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Construction and characterization of murine neuroblastoma cell clones allowing inducible and high expression of the prion protein

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A tetracycline-inducible expression system has been established for the prion protein (PrP) in murine neuroblastoma cells (N2a). For this purpose, N2a cells were first stably transfected with either the tetracycline-controlled transactivator or the reverse transactivator. After selection of N2a clones which carried one of these transactivators, the murine PrP gene (*Prnp*) was introduced under the control of the transactivator-responsive promoter in a second round of stable transfection. Stably double-transfected N2a clones carrying the reverse type but not the normal transactivator were found to be fully inducible, giving a low background of *Prnp* expression before induction and high expression after induction. Stably double-transfected N2a cells were at least as productive as N2a cells over-expressing *Prnp* permanently under the control of a strong viral promoter. Furthermore, the selected N2a clones allowed the *Prnp* expression level to be quantitatively controlled by varying the level of the effector substance, the tetracycline-derivative doxycycline. The clones were fully controllable, as over-expression could be switched on and off as desired. These N2a clones may become an important tool for elucidation of the cellular function of PrP and may pave the way for the tetracycline-inducible expression of many genes in this neuroblastoma cell line.

Introduction

An abnormal isoform of the prion protein (PrP), a neuronal glycoprotein, is an essential factor in the transmission and pathogenesis of prion diseases such as Creutzfeldt–Jakob disease in humans and bovine spongiform encephalopathy (BSE) in cattle. Yet the biological function of the normal, cellular PrP, which is a copper-binding protein (Brown *et al.*, 1997*a*), is poorly understood. Recent data point to a function either in synaptic interaction or in the sensitivity of neurons to oxidative stress (Brown *et al.*, 1997*a*, *b*; Collinge *et al.*, 1994; Herms *et al.*, 1995; Lledo *et al.*, 1996; Tobler *et al.*, 1996). Further insight into these functions may be obtained through comparative analysis of the expression of normal and mutated PrP genes in tissue culture. The aim of this study was to set up a highly efficient and controllable expression system for the PrP gene of the mouse (*Prnp*) in a murine neuroblastoma cell line (N2a).

We decided to employ the binary tetracycline (Tc)-inducible expression system (Gossen & Bujard, 1992), which consists of a tetracycline-controlled transactivator (tTA) and the PrP gene under the control of a transactivator-responsive promoter ($P_{hCMV*\text{-}1}$). The tTA itself is a fusion protein composed of the *tet* repressor (tetR) and the transactivating domain of herpes simplex virus protein 16 (VP16). In the presence of Tc, tTA cannot bind to the *tet* operon sequences in $P_{hCMV*\text{-}1}$. In the absence of Tc, tTA binds with high affinity and specificity to $P_{hCMV*\text{-}1}$ and initiates transcription at the minimal hCMV promoter moiety of $P_{hCMV*\text{-}1}$. In parallel, we used the reverse system, in which the *tet* repressor is mutated at four amino acids and shows the reverse phenotype, binding only in the presence of tetracycline derivatives (Hillen & Berens, 1994; Gossen *et al.*, 1995).

N2a clones carrying either the tTA or the reverse tTA (rtTA) were carefully selected, and the murine PrP gene (*Prnp*) was introduced. Stably double-transfected cell lines were analysed for their expression characteristics.

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Methods

■ **Vector construction.** The plasmid pUHD15-1neo carries both the tTA coding sequences and the neomycin resistance gene (neo^R), and pUHD172-1neo contains the rtTA coding sequences and neo^R. pUHG16-3

has the β-galactosidase gene under the control of $P_{hCMV*-1}$. pUHD10-3 contains $P_{hCMV*-1}$ followed by a multiple cloning sequence. All four constructs were generous gifts from H. Bujard, Zentrum für Molekulare Biologie, Heidelberg (Gossen & Bujard, 1992; Gossen *et al.*, 1995; Resnitzky *et al.*, 1994). The plasmid pHA58 carries the hygromycin resistance gene under the control of the murine pgk-1 promoter and was a generous gift of B. Zevnik (University of Essen, Germany).

