Existence of two translation initiation sites leading to the expression of two proteins from the rat high-affinity neurotensin-receptor cDNA: possible regulation by the 5' end non-coding region

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This work demonstrates the expression of two different proteins (47 and 44 kDa) from the rat high-affinity neurotensin receptor cDNA, observed after both translation *in vitro* and transient transfection into eukaryotic COS-7 cells. These two proteins are the consequence of two initiation sites of translation present in the corresponding mRNA. Site-directed mutagenesis indicated that the 47 kDa protein was generated from the first AUG codon (Met¹). In contrast, suppression of the second AUG codon found

in the sequence (Met²⁷) did not modify the expression of the two proteins previously observed with the wild-type neurotensin receptor. Therefore this second translation site could correspond to a non-AUG codon. Moreover, when the 5' end untranslated region of the neurotensin receptor mRNA is deleted, the expression of the higher-molecular-mass protein is enhanced, indicating that this region could be involved in the regulation of expression of these two proteins.

INTRODUCTION

Murine brain membranes have been described as containing two independent neurotensin (NT)-binding sites that differ in their affinities [1] and in their sensitivities to the H1 antihistaminic drug levocabastine [2]. Indeed, the low-affinity binding component was described as being totally and selectively inhibited by levocabastine, whereas the high-affinity binding component is insensitive to the drug [2]. The physical existence of these two binding sites was confirmed by molecular cloning of the high-affinity NT receptor (NTR) from rat brain [3] and of the low-affinity, levocabastine-sensitive, NTRs from mouse and rat brain [4,5].

The role of the cloned levocabastine-insensitive high-affinity rat NTR in the regulation of several intracellular effectors has been well established. Indeed, this receptor type was shown to be involved in the regulation of intracellular Ca²⁺ and cAMP levels [6,7]. From biochemical properties described in the LTK cells expression system [6], two proteins with molecular masses of 50 and 60 kDa were detected by affinity cross-linking of iodinated NT to whole cells stably transfected with the single cDNA encoding the rat NTR. These two proteins displayed the same affinity for NT because a single binding site was observed in saturation experiments performed on membrane preparations and on intact transfected LTK cells [6].

Previous photoaffinity labelling experiments performed on NTRs in rat brain synaptic membranes led to the specific labelling of two proteins with molecular masses of 49 and 51 kDa [8]. The relationships between the two proteins labelled in rat brain synaptic membranes and in LTK cells is still a matter of speculation. Indeed, in LTK cells, the expression of two proteins from a single receptor cDNA could reflect either the existence of a post-translational modification of the initial protein or the presence of two initiation sites for the translation of the NTR mRNA. In contrast, in brain membranes, the presence of two proteins might be due to (1) the existence of two different NTRs,

(2) alternative mRNA splicing or (3) partial proteolytic cleavage of a single receptor protein.

The cDNA encoding the high-affinity NTR possesses a second Met codon (Met²⁷) in the coding sequence, which could serve also as an initiation site to generate a second protein truncated at the N-terminal level. To study the expression characteristics of this cloned rat brain receptor both with a translation system *in vitro* and in eukaryotic cells, point mutations of Met¹ or Met²⁷ codons were performed by site-directed mutagenesis, and the binding and molecular properties of both mutated and wild-type NTRs were compared after expression *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

Neurotensin was purchased from Peninsula Laboratories. Monoiodoneurotensin radio-iodinated on Tyr³ (¹²⁵I-Tyr³-NT) was prepared and purified as previously described [9]. The pBluescript[®] KS plasmid and the VCS-M13 helper phage were from Stratagene. The expression vector pcDNA I was from Invitrogen. Dulbecco's modified Eagle's medium was from Gibco. Gentamycin, 1,10-phenanthroline and disuccinimidyl suberate (DSS) were from Sigma France. Fetal calf serum, uridine and PCR reagents were from Boehringer Mannheim. Oligonucleotides and restriction endonucleases were purchased from Eurogentec. [³⁵S]Methionine (translation grade) was from ICN Pharmaceuticals.

Construction and expression of wild-type and mutant NTRs

The open reading frame with (1.44 kb) or without (1.27 kb) the 5' end non-coding sequence of the rat NTR cDNA was isolated by PCR with 5' and 3' oligonucleotides bearing a *Hind*III and a *Not*I restriction site respectively, and the original cDNA encoding the rat NTR (gift from Dr. S. Nakanishi) as template [3]. Total

Abbreviations used: DSS, disuccinimidyl suberate; ¹²⁵I-Tyr³-NT, monoiodoneurotensin radio-iodinated on Tyr³; NT, neurotensin; NTR, NT receptor; VSV-G, vesicular-stomatitis-virus glycoprotein.

