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Alternative polyadenylation of the amyloid protein precursor mRNA regulates translation

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The sequence of several cDNAs encoding the amyloid protein precursor showed that two polyadenylation sites of the mRNA are utilized; RNA blot analysis with different riboprobes indicated that this explains the difference between the two major 3.2 and 3.4 kb mRNAs found in the human brain. These two mRNAs, which contain the whole sequence of the natural molecules, were synthesized by *in vitro* transcription and translated in *Xenopus* oocytes. The long mRNA using the second polyadenylation site produced more protein than the short mRNA. The sequence contained within the two polyadenylation sites used in the 3' untranslated region of the amyloid protein precursor mRNA was also able to increase the production of the chicken lysozyme or the chloramphenicol acetyl transferase, as demonstrated by *in vivo* translation of different chimeric mRNAs obtained by *in vitro* transcription. This difference in protein production was also observed when chimeric cDNA constructs were transfected into Chinese hamster ovary cells. Since long mRNAs are not more stable than short mRNAs, the sequence contained within the two polyadenylation sites of the amyloid protein precursor mRNA increases the translation.

Key words: Alzheimer's disease/amyloid protein precursor/translation efficiency

Introduction

The βA4 peptide is a major constituent of the amyloid deposits found in senile plaques and cerebrovascular angiopathy (Glenner and Wong, 1984; Masters *et al.*, 1985). These lesions are, together with neurofibrillary tangles, characteristic neuropathological features of Alzheimer's disease. The βA4 peptide comprises part of the membrane-spanning and extracellular domains of a large precursor protein containing 695 amino acids (APP695), which resembles a transmembrane receptor (Kang *et al.*, 1987). Several other amyloid protein precursor (APP) isoforms have been identified, including APP751, APP770 and APP563 (Kitaguchi *et al.*, 1988; Ponte *et al.*, 1988; Tanzi *et al.*, 1988; de Sauvage and Octave, 1989). They differ from APP695 in the presence of additional sequences in the N-terminal domain of the protein, which are homologous to inhibitors of serine proteases. In addition, APP563 lacks the C-terminal domain, including the transmembrane region, that is present in other APPs.

In cell cultures, proteolytic cleavage of APP occurs between amino acids 15 and 17 of the βA4 peptide, generating a large extracellular soluble fragment and a short intracellular C-terminal fragment (Esch *et al.*, 1990). This splicing is performed by a protease called APP secretase, which has not been identified. In Alzheimer's disease, production of full-length βA4 peptide might result from the inactivation of APP secretase and the activation of alternative pathways generating an amyloidogenic C-terminal APP fragment (Estus *et al.*, 1992; Golde *et al.*, 1992).

Mutations of the APP gene, as found in some cases of familial Alzheimer's disease (Chartier-Harlin *et al.*, 1991; Goate *et al.*, 1991; Murrell *et al.*, 1991) and in Dutch-type cerebrovascular angiopathy (Levy *et al.*, 1990; Van Broeckhoven *et al.*, 1990) might lead to abnormal processing of APP and to the formation of amyloid deposits. A change in the expression of the APP in Alzheimer's disease has also been proposed as a mechanism involved in the generation of amyloid deposits. The increase in APP gene expression seen in trisomy 21 (Rumble *et al.*, 1989) suggests that this might be related to the formation of the abundant senile plaques found in Down's syndrome patients older than 35 years. In Alzheimer's disease, such a gene dosage effect has not been established (Podlisny *et al.*, 1987). However, an increase in the expression of APP mRNA (Cohen *et al.*, 1988; Higgins *et al.*, 1988) or modifications in the differential expression of APP isoforms (Palmert *et al.*, 1988; Johnson *et al.*, 1989; Neve *et al.*, 1988, 1990) have been reported in Alzheimer's disease.

Another factor which might affect the level of APP is the efficiency of translation of APP transcripts. There is some evidence that 5' and 3' untranslated regions of mRNAs are involved in the control of translation or mRNA stability (Kruys *et al.*, 1987, 1989, 1992; Casey *et al.*, 1988; Strickland *et al.*, 1988; Han *et al.*, 1990; Rouault *et al.*, 1991). In this report, we demonstrate that the two major APP mRNAs found as 3.2 and 3.4 kb bands in normal brain correspond to the use of two polyadenylation sites in the 3' untranslated region of the mRNA. Furthermore, the sequence contained between them increases translation of the APP mRNA as well as that of reporter mRNAs coding for chicken lysozyme or chloramphenicol acetyl transferase.