To generate phCMV*-*Prnp*, the complete coding region of *Prnp* (amino acids 1–254; Westaway *et al.*, 1994) was first amplified from mouse genomic DNA by PCR with primers 787W (5′ GGGAAGCT-TCAGTCATCATGGCGAACCTTGG 3′) and 786W (5′ TTCGAATT-CCCTCATCCCACGATCAGGAA 3′). The PCR product was cloned into the *Hin*dIII and *Eco*RI sites of pBluescript II KS(+) (Stratagene) by using restriction sites at the 5′-ends of primers 787W and 786W, resulting in the plasmid pBs-*Prnp*. The sequence of the insert was verified by dideoxy sequencing and was found to correspond to the $Prnp^a$ allele (Westaway *et al.*, 1994). The insert was excised from pBs-*Prnp* with *Hin*dIII and *Bam*HI, its 5′-protruding ends were blunt-ended with the Klenow fragment of DNA polymerase I and the fragment was cloned into the Klenow-treated *Eco*RI site of pUHD10-3, to produce phCMV*-*Prnp*. The vector pCI-*Prnp* was produced by excising the insert of pBs-*Prnp* with *Xho*I and *Xba*I and introducing it into the corresponding restriction sites of the multiple cloning site of pCI-neo (Promega).

■ **Transfection of N2a cells.** The murine neuroblastoma cell line N2a (Klebe & Ruddle, 1969) was obtained from the ATCC. N2a cells which expressed tTA (or rtTA) were produced as follows. Twenty μg pUHD15-1neo (or pUHD172-1neo) was transfected into 5×10^5 cells using the calcium phosphate method (Graham & van der Eb, 1973). Cells resistant to geneticin (400 μg/ml G418) were selected and cloned by limiting dilution. Selected clones were screened for transactivation of $P_{hCMV*-1}$ by transient transfection with pUHG16-3. Three selected clones from each first round of transfection were subjected to a second round of calcium phosphate-mediated transfection with 19 μg phCMV*-*Prnp* and 1 μg pHA58. Cells resistant to hygromycin (200 μg/ml) were selected and again cloned by limiting dilution. The second selection was performed in the presence of geneticin (200 μg/ml) and, in the case of N2a-tTA cells, with doxycycline (dox; 100 ng/ml) to suppress *Prnp* expression during the selection procedure.

N2a cells that permanently over-expressed *Prnp* were produced by calcium phosphate-mediated transfection of pCI-*Prnp* and subsequent selection with geneticin (400 μg/ml G418). Cloning was performed by limiting dilution.

■ **β-Galactosidase assay.** Expression of tTa or rtTA after the first round of transfection was assayed by transient expression of the *lac*Z gene in pUHG16-3. In this assay, 5 μg pUHG16-3 was transfected by the calcium phosphate method into 5×10^5 cells. After 24 h, 2 μg/ml dox was either added to or omitted from parallel dishes. β-Galactosidase activity was assayed after 48 h according to the standard assay system of Promega. Hydrolysis of *O*-nitrophenyl-β-D-galactopyranoside was followed by monitoring the absorbance at 420 nm with a spectrophotometer.

■ **Western blot analysis.** *Prnp* expression was examined after the second round of transfection by Western blot analysis. Cells and tissues were lysed in a buffer containing 150 mM NaCl, 50 mM Tris–HCl (pH 7·5), 0·5% NP40 and 0·5% sodium deoxycholate, supplemented with 1 mM PMSF. Proteins were separated by SDS–PAGE, transferred to a PVDF membrane and further processed as described previously (LeGendre, 1990). The primary antiserum used was Kan72, which is directed against amino acids 89–103 of murine PrP (Hölscher *et al.*, 1998), at a dilution of 1:2000. This was followed by detection with alkaline phosphatase (AP)-conjugated goat anti-rabbit serum (1:600, Dako). Immunopositive signals were developed by using the chemiluminescent substrate CDP-*Star* (Tropix) according to the manufacturer's instructions. Signals were monitored, documented and quantified with a CCD-video system (raytest, Straubenhardt, Germany) and the accompanying software (DIANA, TINA).