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Figure 1 Gene cassettes of the NTR

This representation shows the NTR gene cassettes. The open reading frame (ORF) is located between nucleotides 1 and 1272. The 5' untranslated region (5' UTR) is between nucleotides -171 and 1. Putative AUG codons are positioned in the NTR sequence. The VSV epitope (VSV Tag) is located just after the last amino acid codon. Abbreviations: Met₁Gly, Met¹ → Gly NTR; Met₂₇Gly, Met²⁷ → Gly NTR; wt, wild-type NTR.

sequencing of amplified products showed no change in the initial sequence. The 1.44 kb fragment was inserted into the pBluescript® KS plasmid. Single-stranded DNA corresponding to the coding strand was generated with VCS-M13 helper phage in the presence of uridine in the CJ236 bacterial strain (gift from Dr. L. Counillon). This uracil-containing single strand served as the template for oligonucleotide-directed mutagenesis in accordance with the Kunkel method [10]. Identification of mutant DNA species was performed by dideoxynucleotide sequencing [11].

The receptor was epitope-tagged for Western-blot analysis as follows. The codon for Arg⁴²⁰ was changed by PCR to eliminate the *Sma*I restriction site present at this position. Then an *Sma*I site was added just after the codon of the last residue, Tyr⁴²⁴, and the corresponding *Hind*III–*Sma*I fragment (1.44 kb) was subcloned into the pBluescript® KS vector–VSV-G (a gift from Dr. L. Counillon) in frame with the nucleotide sequence coding for a polypeptide composed of a spacer (RGPPGP) and the vesicular-stomatitis-virus glycoprotein (VSV-G) C-terminal end (Figure 1). The VSV-G sequence was further used as epitope recognized by a mouse monoclonal antibody [12] kindly provided by Dr. Ph. Lenorman.

For eukaryotic expression, the *Hind*III–*Not*I fragments of the wild-type and mutated NTRs were inserted into the pcDNA I vector containing the cytomegalovirus promoter (Invitrogen). The pcDNA I–NTR constructs were transfected into semi-confluent COS-7 cells (0.5 µg of plasmid per 100 mm culture dish) with the DEAE-dextran method [13]. Cells were then grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and 50 µg/ml gentamicin for 48–72 h, a time corresponding to a maximal expression of transfected NTRs.

Translation experiments *in vitro*

Promega's TNT® coupled reticulocyte lysate system kit was used with [³⁵S]methionine as recommended by the manufacturer. Reactions were performed with 1 µg of the expression vector pcDNA I containing the different constructs of NTRs with the T7 RNA polymerase and the reticulocyte lysate. The ³⁵S-labelled

proteins were separated by SDS/PAGE and analysed by autoradiography.

Preparation of cell homogenates and binding experiments

Transfected COS-7 cells were scraped in 50 mM Tris/HCl buffer, pH 7.5, containing 250 mM sucrose and 5 mM EDTA. Cells were pelleted in Microfuge tubes at 15000 *g* for 15 min at 4 °C and resuspended in a hypotonic 5 mM Tris/HCl buffer, pH 7.5, containing 5 µg/ml DNase I for 1 h at 4 °C. Membrane homogenates were recovered by centrifugation at 100000 *g* for 30 min at 4 °C in a Beckman model TL100 centrifuge. Homogenates were resuspended in 5 mM Tris/HCl, pH 7.5, and protein concentration was determined by the Bio-Rad procedure with albumin as a standard.

Competition experiments were performed in 250 µl of 50 mM Tris/HCl, pH 7.5, containing 0.1% BSA and 0.8 mM 1,10-phenanthroline to prevent ligand degradation. Membrane homogenates (5–10 µg) were incubated with 0.1 nM [¹²⁵I]-Tyr³-NT (2000 Ci/mmol) and increasing concentrations of unlabelled NT for 20 min at 25 °C. Binding assays were terminated by the addition of 2 ml of ice-cold binding buffer and rapid filtration through cellulose acetate filters (0.2 µm; Sartorius) and washing of filters twice with 2 ml of buffer. Radioactivity retained on filters was counted with a γ-radiation counter.