Results

Identification of the two polyadenylation sites utilized in the 3' untranslated region

A λGT11 human brain cDNA library was screened with a 1 kb cDNA probe (positions 1796-2850 in the Kang sequence) isolated by restriction digestion of the CTLL-704

A

CTGCAGGATGATTGTAGAGAATCATTGCTTATGACATGATCGCTTTCTACACTGTATTAC 2930
PstI

ATA AATAAA TTA AATAAA AT-poly(A)tail
1 2

B

CTGCAGGATGATTGTAGAGAATCATTGCTTATGACATGATCGCTTTCTACACTGTATTAC 2930
PstI

ATA AATAAA TTA AATAAA ATAACCCCGGGCAAGACTTTTCTTTGAAGGATGACTAC 2986
1 2

AGACATTAAATAATCGAAGTAATTTTGGGTGGGGAGAAGAGGCAGATTCAATTTTCTTTA 3046

ACCAGTCTGAAGTTTCATTTATGATACAAAAGAAGATGAAAATGGAAGTGGCAATATAAG 3106

GGGATGAGGAAGGCATGCCTGGACAAACCCTTCTTTTAAGATGTGTCTTCAATTTGTATA 3166

AAATGGTGTTTTCATGTA AATAAA TACATTCTTGGAGGAGC-poly(A)tail
3

Fig. 1. Nucleotide sequence of the fragments of the two possible 3′ UTRs of the APP mRNA used for chimeric constructs. The nucleotide sequence proceeds in a 5′ to 3′ direction. The positions corresponding to the Kang sequence and the *Pst*I site used for the constructs are indicated. The three consensus sequences for the polyadenylation sites used are indicated; sequencing data indicate that the first and third polyadenylation sites are utilized. The sequence synthesized to obtain a riboprobe between the two utilized polyadenylation sites is underlined.

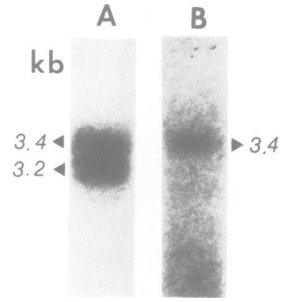

Fig. 2. Northern blot analysis of human brain RNA. The antisense riboprobes used for hybridization were the APP riboprobe (**A**) or the 40 nt riboprobe encompassing the sequence between the two polyadenylation sites (**B**).

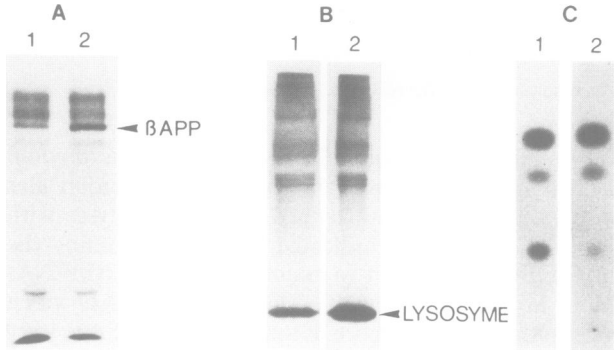

Fig. 3. Typical autoradiograms obtained after 6 h of *in vivo* translation of APP mRNA (**A**), the lysozyme mRNA construct (**B**) or the CAT mRNA construct (**C**). In (A), the labelled proteins obtained after translation of the APP mRNA containing the short (1) or the long (2) 3′ UTR sequence were immunoprecipitated using an anti-APP antibody and analysed by SDS–PAGE. In (B), the labelled proteins obtained after translation of the lysosyme mRNA construct containing the short (1) or the long (2) 3′ UTR sequence of the APP mRNA were immunoprecipitated by an anti-lysozyme antibody and analysed by SDS–PAGE. CAT activity was measured after translation of the CAT mRNA construct (C) with the short (1) or the long (2) 3′ UTR sequence of the APP mRNA.

APP cDNA clone (Vitek *et al.*, 1988) with *Eco*RI. Of the 12 clones isolated, two showed an unusual pattern when digested with *Eco*RI. Sequencing data indicated that these clones, corresponding to APP695 and APP770, use the first polyadenylation site, present at position 2934, and that a poly(A) chain starts at position 2950 (Figure 1). Consequently, this cDNA sequence is 258 nucleotides shorter than the Kang sequence (Kang *et al.*, 1987).