Results

Selection of tTa- and rtTA-expressing N2a clones

The first component of the Tc-responsive expression system was introduced by stably transfecting N2a cells with pUHD15-1neo (tTA) or pUHD172-1neo (rtTA). After selection for G418 resistance, 40 individual clones were selected for each construct. Verification of production of a functionally active transactivator by these clones was performed by transient transfection of pUHG16-3. This plasmid carries the *lacZ* gene under the control of the transactivator-responsive promoter $P_{hCMV*-1}$. Transfections were performed in parallel dishes, with or without dox, and β-galactosidase activity was measured in extracts of transfected cells. There was dramatic variation in the level of expression after induction, as well as in the uninduced background expression, between individual N2a-tTA or N2a-rtTA clones. Three clones of each transactivator that showed a wide range of induction levels (Fig. 1) were chosen for further manipulation. Comparison of N2a-tTA with N2a-rtTA clones revealed a much higher reporter gene activity for the clones carrying tTA (26–61 U; Fig. 1*a*) than for the clones carrying rtTA (11–15 U; Fig. 1*b*). In addition to the three N2a-tTA clones chosen, at least five other N2a-tTA clones showed an equally large range of induction (data not shown), but only the three selected N2a-rtTA clones combined high expression and low background activity. The overall results from the first round of stable transfection were that suitable N2a-tTA clones were easier to obtain than N2a-rtTA clones and that they supported a higher expression of genes under the control of the $P_{hCMV*-1}$ promoter.

Selection of N2a-tTA and N2a-rtTA clones over-expressing *Prnp*

The second component of the Tc-responsive expression system for *Prnp* was introduced by co-transfection of phCMV*-*Prnp* (*Prnp* under the control of the Tc-responsive promoter) and pHA58 (carrying the hygromycin resistance gene). After selection for hygromycin resistance, 94 clones based on tTA and 61 clones based on rtTA were selected and screened for *Prnp* over-expression in the fully induced state (0 μg/ml dox in the case of tTA clones and 2 μg/ml dox for rtTA clones). Comparison with the endogenous level of *Prnp* expression in untransfected N2a cells revealed that only 17 clones based on tTA showed over-expression (18%), whereas 26 clones based on rtTA exhibited *Prnp* over-expression (43%). In contrast to the selection of suitable N2a clones carrying the first component, the search for cells expressing the gene of the

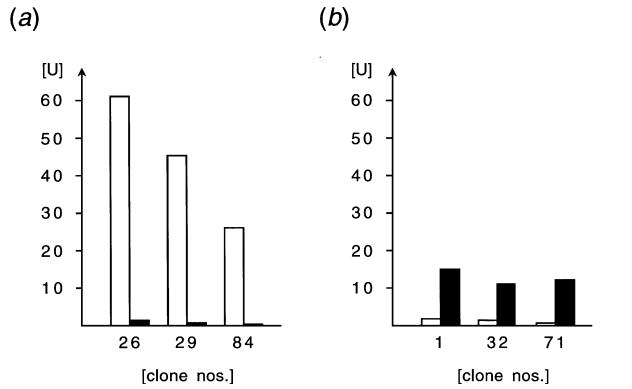

Fig. 1. β-Galactosidase activity of selected N2a-tTA (*a*) or N2a-rtTA (*b*) cell lines after transient transfection of the plasmid pUHG16-3. β-Galactosidase activity is shown in the absence (unfilled bars) or the presence (filled bars) of 2 µg/ml dox.

