMOS, as described previously [21]. Mean ± SD are indicated. Statistical analyses were performed by t test and asterisks indicate significant difference ($p < 0.05$).

of mutant htt and AT-1 on general transcription were basically similar (Fig. 3).

We further asked whether the presence of inclusions is related to general transcription level. We compared nuclear intensities of BrU between inclusion-positive and -negative neurons by using the software for quantifying signals in the area of interest under the microscope view (Fig. 4). Statistical analyses indicated that the BrU uptake was not different between two groups both in cortical and cerebellar neurons. The result meant that general transcription is not repressed specifically in neurons carrying inclusion bodies. Collectively with the result of Fig. 3, we concluded as follows. Mutant htt and AT-1 repress general transcription. The repression initiates before the proteins compose aggregates, and the level of repression is basically similar between inclusion-negative and -positive neurons.

Our approach using BrU-uptake theoretically detects gene expression either by RNA polymerase I, II or III (pol I–III). Therefore, our results have not shown whether a specific type of transcription is downregulated by polyQ proteins or all types of transcription is equivalently affected. To answer this question, we conducted electrophoresis of total RNA and microarray analysis. As shown in Fig. 5, we could not find obvious change in the ratio of ribosomal RNA to total RNA. It suggests that pol I-dependent transcription are equally affected with non-pol I dependent transcription. We could not use microarray analysis for detecting difference between three types of transcription because DNA chips are designed only for pol II-dependent transcription and they do not contain genes transcribed by pol I or pol III. Furthermore, principally, microarray analysis can only show relative expression of a gene (= an RNA transcript) to total RNA transcripts whose sum is assumed to be constant among the compared groups. However, among the pol II-dependent genes, we found that the relative expression of housekeeping genes was increased by mutant polyQ (Table 1). It suggested that genes other than constitutive housekeeping genes were actually decreased.

Discussion

In this study, we show that transcription is generally repressed by expression of abnormal polygutamine

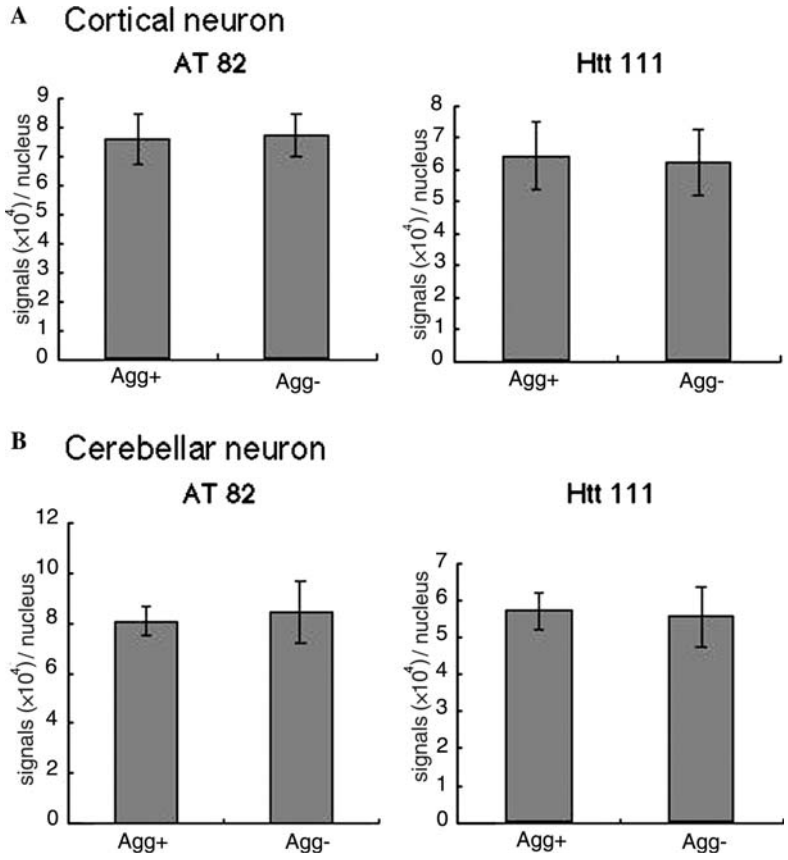

Fig. 4. Comparison of BrU uptake between aggregate-positive and -negative neurons. Statistical analyses were performed by *t* test and no difference was observed between the two groups in each gene expression.