Affinity labelling experiments

Membrane homogenates (100 µg) were resuspended in 50 mM Hepes buffer, pH 7.5, containing 0.1% BSA and 0.8 mM 1,10-phenanthroline and then incubated for 20 min at 25 °C with 0.4 nM [¹²⁵I]-Tyr³-NT in the absence or the presence of 1 µM unlabelled NT. The incubation medium was then centrifuged at 15000 *g* for 15 min at 4 °C. Membrane pellets were resuspended at 0 °C in 200 µl of 50 mM Hepes, pH 8.5, and DSS was added to a final concentration of 50 µM by a 1:100 dilution of a 5 mM stock solution in DMSO. After 10 min at 0 °C the reaction was terminated by the addition of 10 µl of non-titrated 1 M Tris base and centrifugation as above. Pellets were homogenized in 1 ml of incubation buffer containing 1 M NaCl for 45 min at room

temperature to dissociate the non-covalently bound labelled peptide. Washed membranes were finally recovered by centrifugation before analysis by SDS/PAGE.

SDS/PAGE

Membranes were solubilized, boiled and analysed by SDS/PAGE by the procedure of Laemmli [14] with a 10 % polyacrylamide resolving gel and with high-range protein markers (Bio-Rad) as standards. The gel was fixed for 10 min in 10 % (v/v) acetic acid and dried under vacuum before autoradiography either for 1 week on Kodak X-OMAT-AR film for affinity labelling, or for 2 h on Hyper Film β max (Amersham) for translation *in vitro*.

Western blot analysis

Aliquots (100 μ g) of cell homogenates were subjected to electrophoresis as described above. Separated proteins were then blotted on nitrocellulose sheets (Schleicher & Schuell) in accordance with the described procedure [15]. The efficiency of the blotting was checked by revealing the transferred proteins with 0.2 % Ponceau Red in 3 % (v/v) trichloroacetic acid. Nitrocellulose was destained with 140 mM NaCl/20 mM Tris/HCl (pH 7.5) (buffer A) and incubated for 4 h at room temperature in buffer A containing 5 % (w/v) skimmed milk. Nitrocellulose was then exposed overnight at 4 °C to a 1:1000 dilution of the monoclonal antibody directed against the terminal sequence of the VSV-G (Figure 1) in buffer A containing 5 % skimmed milk. Sheets were rinsed twice for 10 min with buffer A containing 5 % skimmed milk and 0.05 % Tween-20, and exposed for 2 h to a 1:7500 dilution of rabbit anti-mouse immunoglobulins coupled to alkaline phosphatase. Nitrocellulose was finally rinsed as above and the IgG-antigen complexes were revealed with the alkaline phosphatase kit from Promega.

RESULTS

All the NTR gene cassettes (Figure 1) were inserted into the pcDNA I expression vector containing the T7 RNA promoter in the 5' region of the cDNA sequence. Translation experiments performed *in vitro* on the 1.44 kb NTR cDNA revealed the translation of two products with molecular masses of 44 and 47 kDa (Figure 2a, lane 1). Under standard conditions the

Figure 2 Translation *in vitro* of the wild-type and mutated NTRs

The TNT[®] coupled reticulocyte lysate system kit from Promega was used with 1 μ g of the expression vector pcDNA I containing the different constructs of NTR and the T7 RNA polymerase. Each lane corresponds to ³⁵S-labelled proteins detected after SDS/PAGE and autoradiography. (a) Lane 1, wild-type NTR; lane 2, pcDNA I alone. (b) Lane 1, wild-type NTR; lane 2, Met¹ → Gly NTR; lane 3, Met²⁷ → Gly NTR.

Figure 3 Competition experiments between ¹²⁵I-Tyr³-NT and unlabelled NT for binding to the wild-type and mutated forms of the rat NTR cDNA transiently expressed in COS-7 cells

Membranes prepared from COS-7 cells transfected with wild-type (Δ), Met¹ → Gly ($\bullet$) or Met²⁷ → Gly ($\circ$) rat NTR cDNA species were incubated with 0.1 nM ¹²⁵I-Tyr³-NT and increasing concentrations of unlabelled NT. Results are expressed as the percentage of total specific ¹²⁵I-Tyr³-NT binding. Curves were obtained from three independent experiments performed in duplicate.