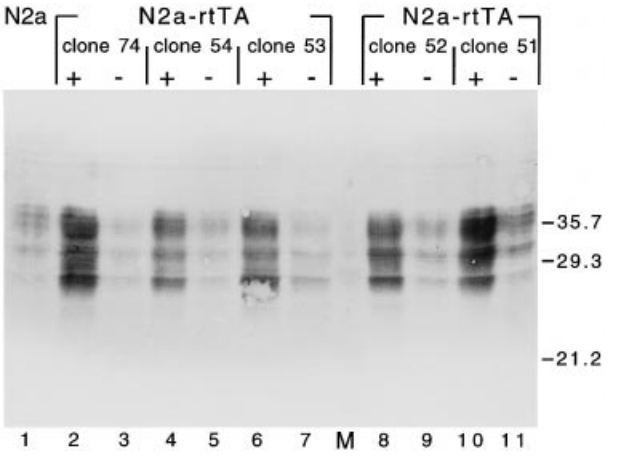

Fig. 2. Inducible expression of *Prnp* in various clones of stably double-transfected N2a-rtTa cells. Expression of *Prnp* was analysed by Western blotting with Kan72 anti-PrP serum and an AP-coupled goat anti-rabbit antibody. Five clones (lanes 2–11) were tested before (−) and after (+) induction with 2 µg/ml dox for 48 h. Equal amounts (90 µg) of protein were loaded in all lanes. A cell lysate from normal N2a cells was included as a control (lane 1). Molecular masses of marker proteins (lane M) are indicated in kDa to the right.

second component was much easier for N2a cells carrying rtTA rather than tTA.

Inducible expression of *Prnp*

Several stably double-transfected N2a clones were examined for their expression levels of *Prnp* in the presence or absence of dox (2 µg/ml medium; Fig. 2). In all N2a-rtTA clones, Western blot analysis revealed a level of *Prnp* expression before the addition of dox comparable to that in untransfected N2a cells (Fig. 2). After induction for 48 h, the level of *Prnp* expression had increased about fourfold. The size and the glycosylation pattern of PrP remained unchanged, showing an unglycosylated isoform (27 kDa), a range of monoglycosylated forms (about 30 kDa) and a broad range of