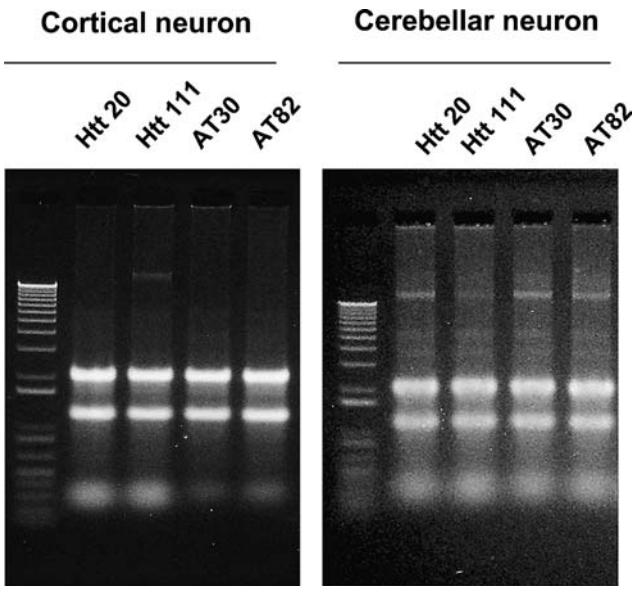

Fig. 5. Analyses of total RNA prepared from cortical and cerebellar neurons 3 days after infection of adenovirus vectors for normal and mutant htt or ataxin-1. As much as 500 ng of total RNA samples was separated on 1% agarose gel. Intensities of 28S and 18S ribosomal RNA bands show no remarkable change, suggesting that relative activities of pol I and non-pol I (pol II + pol III) do not change remarkably under expression of normal polyQ protein and that of mutant polyQ.

disease proteins, htt and AT-1. A criticism for our results would be that repression of general transcription might be a result of loss of cell viability and therefore transcriptional repression may not be the cause of polyglutamine disease pathology. However, we do not take this idea because of the following reasons. The first reason is that transcriptional repression was observed both in cortical and cerebellar neurons. Repression should be more prominent in htt-sensitive cortical neurons than in htt-insensitive cerebellar neurons (Tagawa et al, unpublished observation) if transcription were repressed as a result of cell death. The second reason comes from the data of other groups. Several groups have reported that HDAC inhibitors, which activate general transcription, rescued neurons from the toxicity of mutant proteins in mouse and *Drosophila* models [16–18]. If repression were secondary, HDAC inhibitors should be non-effective.

Our results suggest that target genes of polyglutamine disease proteins in transcription are ubiquitous. So far, it has been suggested that transcription is repressed through dysfunction of specific transcription factors sequestered into inclusion bodies. Among such transcription factors, many data support sequestration of CBP that plays a critical role in histone acetylation (for example, [23,24]). Dysfunction of CBP might be able to

Table 1
Microarray expression profiling data showing relative expression levels (signal intensities scanned by array reader) of housekeeping genes under normal and mutant polyQ expression

	AT82	AT30	Ratio	Htt111	Htt20	Ratio
Cortical neuron						
GAPDH	3999	2105	1.9	3121	2331	1.334
Actin	3953	2994	1.32	2707	2081	1.3
Vascular actin	0	0	0	0	0	0
NF-L	1221	1013	1.206	1134	724	1.57
Cerebellar neuron						
GAPDH	2806	1877	1.495	8628	6096	1.415
Actin	4962	4107	1.208	10200	7446	1.37
Vascular actin	0	0	0	0	0	0
NF-L	2033	1745	1.166	5082	3083	1.65

Signals after correction with total chip signals were increased under the mutant protein expression. Ratios indicate AT82 intensity/AT30 intensity and htt111 intensity/htt20 intensity, respectively.

affect the level of general transcription although it is not known exactly how much percentage of histone acetylation is mediated by CBP among numerous HAT-activity-possessing nuclear proteins (see review [9]). It could be also possible that general transcriptional repression observed in this study is the summative result of specific gene repressions. However, at least, our results clearly ruled out that polyglutamine proteins suppress only a few transcription factors which function in specific gene regulation.

It is rather surprising that transcription level was not linked to the presence of inclusion bodies (Fig. 4). In the expression of htt, the mean value of transcription level was higher in aggregation-positive cells although statistical difference was not confirmed. These results showed that aggregates are not essential to suppress general transcription. Therefore, sequestration theory that transcription is repressed via sequestration of transcription factors into inclusions is not true at least in general transcription. Because transcription factors are incorporated to different types of nuclear bodies and because mutant proteins do not accumulate in all types of nuclear bodies, mutant proteins are not likely to affect all transcription factors. It seems more plausible that mutant proteins non-specifically disturb nuclear factors in the nucleoplasm outside of inclusion bodies. In this sense, our data support a new scheme emerging recently that transcription is disturbed in the nucleoplasm. We could not rule out the possibility that general transcription is repressed by dysfunction of a few critical factors involved in the core transcriptosome which are not necessarily sequestered into inclusions to repress transcription. As such candidates, we could even assume CBP. We still need further analysis to answer all these new questions arisen in this study.

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