relative intensity of the lower-molecular-mass component was more pronounced. Knowing that the NTR cDNA contains a second AUG codon (Met²⁷) in the reading frame, we made two different mutations by replacement of either the Met¹ or the Met²⁷ codon by a Gly codon (Figure 1). Products of translation of these two mutant constructs *in vitro*, analysed by SDS/PAGE and autoradiography, were compared with those of the wild-type NTR (Figure 2b). The NTR cDNA mutated at Met¹ (Met¹ → Gly) was transcribed *in vitro* and translated into a single protein with an apparent molecular mass of 44 kDa (Figure 2b, lane 2), whereas the NTR cDNA modified at Met²⁷ was expressed as two distinct proteins with molecular masses identical with those of the wild-type NTR (Figure 2b, lanes 1 and 3).

To determine whether both forms specifically recognized ¹²⁵I-Tyr³-NT, competition by unlabelled NT of ¹²⁵I-Tyr³-NT binding to membrane preparations from cells transfected with the wild-type, Met¹ → Gly and Met²⁷ → Gly NTRs are shown in Figure 3. The wild-type and mutated NTRs displayed the same affinity for NT (IC_{50} 0.34 $\pm$ 0.02 nM, mean $\pm$ S.E.M.; n = 3).

To demonstrate that the two forms detected by translation experiments *in vitro* were expressed after transient transfection into COS-7 cells, affinity labelling experiments were performed on membrane homogenates from COS-7 cells transiently transfected with the wild-type, the Met¹ → Gly and the Met²⁷ → Gly NTRs. The autoradiographic analysis of proteins covalently labelled with ¹²⁵I-Tyr³-NT by using DSS is illustrated in Figure 4. The results in lanes A and B were obtained from the wild-type NTR. Two different proteins with molecular masses of 47 and 50 kDa were specifically labelled (lane B), the labelling being totally abolished in the presence of 1 μ M unlabelled NT (lane A). When cross-linking experiments were performed with the Met¹ → Gly NTR, only one protein of 47 kDa was radioactively labelled (Figure 4, lanes C and D). In comparison, the mutation Met²⁷ → Gly in the NTR did not abolish the labelling of either the 47 kDa or the 50 kDa protein (Figure 4, lanes E and F).

To verify that the labelling of two bands in cross-linking experiments was not the consequence of a covalent bond between several iodinated NT molecules, both the wild-type and the

Figure 4 Covalent labelling of transiently expressed NTRs

Membranes (100 μ g) prepared from COS-7 cells transfected with wild-type (lanes A and B), Met¹ $\rightarrow$ Gly (lanes C and D) or Met²⁷ $\rightarrow$ Gly (lanes E and F) NTR cDNAs were incubated with 0.4 nM [¹²⁵I]-Tyr³-NT in the presence (lanes A, C and E) or the absence (lanes B, D and F) of 1 μ M unlabelled NT. Washed pellets were then incubated with 50 μ M DSS before analysis by SDS/PAGE and autoradiography. The positions of molecular-mass markers are shown at the left.

mutant forms of the NTR were epitope-tagged by introducing the C-terminal sequence of VSV-G (Figure 1). A Western blot analysis of COS-7 cells transfected with VSV-G-tagged NTRs is shown in Figure 5. Again, the wild-type VSV-G tagged NTR was expressed as two proteins with apparent molecular masses of 44 and 47 kDa (Figure 5, lane 1). Identical proteins were detected with cells transfected with the VSV-G-tagged Met²⁷ $\rightarrow$ Gly NTR (Figure 5, lane 3), whereas only the lower-molecular-mass component was revealed in cells expressing the VSV-G-tagged Met¹ $\rightarrow$ Gly NTR (Figure 5, lane 2). Non-transfected COS-7 cells were totally devoid of cross-reaction with the monoclonal antibody used in these studies (results not shown).

Finally, to try to explain the overexpression of the lower-molecular-mass component, i.e. the 44 kDa protein, compared with the 47 kDa protein, we deleted the 5' end non-coding region from the 1.44 kb gene cassette of the rat NTR and studied the expression of proteins after transcription-translation experiments *in vitro*. The expression products obtained with the gene cassette lacking the 5' end non-coding region (Figure 6, lane 2) were detected at molecular masses identical with those obtained with the 1.44 kb cDNA: 44 and 47 kDa (Figure 6, lane 1). However,

Figure 5 Western-blot analysis of tagged NTRs transiently expressed in COS-7 cells

Membranes (100 μ g) prepared from COS-7 cells transfected with the VSV-tagged wild-type (WT-VSV, lane 1), the Met¹ $\rightarrow$ Gly (Met1Gly-VSV, lane 2) and the Met²⁷ $\rightarrow$ Gly (Met27Gly-VSV, lane 3) NTR cDNAs were separated by SDS/PAGE, blotted on nitrocellulose and incubated with the monoclonal antibody directed against the VSV sequence before being revealed with the alkaline phosphatase kit from Promega.