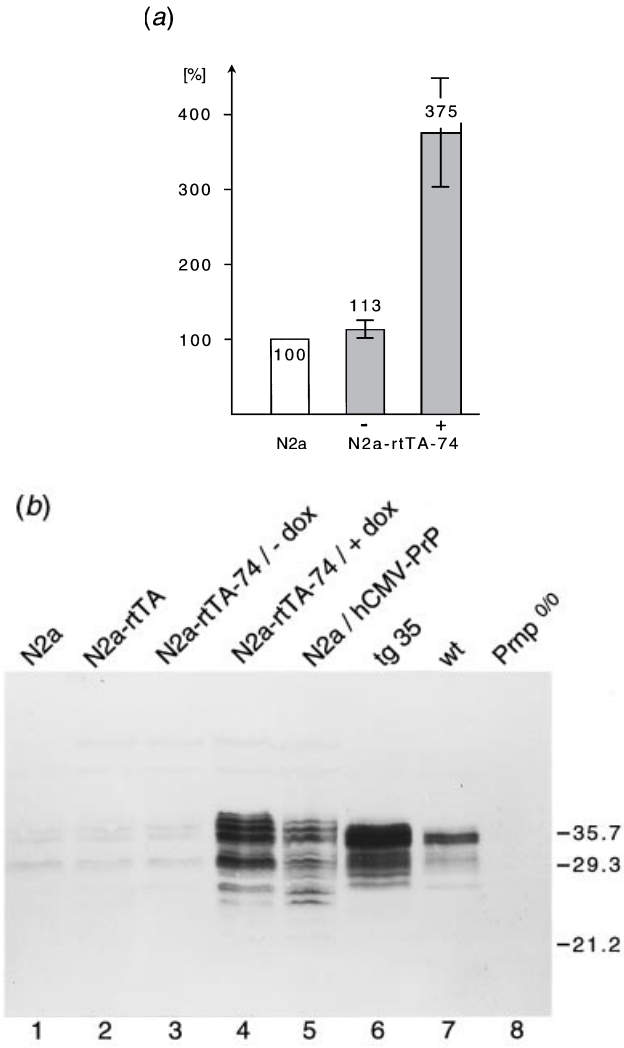

Fig. 3. Quantitative analysis of induced *Prnp* expression in N2a-rtTA-74. (*a*) Quantitative measurement of *Prnp* expression by direct summation of the chemiluminescence signals of three independent Western blot experiments. Expression is given as a percentage of the endogenous level in normal N2a cells (unfilled bar), before (−) and after (+) induction with dox (filled bars). Error bars represent SD. (*b*) Comparative analysis of *Prnp* expression in various types of N2a cells (lanes 1–5) and brain homogenates (lanes 6–8). Equal amounts (100 µg) of lysates from the following were analysed by Western blot with the antiserum Kan72: lanes 1, normal N2a cells; 2, N2a cells stably transfected with the first component of rtTA system (N2a-rtTA); 3–4, N2a-rtTA-74 incubated either without (-dox, lane 3) or with (+dox, lane 4) 2 µg/ml dox for 48 h; 5, N2a cells permanently over-expressing *Prnp* (N2a/hCMV-PrP); 6–8, brain homogenates of mice over-expressing *Prnp* (tg 35, lane 6), wild-type mice (wt, lane 7) and *Prnp*-null mice ($Prnp^{0/0}$, lane 8). Molecular masses of marker proteins are indicated in kDa to the right.

diglycosylated isoforms (about 33–40 kDa) (Fischer *et al.*, 1996). Parallel analysis of clones based on tTA showed a high level of expression of PrP in many clones, irrespective of whether dox was absent (tTA binds the *tet* operon) or present (tTa is not bound). Only a small number of clones displayed any inducibility, but all of them had a high background before induction (data not shown). We therefore concluded that tTA,

while allowing the over-expression of *Prnp* in N2a cells, does not permit significant regulation of expression of this particular gene in these cells. Further analyses were performed with two clones based on rtTA (N2a-rtTA-74, Fig. 2, lanes 2 and 3; N2a-rtTA-51, lanes 10 and 11). The clones did not differ significantly and further analysis of N2a-rtTA-74 is described in the following paragraphs.

PrP expression level

A quantitative analysis of the increase in expression of *Prnp* after induction was performed in three independent Western blot experiments. The chemiluminescence signals were detected and quantified directly and showed a level of expression four times higher in the induced versus the uninduced cells (Fig. 3*a*). Furthermore, we compared the *Prnp* level in these cells with brain tissue of various mouse lines and N2a cells that permanently over-express *Prnp* (Fig. 3*b*). The expression level of *Prnp* in N2a-rtTA-74 after induction with dox (Fig. 3*b*; lane 4) was comparable to that in the brain of a transgenic mouse over-expressing *Prnp* (line tg 35, Fischer *et al.*, 1996) (Fig. 3*b*, lane 6) and much higher than that in normal mouse brain (Fig. 3*b*, lane 7). Comparison with an N2a clone permanently over-expressing *Prnp* under the control of the hCMV promoter (Fig. 3*b*, lane 5) indicated that the Tc-inducible system was slightly superior in terms of PrP production to this conventional system.

Quantitative control and kinetics of *Prnp* expression

The quantitative regulation of *Prnp* expression was examined by inducing expression in N2a-rtTA-74 for 48 h with various concentrations of dox (0–2000 ng/ml medium). Cell extracts were then analysed by Western blot (Fig. 4*a*). At 5 ng/ml dox, an increase in *Prnp* expression was visible by comparison of the signal intensity (Fig. 4*a*, lane 4) with the uninduced state (Fig. 4*a*, lane 2). The signal intensity reached saturation at dox concentrations around 50–150 ng/ml (Fig. 4*a*, lanes 7 and 8). The level of *Prnp* expression was clearly quantitatively controllable within a range of dox concentrations from 1 to 50 ng/ml (Fig. 4*b*).