Northern blot analysis

An antisense riboprobe, encompassing 40 nucleotides between the two utilized polyadenylation sites (Figure 1), was used for Northern blot analysis. The results presented in Figure 2 indicate that this 40 nucleotide riboprobe recognizes only the 3.4 kb band and not the two bands recognized by the riboprobe corresponding to the cDNA probe which was used for screening of the cDNA library. These results demonstrate that the two bands corresponding to the 3.2–3.4 kb mRNA doublet result from alternative polyadenylation of the APP mRNA.

Translation of the APP mRNAs

We have studied the influence of the sequence contained within the two polyadenylation sites used in the 3′ untranslated region (UTR) of the APP on mRNA translation. The two possible APP cDNAs were subcloned into an SP64 plasmid. Messengers obtained by *in vitro* transcription were translated in *Xenopus* oocytes incubated in the presence of [^{35}S]methionine (Hames and Higgins, 1984). The translation products were then immunoprecipitated using monospecific antibodies. When the messengers obtained by *in vitro* transcription were translated for 6 h in *Xenopus* oocytes, more protein had been synthesized from the mRNA containing the long 3′ UTR sequence than from the mRNA containing the short 3′ UTR sequence (Figure 3A). Scanning of the autoradiogram using a Chromoscan 3 (Joyce Loebl) indicated that long mRNA produces 3.0 times more protein than short mRNA.

In vivo translation of chimeric mRNAs

In order to investigate whether the APP 3′ UTR was able to influence the protein production independently from the upstream sequences, the two possible fragments of the 3′ UTR sequences of the APP mRNA [from the *Pst*I site to the poly(A) tail] were subcloned into a SP64 plasmid (Figure 4), downstream of the coding region of the chicken lysozyme gene which is translated well in *Xenopus* oocytes (Kruys *et al.*, 1987). When the chimeric messengers obtained by *in vitro* transcription were translated for 6 h in *Xenopus* oocytes, more protein had been synthesized from the mRNA containing the long 3′ UTR sequence than from the mRNA containing the short 3′ UTR sequence (Figure 3B). Scanning of several autoradiograms indicated that long mRNA produces 3.3 ± 0.6 (n = 3) times more protein than short mRNA. This effect was not related to the