Figure 6 Translation *in vitro* of the 1.44 and 1.27 kb gene cassettes of NTR cDNA species

The TNT[®] coupled reticulocyte lysate system kit from Promega was used as described in the legend to Figure 2. Lane 1, 1.44 kb NTR; lane 2, 1.27 kb NTR; lane 3, pcDNA alone.

the relative intensities of the two translation products were reversed in the absence of the 5' untranslated sequence (Figure 6, lane 2).

DISCUSSION

Since the molecular cloning of the rat high-affinity NTR [3], several studies on eukaryotic and prokaryotic expression systems have been conducted to assess the pharmacological and biochemical properties of this receptor type [6,7,16]. The expression of the cloned NTR in stably transfected LTK cells [6] led to the detection by photoaffinity labelling of two different proteins with molecular masses of 50 and 60 kDa and raised the problem of the origin of these two components. The generation of two proteins from a single cDNA could be the consequence of either a partial post-translational modification or the existence of two translation initiation sites. To investigate the latter possibility we individually modified the two putative initiation codons, Met¹ and Met²⁷, that are present in the mRNA sequence of the NTR by using site-directed mutagenesis. The expression products of these modifications were compared with the wild-type receptor in several assays including translation *in vitro*, binding assays, affinity labelling and immunological detection after transient expression into eukaryotic cells. This investigation allowed us to clearly demonstrate, by using a combined transcription-translation coupled system *in vitro*, that the native NTR cDNA was translated into two different proteins with molecular masses of 44 and 47 kDa. These two proteins were also recovered after transient expression into eukaryotic COS-7 cells as demonstrated by Western blotting and covalent cross-linking of [¹²⁵I]-Tyr³-NT on membrane homogenates by using DSS, and they bound NT with the same affinity. Taking into account the molecular mass of iodinated NT (about 1850 Da), we can conclude from the affinity-labelling experiments that the intrinsic molecular masses of these two expressed proteins are very close to those observed after translation experiments *in vitro*.

Although the receptor contains several N-glycosylation consensus sequences (Asn⁴, Asn³⁸, Asn⁴² and Asn²¹²), the detection by Western blotting of receptor proteins with molecular masses identical with those observed by translation *in vitro* certainly indicates that COS-7 cells are devoid of the N-glycosylation enzymes responsible for the 60 kDa molecular form detected in

LTK cells [6]. The latter form could correspond to the product of translation starting at the level of the Met¹ codon, whereas the 50 kDa form could represent the protein expressed from the second translation initiation site, also partly glycosylated. Therefore, in LTK cells, the molecular mass difference could be due to the loss of an N-glycosylation site as the consequence of the use of the second initiation site.

When the AUG codon corresponding to the N-terminal Met residue of the receptor molecule [3] was mutated into a Gly codon (Met¹ → Gly-NTR), only the lower-molecular-mass component (44 kDa) was recovered both after translation *in vitro* and after eukaryotic expression/labelling experiments. This result confirms that one of the two proteins expressed in these systems, i.e. the 47 kDa component, is effectively initiated at the level of the Met¹ codon. In contrast, mutation of the second AUG codon (Met²⁷ → Gly-NTR) did not affect the expression of the two proteins in either the prokaryotic or the eukaryotic system. This result indicates that the second initiation site is not located at the level of the Met²⁷ codon. Thus the expression of two proteins from a single cDNA is certainly due to two initiations of translation, the first initiation site being Met¹ as initially proposed [3] and the second site, probably between amino acid codons 20 and 35, given the molecular mass of the second protein detected both by translation *in vitro* and affinity labelling after expression in COS-7 cells. The origin of this site corresponds either to a non-AUG translation initiation codon, as observed in several cases for mammalian genes [17], or to an AUG codon generated by post-transcriptional RNA editing [18]. However, the fact that the cDNA sequence [3] is identical with that of the recently described genomic sequence [19] is not in accordance with an RNA editing mechanism.