Monitoring of induction was generally performed after 48 h of dox treatment. To examine the time-course of *Prnp* expression, we harvested cells at different times after induction and analysed *Prnp* expression in a quantitative manner. Expression started to rise above the background level between 3 and 5 h after induction and reached a plateau at around 20 h (Fig. 4*c*). There was no further increase after extended induction periods of up to 10 days (data not shown).

Prnp expression is reversible

It has been suggested that induction of expression by the Tc system is reversible (Gossen & Bujard, 1992), although very few examples have been shown to support this statement. We examined N2a-rtTA-74 in this respect by adding and with-

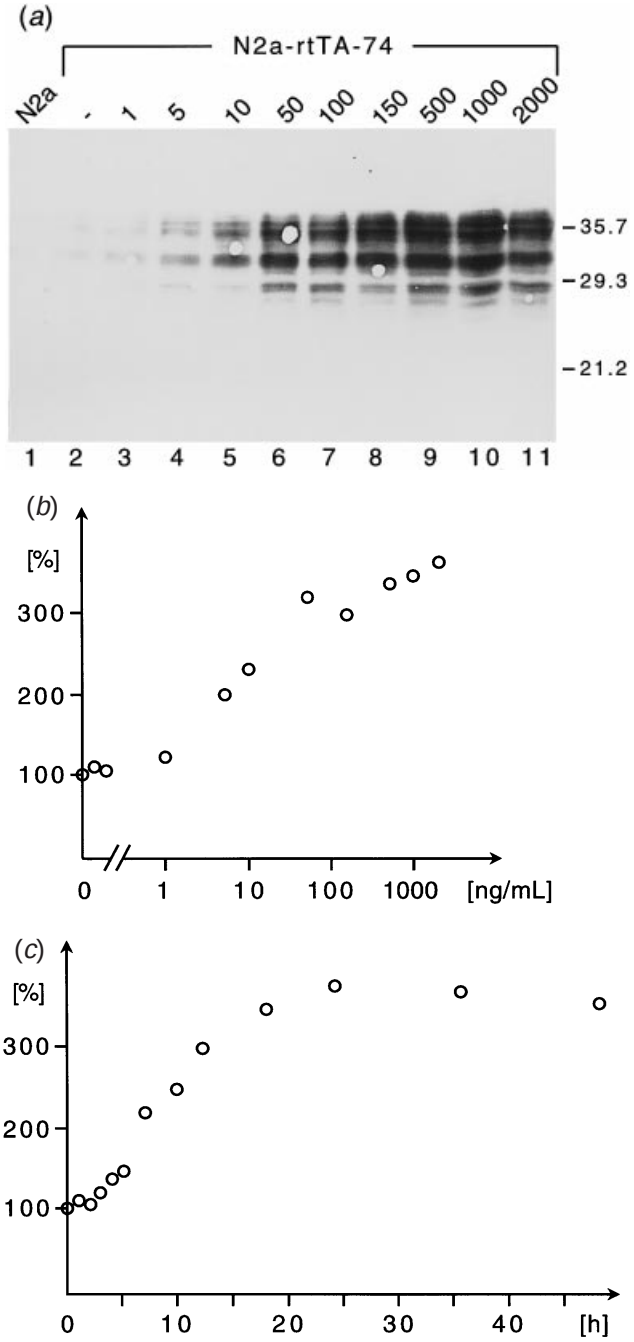

Fig. 4. Regulation and kinetics of *Prnp* expression. (*a*) Equal amounts (90 µg) of protein lysates from N2a-rtTA-74 cells (lane 2–11) and normal N2a cells (lane 1) were analysed by Western blotting. N2a-rtTA-74 cells were incubated in the presence of various dox concentrations for 48 h; dox concentrations are indicated above the individual lanes in ng/ml. Molecular masses of marker proteins are indicated in kDa to the right. (*b*) PrP-specific signals from the Western blot of Fig. 4 (*a*) were quantified and are given as percentages of expression in uninduced cells. (*c*) Individual dishes of N2a-rtTA-74 cells (5×10^5 cells per 60 mm diameter dish) were incubated with 2000 ng/ml dox at time 0. The cells were harvested at the time-points indicated and equal amounts of protein (100 µg) were examined by Western blot. PrP-specific signals were quantified and are given as percentages of the signal from uninduced cells.

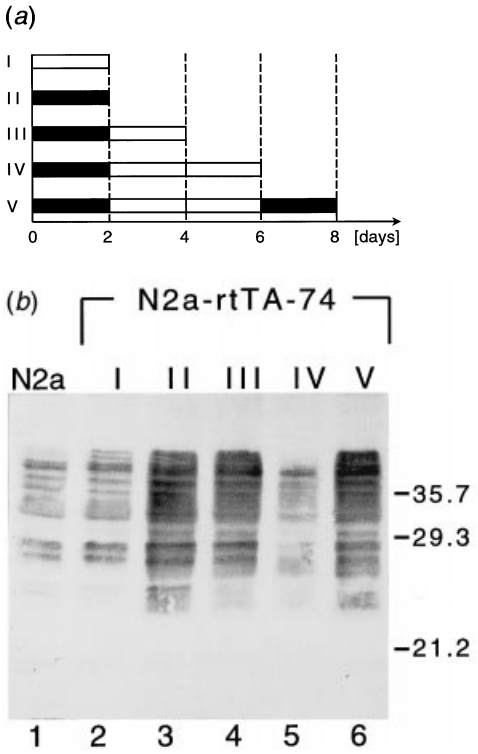

Fig. 5. Reversibility of *Prnp* expression. (*a*) Scheme for the test of reversibility. Individual dishes (I–V) of N2a-rtTA-74 cells (5×10^5 cells per 60 mm diameter dish) were treated with 2 μg/ml dox (filled bars) or grown in the absence of dox (unfilled bars) for the time-periods indicated. (*b*) Western blot analysis of equal amounts of protein lysates of cells from dishes I–V (lanes 2–6) and normal N2a cells (lane 1). Molecular masses of marker proteins are indicated in kDa to the right.