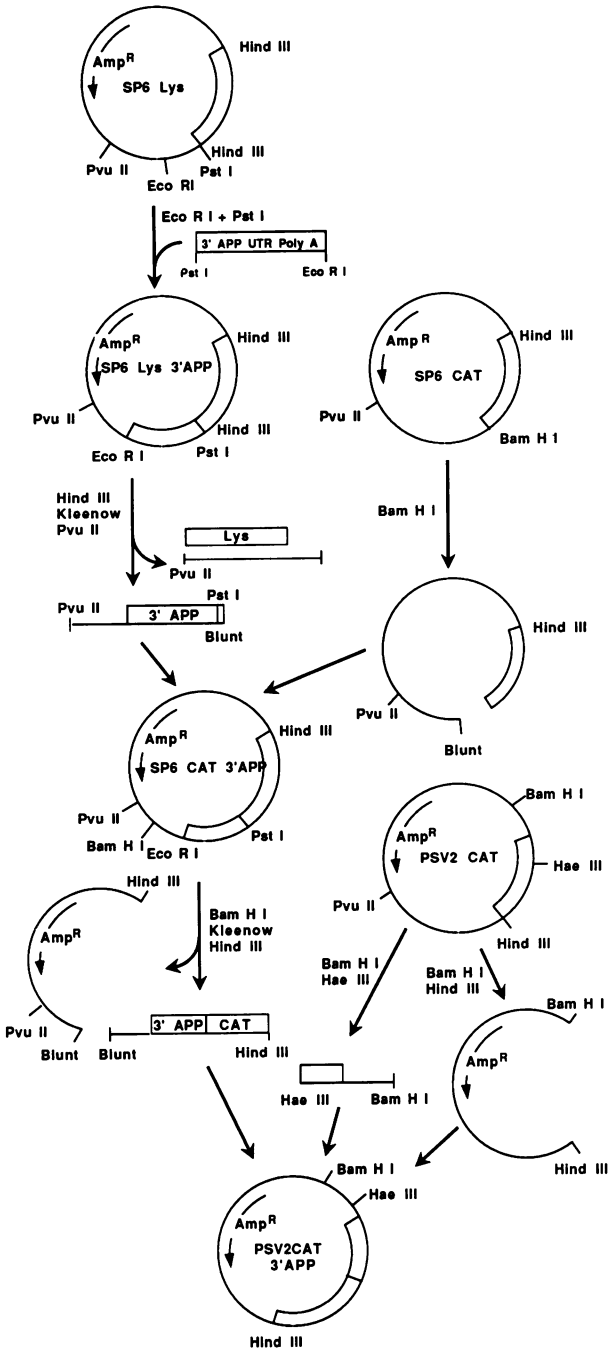

Fig. 4. Overview of plasmid construction. From the SP6 lysozyme and the SP6 CAT plasmids, the SP6 Lys 3'APP, the SP6 CAT 3'APP or the pSV2 CAT 3'APP constructs containing the two possible 3' UTR sequences of the APP were obtained as described in Materials and methods.

mRNA length since the same results were obtained when the short construction was linearized with *Pvu*II (Figure 4) instead of *Eco*RI before *in vitro* transcription.

The difference in protein production described above could have two explanations: either the 3' UTR sequence directly affects mRNA translation, or the short mRNA is degraded more rapidly. In order to test the latter hypothesis, the stability of lysozyme chimeric RNA in oocytes was studied by Northern blot analysis at different time intervals after microinjection. Figure 5 shows that mRNA with the short APP 3' UTR was stable for at least 24 h, whereas partial

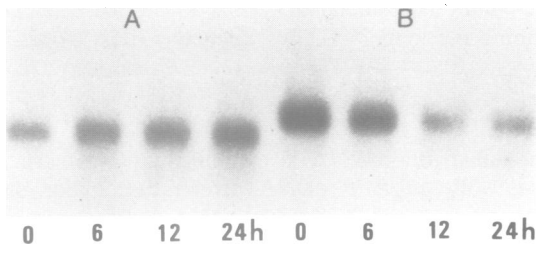

Fig. 5. Analysis of the stability of the chimeric RNAs containing the short (**A**) or the long (**B**) 3' UTR of APP mRNA. Northern blot analysis using a lysozyme specific RNA probe was performed on RNA isolated from oocytes O, 6, 12 or 24 h after injection with the lysozyme mRNA construct.

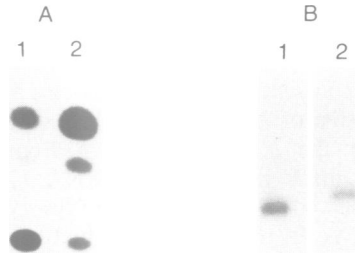

Fig. 6. The difference in the CAT activity (**A**) measured after transfection of CHO cells with the CAT cDNA construct containing the short (1) or the long (2) 3' UTR sequence of the APP mRNA is not related to a difference in the expression of the mRNA (**B**) containing the short (1) or the long (2) 3' UTR.

degradation of mRNA with the long 3'APP UTR was observed as early as 6 h after microinjection of the cells.

Other mRNA constructs were obtained by inserting the two different 3' UTR sequences of the APP downstream the sequence coding for CAT (Figure 4). After 6 h of *in vivo* translation in *Xenopus* oocytes, the CAT activity (Figure 3C) was more abundant in cells microinjected with mRNA containing the large 3' UTR sequence of the APP. When quantifying the results by counting the spots cut from the CAT assay chromatogram, CAT activity was 1.9 ± 0.3 (n = 3) times more abundant in oocytes injected with mRNA containing the long 3' UTR sequence.

Transfection of chimeric cDNA constructs

To study whether the effect of the 3' UTR of the APP on the mRNA translation could be reproduced in cell types other than *Xenopus* oocytes, cDNA constructs were obtained by inserting the two different fragments of the APP 3' UTR sequences downstream of the sequence coding for CAT (Figure 4), in a plasmid containing the SV40 promoter. After transfection of CHO cells, the CAT activity (Figure 6A) was more abundant in cells transfected with the construct containing the large 3' UTR sequence of the APP, while Northern blot analysis did not show more of the corresponding mRNA than of the short mRNA found in the other transfected cell population (Figure 6B). When quantifying the results by counting the spots cut from the CAT assay chromatogram, CAT activity was 3.3 ± 0.3

($n = 3$) times more abundant in cells transfected with cDNA containing the long 3′ UTR sequence.