In conclusion, the cloned high-affinity NTR is expressed in transfected cells as two membrane proteins with different molecular masses (44 and 47 kDa). This peculiarity could be responsible for the detection of two proteins in rat brain synaptic membranes [8]. It could also account for the immunological detection of two proteins with molecular masses of 54 and 56 kDa in rat cerebral cortex by using an antipeptide antibody raised against a fragment of the third intracellular loop of the cloned high-affinity NTR [20]. The existence of a second translation initiation site could have a role in the regulation of NTR expression. Indeed, as observed in Figure 6, when the 5' end non-coding segment was removed, the expression of the higher-molecular-mass component was enhanced, suggesting a negative influence of this region on the initiation of translation at Met¹. This hypothesis is confirmed by the secondary structure analysis of the NTR mRNA, which reveals the possibility of several

strong annealings of the 5' untranslated segment (171 nt) at the level of the domain surrounding the first AUG codon (results not shown) and then limiting the translation efficiency at this level. The presence of a second initiation site would permit the translation of the receptor protein in all conditions. This hypothesis is corroborated by the observation that the expression of the lower-molecular-mass component is always greater than that of the component generated by initiation at the first Met residue (47 kDa). The identity and location of the second initiation site remains to be elucidated.

We thank Dr. J. Chabry for fruitful discussions and comments, Dr. S. Nakanishi for the gift of the rat neurotensin receptor cDNA, Dr. A. Beaudet for carefully reading this manuscript, and Franck Aguila for illustrations. This work was supported by the Centre National pour la Recherche Scientifique (CNRS). J.M.B. is a fellowship recipient of the Association pour la Recherche sur le Cancer (ARC).

REFERENCES

- 1 Mazella, J., Poustis, C., Labbé, C., Checler, F., Kitabgi, P., Granier, C., Van Rietschoten, J. and Vincent, J. P. (1983) *J. Biol. Chem.* **258**, 3476–3481
- 2 Schotte, A. and Laduron, P. (1987) *Brain Res.* **408**, 326–328
- 3 Tanaka, K., Masu, M. and Nakanishi, S. (1990) *Neuron* **4**, 847–854
- 4 Mazella, J., Botto, J. M., Guillemare, E., Coppola, T., Sarret, P. and Vincent, J. P. (1996) *J. Neurosci.* **16**, 5613–5620
- 5 Chalon, P., Vita, N., Kaghad, M., Guillemot, M., Bonnin, J., Delpech, B., Lefur, G., Ferrara, P. and Caput, D. (1996) *FEBS Lett.* **386**, 91–94
- 6 Chabry, J., Labbé-Jullié, C., Gully, D., Kitabgi, P., Vincent, J. P. and Mazella, J. (1994) *J. Neurochem.* **63**, 19–27
- 7 Hermans, E., Maloteaux, J.-M. and Octave, J.-N. (1992) *Mol. Brain Res.* **15**, 332–338
- 8 Mazella, J., Kitabgi, P. and Vincent, J.-P. (1985) *J. Biol. Chem.* **260**, 508–514
- 9 Sadoul, J. L., Mazella, J., Kitabgi, P. and Vincent, J.-P. (1984) *Biochem. Biophys. Res. Commun.* **120**, 812–819
- 10 Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 488–492
- 11 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- 12 Kreiss, T. E. (1986) *EMBO J.* **5**, 931–941
- 13 Cullen, R. B. (1987) *Methods Enzymol.* **152**, 684–695
- 14 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 15 Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- 16 Watson, M. A., Yamada, M., Yamada, M., Cusack, B., Veverka, K., Bolden-Watson, C. and Richelson, E. (1992) *J. Neurochem.* **59**, 1967–1970
- 17 Nagpal, S., Zelent, A. and Chambon, P. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2718–2722
- 18 Cattaneo, R. (1991) *Annu. Rev. Genet.* **25**, 71–88
- 19 Maeno, H., Yoshimura, R., Fujita, S., Su, Q., Tanaka, K., Wada, K. and Kiyama, H. (1996) *Mol. Brain Res.* **40**, 97–104
- 20 Boudin, H., Grauz, G. A., Faure, M. P., Forgez, P., Lhiaubet, A. M., Dennis, M., Beaudet, A., Rostene, W. and Pelaprat, D. (1995) *Biochem. J.* **305**, 277–283