drawing dox (2 μg/ml) for different time periods (Fig. 5*a*). After 2 days of induction (Fig. 5*b*, lane 3), it was possible to reduce *Prnp* expression to near-basal activity by withdrawing dox for 4 days (Fig. 5*b*, compare lane 5 with lane 2), while 2 days of withdrawal was not sufficient to reduce induced expression to the basal level (Fig. 5*b*, lane 4). The system was shown to be fully reversible, as after a second round of induction for 2 days following complete reversion, the level of *Prnp* expression had returned to full over-expression (Fig. 5*b*, lane 6).

Discussion

We have established a highly controllable expression system for PrP^C in order to facilitate future functional analysis of this protein. As a first step, we generated host cells that expressed either tTA (N2a-tTA cells) or the transactivator of the reverse system, rtTA (N2a-rtTA cells). To our knowledge, this is the first time that such clones have been generated from this murine neuroblastoma cell line. They may become a resource not only for the expression of *Prnp* and mutants thereof, but also for the functional analysis of other genes (Schmid, 1995). *Prnp* was introduced by a second round of transfection and expression of *Prnp* was inducible in N2a cells carrying rtTA but not tTA. In many tTA-carrying cells, maximal *Prnp* expression was achieved irrespective of whether dox was present in the medium. This was in contrast to the selection of N2a cells after the first transfection step with a reporter gene construct, which clearly showed the ability of the selected N2a-tTA clones to support controllable expression of *lacZ* from the $P_{hCMV^{*}\text{-}1}$ promoter (Fig. 1*a*). Therefore, the failure to control *Prnp* expression seemed to be a consequence of the introduction of the second component, *Prnp* and the hygromycin resistance gene. At present it is difficult to unravel this problem, but for future applications we would recommend employing both tTA and rtTA, as only one of them may give the desired result.