Discussion

When human brain RNA is examined by Northern blot analysis with an APP cDNA probe, two major bands corresponding to a 3.2−3.4 kb mRNA doublet are found. Different APP mRNAs appear to arise by alternative splicing of a single gene which does or does not contain an insert coding for serine protease inhibitor activity (Kitaguchi *et al.*, 1988; Ponte *et al.*, 1988; Tanzi *et al.*, 1988; de Sauvage and Octave, 1989). While this inserted sequence might be responsible for the doublet detected by Northern blot analysis, we demonstrate herein that the 3.2−3.4 kb mRNA doublet expressed in normal brain and in other tissues is due to alternative use of two polyadenylation sites at the 3′ UTR of the APP mRNA. Furthermore, the sequence contained within the two polyadenylation sites causes a 3-fold increase of the translation of APP mRNA. This stimulation of the translation by the 3′ UTR of the APP mRNA is not influenced by the 5′ region of the mRNA, since a similar effect was observed on the translation of lysozyme and CAT reporter mRNAs. By measuring the stability of the lysozyme chimeric RNAs injected into oocytes, we have observed that the more efficiently translated mRNA is not more stable, indicating that the increased production of protein is not related to mRNA stability but to increased translation.

The 3′ UTR of certain mRNAs has been reported to influence their translation. For instance, the 3′ UTR of human interferon-β and tumour necrosis factor mRNA has an inhibitory effect on translation (Kruys *et al.*, 1987; Han *et al.*, 1990), which is not related to rapid mRNA degradation (Kruys *et al.*, 1988). As determined by computer aided analysis (Zucker and Stiegler, 1981), the last 66 nucleotides of the 3′ UTR sequence found within the two utilized polyadenylation sites of the APP mRNA yield putative stem−loop structures with calculated free energies of formation of −40.3 kcal/mol. These secondary structures could be involved in the interaction with cellular proteins present in *Xenopus* oocytes and CHO cells.

In Alzheimer's disease, abnormal metabolism of APP leads to the extraneuronal deposition of the βA4 peptide in abundant senile plaques. While the mechanism by which the βA4 peptide is generated from APP is unknown, overexpression of the APP in Alzheimer's disease has been proposed as a possible mechanism of APP metabolism disturbance (Cohen *et al.*, 1988; Higgins *et al.*, 1988; Palmert *et al.*, 1988; Tanzi *et al.*, 1988; Johnson *et al.*, 1989, 1990; Neve *et al.*, 1988, 1990). In this way, the 150% increase in APP gene expression in trisomy 21 might be related to the formation of the abundant senile plaques found in the cerebral cortex of Down's syndrome patients (Rumble *et al.*, 1989). We demonstrate here that different APP isoforms are translation products of mRNA which utilize two polyadenylation sites. Alternative polyadenylation of the APP mRNA can regulate its translation, the 3.4 kb mRNA producing more protein than the 3.2 kb mRNA. The preferential use of the second polyadenylation site of the APP mRNA in Alzheimer's disease awaits further investigation, since it could induce a factor of amplification, which might be of significance *in vivo* for amyloid pathology.

Materials and methods

Analysis of cDNA clones

Complementary DNAs were isolated from a library constructed from mRNA from the cerebral cortex of a 54 year old patient with Alzheimer's disease. Oligo(dT) primed double-stranded cDNA was ligated with linkers into the *Eco*RI site of the λGT11 vector (Young and Davis, 1983). About 2×10^6 recombinant phages from the library were screened using a cDNA fragment (positions 1796−2851 of the Kang sequence) (Kang *et al.*, 1987) labelled by multipriming nick-translation (Amersham). Of the 12 hybridizing clones, two were subcloned in the M13 vector for sequence analysis using the dideoxynucleotide termination method (Sanger *et al.*, 1977).