Quantitative analysis of the stably double-transfected N2a-rtTA cells revealed an expression level for *Prnp* which was four times higher in the induced state than in the uninduced state or in normal N2a cells. This may seem small compared to the 1000-fold induction of reporter gene activities described for the rtTA system (Gossen *et al.*, 1995), but the endogenous level of PrP in N2a cells is relatively high. The increase after induction may therefore be masked by the endogenous PrP level, particularly since the sensitivity of Western blot analysis in detecting PrP is much lower than the sensitivity of the assays for the reporter genes.

A comparison between the expression levels of Tc-inducible N2a cells and N2a cells permanently over-expressing *Prnp* under the control of the hCMV promoter revealed a slightly higher expression level in the Tc-induced cells. This is in agreement with data from another study (Yin *et al.*, 1996) but in contrast to that study, where the Tc system was reported to give 35-fold higher expression, the difference in terms of absolute expression was not very great in our system. *Prnp* expression in N2a cells may have an upper limit, in contrast to the reporter gene activity that was monitored in Yin's study (Yin *et al.*, 1996). In an attempt to confirm this suggestion, we analysed 42 N2a clones over-expressing *Prnp* with the Tc system and about 20 clones permanently over-expressing *Prnp* under the control of the hCMV promoter (C. Behrens & A. Römer, unpublished results) and found no clones with an expression level higher than those reported here. Recently, the hamster PrP gene has been over-expressed 14-fold compared with the PrP^C content in normal hamster brain by using the glutamine synthetase system in Chinese hamster ovary (CHO) cells (Blochberger *et al.*, 1997). Whereas purification of PrP^C from this source now seems rather promising, the use of CHO cells for physiological studies is limited and a successful infection of CHO cells has never been reported. In both respects the N2a cells are superior and, in particular, comparison with scrapie-infected N2a cells (Race *et al.*, 1987; Butler *et al.*, 1988) allowed insights into changes in the biochemistry of the prion protein (Vey *et al.*, 1996) and cell physiology (Wong *et al.*, 1996). However, N2a cells synthesize endogenous PrP, which could cause difficulties in the interpret-

ation of results after the expression of mutant PrPs. While the mutant protein could be labelled (i.e. epitopically tagged) and therefore identified unequivocally, its effects on cell physiology before and after scrapie infection would be influenced by endogenous PrP. It is hoped that cells over-expressing wild-type *Prnp*, as presented in this paper, will be an appropriate control for most effects of over-expressed mutant PrPs, but there are clearly conceivable situations where this will not be the case. All currently known cell lines derived from neurons show PrP gene expression and so there is no way to circumvent this problem. Clearly, a cell line of neuronal origin derived from *Prnp*-knockout mice (Büeler *et al*., 1992) would be highly desirable but to our knowledge nobody has succeeded in producing such a cell line.

We have shown that *Prnp* expression in stably double-transfected N2a-rtTA cells is correlated with the dox concentration and that dox quantitatively controls PrP production. Furthermore, induced *Prnp* expression was reversed within 96 h of the depletion of dox, but was not reversed only 48 h after depletion. Hence, reversion took more than twice as long as induction, which reached a plateau within 20 h after the addition of dox. Reversion has not been given much attention in most publications so far. In the tTA system, a reversion as fast as the induction was mentioned (Gossen & Bujard, 1992), whereas in the rtTA system, a time-scale similar to the one described here has been reported in one case (Bohl *et al*., 1997).

Future experiments will aim to use the inducible PrP expression system to solve some of the problems in prion research, for example the metabolism and the function of PrP^{C} and the kinetics of PrP^{Sc} accumulation following infection of these recombinant N2a cells.

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