Northern blot analysis

Northern blot analysis of total human brain RNA was performed using antisense riboprobes. The first riboprobe was obtained by cloning the *Eco*RI fragment of the APP cDNA into the *Eco*RI site of the M13-T7 mlc10 vector from Amersham. RNA was labelled using T7 RNA polymerase, [^{32}P]UTP and protocols suggested by the manufacturer. An oligonucleotide corresponding to a portion of the sequence present between the two possible polyadenylation sites (positions 3144−3183) (Figure 1) and its complementary strand were chemically synthesized by β-cyanoethyl phosphoramidite chemistry on an Applied Biosystems DNA synthesizer with a *Pst*I restriction site at the 5′ end and an *Eco*RI restriction site at the 3′ end. After hybridization, the double-stranded oligonucleotide was cloned between the *Eco*RI and *Pst*I sites of M13-T7 mlc10 and 11. Single-stranded viral DNA was used as a template for DNA synthesis in the presence of the Klenow fragment of DNA polymerase, and transcription was initiated according to Eperon (1986) using T7 RNA polymerase. Total RNA was prepared by the guanidinium/CsCl method (Chirgwin *et al.*, 1979). Ten micrograms of glyoxal denatured RNA was fractionated on a 1% agarose gel, in 10 mM sodium phosphate buffer (pH 7), as described by Thomas (1980) and transferred on to Hybond N membranes (Amersham). Prehybridization (4 h) and hybridization with the riboprobes (16 h) were carried out at 45°C or 65°C in 5×SSC, 50% formamide, 10×Denhardt's, 0.1% SDS. After hybridization, the filters were washed three times for 30 min at 65°C in 2×SSC, 0.1% SDS, incubated for 15 min at 37°C in 2×SSC containing 50 μg/ml RNase, and washed for 30 min at 65°C in 0.2×SSC, 0.1% SDS.

Construction of the different plasmids used for in vitro transcription

Chicken lysozyme SP6 plasmid (SP64LYS) was obtained from Amersham. The lysozyme 3′ APP constructions were obtained by inserting the two different *Pst*I−*Eco*RI fragments of the 3′ UTR sequences of the APP in the SP64LYS digested with *Pst*I and *Eco*RI. The resulting plasmids were linearized by *Eco*RI prior to *in vitro* transcription.

CAT SP6 plasmid (SP64CAT) was obtained by the following procedure. The CAT gene was isolated by digesting the psV2CAT plasmid (Gorman *et al.*, 1982) with *Hin*dIII and *Sau*3AI. The 768 bp fragment corresponding to the coding region of CAT was then inserted into the SP64 plasmid previously digested with *Hin*dIII and *Bam*HI. The two possible 3′ UTR sequences of the APP were recovered by digestion of the lysozyme 3′ APP constructions by *Hin*dIII; the extremities were then filled in with the Klenow fragment of DNA polymerase, and the plasmid was digested with *Pvu*II. The SP64CAT was digested with *Bam*HI, filled in and digested with *Pvu*II. The CAT 3′-APP constructions were obtained by insertion of the *Hin*dIII filled-in *Pvu*II fragments of the lysozyme 3′-APP constructions in the SP64CAT vector between the *Bam*HI filled-in and *Pvu*II sites. The resulting constructions were linearized by *Eco*RI for *in vitro* transcription. The SP6 CAT 3′- APP constructs were digested with *Bam*HI, filled-in and digested with *Hin*dIII. This gave the first fragment of a three component ligation. The two other fragments were obtained by digesting pSV2CAT with *Bam*HI and *Hin*dIII or by *Bam*HI and *Hae*III. The pSV2CAT 3′−APP was obtained by ligating these three fragments.

Injection into oocytes

Oocytes were injected with 50 nl of mRNA dissolved in water and adjusted to a concentration of 0.1 mg/ml. The injection procedure and the incubation of the oocytes have been described by Gurdon *et al.* (1971). After injection, the oocytes were incubated for 6 h at 18°C in Barth's medium (0.01 ml per oocyte) containing [^{35}S]methionine (9 μCi per oocyte), 10% bovine serum albumin, 1% trasylol. Oocytes were lysed and immunoprecipitated with monospecific antibodies according to the method described by Huez *et al.* (1983). Analysis of immunoprecipitated proteins was performed by SDS−PAGE.

CAT assay

CAT activity was measured as described by Gorman *et al.* (1982) in oocytes injected with the chimeric CAT mRNA or in CHO cells transfected with the chimeric CAT cDNA constructs.

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